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The *tfdR* Gene Product Can Successfully Take Over the Role of the Insertion Element-Inactivated TfdT Protein as a Transcriptional Activator of the *tfdCDEF* Gene Cluster, Which Encodes Chlorocatechol Degradation in *Ralstonia eutropha* JMP134(pJP4)

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The *tfdT* gene is located upstream of and transcribed divergently from the *tfdCDEF* chlorocatechol-degradative operon on plasmid pJP4 of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134. It is 684 bp long and encodes a 25-kDa protein. On the basis of its predicted amino acid sequence, the TfdT protein could be classified as a LysR-type transcriptional regulator. It has the highest degree of similarity with the proteins TcbR, ClcR, and TfdR, which are involved in the regulation of chloroaromatic breakdown. Despite this homology, the TfdT protein failed to activate the expression of its presumed target operon, *tfdCDEF*. This failure could be attributed to the inability of TfdT to bind the *tfdC* promoter region, an absolute requirement for transcriptional activation. Sequence analysis downstream of the *tfdT* gene revealed the presence of an insertion element-like element. We postulate that this element disrupted the *tfdT* open reading frame, leading to a premature termination and the production of a truncated, disfunctional TfdT protein. As an alternative to the inactivated TfdT protein, we propose that the product of the *tfdR* gene (or its identical twin, *tfdS*), located elsewhere on plasmid pJP4, can successfully take over the regulation of *tfdCDEF* expression. The TfdR protein was capable of binding to the *tfdC* promoter region and activated *tfdCDEF* gene expression by a factor of 80 to 100 when provided in *cis* as a *tfdR-tfdCDEF* hybrid regulon. Although to a lesser extent, induction of *tfdCDEF* expression was also observed when no functional TfdR protein was provided, implying cross-activation by chromosomally encoded regulatory elements in *R. eutropha* JMP134(pJP4).

The chlorocatechol (CC)-degradative pathway is often found in bacteria that can use chlorinated aromatic compounds as carbon and energy sources. In these bacteria, the combined action of the CC-degradative pathway with one or more peripheral pathways ensures the complete digestion of the chloroaromatic substrate via CC as an intermediate metabolite (43). To date, three different gene clusters that encode a complete set of enzymes for the CC-degradative pathway have been identified. They are the *tfdCDEF* gene cluster of plasmid pJP4 from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134 (7, 29), the *tcbCDEF* gene cluster of plasmid pP51 from *Pseudomonas* sp. strain P51 (44), and the *clcABD* gene cluster, which is found on plasmid pAC27 from *Pseudomonas putida* (15), in *Pseudomonas* sp. strain B13 (16), and in *Alcaligenes* sp. isolate JS705 (42). All three gene clusters have a similar operonic organization and a high degree of identity in their nucleotide sequences (44). Furthermore, the enzymes that they encode resemble each other very much in terms of amino acid sequence and substrate specificity (38, 44).

Another characteristic shared by these operons is the presence of a regulatory element located upstream of and transcribed divergently from the first gene of the operon. For *tcbCDEF* and *clcABD*, these are the *tcbR* (45) and *clcR* (5) genes, respectively. In the case of the *tfdCDEF* operon, a partially sequenced open reading frame (ORF), located upstream and designated *tfdT*, has been suggested to encode the regulatory protein (29, 47). Interestingly, the involvement of a second protein, TfdR (or TfdS), in the regulation of *tfdCDEF* expression has been proposed and rejected in several mutually contradicting reports (21, 48). What is clear, though, is that the TfdR protein is involved in the activation of *tfdA* and *tfdB* gene expression (22, 48). These genes encode enzymes for the conversion of 2,4-dichlorophenoxyacetic acid (2,4-D) (40) and 2,4-dichlorophenol (29) which, in concert with the CC-degradative pathway encoded by *tfdCDEF*, enable host strain *R. eutropha* JMP134(pJP4) to grow on 2,4-D. The identical *tfdR* and *tfdS* genes, which code for the TfdR protein, are located about 8 kb away from the *tfdCDEF* operon on plasmid pJP4 (27, 48). The *tfdS* gene is oriented divergently from the *tfdA* gene (40, 48). The *tfdR* gene is organized in a fashion similar to that of *tfdS*, with a *tfdD*-like gene ($tfdD_{II}$) on plasmid pJP4 (27). The TcbR, ClcR, TfdR, and partial TfdT proteins have a very high degree of identity in their overlapping predicted amino acid sequences (45, 48). They all belong to the LysR-type family of transcriptional regulators (19, 37). The TcbR and ClcR regulatory proteins have already been studied in great detail. The binding of the proteins to their target promoter DNA has been reported and described meticulously (4, 25). Binding of the TfdR protein to the $tfdD_{II}$ promoter region has also been demonstrated (27), but regulation of this gene by TfdR has not yet been established.

By analogy with *tcbR-tcbCDEF* and *clcR-clcABD*, it would be reasonable to assume that the *tfdT* gene and *tfdCDEF* operon also constitute a regulon specialized for the degradation of CC. We therefore decided to complete the sequencing of the *tfdT* gene, compare its gene product with proteins like TcbR and ClcR, and establish its role as an activator of *tfdCDEF* expression. Additionally, in response to previously contradicting re-

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ports on the role of the *tfdR* gene product, we reevaluated the possible involvement of TfdR in the regulation of the *tfdCDEF* operon.

MATERIALS AND METHODS

Bacterial strains. *Pseudomonas putida* KT2442 (14) is a rifampin-resistant, plasmid-free derivative of *P. putida* mt-2. *Pseudomonas* sp. strain B13 (9) can grow on 3-chlorobenzoate (3-CBa) as its sole carbon and energy source. *Ralstonia eutropha* JMP134 (6, 7) harbors plasmid pJP4 and is able to degrade 2,4-D. *R. eutropha* JMP289 (6) is a derivative of strain JMP134 that is cured of pJP4 and resistant to rifampin. *Alcaligenes* sp. isolate JS705 (42) can grow on monochlorobenzene. *Escherichia coli* DH5α and TG1 (35) were used for routine cloning experiments with plasmids and M13 phages, respectively. *E. coli* HB101 (pRK2013) (13) was used as a helper strain for mobilizing pKT230-derived plasmids in triparental matings with *P. putida* KT2442 or *R. eutropha* JMP289. *E. coli* BL21(DE3)(pLysS) (41), which carries the T7 RNA polymerase gene under control of the *lacUV5* promoter, was used for the T7-directed expression of pET8c-derived plasmids (33).

Media and growth conditions. *E. coli* strains were cultivated at 37°C in Luria broth (LB) (35). Where appropriate, 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactoside or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium. *P. putida* and *R. eutropha* strains were grown at 30°C on LB, on *Pseudomonas* mineral medium (18) supplied with an appropriate C source, or on modified *Pseudomonas* undefined medium PPUM (21). PPUM consisted of 1 g of $(NH_4)_2SO_4$, 5.24 g of Na_2HPO_4, 2.77 g of KH_2PO_4, 0.5 g of yeast extract, and 20 ml of Hutner trace solution (18) per liter of medium. Antibiotics were added in the following final concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; rifampin, 5 μg/ml; streptomycin, 50 μg/ml for *E. coli* and 250 μg/ml for the *P. putida* and *R. eutropha* strains.

DNA manipulations and sequence analysis. Plasmid DNA isolations and transformations and other DNA manipulations were carried out according to established procedures (35). Restriction enzymes and other DNA-modifying enzymes were obtained from GIBCO/BRL Life Technologies Inc. (Gaithersburg, Md.), Pharmacia LKB Biotechnology (Uppsala, Sweden), or Appligene (Illkirch, France) and used according to the specifications of the manufacturer. Oligonucleotides for the PCR were obtained from Pharmacia or MWG-BIOTECH AG (Ebersberg, Germany). PCR mixtures contained 2 μg of each primer per ml, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.05% W1, 0.2 mM each deoxynucleoside triphosphate, 0.2 mg of bovine serum albumin (BSA) per ml, and 20 U of *Taq* DNA polymerase (Life Technologies) per ml. Amplification was carried out in a Crocodile II thermocycler (Appligene) with the following standard settings: 30 cycles of 1 min at 93.5°C, 1 min at 45 to 55°C (depending on the melting temperatures of the primers used), and 1.5 min at 72°C, with a final extension of 4 min at 72°C. DNA sequencing was performed as described by Sanger et al. (36) with a Sequenase kit (version 2.0; United States Biochemical Corp., Cleveland, Ohio) with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham International plc, Amersham, United Kingdom). Computer analysis of DNA and amino acid sequences was performed with the programs PC/GENE (Genofit, Geneva, Switzerland) and GCG (J. Devereux, University of Wisconsin).

Plasmids. Restriction maps of the most relevant plasmids are presented in Fig. 1. Plasmids pUC18 and pUC19 (46) were used as general cloning vehicles. For sequencing, M13mp18 and M13mp19 were used (46). Plasmid pET8c (33) is an ATG vector derived from pBR322 which contains the ϕ_{10} promoter, ribosome binding site, and terminator and is optimized for T7-directed expression. Plasmid pGEM-5Zf(+) (Promega, Madison, Wis.) contains a multiple cloning site region and was used as a general cloning vector. Plasmid pKT230 (1) is a mobilizable, broad-host-range vector. Plasmid pKT231-*Sst*I-pJP4 (=pCBA1) (8) contains the 8-kb *Sst*I fragment of pJP4, encompassing the complete *tfdCDEF* gene cluster, a 3.4-kb region upstream, and an incomplete *tfdB* gene downstream. The same *Sst*I fragment was cloned into *Sst*I-cut pUC19, producing pCBA4, which was used as a source for further subcloning of *tfd* genes or gene fragments and for the sequencing of *tfdT*, ISJP4, and the downstream region.

For overexpression of the regulatory genes *tcbR*, *clcR*, *tfdT*, and *tfdR* in *E. coli*, translational fusions of these genes were constructed by using the ATG triplet in the *Nco*I site located downstream of the ϕ_{10} promoter and ribosome binding site on pET8c as the start codon. Plasmid pTCB77 (45) contains a complete *tcbR* gene in pET8c. Plasmid pTCB77Δ (45) is identical to pTCB77 except that it has a frameshift mutation in *tcbR*. For overexpression of the *clcR* gene, we amplified a DNA fragment containing a complete *clcR* gene by using the PCR on total DNA of *Pseudomonas* sp. strain B13. The primers used for amplification of *clcR* were synthesized in such a way that the forward primer contained an *Nco*I site around the *clcR* ATG start codon (5′-GTTCCATGGAATTTCGGCAGCT TCG-3′ [the *Nco*I site is underlined]) and the reverse primer contained a *Bam*HI site (5′-GCGGGATCCACAACCTAACGATTGGC-3′ [the *Bam*HI site is underlined]) to facilitate cloning into pET8c. A 0.9-kb PCR product was obtained, cut with *Bam*HI, partially digested with *Nco*I, and ligated into pET8c, resulting in plasmid pCBA13. The determined nucleotide sequence of the insert was identical to that of the *clcR* gene of *P. putida* (pAC27) (5). A frameshift-mutated *clcR* gene was created by digestion and filling in of the 3′-overhanging ends of the internal *Bgl*II site on pCBA13 (yielding plasmid pCBA13Δ). The *tfdT* gene was also cloned into pET8c by using the PCR. The forward primer (5′-AACGGGACGGACCATGGAAATAAG-3′ [the *Nco*I site is underlined]) was directed against the 5′-end sequence of the *tfdT* gene (29) and included an *Nco*I site around the *tfdT* ATG start codon. As a reverse primer we used an oligonucleotide directed against the multiple cloning site of pUC plasmids (5′-AATTCGAGCTCGGTACCC-3′). The template DNA in the PCR was plasmid pCBA27, which contains a complete *tfdT* gene on a 1.7-kb *Sal*I fragment. From the 1.1-kb PCR product we used only the first 150 bp of *tfdT*, whereas the remaining part of *tfdT* was cloned directly. To do this, the PCR product was cut with *Nco*I and *Hin*dIII, and a 0.15-kb *Nco*I-*Hin*dIII fragment was isolated and ligated with the 0.9-kb *Hin*dIII-*Bam*HI fragment of pCBA27 into *Nco*I-*Bam*HI-cut pET8c. This resulted in plasmid pCBA28. The nucleotide sequence of the PCR-amplified *Nco*I-*Hin*dIII DNA region was verified. The *tfdR* gene was reassembled from a partial *Bam*HI library of plasmid pJP4: the 0.6-kb *Bam*HI-*Xba*I segment of pJP4 *Bam*HI fragment E, containing the 5′ end of the *tfdR* gene (27), was ligated with the 0.6-kb *Sst*I-*Bam*HI segment of pJP4 *Bam*HI fragment F, which contains the 3′ end of *tfdR* (27), into *Sst*I-*Xba*I-cut pUC18, yielding pCBA39. To add an *Nco*I site to the start of *tfdR*, we again used the PCR, with pCBA39 as the template DNA, in a manner similar to that used for *clcR* and *tfdT*. A forward primer (5′-CCAGGAGTGAACCATGGAGTTTCG-3′ [the *Nco*I site is underlined]) was directed against the 5′ end of the *tfdR* gene, whereas the reverse primer was directed against a pUC-located sequence (5′-TGAGCGG ATAACAATTTC-3′). The 1.4-kb PCR product was digested with *Nco*I and *Sst*I, after which it was inserted into *Nco*I-*Sst*I-cut pGEM-5Zf(+), yielding plasmid pCBA53. The PCR-generated 0.7-kb *Bam*HI-*Sst*I fragment of this plasmid was exchanged with the corresponding 0.7-kb *Bam*HI-*Sst*I fragment of pCBA39, resulting in pCBA54. The nucleotide sequence of the PCR-generated *Nco*I-*Bam*HI fragment of pCBA53 was verified. Insertion of the 1.2-kb *Nco*I-*Bgl*II fragment of plasmid pCBA54 with a complete *tfdR* gene into *Nco*I-*Bam*HI-digested pET8c finally resulted in plasmid pCBA55. To create a frameshift mutation in the *tfdR* gene, the internal *Sph*I site on pCBA55 was digested and its 3′-overhanging ends were removed by the exonuclease activity of Klenow enzyme. Religation generated plasmid pCBA55Δ.

For in vivo expression studies, we constructed several pKT230-derived plasmids with the different CC-degradative regulons. Plasmid pTCB75 (45) contains a complete *tcbR-tcbCDEF* regulon on a 10-kb *Hpa*I-*Sst*I fragment. Plasmid pTCB74 (45) is identical to pTCB75 except for a 2-bp deletion in the *tcbR* gene, which causes a premature ending of the reading frame. A complete *tfdT-tfdCDEF* regulon was cloned in pKT230 as follows. Plasmid pCBA26 contained a 1.7-kb *Sal*I fragment with a complete *tfdT* gene and the *tfdT-tfdC* intergenic region. This fragment was extended with the *tfdC* gene, yielding plasmid pCBA44. This made it possible to assemble a complete regulon in pKT230 by a three-point ligation of a 2.4-kb *Cla*I-*Hin*dIII (partial *Hin*dIII) fragment of pCBA44 (containing *tfdT-tfdC*) with a 3.8-kb *Bgl*II-*Cla*I fragment of pCBA4 (containing *tfdDEF*) and *Bam*HI-*Hin*dIII-digested pKT230 (yielding plasmid pCBA49). Plasmid pCBA49Δ is identical to pCBA49 except for a frameshift mutation in the *tfdT* gene, which was introduced by filling in of the internal *Hin*dIII site. This plasmid was derived from several intermediate constructs and obtained in a way similar to that described for pCBA49.

To test whether *tfdR* could take over the role of *tfdT* as a regulator of *tfdCDEF* expression, we constructed a *tfdR-tfdCDEF* hybrid regulon by replacing the *tfdT* ORF with that of *tfdR*. This construct, pCBA59, was assembled as follows. With the use of PCR, we first amplified the *tfdT-tfdC* intergenic region and created an *Nco*I restriction site at the start of *tfdT*. This DNA fragment could then be digested with *Nco*I and inserted into the previously described plasmid pCBA54, which contains a *tfdR* gene with an introduced *Nco*I site. This new plasmid, pCBA57, thus contained a *tfdR-tfdC* hybrid in which the *tfdT-tfdC* intergenic region and the *tfdR* gene were fused at the position of the ATG start codon of *tfdT*. We verified the nucleotide sequence of this region and the connection to *tfdR*. After one more cloning step to strategically position restriction sites, this complete fragment was combined with a DNA fragment from pCBA4 containing *tfdCDEF* and ligated into pKT230. This resulted in plasmid pCBA59. In plasmid pCBA59Δ a frameshift mutation was introduced in the *tfdR* gene by removing an internal *Sph*I site using the exonuclease activity of Klenow enzyme.

Expression of *tcbR*, *clcR*, *tfdT*, and *tfdR* in *E. coli*. *E. coli* BL21(DE3)(pLysS) strains harboring pET8c-derived plasmids were grown in 50 ml of LB to an optical density at 600 nm of 0.25 to 0.35. Cells were then induced by the addition of 1 mM IPTG and grown for an additional 4 h. The cultures were then centrifuged, washed once with 20 mM Tris-HCl (pH 7.5), and resuspended in 0.7 ml of lysis buffer, containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 μg of DNase I (Fluka AG, Buchs, Switzerland) per ml. Samples of 50 μl were taken from these cell suspensions for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed according to the method of Laemmli (24). The remainder of the cell suspensions was transferred to screw-cap Eppendorf tubes containing 0.4 g of glass beads (0.10 to 0.11 mm in diameter). Disruption of the cells was established by a 1-min treatment carried out twice in a Cell Homogenizer MSK (B. Braun Melsungen AG, Melsungen, Germany) at 4,000 rpm; tubes were placed on ice before, between, and after treatments. Centrifugation at 4°C for 5 min at 11,000 × *g* allowed removal of the glass beads, after which another centrifugation at 4°C for 20 min at 15,000 × *g* followed. The resulting supernatants, referred to as cell

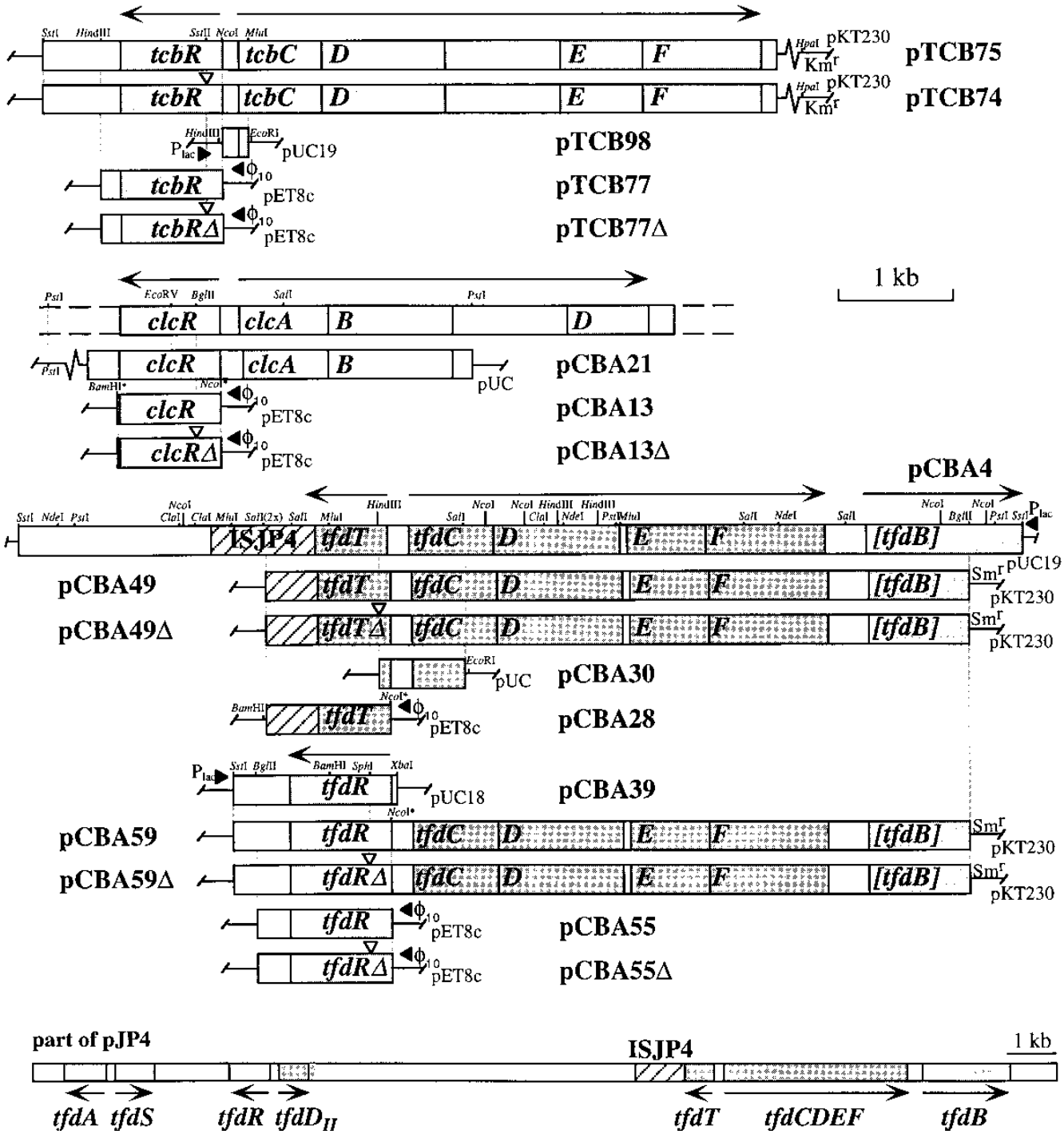

FIG. 1. Restriction maps of plasmid constructs most relevant to this study. The construction of plasmids pTCB74 (45), pTCB75 (45), pTCB77 (45), pTCB77Δ (45), and pTCB98 (25) has been described previously. All pCBA plasmids depicted here were constructed as described in Materials and Methods. For each plasmid, the replicon is given together with the direction of vector-based promoters and antibiotic resistance markers, if applicable. Shaded boxes represent genes; hatched boxes represent sections containing ISJP4 DNA. A gene name in brackets indicates that the gene is incomplete; where the name is followed by a Δ, the gene contains a frameshift mutation. The positions of restriction sites relevant to the construction of the various plasmids are indicated. Restriction sites marked with an asterisk were introduced artificially by PCR (see Materials and Methods). Arrows indicate the direction of transcription. A white triangle indicates the position at which a frameshift mutation was introduced into a particular gene (see Materials and Methods). Also depicted is part of plasmid pJP4, showing the locations of the various *tfd* genes with respect to one another.

extracts, were used in gel mobility shift DNA binding assays. Protein concentrations were determined as described by Bradford (3), by using BSA as a standard.

Gel mobility shift DNA binding assays. DNA fragments for use in gel mobility shift assays were generated from plasmids pTCB98 (25), pCBA21 (42), and pCBA30 (this paper), containing the *tcbR-tcbC*, *clcR-clcA*, and *tfdT-tfdC* intergenic regions, respectively. Plasmid pTCB98 was digested with *Eco*RI and *Hin*dIII, pCBA21 was digested with *Eco*RV and *Sal*I, and pCBA30 was digested with *Eco*RI and *Hin*dIII. The resulting DNA fragments, 0.2, 0.8, and 0.7 kb in

size, respectively, were isolated and end labeled by using Klenow polymerase and [α-^{32}P]dATP (Amersham). Gel mobility shift DNA binding assays were performed as described previously (45).

In vivo expression studies. In order to examine whether *tfdT* or *tfdR* could regulate *tfdCDEF* expression, we measured induction of TfdC enzyme activity in *P. putida* KT2442 or *R. eutropha* JMP289 with the different *tfd* plasmids pCBA49, pCBA49Δ, pCBA59, and pCBA59Δ. The differences in enzyme induction after growth in the presence and absence of inducer were taken as a measure for transcriptional activation. All cultures to be induced were grown in the presence of 3-CBa, which is converted by both strains to 3-chlorocatechol (3-CC) (8, 31). With a functional regulatory gene complemented on a plasmid in these strains, 3-CC, or a catabolite thereof, can act as an inducer of *tfdCDEF* expression. For comparisons we included induction experiments with strains harboring plasmids pTCB75 (*tcbR-tcbCDEF*) and pTCB74 (*tcbR*Δ*-tcbCDEF*). Cultures of the respective strains were grown in flasks containing 50 ml of PPUM plus 250 μg of streptomycin or 50 μg of kanamycin per ml and were inoculated from an overnight LB culture to give a starting A_{600} of 0.01 to 0.05. 3-CBa was added to a final concentration of 0, 1, 5, or 10 mM. The cultures were incubated at 30°C with rotary shaking until they reached stationary growth phase; this took 15 to 17 h for the *P. putida* cultures and 20 to 22 h for the *R. eutropha* cultures. Cells were then harvested and washed, and cell extracts were prepared as described above for *E. coli* BL21 cultures. Activities of 3-chlorocatechol- and catechol 1,2-dioxygenase (C1,2-D) in the cell extracts were measured spectrophotometrically by the formation of 2-chloromuconic acid or muconic acid, respectively (10, 11). The reaction buffer contained 39 mM Tris-HCl (pH 8.0), 0.3 mM EDTA, and 0.1 mM 3-CC or catechol. Typically, 5 to 20 μg of protein was used in a 0.5-ml assay. The reaction was carried out in a quartz cuvette equilibrated at 30°C, and the increase in A_{260} was measured in time. Specific activities, expressed as nanomoles of (2-chloro)muconic acid per minute per milligram of protein (milliunits per milligram of protein), were calculated by using the molar extinction coefficients of 2-chloromuconic acid and muconic acid, which are 17,100 and 16,800 liters $\cdot$ mol^{-1} $\cdot$ cm^{-1}, respectively (10, 11). 3-CC was a kind gift of Barbara Jakobs, GBF, Braunschweig, Germany. To obtain statistically sound data, induction experiments were repeated independently at least three times.

Nucleotide sequence accession number. The nucleotide sequence presented in this article has been deposited in the GenBank database under accession number AE16782.

RESULTS AND DISCUSSION

The *tfdT* gene encodes a truncated LysR-type transcriptional regulator. The ORF designated *tfdT*, which was previously characterized partially and proposed to encode a regulatory protein of the *tfdCDEF* gene cluster on plasmid pJP4 (29, 47), was completely sequenced here (Fig. 2A). The ORF is 684 bp long, with an ATG start codon and a TAA stop codon. Thus, it theoretically encodes a polypeptide of 228 amino acids, with a predicted molecular mass of 24.9 kDa. This is in fair agreement with the bulk production of a 25-kDa polypeptide which we found by SDS-PAGE analysis in *E. coli* BL21 with a plasmid construct (pCBA28) overexpressing the *tfdT* gene (Fig. 3A, lane 7). The predicted TfdT amino acid sequence indeed revealed homology with several members of the LysR-type family of transcriptional regulators. The highest degrees of identity were found with ClcR, TcbR, and TfdR (=TfdS), i.e., 53, 52, and 47%, respectively, in a 210-amino-acid overlap (Fig. 2C). These three proteins are all involved in the regulation of chloroaromatic degradation. The sequence identity of TfdT with CatR of *P. putida* (34) was 32%, and its sequence identity with CatM of *Acinetobacter calcoaceticus* (32) was 30%. CatR and CatM are both involved in the regulation of catechol degradation. Additionally, TfdT showed a 30% sequence identity (210-amino-acid overlap) with the putative gene product of *bphR*, which supposedly is involved in the regulation of biphenyl breakdown (23).

Despite a high sequence homology with these LysR-type regulators, the TfdT protein distinguished itself by its significantly shorter length of 228 amino acids, compared with an average length of approximately 300 amino acids for the others. Another striking observation was that the C-terminal 18 amino acids of the TfdT protein (underlined with dashes in Fig. 2A) had no notable similarity to the corresponding regions of TcbR, ClcR, and TfdR (Fig. 2C). Directly downstream of the *tfdT* gene we found a 0.9-kb DNA sequence with 78% identity to insertion element IS*402* from *Pseudomonas cepacia* (12). This DNA sequence was designated ISJP4 and is the subject of a separate study (26). The *tfdT* gene and element ISJP4 partially overlap: the terminal 18 amino acids of the TfdT protein are encoded by the first 54 bp of the 5′ end of ISJP4 (Fig. 2A). The presence of the insertion element-like element apparently introduced a TAA stop codon in the *tfdT* ORF, which causes a premature ending of the TfdT protein. We analyzed the translation products of all three forward frames directly downstream of ISJP4 to see if they showed homology with LysR-type sequences (Fig. 2B). However, we found no indications that these frames encode a polypeptide representing the "missing" C-terminal portion of TfdT, as judged from individual homology comparisons with the C-terminal domains of TcbR, ClcR, and TfdR.

The *tfdT* gene no longer codes for a functional regulatory protein of *tfdCDEF* expression. For the purpose of verifying the hypothesis that the *tfdT* gene was indeed insertionally inactivated by the insertion element-like element ISJP4, we performed two experiments to establish its role, if any, in the regulation of *tfdCDEF* expression. First, we tested if the *tfdT* gene product was capable of binding the *tfdC* promoter region in a gel mobility shift assay. Cell extracts of *E. coli*(pCBA28) overexpressing the *tfdT* gene exhibited no binding activity towards the *Eco*RI-*Hin*dIII fragment of pCBA30 containing the *tfdT-tfdC* intergenic region (Fig. 3B, lane 7), providing a first clue to the incompetence of TfdT.

Secondly, we performed in vivo induction experiments. For this purpose, we constructed plasmids pCBA49, which harbors a wild-type *tfdT-tfdCDEF* gene cluster, and pCBA49Δ, which is identical to pCBA49 except for a frameshift mutation in the *tfdT* gene. This plasmid would thus express a mutated protein (TfdTΔ). These plasmids were then introduced into *R. eutropha* JMP289 (6). This strain is a pJP4-cured derivative of *R. eutropha* JMP134 and as such has a chromosomal background native to the *tfdT-tfdCDEF* gene cluster. It can convert 3-CBa into 3-CC by chromosomally encoded enzymes (8); however, 3-CC is not converted further. If the *tfdT* gene encodes a functional activator, an elevated level of *tfdCDEF* gene expression would be expected in the presence of 3-CBa. We measured *tfdCDEF* expression by determining TfdC chlorocatechol 1,2-dioxygenase activity in cell extracts of uninduced and induced cultures (Fig. 4A).

We saw no difference in the induction of TfdC activity between *R. eutropha* JMP289 harboring plasmid pCBA49 or pCBA49Δ. In the absence of 3-CBa, both strains had a low basal level of TfdC activity (20 and 24 mU/mg of protein, respectively). In the presence of 1 mM inducer, TfdC activities were elevated to 333 and 352 mU/mg of protein. When exposed to 5 or 10 mM 3-CBa, the cells exhibited severely retarded growth, accompanied by a brown coloring of the medium. This probably was caused by polymerization of accumulating 3-CC, which is known to have a toxic effect on cell metabolism (17). In extracts of these cells no TfdC activity could be detected. The same inhibitory effect was observed with *R. eutropha* JMP289 cells that carried plasmid pTCB74 (*tcbR*Δ*-tcbCDEF*) (Fig. 4A). Very much in contrast, *R. eutropha* JMP289 cells harboring plasmid pTCB75 (expressing the functional CC-degradative regulon *tcbR-tcbCDEF*) exhibited normal growth in the presence of 0, 1, 5, and 10 mM 3-CBa; we measured TcbC activities of 237, 62, 553, and 1344 mU/mg of protein, respectively.

These results led us to conclude that the *tfdT* gene does not code for an active regulator of *tfdCDEF* expression. Our hypothesis is that ISJP4 at one time or another inserted itself into

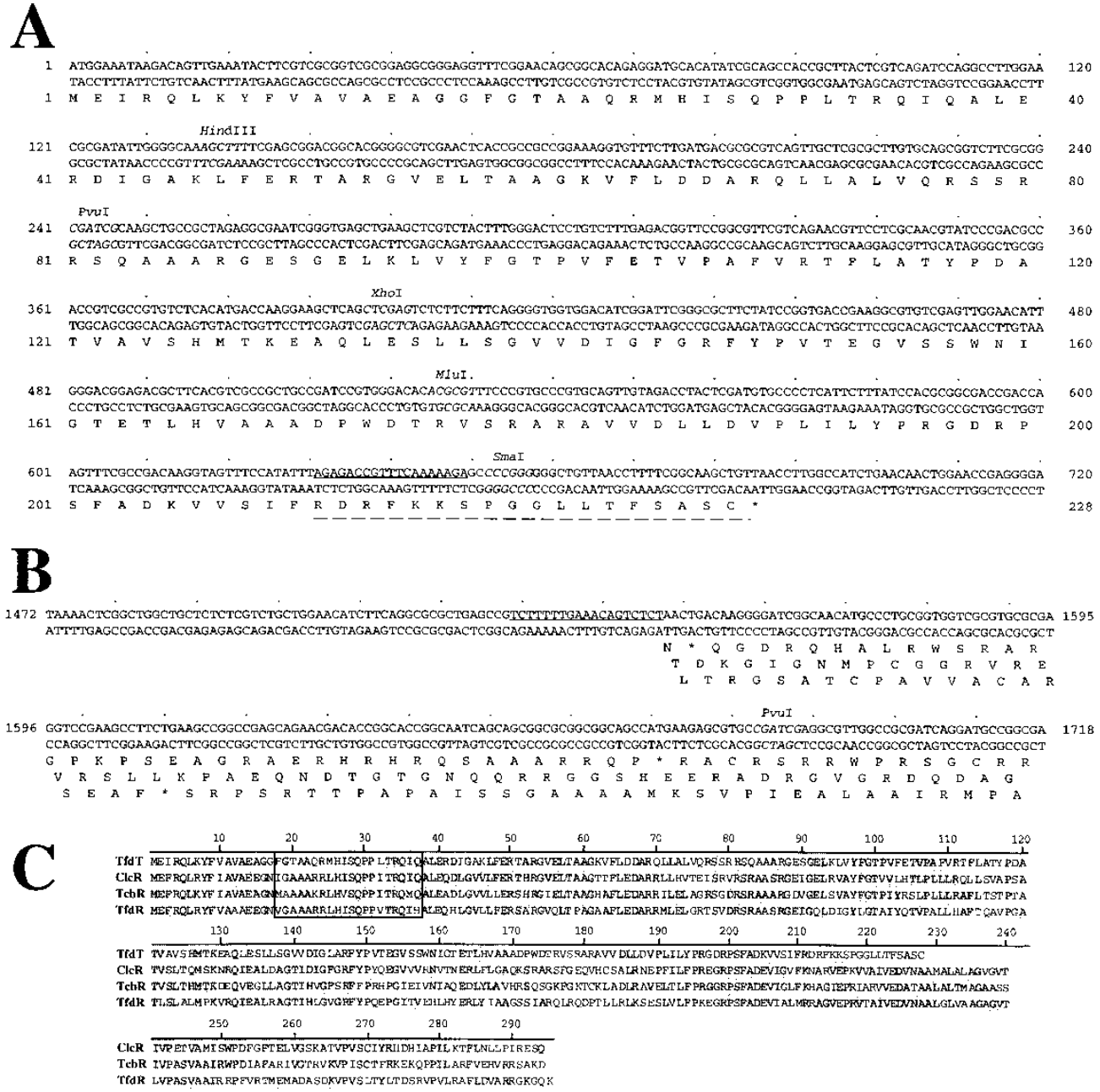

FIG. 2. (A) Nucleotide sequence of the *tfdT* gene and its translation product. Bases are numbered relative to the ATG start codon of *tfdT*. Restriction sites are shown in italics above the nucleotide sequence. The nucleotide sequence of bases 1 to 141 has been reported previously (29). The solid underlined sequence indicates the left inverted repeat sequence of ISJP4 (26). The deduced amino acid sequence for the *tfdT* gene is given below the nucleotide sequence. The part underlined with the dashed line indicates the portion of the TfdT protein encoded by ISJP4 DNA. (B) Nucleotide sequence of the DNA region downstream of ISJP4. Bases are numbered relative to the ATG start codon of *tfdT*. The underlined sequence indicates the right inverted repeat sequence of ISJP4 (26). The deduced amino acid sequences in all three forward frames (relative to the direction of *tfdT* transcription) are given below the nucleotide sequence. (C) Alignment of the amino acid sequences of the TfdT, ClcR, TcbR, and TfdR proteins. Shaded amino acids indicate residues that are identical in at least two proteins. The presumed helix-turn-helix motifs are boxed.

a complete and functional original copy of the *tfdT* gene. Circumstantial evidence for such an event comes from comparison with the *tfdT-tfdCDEF* gene cluster on plasmid pMAB1 from *Pseudomonas cepacia* CSV90 (2). This cluster lies on an 8-kb *Hin*dIII-*Sst*I fragment whose restriction map is indistinguishable from that of pJP4 (see reference 2). In addition, the DNA sequence of a small part of pMAB1 containing *tfdC* was identical to that of *tfdC* on pJP4. Plasmids pMAB1 and pJP4 were otherwise clearly different. Interestingly, the resemblance in the restriction map stops abruptly in the region where on pJP4 element ISJP4 is inserted into the *tfdT* gene. Perhaps pMAB1, unlike pJP4, still encodes a functional TfdT regulator of *tfdCDEF* expression.

Cross-activation of *tfdCDEF* by heterologous, chromosomally encoded host factors. We observed that in *R. eutropha* JMP289 cells carrying plasmid pCBA49 (*tfdT-tfdCDEF*) or

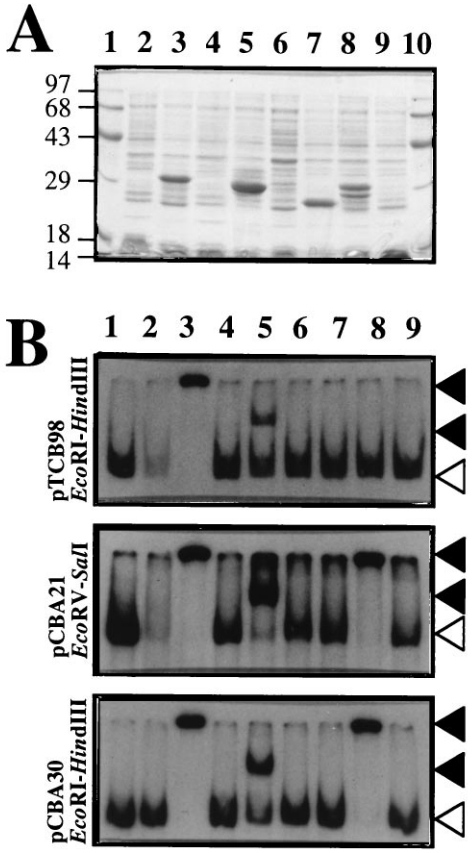

FIG. 3. (A) Overexpression of the *tcbR*, *tfdR*, *clcR*, and *tfdT* genes in *Escherichia coli*. Total cell extracts of IPTG-induced *E. coli* BL21(DE3)(pLysS) harboring different pET8c-derived plasmids were analyzed by SDS-PAGE. Lanes: 1 and 10, molecular weight standards (sizes of markers are given on the left); 2, pET8c (vector control); 3, pTCB77 (*tcbR*); 4, pTCB77Δ (*tcbR*Δ); 5, pCBA13 (*clcR*); 6, pCBA13Δ (*clcR*Δ); 7, pCBA28 (*tfdT*); 8, pCBA55 (*tfdR*); 9, pCBA55Δ (*tfdR*Δ). (B) DNA binding activity and specificity of the *tcbR*, *clcR*, *tfdT*, and *tfdR* gene products. In a gel mobility shift assay, 2 μg of protein of cell extract from the *E. coli* BL21(DE3)(pLysS) strains described for panel A was added to three different DNA fragments, each containing the promoter/operator region of either *tcbC*, *clcA*, or *tfdC*. Lane 1, no cell extract added. Lanes 2 through 9, cell extract of *E. coli* BL21(DE3)(pLysS) harboring pET8c (lane 2), pTCB77 (lane 3), pTCB77Δ (lane 4), pCBA13 (lane 5), pCBA13Δ (lane 6), pCBA28 (lane 7), pCBA55 (lane 8), or pCBA55Δ (lane 9). Open arrowheads indicate unbound DNA, and solid arrowheads indicate bound DNA.

pCBA49Δ (*tfdT*Δ-*tfdCDEF*), TfdC activity was induced in the presence of 1 mM 3-CBa (Fig. 4A). This induction cannot be mediated by a plasmid-encoded element and is, thus, likely caused by a chromosomally encoded regulatory protein. It is quite conceivable that one or more regulatory elements of the benzoate/catechol utilization pathway in *R. eutropha* are responsible for this induction by cross-binding to the *tfdC* promoter region. A very similar case of heterologous cross-activation has been described for *P. putida* (pAC27) (28). 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. This promoter directs the expression of the *clcABD* genes encoding CC breakdown and is normally controlled by its cognate regulatory protein, ClcR (5). Regulation of the benzoate/catechol pathway in *R. eutropha* is clearly closely intertwined with that of CC breakdown, since we measured induced activity of chromosomally encoded C1,2-D in extracts of JMP289 cells that were grown in the presence of 1 mM 3-CBa (Fig. 4C). The natural inducer of C1,2-D activity in *R. eutropha* is benzoate (20); induction with 3-CBa may indicate a relaxed specificity of the responsible regulator. This protein might also be responsible for the cross-induction of the *tfdC* promoter.

The *tfdR* gene product is functionally related to TcbR and ClcR and is capable of binding the *tfdC* promoter region. If TfdT is no longer regulating *tfdCDEF* expression, could it be that TfdR is capable of doing so? A couple of notions support this idea. First, TfdR has high similarity with TfdT (47% identity) but also with TcbR and ClcR, which are involved in the regulation of CC breakdown. Second, the *tfdR* (and *tfdS*) gene is located on the same plasmid pJP4 as the *tfdCDEF* genes. Third, the *tfdC* promoter/operator region resembles known target promoters of TfdR, such as *tfdA* and $tfdD_{II}$ (an alignment is shown in reference 27). To verify whether the homology of TfdR with ClcR and TcbR indeed reflects similar functionality, we compared the DNA binding specificities of the TfdR, TcbR, and ClcR proteins. To this end, we first overexpressed the *tfdR*, *tcbR*, and *clcR* genes in *E. coli*. In the total cell extract of *E. coli* BL21(pCBA55) overexpressing the *tfdR* gene, two overproduced polypeptides with sizes of 30 and 27 kDa were observed by SDS-PAGE analysis (Fig. 3A, lane 8). Both protein bands were absent in the total cell extract of *E. coli* BL21(pCBA55Δ), harboring a frameshift mutant of *tfdR* (Fig. 3A, lane 9). We suppose that the 30-kDa polypeptide represents the product of the 888-bp *tfdR* gene, which theoretically encodes a protein of 32 kDa, whereas the 27-kDa polypeptide is either a highly specific degradation product of the TfdR protein or is encoded from a *tfdR*-internal ATG or GTG start codon (e.g., the codons located 132, 162, and 210 bp downstream of the proposed *tfdR* ATG start codon [27, 48]). *E. coli* BL21 cells that carried plasmid pTCB77 overproduced the TcbR protein, with a size of about 32 kDa (Fig. 3A, lane 3). This polypeptide was absent in the total extract of *E. coli* BL21 (pTCB77Δ) cells, which overexpressed a *tcbR* gene with a frameshift mutation (Fig. 3A, lane 4). Overexpression of the *clcR* gene in *E. coli* BL21(pCBA13) resulted in the production of a 30-kDa polypeptide (Fig. 3A, lane 5). In total extracts of *E. coli* BL21 cells that fostered plasmid pCBA13Δ, carrying a frameshift-mutated *clcR* gene, this polypeptide was absent (Fig. 3A, lane 6).

We then compared the DNA binding specificities of TfdR, TcbR, and ClcR in cell extracts of overproducing *E. coli* BL21 cultures in gel mobility shift assays. We observed binding of the TcbR and ClcR proteins to their respective target DNAs, i.e., the promoter/operator regions of *tcbC* and *clcA* (Fig. 3B, lane 3, top panel, and lane 5, middle panel). The specificity of binding of these proteins to their cognate target sequences had been established before (4, 25). Interestingly, both proteins were also capable of binding to each other's target DNA (Fig. 3B, lane 3, middle panel, and lane 5, top panel). The TfdR protein could bind a DNA fragment containing the presumed operator/promoter region of *tfdC* (29). In addition, we observed that it could bind the promoter/operator region of the *clcA* gene (Fig. 3B, lane 8, middle panel) but not that of *tcbC* (Fig. 3B, lane 8, top panel). TcbR and ClcR were capable of binding the promoter/operator region of the *tfdC* gene, too (Fig. 3B, lanes 3, 5, and 8, bottom panel). The specificity of all these protein-DNA interactions was demonstrated in control reactions with cell extracts of *E. coli* BL21 harboring pET8c or overexpressing truncated proteins (pCBA55Δ, pTCB77Δ, and pCBA13Δ). No binding was observed in any of these reactions (Fig. 3B, lanes 2, 4, 6, and 9, all panels). The small migration distance of TcbR-DNA and TfdR-DNA complexes compared to ClcR-DNA may be explained by the significant difference in pI between TcbR and TfdR (calculated as 10.7 and 11.0, re-

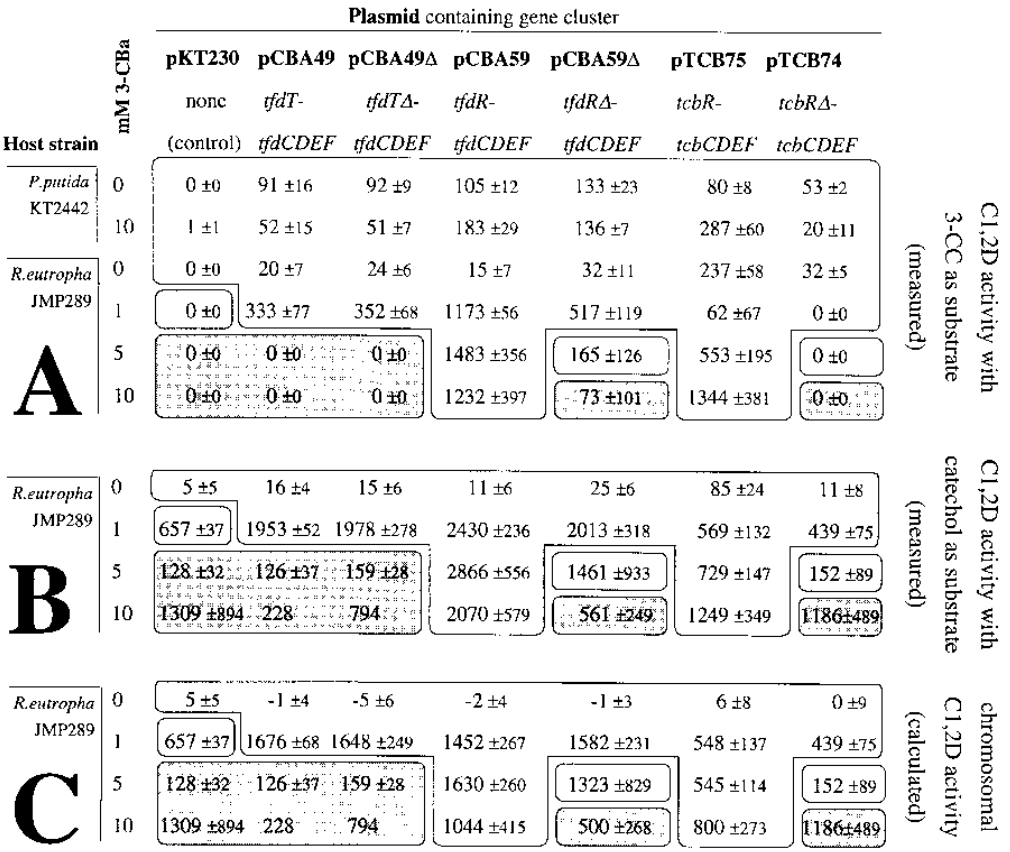

FIG. 4. Expression and induction of CC-degradative regulons in two different host strains. Given are specific C1,2-D activities in milliunits per milligram of protein with either 3-CC (A) or catechol (B) as a substrate in the enzyme assay. These activities were measured in cell extracts of *P. putida* KT2442 or *R. eutropha* JMP289, harboring different plasmid-borne regulons, and were induced with various concentrations of 3-CBa. Chromosomally encoded C1,2-D activity (C) was calculated by subtracting the activity that could be attributed to TfdC or TcbC from the total activity measured in the same cell extract with catechol as the substrate. The relative contribution of TfdC was calculated from the measured activity on 3-CC by using the specificity factor 1.2 (TfdC) (30) or 2.5 (TcbC) (42) for 3-CC as the substrate compared with catechol. All values represent the average and standard deviation of measurements of at least three independent induction experiments. Degrees of shading represent cell culture characteristics. No shading, normal growth; light shading, slightly retarded growth and brownish medium; dark shading, severely retarded growth and dark brown medium.

spectively) and ClcR (9.3): the more positively charged the binding protein is, the more it reduces the overall negative charge of the protein-DNA complex and consequently its speed in an electrical field. These results showed that TfdR, TcbR, and ClcR are functionally very similar. The capability of TfdR to bind the promoter region of the *tfdCDEF* operon (and of *clcA*) implied that TfdR could also act as a regulator of CC breakdown.

TfdR acts as a positive regulator of *tfdCDEF* expression. To test the possibility that TfdR would regulate *tfdCDEF* expression, we performed in vivo expression studies in *R. eutropha* JMP289 with cloned *tfdR* and *tfdCDEF* genes. Initial attempts to add the *tfdR* gene in *trans* to a cloned *tfdT-tfdCDEF* gene cluster, thus mimicking the natural genetic organization on plasmid pJP4, failed. This led us to construct an artificial hybrid *tfdR-tfdCDEF* regulon in which the *tfdR* gene was provided in *cis* by exchanging the *tfdT* ORF with that of *tfdR* (see Materials and Methods). Apart from a plasmid harboring this *tfdR-tfdCDEF* gene cluster (pCBA59), we constructed one (pCBA59Δ) which carried a frameshift mutation in the *tfdR* gene, thus encoding a truncated TfdR protein (TfdRΔ). The results of the in vivo induction experiments with these plasmids are given in Fig. 4A. Grown in the absence of 3-CBa, *R. eutropha* JMP289 cells that harbored a *tfdR-tfdCDEF* or *tfdR*Δ-*tfdCDEF* regulon gave TfdC activities of 15 and 32 mU/mg of protein, respectively. In the presence of 1, 5, and 10 mM 3-CBa, *tfdC* expression in *R. eutropha* JMP289(pCBA59) was induced by a factor of 80 to 100 (TfdC activities of 1,173, 1,483, and 1,232 mU/mg of protein, respectively). In clear contrast, extracts of *R. eutropha* JMP289 with plasmid pCBA59Δ revealed much lower TfdC activities of 517, 165, and 73 mU/mg of protein, respectively. The fact that we still saw induced activity with this plasmid at 1 mM 3-CBa may be attributed to chromosomally encoded cross-activation. We saw no induction with cells carrying a *tfdR*Δ-*tfdCDEF* regulon (pCBA59Δ) at 5 and 10 mM 3-CBa: just like *R. eutropha* JMP289 cells harboring pCBA49 (*tfdT*) or pCBA49Δ (*tfdT*Δ), these cells exhibited impaired growth. From these results we concluded that the gene product of *tfdR* (or *tfdS*) can indeed act as a positive regulator of *tfdCDEF* expression. The extent of induction of TfdR on *tfdCDEF* clearly exceeds that of heterologous, chromosomally encoded regulatory factors.

Previous reports on the regulation of *tfdCDEF* on plasmid pJP4 have been both mutually contradictory and speculative (21, 48). With hindsight, this can be attributed to a lack of detailed knowledge of where regulatory genes were located (*tfdR*) and whether they were intact (*tfdT*) and of the presence of multiple gene copies (identical *tfdR* and *tfdS* genes). However, part of the problem in interpretation also arose because heterologous hosts were used to study gene expression (e.g.,

Pseudomonas aeruginosa, *P. cepacia*, and *P. putida*). For example, when we used *P. putida* KT2442 to host the various *tfd* regulons, the induction data (summarized in Fig. 4A) looked quite different from those for *R. eutropha* JMP289. First, the *tfdC* promoter showed a higher basal level of expression under uninduced conditions. Host-dependent expression was also something we noticed when testing the *tcbC* promoter under uninduced conditions in the two heterologous hosts (Fig. 4A). Secondly, the presence of *tfdR* caused induction of the expression of *tfdCDEF* in *P. putida* only by a factor of 1.5 (although this was statistically significant). Similarly, the extent of induction of *tcbCDEF* by TcbR was clearly less obvious in *P. putida* than in *R. eutropha*. Such differences between strains will clearly lead to different interpretations. For example, the observation that *P. cepacia* cells carrying a deletion derivative of plasmid pJP4, lacking both the *tfdR* and *tfdS* genes, could grow on 3-CBa was interpreted as indicating that TfdR (TfdS) is not a regulator of *tfdCDEF* expression (48). However, a high basal level of expression of *tfdCDEF* in this strain would eliminate the need for an activator protein and still allow growth of the strain on 3-CBa. Such a high level of basal expression of *tfdCDEF* in *P. cepacia* may perhaps be promoted by the presence of ISJP4 in *tfdT*. In this respect, it is interesting that ISJP4 is closely related to IS*402* (12), an insertion element which is known to increase the level of downstream-located genes considerably in *P. cepacia* (39).

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