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Characterization of the *Pseudomonas* sp. Strain P51 Gene *tcbR*, a LysR-Type Transcriptional Activator of the *tcbCDEF* Chlorocatechol Oxidative Operon, and Analysis of the Regulatory Region

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Plasmid pP51 of *Pseudomonas* sp. strain P51 contains two gene clusters encoding the degradation of chlorinated benzenes, *tcbAB* and *tcbCDEF*. A regulatory gene, *tcbR*, was located upstream and divergently transcribed from the chlorocatechol oxidative gene cluster *tcbCDEF*. The *tcbR* gene was characterized by DNA sequencing and expression studies with *Escherichia coli* and pET8c and appeared to encode a 32-kDa protein. The activity of the *tcbR* gene product was analyzed in *Pseudomonas putida* KT2442, in which it appeared to function as a positive regulator of *tcbC* expression. Protein extracts of both *E. coli* overproducing TcbR and *Pseudomonas* sp. strain P51 showed specific DNA binding to the 150-bp region that is located between the *tcbR* and *tcbC* genes. Primer extension mapping demonstrated that the transcription start sites of *tcbR* and *tcbC* are located in this region and that the divergent promoter sequences of both genes overlap. Amino acid sequence comparisons indicated that TcbR is a member of the LysR family of transcriptional activator proteins and shares a high degree of homology with other activator proteins involved in regulating the metabolism of aromatic compounds.

Pseudomonas sp. strain P51 is a recently isolated bacterium able to use chlorobenzenes as sole carbon and energy sources (49, 50). With the current interest in environmental pollution, increasing numbers of bacterial strains that degrade organic chemicals are being described (35). These strains offer the unique possibility of studying the evolution of bacterial metabolism in response to new substrates, such as xenobiotic compounds.

Bacteria that degrade chlorinated catechols via the chlorocatechol oxidative pathway (35), such as *Pseudomonas* sp. strain P51 (49, 50) and strain B13 (11, 12), *Pseudomonas putida*(pAC27) (7, 8), and *Alcaligenes eutrophus* JMP134 (pJP4) (10, 43), express specialized enzymes capable of converting chlorinated substrates. Sequence analysis showed a strong homology among the *tcbCDEF* (48), *clcABD* (16), and *tfdCDEF* (18, 19, 32, 33) gene clusters. The high similarity in the functions and deduced sequences of the key enzymes in this metabolic pathway, such as catechol 1,2-dioxygenases (12, 18, 21, 28, 33, 48), cycloisomerases (19, 26, 33, 43, 48), and hydrolases (33, 43, 44, 48), suggests that the chlorocatechol oxidative pathway originated from common metabolic pathways, such as that of catechol and protocatechuate degradation in *Acinetobacter calcoaceticus* (21, 28, 30) or pseudomonads (3, 30). However, new pathways, such as the chlorocatechol oxidative pathway, need, in addition to altered enzymatic activities (12, 26, 35, 43, 44, 48), fine-tuning of regulatory functions, such as inducer recognition. Mutation studies with TOL plasmid-encoded regulatory gene *xylS* showed that new metabolic substrates for the TOL pathway could be selected on the basis of their ability to function as inducers for the altered XylS protein (1, 34).

Preliminary studies on the regulation of the *tfdCDEF* cluster and of the *tfdA* and *tfdB* genes (20, 24, 25) and (partial) sequence analysis of flanking regions of the *tfdCDEF* (32, 33) and *clcABD* (16, 32) clusters indicated that the expression of those gene clusters was regulated by proteins that showed homology to the LysR family of transcriptional activator proteins (22, 23). This group also includes the well-studied NahR (41, 42, 52), CatR (37), and CatM (29) proteins, which are all involved in regulating the metabolism of aromatic compounds. In previous studies, preliminary evidence for the presence of a regulatory gene of the *tcbCDEF* gene cluster which would be located upstream of *tcbC* was obtained (48, 50). This paper describes the cloning and characterization of this regulatory gene, *tcbR*, as well as an analysis of the promoter regions of both the *tcbC* and *tcbR* genes, on which TcbR exerts its activity. The results show that TcbR is a member of the LysR family of transcriptional activator proteins.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Pseudomonas* sp. strain P51 (49, 50) contains plasmid pP51 and is able to use dichlorobenzenes (Dcb^+) and 1,2,4-trichlorobenzene (Tcb^+) as sole carbon and energy sources. *P. putida* KT2442 (15) is a rifampin-resistant (Rif^r), plasmid-free derivative of strain mt-2 and was used as a recipient strain for pKT230-derived plasmids containing pP51 DNA fragments. *Escherichia coli* DH5α and TG1 (38) were used for routine cloning experiments with plasmids and M13 phages, respectively. *E. coli* BL21(DE3) carrying the T7 RNA polymerase gene under the control of the *lacUV5* promoter and harboring plasmid plysS, which expresses the T4 lysozyme gene (47), was used for the T7-directed expression of pET8c-derived plasmids (36). *E. coli* HB101(pRK2013) (13) was the helper strain used for mobilizing pKT230-derived plasmids in triparental mat-

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ings with *P. putida* KT2442. Plasmids pUC18 and pUC19 (51) were used as general cloning vehicles. Plasmid pKT230 (5) is a mobilizable broad-host-range vector. pET8c (36), an ATG vector derived from pBR322, contains the φ10 promoter, ribosome binding site, and terminator and is optimized for T7-directed expression. For sequencing, we used M13mp18 and M13mp19 (51). pTCB1 and pTCB45 (50) contain the *tcbCDEF* chlorocatechol oxidative gene cluster and the *tcbR* gene of plasmid pP51 and were used as the sources for the cloning and expression experiments described here. Plasmids pTCB66 and pTCB77 contain an intact *tcbR* gene (see Fig. 1A). A frameshift mutation was introduced into the *tcbR* gene of plasmid pTCB77, resulting in plasmid pTCB77Δ. This was done by removing the 3′-protruding ends of the *Sst*II-linearized plasmid by using the exonuclease activity of Klenow polymerase, recircularizing the plasmid, and transforming *E. coli*. Plasmids pTCB75 and pTCB76 carry the *tcbCDEF* gene cluster and an intact *tcbR* gene on a 10.0-kb *Hpa*I-*Sst*I fragment isolated from pTCB45. This fragment was inserted into pKT230 which was digested with *Hpa*I and *Sst*I (pTCB75) or into pKT230 which was first digested with *Eco*RI, then subjected to Klenow polymerase treatment, and finally digested with *Sst*I (pTCB76). The *tcbR* gene was inactivated in plasmid pTCB74 (see Fig. 1B). The mutation was introduced in *tcbR* by first cloning the 1.5-kb *Eco*RI-*Sst*I fragment containing *tcbR* separately in pUC19 (pTCB56) and subsequently digesting the resulting plasmid with *Sst*II and treating it with Klenow polymerase (pTCB56Δ). The 1.5-kb *Eco*RI-*Sst*I fragment of pTCB56Δ was then isolated and ligated with the 8.5-kb *Hpa*I-*Eco*RI fragment of pTCB45 containing *tcbCDEF* and with pKT230 which was digested with *Hpa*I and *Sst*I. After transformation in *E. coli*, plasmid pTCB74 resulted.

Media and culture conditions. *Pseudomonas* sp. strain P51 was grown on minimal medium containing 3.2 mM 1,2,4-trichlorobenzene (1,2,4-TCB) or 10 mM succinate at 30°C (50). *P. putida* was grown at 30°C on LB (38) or on M9 minimal medium (38) containing one of the following carbon sources: 10 mM succinate, 10 mM 3-chlorobenzoate (3-CB), or 10 mM benzoate. *E. coli* was cultivated at 37°C on LB. Antibiotics were added in the following amounts: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and rifampin, 50 μg/ml. When necessary, media were supplemented with 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactoside or 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

DNA manipulations and sequence analysis. Plasmid DNA isolations, transformations, conjugative crosses, and other DNA manipulations were carried out as described earlier (50) or by established procedures (38). DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (39) as described elsewhere (48). Computer analysis and processing of sequence information were done with the program PC/GENE (Genofit, Geneva, Switzerland) and the GCG package (J. Devereux, University of Wisconsin). Restriction enzymes and other DNA-modifying enzymes were obtained from Life Technologies Inc. (Gaithersburg, Md.) or Pharmacia LKB Biotechnology (Uppsala, Sweden).

RNA isolation and primer extension studies. RNA was isolated from 500-ml cultures of *Pseudomonas* sp. strain P51 cultivated on 1,2,4-TCB or succinate and harvested in the logarithmic phase by the acid phenol extraction procedure of Aiba et al. (2). For primer extension experiments, 0.2 μg of a synthetic oligonucleotide was annealed to 10 or 30 μg of RNA. Oligo 11 (5′ GAGGGTCTTCTGGATCG 3′) was complementary to a region between 42 and 60 nucleotides downstream from the ATG codon of *tcbC*; oligo 12 (5′ TGCAGCCATGTTCCCTG 3′) was complementary to a region between 43 and 60 nucleotides downstream from the putative start of *tcbR* (see Fig. 2). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch) and end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham International plc., Amersham, United Kingdom) by using T4 kinase. The primer-RNA hybrid was extended with 200 U of Moloney murine leukemia virus reverse transcriptase for 1 h at 37°C (38). Extension products were separated on a 6% denaturing polyacrylamide gel and compared with the products derived from DNA sequencing reactions primed with the same oligonucleotides.

Catechol 1,2-dioxygenase activity measurements. For studying the induction of *tcbC* in *P. putida*, were grown 100-ml cultures to an A_{620} of 1.0, harvested by centrifugation, washed once in 50 ml of 50 mM Tris hydrochloride (pH 7.5), resuspended in 1.0 ml of the same buffer, and subsequently disrupted by sonication (50). Catechol 1,2-dioxygenase *tcbC* was induced with either 3-CB or benzoate as the sole carbon source in the growth medium. Catechol 1,2-dioxygenase activity was assayed with 3-chlorocatechol (3-CC) as a specific substrate for *tcbC*-mediated activity (12, 50) and catechol for both endogenous and *tcbC*-derived catechol 1,2-dioxygenase activities.

DNA binding experiments. Cell extracts of *Pseudomonas* sp. strain P51 or *E. coli* harboring cloned pP51 DNA fragments with the *tcbR* gene were tested for DNA binding activity by an electrophoretic mobility shift assay. Crude cell extracts were prepared from exponentially growing cultures of *Pseudomonas* sp. strain P51 on 1,2,4-TCB as described previously (50). *E. coli* BL21(DE3) was grown to an A_{620} of 0.6, after which IPTG was added and incubation was continued for another 2 h. Subsequently, cells were harvested, washed, and disrupted as described above. Crude cell extracts were then cleared by centrifugation at 30,000 rpm (80,000 × *g*) for 30 min at 4°C and kept on ice until further use. The DNA binding assay was performed with a total volume of 15 μl of 10 mM HEPES buffer (pH 7.9) containing 10% glycerol, 100 mM KCl, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM dithiothreitol, 2 mM $MgCl_2$, 1.5 μg of bovine serum albumin, and 1 μg of poly(dI-dC) (Boehringer GmbH, Mannheim, Germany). Typically between 1 and 10 μg of protein was used in the assay. DNA fragments tested for binding were labeled with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham) by filling in of 3′-recessive ends with Klenow DNA polymerase (38). In each assay, approximately 10,000 cpm of a labeled fragment was used. Binding reactions were carried out for 15 min at 20°C, after which the samples were electrophoresed through a 5% native polyacrylamide gel. Subsequently, the gels were dried and exposed to X-ray film.

Protein determinations. Concentrations of proteins in cell extracts were determined as described by Bradford (6).

Nucleotide sequence accession number. The nucleotide sequence presented in this article has been deposited at GenBank under accession number M57629.

RESULTS

DNA sequence and expression analysis of *tcbR*. In a previous study (48), we showed that the expression of the *tcbCDEF* gene cluster in *E. coli* was affected by an upstream region. Therefore, the region of plasmid pP51 immediately preceding the *tcbCDEF* chlorocatechol oxidative gene cluster (48, 50) was analyzed for the presence of a putative

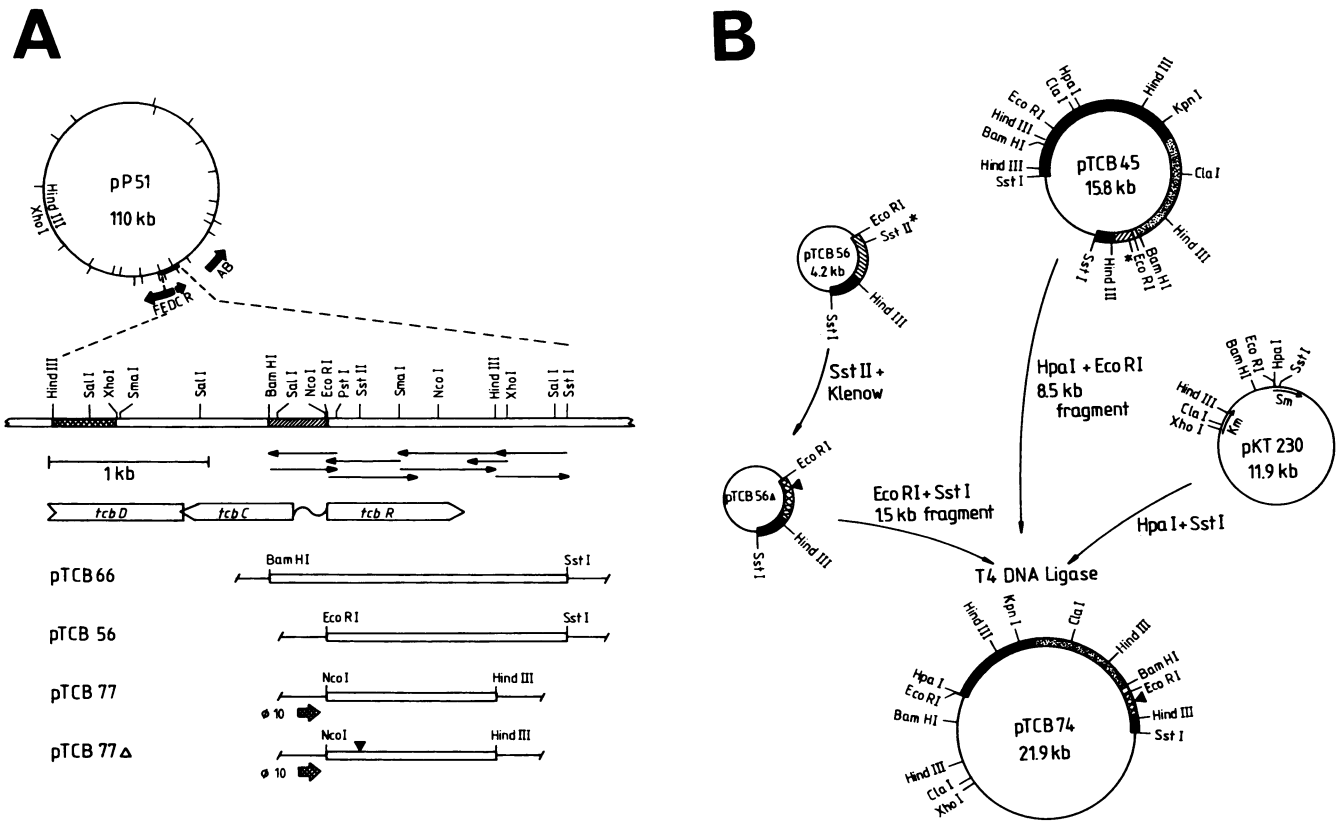

FIG. 1. (A) Enlarged region of the part of catabolic plasmid pP51 containing the *tcbR* gene and the start of the *tcbCDEF* gene cluster (48, 50). The relevant restriction sites are shown. The directions and sizes of DNA fragments used for sequencing are indicated by arrows. Open bars represent the locations and directions of the determined genes. The names of the plasmids refer to the constructs used in this study. The replicons of pTCB77 and pTCB77Δ are from pET8c (36), and the direction of the vector-localized ϕ10 promoter is indicated. Symbols: ▨, fragment containing the promoter regions of *tcbC* and *tcbR* used in the electrophoretic mobility shift assay; ▩, negative control fragment used in the binding assay; ▼, position of the *Sst*II site which was removed in pTCB77Δ. (B) Cloning strategy of plasmid pTCB74 carrying a frameshift mutation in *tcbR*. The strategy is explained in the text. Symbols: *, position of the *Sst*II site to be mutated in *tcbR*; ▼, *Sst*II site removed in pTCB56Δ and pTCB74; ▩, region of *tcbCDEF* on plasmid pTCB45 and pTCB74 (50); ▨, region of *tcbR*; ▩, mutated *tcbR* expressing a truncated protein; ■, remaining parts of pP51 insert; □, promoter region of *tcbC* and *tcbR*. Relevant restriction sites and sizes are indicated.

regulatory gene (Fig. 1A). The nucleotide sequence of the 1,877-bp *Bam*HI-*Sst*I fragment of pP51 DNA preceding the *tcbCDEF* gene cluster was determined. The results (Fig. 2) revealed one large open reading frame from positions 357 to 1241 (designated *tcbR*; see below), starting with an ATG start codon and preceded by a potential ribosome binding site (45). The open reading frame was found to be oriented in the opposite direction to the *tcbCDEF* gene cluster. The *tcbR* gene could encode a protein of 294 amino acid residues with a calculated molecular mass of 32,025 daltons. A second possible start codon was found at position 408 and was also preceded by a potential ribosome binding site.

Expression of the *tcbR* gene was analyzed in *E. coli* BL21(DE3) by cloning of the 1.1-kb *Nco*I-*Hin*dIII fragment of pTCB66 in vector pET8c, which had been digested with *Nco*I and *Hin*dIII, yielding plasmid pTCB77. In this construction, the *tcbR* coding region is fused with the ATG start codon present on the T7 expression vector pET8c (36). The expression of a polypeptide of approximately 30 kDa could be seen upon induction of *E. coli* BL21(DE3) harboring plasmid pTCB77 (Fig. 3). This size was in agreement with that predicted from the sequence of the *tcbR* gene. When a frameshift mutation was introduced into the *tcbR* gene, as in plasmid pTCB77Δ, the 30-kDa protein was no longer produced. Instead, a smaller, truncated protein of 12 kDa was synthesized (Fig. 3); this size corresponded to that predicted from the out-of-frame gene fusion from the first ATG at position 357. Both protein bands were absent in *E. coli* BL21(DE3) harboring the vector pET8c. Furthermore, a second protein band of 19 kDa was observed upon induction of *E. coli* BL21(DE3) harboring plasmid pTCB77. Because the introduction of the frameshift mutation abolished the synthesis of this protein (Fig. 3, lane 4), we assume that it was a degradation product of TcbR.

Homology of TcbR with LysR-type transcriptional regulators. Comparison of the deduced amino acid sequence of TcbR with those of members of the LysR family by a FASTA alignment (31) revealed clear similarities (Fig. 4). Assuming that the *tcbR* gene starts at position 357 (Fig. 2), the homology was highest in the N-terminal helix-turn-helix motif which is presumed to be the DNA binding region of these proteins (22, 23). Good homology was observed with amino acid sequences deduced from regulatory genes involved in aromatic metabolism, such as those for CatR of *P. putida* (31.4% identity in 283 amino acids [37]), CatM of *A. calcoaceticus* (31.2% in 247 amino acids [29]), or NahR of *P.*

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      BamHI                                SalI
  1   GGATCCGCGCGTGGCCTGGGCTTTGAGGTCGACGATCGTGTGGTTGAAGAACGCGTCCAG
      CCTAGGCGCGCACCGGACCCGAAACTCCAGCTGCTAGCACACCAACTTCTTGCGCAGGTC

 61   CAGGACAGCCACTTCCTTGGCCTCGGCCAGTTTCATCATGTAGCCGACGCCCGCGCGCCA
      GTCCTGTCGGTGAAGGAACCGGAGCCGGTCAAAGTAGTACATCGGCTGCGGGCGCGCGGT

121   CTCTTCTTCCGTCACGCGTTGCTCCGTGAGGGTCTTCTGGATCGCATCCACCAATGCGGA
      GAGAAGAAGGCAGTGCGCAACGAGGCACTCCCAGAAGACCTAGCGTAGGTGGTTACGCCT

181   CGCAACCTGCTTCACTCGTTCGTTCATCCCGGTCTCCTTTGTCGGTTTGCCCGGTCCGGA
      GCGTTGGACGAAGTGAGCAAGCAAGTA   start tcbC

241   TTGACGCGGGACGAGTGAATGCTAGGCAGCGCCGGCCATGCCGTCCAATACCAAATTAGT
                                                          NcoI
301   CAGCCATCGTTACGGTTTGCGTAATATAGCGCACATCACCAACTCCGAAAGCGCGCCATGG
  1                                                start tcbR     M E
      EcoRI                                                  PstI
361   AATTCCGGCAGCTCAAGTATTTCATCGCCGTCGCGCAAGCAGGGAACATGCCTGCAGCAG
  3     F  R  Q  L  K  Y  F  I  A  V  A  E  A  G  N  M  A  A  A  A

421   CCAAGCGCCTGCACGTCTCACAGCCCCCCATCACGCGGCAGATGCAAGCCCTGGAAGCGG
 23     K  R  L  H  V  S  Q  P  P  I  T  R  Q  M  Q  A  L  E  A  D
                                    SstII
481   ACTTGGGCGTCGTGCTTCTGGAGCGAAGCCACCGCGGGGATCGAACTCACCGCCGCAGGTC
 43     L  G  V  V  L  L  E  R  S  H  R  G  I  E  L  T  A  A  G  H

541   ACGCTTTCCTGGAGGATGCGCGCCGCATCCTGGAGTTGGCAGGCCGTTCGGGTGATCGCT
 63     A  F  L  E  D  A  R  R  I  L  E  L  A  G  R  S  G  D  R  S

601   CCCGCGCCGCCGCGCGGGGCGACGTGGGCGAGTTGAGCGTGGCGTATTTCGGAACGCCCA
 83     R  A  A  A  R  G  D  V  G  E  L  S  V  A  Y  F  G  T  P  I

661   TCTACCGCAGCCTGCCCCTTTTGCTGCGCGCTTTCCTTACGTCCACGCCTACGGCGACGG
103     Y  R  S  L  P  L  L  L  R  A  F  L  T  S  T  P  T  A  T  V

721   TATCCCTCACGCACATGACCAAGGACGAGCAGGTGGAGGGCCTGCTCGCGGGGACCATCC
123     S  L  T  H  M  T  K  D  E  Q  V  E  G  L  L  A  G  T  I  H
                                    SmaI
781   ACGTGGGCTTCAGCCGCTTCTTTCCCCGGCATCCCGGGGATCGAGATCGTCAACATCGCGC
143     V  G  F  S  R  F  F  P  R  H  P  G  I  E  I  V  N  I  A  Q

841   AAGAGGATCTCTACCTCGCGGTGCACCGCTCCCAGTCGGGGAAGTTCGGCAAGACCTGCA
163     E  D  L  Y  L  A  V  H  R  S  Q  S  G  K  F  G  K  T  C  K

901   AGCTCGCGGACCTGCGCGCGGTGGAACTCACACTGTTCCGCGCGGCGGCCGGCCGAGCT
183     L  A  D  L  R  A  V  E  L  T  L  F  P  R  G  G  R  P  S  F

961   TCGCCGATGAGGTGATCGGCCTGTTCAAGCACGCGGGTATCGAGCCCCGCATCGCCCGAG
203     A  D  E  V  I  G  L  F  K  H  A  G  I  E  P  R  I  A  R  V
                                    NcoI
1021  TCGTGGAGGACGCCACGGCCGCGCTCGCCCTGACCATGGCCGGTGCGGCGTCGAGCATCG
223     V  E  D  A  T  A  A  L  A  L  T  M  A  G  A  A  S  S  I  V

1081  TCCCTGCGTCTGTCGCAGCGATCCGTTGGCCGGATATCGCGTTCGCGCGGATCGTCGGGA
243     P  A  S  V  A  A  I  R  W  P  D  I  A  F  A  R  I  V  G  T

1141  CACGGGTCAAGGTGCCCATCAGCTGCACCTTCCGCAAGGAGAAACAGCCGCCCATCCTGG
263     R  V  K  V  P  I  S  C  T  F  R  K  E  K  Q  P  P  I  L  A

1201  CAAGGTTCGTGGAACACGTGCGGCGATCCGCGAAGGACTGAGGCAAATGGTTATTCGCTC
283     R  F  V  E  H  V  R  R  S  A  K  D  -  end tcbR          294

1261  AACCACGTTTGAGTTCGCAAGGTCCGATCGTCACGCTGGACAGGGATCGCGTTGCGGTCG
1321  CAAGCTACGAGTTCGTAGCCCAGCCTTCATCGCCCAGCCCGCCGTCACAGATGCAAAAGA
                                                          HindIII
1381  GTCAGAAAGGTTGCAGTTCTGATGGCAAGCATTCGTAGCTACCGTCAAGCAAGCTTATCG
1441  GATCACTTGAGGTGGGCTCGATTGACTCGCTCGATGGTCTGCGAGGGTAGTTCCCCAAAC
      XhoI
1501  TCGAGTCGGTACTCTTCAGAGAAACCCCCCAGGTGATGCAGTGCCGCATTCTCCAGCCACC
1561  TCCGTGACGGAAGCGCGAGCTTGCGTCGATCAGCATCTTTCTAGCCTGCGTCAATCGGT
1621  GGAGGCGCAGGTACGCCATAGGACTCGTATTTCGAAATCGCCTGAAATTTATACAAAGGC
1681  AGCGCTTAGTTACTCCGGCGGCTGACGCAACCTCGGCCAGCGTAATCGCTTCCCGAAAGT
1741  TCTCGCGCATGAAGGCTTCCGCTTTTTGCACATGGAACGGCACGGCGCCCAAGCAACGTC
      SalI
1801  GACCTGATTCGGCCGGAAGATAACGAATCATCAGGTCAACCATCAGATCGGCCATCCGCT
                    SstI
1861  CGGACGTGCGCGAGCTC  1877
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FIG. 2. Nucleotide sequence of the 1,877-bp *Bam*HI-*Sst*I fragment of pP51 containing *tcbR*. Also shown is the derived amino acid sequence for the largest open reading frame, designated *tcbR*. Putative consensus ribosome binding sites are indicated by double underlining. Nucleotides printed in italic type represent those of the divergently transcribed *tcbC* gene. Relevant restriction sites are indicated above the nucleotide sequence (nucleotides are printed in boldface type). Bases are numbered relative to the initial *Bam*HI site; amino acid residues are numbered relative to the putative start site of *tcbR*.

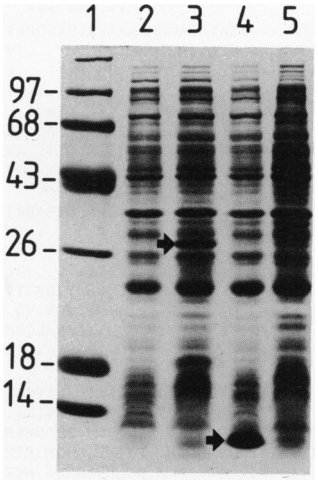

FIG. 3. Expression of the *tcbR* gene in *E. coli* BL21(DE3) cell extracts visualized on a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel. Lanes: 1, molecular weight markers (in kilodaltons); 2, *E. coli* BL21(DE3)(pET8c) plus IPTG; 3, *E. coli* BL21(DE3)(pTCB77) plus IPTG; 4, *E. coli* BL21(DE3)(pTCB77Δ) plus IPTG; 5, same as lane 3 but without IPTG. The arrows in lanes 3 and 4 indicate the 30-kDa *tcbR* gene product and its 12-kDa truncated form, respectively.

putida (18.2% in 176 amino acids [41, 52]). Very good homologies were found when the TcbR deduced primary sequence was compared with the amino acid sequences translated from parts of open reading frames from upstream regions of the *clcABD* gene cluster of *P. putida* (16) or the *tfdA* gene (46) and *tfdCDEF* gene cluster (18, 19, 32, 33) of *A. eutrophus*, which are also presumed to encode regulatory proteins (20, 24, 25, 32, 37) (76.3% [in 76 residues], 62.6% [in 155 residues], and 68.0% [in 47 residues], respectively).

Analysis of the activity of the *tcbR* gene product in *P. putida*. *P. putida* KT2442 harboring plasmid pTCB1, which contained the *tcbCDEF* gene cluster, had previously been found to be able to degrade 3-CB (50). We decided to use this strain to analyze the function of the *tcbR* gene product. Therefore, plasmids that carried either *tcbCDEF* and an intact *tcbR* gene (pTCB75 and pTCB76) or *tcbCDEF* and an inactivated *tcbR* gene (pTCB74) were constructed (Fig. 1B).

Table 1 shows the expression of catechol 1,2-dioxygenase activity obtained when *P. putida* strains harboring different constructs were grown on different media. To distinguish between endogenous catechol 1,2-dioxygenase activity and the activity of the *tcbC* gene product, we used both catechol and 3-CC as substrates in the enzyme assay (12, 50). Growth on succinate did not induce catechol 1,2-dioxygenase activity in *P. putida*(pKT230). However, *P. putida* harboring *tcbC* showed low catechol 1,2-dioxygenase activity when grown on succinate, which was highest when 3-CC was used as a substrate in the enzyme assay. Since 3-CC is not a substrate for the endogenous catechol 1,2-dioxygenase of *P. putida*, as observed with *P. putida*(pKT230), this result indicated that the activity was due to the expression of *tcbC*. Upon growth on aromatic substrates, such as benzoate or 3-CB, both catechol 1,2-dioxygenase activities were induced (Table 1). The expression of *tcbC* in *P. putida* containing wild-type genes (pTCB1, pTCB75, and pTCB76) was induced at least 3-fold by growth on benzoate and up to 17-fold by growth on 3-CB. The introduction of a mutation in *tcbR* in plasmid pTCB74 affected expression in *P. putida* as

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                    .         .     **  ** ***   **          .
TcbR    1 MEFRQLKY-----FIAVAEAGNMAAAAKRLHVSQPPITRQMQALEADLGV 45
TfdS    1 ......R.------.V.A..E..VG...R...I....V...I....QH... 45
TfdX    1 ..I.....------.V......GFGT..Q.M.I....L...I....R.I.A 45
ClcR    1 ......R.----------.E..IG...R...I........I....Q.H.. 45
CatR    1 ..L.H.R.------.KVL..TL.FTR..EL..IA...LS..ISQ..DQ..T 45
CatM    1 ..L.H.R.------.VT.V.EQSISK..EK.CIA...LS..I.K..EE..I 45
NahR    1 ..L.D.DLNLLVV.NQLLVDRRVSIT.EN.GLT..AVSNALKR.RTS.QD 50
LysR    3 VNL.HIEI------.H..MT..SLTE..HL..T...TVS.ELARF.KVI.L 48
                    .         .         .         .         .
TcbR   46 VLLERSHRGIE-----------LTAAGHAFLEDARRILELAGRSGDRSRA  84
TfdS   46 L.F...A..VQ-----------..P..A........M...GRT.V.....  84
TfdX   46 K.
ClcR   46 ..F..T...V.-----------.....TT.......L.HVT
CatR   46 L.VV..-E.PLR-----------..E..RF.Y.QSCTV.Q.Q-NIS.NT.R  82
CatM   46 Q.F..GF.PAK-----------V.E..MF.YQH.VQ..THTAQASSMAKR  84
NahR   51 P.FV.T.Q.M.PTPYAAHLAEPV.S.M..LRNALQHHESFDPLTSE.TFT 100
LysR   49 K.F..VRGRLH-----------P.VQ.LRLF.EVQ.SWYGLD.IVSAAES  87
                    .         .         .         .         .
TcbR   85 AARGDVGELSVAYFGTPIYRSLPLLLRAFLTSTPTATVSLTHMTKDEQVE 134
TfdS   85 .S..EI.Q.DIG.L..A..QTV.A..H..TQAV.G..L..AL.P.VR.I. 134
CatR   83 IGQ.QRQW.GIGFAPSTL.KV..E.I.E-.RQDSELELG.NE..TLQ... 131
CatM   85 I.TVS-QT.RIG.VSSLL.GL..EIIYL.RQQN.EIHIE.IECGTKD.IN 133
NahR  101 L.MT.I..I---..-------M.R.MDVLAHQA.NCVI.TVRDSSMSLMQ 140
LysR   88 LREFRQ....I.CLPVFSQSF..Q..QP..ARY.DVSLNIVPQESPLLE. 137
                    .         .         .         .         .
TcbR  135 GLLAGTIHVGFSRFFPRHPGIEIVNIAQEDLYLAVHRSQSG------KFG 178
TfdS  135 A.R.....L.VG..Y.QE..
CatR  132 A.KS.R.DIA.G.IRIDD.A.HQQVLCEDP.VAVLPKDHPL------A-S 174
CatM  134 A.KQ.K.DL..G.-LKTD.A.RRIVLHK.Q.K..I.KHHHL-----NQ.A 177
NahR  141 A.QN..VDLAVG.LL.NLQTGFFQRRLLQNH.VCLC.KDHPVTREPLTLE 189
LysR  138 W.S.QRHDL.LTETLHTPA.T.RTELLSL.EVCVLPPGHP------LAVK 181
                    .         .         .         .         .
TcbR  179 -KTCKLADLR---------AVELTLFPRGGRPSFADEVIGLFKHAGIEPR 218
CatR  175 -SPLT..Q.A---------GEAFI.Y.ANP...Y..H.LA..A.H.MSIH 214
CatM  178 A.GVH.SQII---------DEPML.Y.VSQK.N..TFIQS..TEL.LV.S 218
NahR  190 -RF.SYGHV.VIAAGTGHGE.DTYMTRV.I.RDIRL..PHF---.AVGHI 235
LysR  182 -.VLTPD.FQ---------GENYISLS.TD--.YRQLLDQ..TEHQVKR. 219
                    .         .         .         .         .
TcbR  219 IARVVEDATAALALTMAGAASSIVPASVAAIRWPDIAFARIVGTRVKVPI 268
CatR  215 VSQWANELQT.IG.VAV.VGVTL.....QQQHRT..EYVSLLDSGAVS.. 264
CatM  219 KLTEIAEIQL..G.VA..EGVC.....AWILG  end              249
NahR  236 LQ.TDLL..VPIR.ADCCVEPFGLS.LPHPVVL.E..INMFWHAKYHKDL 285
LysR  220 MIVETHS.ASVC.MVR..VGI.V.-NPLT.LDYAASGLV-VRRFSIA..F 267
                    .         .
TcbR  269 SCTFRKEKQPPILARFVEHVRRSAKD end 294
CatR  265 ILSR..GDVS..VQ.CLTLIAQQAE end  289
NahR  288 ANIWLRQLMFDLFTD  end           300
LysR  268 TVSLIRPLHR.SSALVQAFSGHLQAGLPKL
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FIG. 4. FASTA alignment of the deduced amino acid sequences of proteins encoded by regulatory genes of aromatic catabolism within the LysR family of transcriptional regulator proteins relative to TcbR. The ClcR, TfdS, and TfdX sequences are partial sequences that were translated from nucleotide sequences of upstream regions of *clcABD* (16), *tfdA* (46), and *tfdCDEF* (32, 33), respectively, and their homology to LysR was observed previously (23). Symbols: ., identical amino acid residues; -, gaps needed for optimal alignment; *, strongly conserved residues involved in the helix-turn-helix N-terminal parts of the proteins. Numbers indicate the positions of the amino acid residues in the total sequence as well as the total lengths of the deduced proteins.

follows: growth on benzoate still resulted in the induction of *tcbC*-encoded catechol 1,2-dioxygenase activity, whereas growth on 3-CB no longer resulted in the elevated expression of *tcbC*, which was measured with 3-CC as a substrate. Surprisingly, *P. putida*(pTCB74) was still able to grow on 3-CB as the sole carbon and energy source, albeit at a much lower rate than *P. putida* harboring pTCB1, pTCB75, or pTCB76. This growth was probably the result of the residual expression of *tcbC*, also observed under noninducing conditions, such as growth on succinate. We conclude from these results that an active *tcbR* gene product is required for the induction of the expression of *tcbC* upon growth on a chlorinated compound such as 3-CB.

Determination of transcription start sites of *tcbC* and *tcbR*. The transcription start sites of *tcbC* and *tcbR* in *Pseudomonas* sp. strain P51 were determined by primer extension mapping of RNA isolated from strain P51 cultivated on either 1,2,4-TCB or succinate (Fig. 5). A single transcription start site was detected for *tcbC*. The extension product of *tcbC* was only observed with RNA isolated from cultures that had been grown on 1,2,4-TCB and not with RNA isolated from succinate-grown cultures. This result indicated that the transcription of *tcbC* is induced by growth on 1,2,4-TCB but not by growth on succinate. In contrast, when RNA was isolated from cultures grown on 1,2,4-TCB or succinate, the synthesis of an identical extension product specific for *tcbR* resulted. This result indicated that *tcbR* transcription is at least partially constitutive and initiated at the same start site. The start sites of the divergent transcripts were located in close vicinity on the opposite strands.

TABLE 1. Catechol 1,2-dioxygenase activities measured in cell extracts of *P. putida* KT2442 harboring different constructs containing *tcbCDEF* and *tcbR*

Plasmid	Growth substrate[a]	Catechol 1,2-dioxygenase activity[b] with the following substrate:	
		Catechol	3-CC
pKT230	SU	—	—
	BE	644	—
	3-CB	—	—
pTCB1	SU	3	10
	BE	382	48
	3-CB	185	170
pTCB75	SU	3	10
	BE	372	31
	3-CB	105	30
pTCB76	SU	8	20
	BE	500	65
	3-CB	142	69
pTCB74	SU	7	19
	BE	390	39
	3-CB	165	12

[a] SU, succinate; BE, benzoate.
[b] Nanomoles of muconate per milligram of protein per minute. —, not detectable.

DNA binding activity of the *tcbR* gene product. To determine whether the activation of *tcbC* gene expression was due to a direct interaction of the *tcbR* gene product with the mapped *tcbC* promoter region, we performed a series of DNA binding experiments. Cell extracts of *E. coli* BL21 (DE3) harboring pTCB77 and induced by IPTG and of *Pseudomonas* sp. strain P51 were tested for the ability to bind in an electrophoretic mobility shift assay to a 0.38-kb *Eco*RI-*Bam*HI fragment that contained the promoter regions of *tcbC* and *tcbR* (Fig. 1A). As a negative control, a 0.40-kb *Xho*I-*Hin*dIII fragment originating from the *tcbD* gene was used (Fig. 1A). Figure 6A shows a clear shift of the labeled *Eco*RI-*Bam*HI fragment incubated with a cell extract of *E. coli*(pTCB77), in which *tcbR* was expressed. This shift was not observed with the labeled *Xho*I-*Hin*dIII control fragment. Similarly, no retardation was observed with induced *E. coli* cell extracts harboring pET8c alone or pTCB77Δ, which expresses a truncated TcbR protein. The retardation of the *Eco*RI-*Bam*HI fragment by TcbR could be abolished

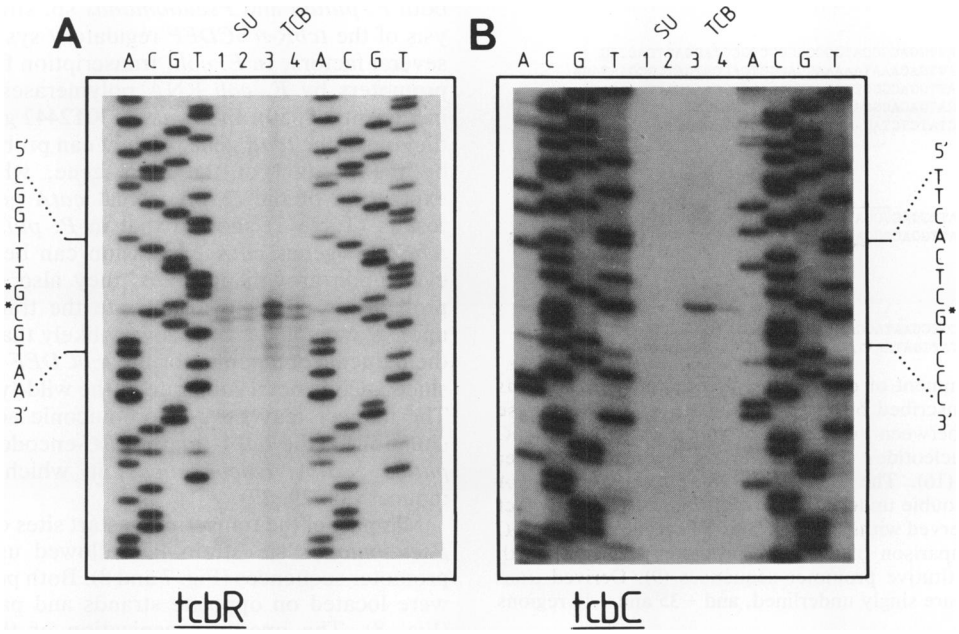

FIG. 5. Primer extension products of RNA transcribed from *tcbR* and *tcbC*. (A) Transcription start sites determined for *tcbR*. (B) Transcription start sites determined for *tcbC*. The relevant DNA sequences are indicated (5′ to 3′ direction), and the determined transcription start sites are marked by asterisks. Lanes: A, C, G, T, sequencing reactions carried out as described in Materials and Methods; SU, cells pregrown on succinate; TCB, cells cultivated on 1,2,4-TCB; 1, 30 μg of RNA; 2, 10 μg of RNA; 3, 30 μg of RNA; 4, 10 μg of RNA.

by adding 0.5 μg of unlabeled plasmid pTCB48 (50), which contains the cloned promoter region and the *tcbC* gene, but not by adding 0.5 μg of unlabeled plasmid pUC18. Preliminary purification of the TcbR DNA binding activity from cell extracts of *E. coli* BL21(DE3) harboring pTCB77 showed that the activity could be attributed to the 30-kDa protein (27). A similar specific retardation of the promoter fragment was observed with cell extracts of *Pseudomonas* sp. strain P51 (Fig. 6B), indicating that this strain contains DNA binding activity most probably caused by the *tcbR* gene product. From these results, we conclude that the TcbR protein binds specifically to a fragment containing the divergently located promoter regions of the *tcbC* and *tcbR* genes.

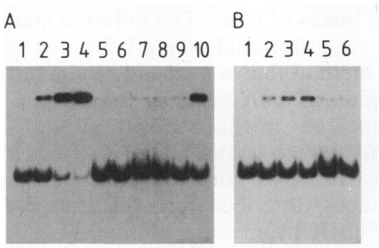

FIG. 6. Electrophoretic mobility shift assays of *tcbC* promoter fragments with cell extracts containing TcbR. (A) Assays performed with IPTG-induced cell extracts of *E. coli* BL21(DE3) containing various expression plasmids and the pTCB48 *Eco*RI-*Bam*HI promoter fragment (48EB) or with the pTCB48 *Xho*I-*Hin*dIII control fragment (48XH). Lanes: 1, 48EB, no extract added; 2, 48EB plus 0.5 μg of cell extract of *E. coli*(pTCB77); 3, 48EB plus 1.0 μg of cell extract of *E. coli*(pTCB77); 4, 48EB plus 2.0 μg of cell extract of *E. coli*(pTCB77); 5, 48EB plus 2.0 μg of cell extract of *E. coli* (pTCB77Δ); 6, 48EB plus 2.0 μg of cell extract of *E. coli*(pET8c); 7, 48XH, no extract added; 8, 48XH plus 2.0 μg of *E. coli*(pTCB77); 9, 48EB plus 1.0 μg of cell extract of *E. coli*(pTCB77) and 0.5 μg of pTCB48; 10, 48EB plus 1.0 μg of cell extract of *E. coli*(pTCB77) and 0.5 μg of pUC18. (B) Assays performed with *Pseudomonas* sp. strain P51 cell extracts and 48EB or with 48XH. Lanes: 1, 48EB, no extract added; 2, 48EB plus 15 μg of cell extract; 3, 48EB plus 30 μg of cell extract; 4, 48EB plus 45 μg of cell extract; 5, 48XH, no extract added; 6, 48XH plus 45 μg of cell extract.

DISCUSSION

In this report, we describe the cloning and characterization of the *Pseudomonas* sp. strain P51 *tcbR* gene, which encodes a regulatory protein involved in expression of the *tcbCDEF* chlorocatechol oxidative gene cluster. The functionality of the *tcbR* gene was confirmed by analysis of the inducible expression of catechol 1,2-dioxygenase encoded by *tcbC* in *P. putida*. Furthermore, the nucleotide sequence of the *tcbR* gene was determined and the deduced 294-amino-acid product of this gene was identified in overproducing *E. coli* as a protein with an apparent mass of 30 kDa.

The TcbR regulatory protein showed all the features of members of the LysR family of regulatory proteins (22, 23): considerable homology with LysR (21.5% identity in 279 amino acids [22]), a helix-turn-helix motif in the N-terminal part, divergent transcription from the gene cluster which is regulated, and specific binding to a DNA fragment containing the promoter region of this cluster. Although the complete sequences of the regulatory genes from chlorocatechol oxidative gene clusters have not been reported, the high homology (62.6 to 76.3%) of the deduced amino acid sequences of the N-terminal parts of ClcR (translated from part of the nucleotide sequence upstream of the *clcABD* operon [16]), TfdS (translated from part of the sequence upstream of *tfdA* [46]), and TfdX (translated from part of the sequence upstream of the *tfdCDEF* gene cluster [32, 33]) strongly suggests that these proteins constitute a subgroup within the

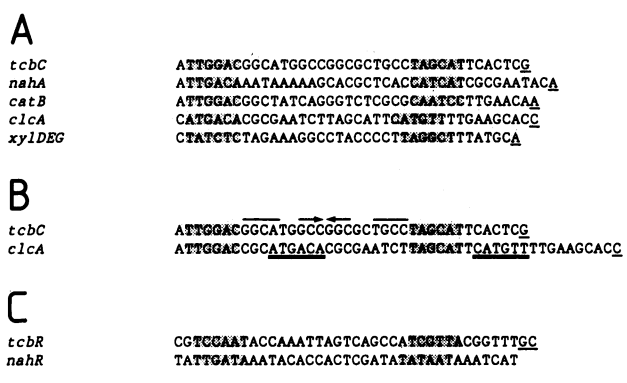

FIG. 7. (A) Alignment of promoter sequences of *Pseudomonas* catabolic genes transcribed by the σ^{70}-activated RNA polymerase (9). (B) Homology between the derived promoter sequence of *tcbC* and a region 9 nucleotides upstream of the deduced promoter sequence of *clcA* (16). The −35 and −10 regions of the *clcA* promoter (16) are double underlined. Arrows indicate a nonperfect inverted repeat observed within the −35 and −10 regions of the *tcbC* promoter. (C) Comparison of the *tcbR* promoter sequences with *Pseudomonas* constitutive promoter sequences (9). Derived transcription start sites are singly underlined, and −35 and −10 regions are shaded.

LysR family. Except for TfdS, which was reported to regulate *tfdB* expression (25), no clear role or function was ascribed to the other two putative proteins. Like TcbR, they could be involved in the regulation of the divergently transcribed gene clusters which start in their immediate vicinity, e.g., *clcABD* and *tfdCDEF*. This possibility, however, is not in agreement with the suggestion that the *tfdCDEF* gene cluster is regulated by the *tfdR* gene, which has been localized elsewhere on plasmid pJP4 (20, 24). Preliminary studies have shown that cell extracts of *E. coli* overproducing TcbR also retard DNA fragments that contain regions immediately upstream of *tfdCDEF* (27), suggesting similar mechanisms of regulation of the chlorocatechol oxidative operons. This possibility would reinforce the close similarity that was also observed for the genetic organization and DNA sequence of these clusters (18, 19, 33, 48).

Among the regulatory proteins of the LysR family, both negative and positive regulators are found. CatM was shown to be a negative regulator (29), as was TfdS (25), whereas NahR (41, 42, 52), CatR (37), and LysR (22) belong to the class of positive regulators. Our results suggest that TcbR is a positive regulator, since in its presence *tcbC*-encoded catechol 1,2-dioxygenase activity was much higher in *P. putida* KT2442 (Table 1). However, low constitutive expression of *tcbC* was observed in the absence of induction in both *P. putida* and *Pseudomonas* sp. strain P51 (50). Analysis of the *tcbR-tcbCDEF* regulatory system is hindered by several factors. In *E. coli*, transcription from *Pseudomonas* promoters by *E. coli* RNA polymerases was found to be inefficient (48, 50). In *P. putida* KT2442 grown on benzoate, the role of the *tcbR* gene product can probably be taken over by the product of the *catR* gene, which regulates the expression of the chromosomal *catA* gene. Although our results (Table 1) showed that in *P. putida* containing the *tcbCDEF* genes *catA* expression can be induced by CatR even upon growth on 3-CB, they also showed that CatR probably is not able to activate the transcription of *tcbC* upon growth on 3-CB. It is very likely that 3-CB itself is not the inducing compound for the *tcbCDEF*-encoded pathway, since 3-CB is not a substrate of the wild-type strain P51 (50). The inducer may be 2-chloromuconic acid, similar to the situation in the *catA*- and *catBC*-encoded pathways of *P. putida* and *A. calcoaceticus*, in which muconate is the inducer (28, 29, 37).

Mapping of the transcription start sites of *tcbC* and *tcbR* in *Pseudomonas* sp. strain P51 allowed us to identify their promoter sequences (Fig. 7 and 8). Both promoter sequences were located on opposite strands and partially overlapped (Fig. 8). The operon organization of the *tcbCDEF* gene cluster, as determined previously (48), suggests that these genes are transcribed from the same, *tcbC*, promoter. The sequence of the promoter region of *tcbC* could be aligned with several promoter region sequences derived for catabolic genes in *Pseudomonas* spp. (Fig. 7) (9). It showed good homology with the *nahA* (40) and *catB* (4) promoter region sequences with respect to the −35 box TTGGAC recognized by the σ^{70}-activated RNA polymerase in *Pseudomonas* spp. (9). The −10 box was more variable among the promoter region sequences, but the sequence TAGCAT of the *tcbC* promoter resembled that of the promoters of both *nahA* and *catB*. A striking difference was found when the *tcbC* promoter region was compared with the reported *clcA* promoter sequence (16). However, we found that the nucleotide sequences of both regions were highly conserved (Fig. 7B) and that the *clcA* region contained sequences identical to the −35 and −10 boxes of *tcbC*. The different start site that was reported for *clcA* (16) could have been due to the different and indirect method that was used in its determination. The promoter region of *tcbR* partially overlapped that of *tcbC*, which was located on the opposite strand. The −35 box TCCAAT and the −10 box TCGTTA of the *tcbR* promoter showed, however, less homology with the consensus promoter sequences described for constitutive *Pseudomonas* promoters (Fig. 7C) (9).

Analogous to other systems, the ability of the TcbR protein to activate the transcription of *tcbC* would depend on

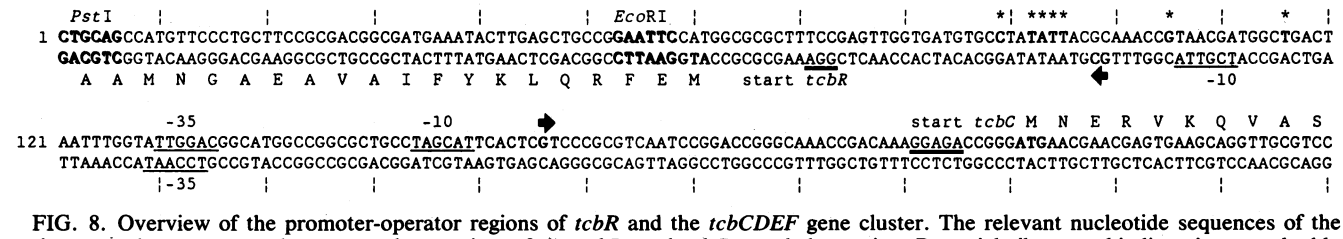

FIG. 8. Overview of the promoter-operator regions of *tcbR* and the *tcbCDEF* gene cluster. The relevant nucleotide sequences of the regions are shown, as are the proposed start sites of the *tcbR*- and *tcbC*-encoded proteins. Potential ribosome binding sites are doubly underlined, and the −35 and −10 regions of the derived promoter sequences are singly underlined. Arrows indicate transcription start sites and directions of transcription; *, nucleotides homologous to regions protected by NahR and NodD from DNase I digestion (14, 41). Relevant restriction sites are indicated as explained in the legend to Fig. 2.

the binding of this protein to a specific target site upstream from the promoter of *tcbC* and after interaction with a suitable inducer, to form an open transcription complex (17). In fact, the TcbR protein binds specifically with a region containing the promoters of *tcbC* and *tcbR*. Comparison of this region with the binding sites of the NahR and NodD proteins (14, 41) revealed a number of consensus nucleotides (Fig. 8) which are known to be involved in the binding of those regulatory proteins.

Further analysis of the specific specialized features of TcbR and the *tcbR-tcbCDEF* regulatory circuit, which arose in response to environmental stress with chlorinated benzenes, may provide more insight into the importance of the fine-tuning of regulatory systems as a means of adaptation to novel substrates.

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