JOURNAL OF BACTERIOLOGY, Nov. 1996, p. 6327–6337 Vol. 178, No. 21
0021-9193/96/$04.00+0

Cascade Regulation of the Toluene-3-Monooxygenase Operon (*tbuA1UBVA2C*) of *Burkholderia pickettii* PKO1: Role of the *tbuA1* Promoter (*PtbuA1*) in the Expression of Its Cognate Activator, TbuT

ARMANDO M. BYRNE AND RONALD H. OLSEN*

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0620

Received 3 May 1996/Accepted 20 August 1996

***Burkholderia pickettii* PKO1 metabolizes toluene and benzene via a chromosomally encoded toluene-3-monooxygenase pathway. Expression of the toluene-3-monooxygenase operon (*tbuA1UBVA2C*) is activated by the regulator, TbuT, in the presence of toluene. We have identified the TbuT coding region downstream of the toluene-3-monooxygenase structural genes by nucleotide sequence analysis and have shown that although TbuT is similar to XylR and DmpR, two members of the NtrC family of transcriptional activators which control toluene-xylene and (methyl)phenol catabolism, respectively, it is significantly different in the domain associated with effector specificity. Using a *tbuA1-lacZ* fusion reporter system, we determined that TbuT is activated not only by aromatic effectors but also the chlorinated aliphatic hydrocarbon trichloroethylene. Expression of *tbuT* and that of the *tbuA1UBVA2C* operon were found to be linked by readthrough transcription of *tbuT* from the toluene-3-monooxygenase promoter. As a result, transcription of *tbuT* is low when the toluene-3-monooxygenase operon is uninduced and high when expression of *tbuA1UBVA2C* is induced by toluene. Thus, the toluene-3-monooxygenase promoter drives the cascade expression of both the toluene-3-monooxygenase operon and *tbuT*, resulting in a positive feedback circuit. Examination of the nucleotide sequence upstream of the toluene-3-monooxygenase operon for promoter-like sequences revealed a −24 TGGC, −12 TTGC sequence, characteristic of σ^{54} (*rpoN*)-dependent promoters. Primer extension and *tbuA1-lacZ* fusion analyses demonstrated that this −24, −12 promoter sequence, referred to as *PtbuA1*, was the toluene-3-monooxygenase promoter. Upstream of *PtbuA1*, a DNA region with dyad symmetry exhibited homology with the XylR-binding site present upstream of the *Pu* promoter. Deletions within this DNA sequence resulted in complete loss of expression from *PtbuA1*, suggesting that this region may serve as the TbuT-binding site.**

Burkholderia (formerly *Pseudomonas*) *pickettii* PKO1 metabolizes benzene and toluene via a toluene-3-monooxygenase pathway (36). The initial step of this pathway involves the hydroxylation of toluene and benzene to *m*-cresol and phenol, respectively, by toluene-3-monooxygenase (36). The phenolic intermediates (phenol and *m*-cresol) are then further hydroxylated to catechol and methylcatechol, respectively, by a phenol hydroxylase, prior to ring cleavage by a meta-fission dioxygenase (25–27). We recently reported on the ability of *B. pickettii* PKO1 to degrade trichloroethylene (TCE) and provided evidence suggesting that this oxidation reaction was catalyzed by this same pathway (29). The toluene-3-monooxygenase is encoded by six tightly clustered chromosomal genes, *tbuA1UBVA2C* (6). On the basis of functional and sequence data, the toluene-3-monooxygenase is similar to the toluene-4-monooxygenase from *Pseudomonas mendocina* KR1 (57, 58) and the toluene/benzene-2-monooxygenase from *Pseudomonas* sp. strain JS150 (24). These enzyme systems together with the toluene-2-monooxygenase from *Burkholderia cepacia* G4 (16, 33) and the toluene/benzene-4-monooxygenase from *Pseudomonas* sp. strain JS150 (23) comprise a family of multicomponent toluene monooxygenases which hydroxylate the aromatic nucleus. The xylene monooxygenase from the TOL plasmid of *Pseudomonas putida* mt-2 also oxidizes toluene; however, it hydroxylates the methyl group rather than the aromatic ring (56).

Previous studies have shown that toluene-3-monooxygenase expression is tightly regulated, with very little activity occurring in the absence of pathway substrates (36). Through a series of complementation studies, the locus encoding a positive regulator, designated *tbuT*, was identified and mapped downstream of the toluene-3-monooxygenase structural genes (36). Nucleotide sequence analysis of the monooxygenase-encoding region revealed part of an open reading frame downstream of *tbuC* showing homology to the 5′ end of *xylR* (5), the gene encoding the transcriptional activator from the TOL plasmid of *P. putida* mt-2. Here we report the nucleotide sequence of the *tbuT* gene and show that TbuT has homology with two members of the NtrC family of transcriptional activators, XylR and DmpR.

DmpR, from *Pseudomonas* sp. strain CF600, and XylR regulate the expression of genes involved in phenol-cresol and toluene-xylene catabolism, respectively (21, 46; for a review, see reference 45). In the presence of pathway substrates, XylR and DmpR activate transcription from the *Pu* promoter of the *upper* TOL operon and the *Po* promoter of the *dmp* operon, respectively (2, 47). Both *Pu* and *Po* are members of a distinct class of promoters that are recognized by RNA polymerase containing the alternative σ^{54} factor (encoded by *rpoN*) and are positively regulated by activators of the NtrC family (30, 46) which bind to specific DNA sequences, often inverted

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-0620. Phone: (313) 764-4380. Fax: (313) 764-3562. Electronic mail address: ronolsen@umich.edu.

repeats, located 120 to 180 bp upstream of the transcriptional start site (28, 32).

In this study, we analyzed the regulation of the toluene-3-monooxygenase operon, *tbuA1UBVA2C*, by its positive regulator, TbuT, and identified and characterized the toluene-3-monooxygenase promoter, *PtbuA1*. Given the distinctive organizational arrangement of *tbuT* relative to *tbuA1UBVA2C*, we demonstrate that unlike expression of the *xylR* and *dmpR* genes, analyzed previously, the novel expression of *tbuT* is driven, in part, by its cognate promoter, *PtbuA1*, by readthrough transcription. By transcriptional fusion analyses, we also describe the effector specificity of TbuT, including its ability to activate *PtbuA1* expression in response to the presence of TCE.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* DH5α (*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* φ80d *lacZ*) (19) was used for construction and maintenance of plasmids. *E. coli* MM294 carrying the mobilization plasmid pRK2013 (15) was used in triparental matings to mobilize plasmid pKRZ1 and its derivatives from *E. coli* DH5α into *Pseudomonas aeruginosa* PAO1. In vivo *trans*-complementation β-galactosidase assays were carried out in *P. aeruginosa* PAO1. Bacteria were routinely grown in Luria-Bertani (LB) medium (43). To select for *Pseudomonas* transconjugants in triparental mating experiments, Vogel-Bonner minimal medium supplemented with glucose (VBG) (54) was used. For growth of bacteria on solid media, agar (Difco Laboratories, Detroit, Mich.) was added to a final concentration of 2.0% (wt/vol). Growth of liquid cultures and incubation of agar plates were always carried out at 37°C. To maintain recombinant plasmids in *E. coli*, media were supplemented with the antibiotics ampicillin (100 μg/ml), tetracycline (25 μg/ml), and kanamycin, (75 μg/ml). In *Pseudomonas* hosts, kanamycin and tetracycline were used at concentrations of 600 and 50 μg/ml, respectively.

Genetic techniques. Recombinant DNA methods were carried out according to published protocols (43). Midi preparations of *E. coli* and *Pseudomonas* plasmid DNAs were made by using a Qiagen plasmid Midi kit (Qiagen Inc., Chatsworth, Calif.) as directed by the manufacturer. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Gel purification of restriction fragments was performed by using agarose gels containing Tris-acetate-EDTA buffer (43) and a Geneclean II kit (Bio 101, Inc., La Jolla, Calif.) as recommended by the manufacturer.

PCR. PCR was performed by using a GeneAmp kit (Cetus) and thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, Conn.). Reactions were done in 100-μl volumes, and the conditions were essentially as recommended by the manufacturer. PCR products were purified with a QIAquick spin PCR purification kit (Qiagen) as directed by the manufacturer. Oligonucleotide primers were synthesized by the DNA Core facility at the University of Michigan and are described below in the following manner: name of the primer, nucleotide sequence, and coordinates of the 5′ and 3′ ends (given in parentheses) relative to the toluene-3-monooxygenase transcriptional start (Fig. 1A). These primers were *Xho*I-352, 5′-ggcgCTCGAGATTCTTACCAATTG-3′ (nucleotides [nt] −211 to −198); *Xho*I-316, 5′-ggcgCTCGAGGCCGGTGGCCTGATCTG-3′ (nt −177 to −161); *Xho*I-291, 5′-ggcgCTCGAGCTGAAATCGCGGGACCG-3′ (nt −152 to −136); *Sma*I-352, 5′-agttCCCGGGGTCCAGTTGGTCG-3′ (nt 135 to 119); *Sma*I-266, 5′-agttCCCGGGGTGTTTTTTGCTTG-3′ (nt 48 to 35); *Sma*I-254, 5′-agttCCCGGGTTGGCGGCTCCAGC-3′ (nt 37 to 24); *Sma*I-239, 5′-agttCCCGGGATCGCGTCCGCGC-3′ (nt 24 to 8); and *Sma*I-204, 5′-agttCCCGGGAGGCCGGTGCCAACC-3′ (nt −14 to −26). *Xho*I and *Sma*I restriction endonuclease sites were engineered at the 5′ ends (underlined), with four additional nucleotides 5′ to the restriction sites (lowercase letters) to ensure the presence of intact restriction endonuclease cleavage sites in the amplified PCR products. Approximately 10 to 100 ng of pKS::352-bp (pBluescript II KS+ derivative carrying the 352-bp *Xho*I-*Ava*I restriction fragment containing the *tbuA1* promoter) was used as template DNA for the PCR.

Plasmids and bacterial strain construction. The series of *tbuA1-lacZ* fusion plasmids was constructed as follows. Oligonucleotides (described above) complementary to targeted sequences within and upstream of the *tbuA1* gene were used with *Taq* polymerase to amplify *PtbuA1* promoter sequences from the template. PCR products were digested with *Xho*I and *Sma*I and ligated into *Sal*I-*Sma*I-digested pKRZ1 (42). Ligated products were introduced by electroporation into electrocompetent *E. coli* DH5α cells by using a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, Calif.) as described by the manufacturer. Sequencing of the cloned PCR products was done as described below to ensure the fidelity of the PCRs. The extents of the promoter sequences present in the resulting seven pKRZ1 derivatives, p352*X*/*S*, p316*X*/*S*, p291*X*/*S*, p266*X*/*S*, p254*X*/*S*, p239*X*/*S*, and p204*X*/*S*, are shown in Fig. 1A.

The pKRZ1 derivatives carrying large DNA fragments containing all or portions of the toluene-3-monooxygenase operon were constructed by conventional subcloning techniques. Plasmids p5.5*X*I-1 and p5.5*X*I-2 were generated by subcloning the gel-purified 5.5-kb *Xho*I fragment, containing the *tbuA1UBVA2C* genes, from pAB1 (pBluescript II KS+::5.5-kb *Xho*I pRO1966), into *Sal*I-digested pKRZ1 in both orientations. Plasmid p4.2*B*I/*E*V, carrying the 4.2-kb *Bcl*I-*Eco*RV DNA fragment from pRO1966 (36), was constructed as follows. *P. aeruginosa* PAO1-derived pRO1966 DNA was first digested with *Eco*RV and then digested partially with *Bcl*I. The *Bcl*I-*Eco*RV-digested DNA was then ligated into *Bam*HI-*Sma*I-digested pKRZ1. The *Sma*I deletion derivative of p4.2*B*I/*E*V, p4.2*B*I/*E*VΔ*S*I, was constructed by digestion of p4.2*B*I/*E*V DNA with *Sma*I and religation. The *Sst*II deletion derivatives of both p5.5*X*I and p4.2*B*I/*E*V were made by digesting plasmid DNAs with *Sst*II and religating, yielding p5.5*X*IΔ*S*II and p4.2*B*I/*E*VΔ*S*II, respectively.

The pKRZ1 derivatives were then mobilized into *P. aeruginosa* PAO1 by triparental mating by utilizing the mobilization functions provided by plasmid pRK2013 present in *E. coli* MM294. Transconjugants were selected on VBG minimal medium with 1,200 μg of kanamycin per ml and screened for plasmid content.

The second plasmid in the complementation experiments consisted of pRO1614 (35) and its *tbuT*-containing derivatives pRO1614::3.1-kb *tbuT* and pRO1614::3.1-kb *tbuT*Δ0.5-kb *Sst*II. Plasmid pRO1614::3.1-kb *tbuT* was constructed by subcloning the 3.1-kb *Eco*RI-*Pvu*II *tbuT*-containing fragment from pRO1966 into *Eco*RI-*Stu*I-digested pRO1614. Plasmid pRO1614::3.1-kb *tbuT*Δ0.5-kb *Sst*II was generated by digesting pRO1614::3.1-kb *tbuT* DNA with *Sst*II, deleting the 0.5- and 1.7-kb *Sst*II fragments, and religating. The 1.7-kb *Sst*II *tbuT*-containing fragment was then cloned back into the *Sst*II deletion derivative of pRO1614::3.1-kb *tbuT*, in effect resulting in an 0.5-kb *Sst*II deletion of the original pRO1614::3.1-kb *tbuT* plasmid. Plasmid pRO1614 and its derivatives were electroporated into *Pseudomonas* strains containing pKRZ1 and its derivatives by the method developed by Smith and Iglewski (48), with the exception that the actual voltage applied to the cells was 2.1 kV rather than the suggested 1.6 kV.

DNA sequence analysis. Double-stranded DNA sequencing was carried out by using the dideoxy-chain termination method (44), a Sequenase version 1.0 kit (U.S. Biochemical, Cleveland, Ohio), and primers (DNA Core facility at the University of Michigan) complementary to regions surrounding the multiple cloning region of pKRZ1 (KRZ1-Right [5′-GTTCTAAAACGACGGCC-3′] and KRZ1-Left [5′-AGGAACACCAGATGTCG-3′]). Primers complementary to the T3 and T7 promoter regions of pBluescript II KS+ were used when DNA sequencing was carried out on fragments cloned into this vector. Sequence analysis was done with MacVector version 4.5.3 (Oxford Molecular, Campbell, Calif.) and the Genetics Computer Group (GCG; University of Wisconsin, Madison) software package, version 8.1 (12, 17). Searches of the GenBank database (release 93) and pairwise sequence comparisons were carried out with the GCG programs TFASTA and BESTFIT, respectively. The nucleotide sequence alignments were constructed by using the GCG multiple sequence alignment program PILEUP.

Quantification of promoter activity in vivo. Promoter activity was monitored by assaying accumulation of β-galactosidase in cells carrying pKRZ1 and its derivatives in *trans* with pRO1614 and its derivatives. *Pseudomonas* strains that harbored test plasmids were grown overnight on LB broth that contained kanamycin (600 μg/ml) and tetracycline (50 μg/ml) to select for retention of the pKRZ1- and pRO1614-based plasmids, respectively. Cells were subsequently diluted 1:50 in the same medium supplemented with the appropriate antibiotics and exposed for 8 h to saturating vapors of toluene. In experiments studying TbuT's effector specificity, similarly grown cells were exposed to either saturating vapors or 2.5 mM concentrations of the indicated compounds. β-Galactosidase assays were done with cells permeabilized with chloroform and sodium dodecyl sulfate as described by Miller (31). The given β-galactosidase activity values represent the averages of at least three independent experiments, each of which was conducted with duplicate samples, with deviations being less than 15%. β-Galactosidase activity values are expressed in units as specified by Miller (31). Restriction endonuclease digestions of plasmid DNA extracted from each test strain was performed at the end of each experiment to verify plasmid content.

RNA isolation and primer extension analysis. Strains grown under the conditions used for β-galactosidase assays were used for extractions of total RNA, using the Trizol reagent (Bethesda Research Laboratories) essentially as directed by the manufacturer. After the cells were exposed to the Trizol reagent, the mixture was incubated at 68°C for 10 min and then allowed to cool to room temperature, at which time the protocol was continued unchanged. Precipitated nucleic acids were resuspended in 100 μl of diethyl pyrocarbonate-treated water and incubated with 10 U of RNase-free DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 1 h at 37°C. The reaction mixtures were extracted with phenol-chloroform, and RNA was precipitated with ethanol. The precipitated RNA was dissolved in 50 μl of diethyl pyrocarbonate-treated water.

The oligonucleotides (primer 1 [*Sma*I-352], 5′-AGTTCCCGGGGTCCAGTTGGTCG-3′ [nt 135 to 119]; and primer 2 [*Sma*I-266], 5′-AGTTCCCGGGGTGTTTTTTGCTTG-3′ [nt 48 to 35]) were 5′ end labeled with [γ-^{32}P]ATP (ICN Biomedicals, Costa Mesa, Calif.) and annealed to approximately 20 μg of total RNA. Primer extension reactions were carried out using Superscript RNase H$^-$ reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) as recommended by the manufacturer.

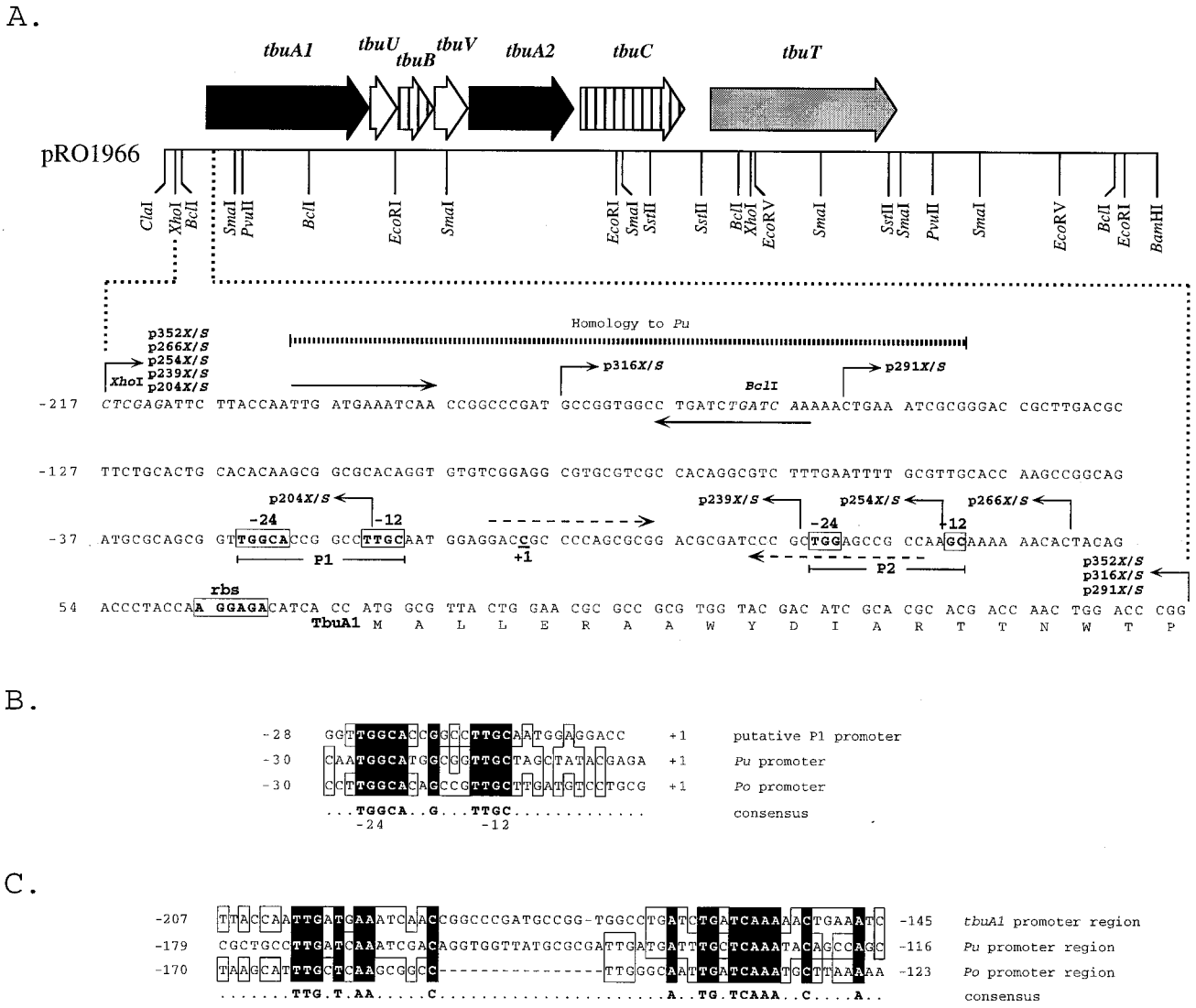

FIG. 1. Analysis of the toluene-3-monooxygenase promoter region. (A) Physical and genetic maps of the 9.4-kb *Cla*I-*Bam*HI fragment in plasmid pRO1966 (36). The arrows above the restriction map represent the locations and orientations of the genes (denoted above the arrows by name) which comprise the toluene-3-monooxygenase operon, *tbuA1UBVA2C*, and the positive regulator *tbuT*. Below the restriction map, the putative *tbuA1* promoter region with relevant flanking DNA is expanded. The DNA region containing the 13-bp imperfect inverted repeats (solid arrows) and with homology to *Pu* is demarcated by the horizontal hatched bar. The −12 and −24 sequences of the putative promoters, P1 and P2 (shown in brackets below the corresponding sequence), are boxed and labeled accordingly. The 15-bp imperfect inverted repeats, partially overlapping the P2 promoter sequence, are indicated by the dashed arrows. The putative ribosome-binding site is boxed and labeled rbs. The first 20 amino acids from the deduced amino acid sequence of the *tbuA1* gene (labeled TbuA1) are shown in one-letter code beneath the corresponding DNA coding region. The underlined C residue with a +1 designation identifies the toluene-3-monooxygenase transcriptional start site as determined by primer extension analysis (described in Results). The right-angled arrows delimit the boundaries of the PCR-generated fragments fused to the *lacZ* gene of pKRZ1, generating plasmids p352*X/S*, p266*X/S*, p254*X/S*, p239*X/S*, p204*X/S*, p316*X/S*, and p291*X/S*. The *Xho*I and *Bcl*I restriction endonuclease recognition sequences are shown in italics. (B) DNA sequence alignment of the σ^{54}-dependent promoter sequences of *Pu*, *Po*, and P1. The −24 and −12 sequences within the consensus sequence are labeled accordingly. (C) DNA sequence alignment of the palindromic region upstream of *tbuA1* shown in panel A (as solid arrows) with palindromic regions containing the XylR-binding site upstream of *Pu* and the proposed DmpR-binding site upstream of *Po*. Nucleotide sequence identities among the three sequences are boxed. Nucleotides conserved in all three sequences are represented as white letters on a black background. The consensus sequences established from the comparison of the regions within and upstream of the *Pu*, *Po*, and P1 sequences are displayed below each alignment. Gaps, indicated by dashes, were introduced to maximize homology.

Nucleotide sequence accession numbers. The sequence data in this report have been submitted to the GenBank data library under accession numbers U04052 and U72645.

RESULTS

Identification of promoter and regulatory sequences upstream of the *tbuA1* gene. The earlier finding of part of an open reading frame downstream of the toluene-3-monooxygenase operon with nucleotide sequence similarity to *xylR* (5) suggested that regulation of the toluene-3-monooxygenase operon might be similar to that described for the *upper* TOL operon, although the pathway structural and regulatory genes differ in composition and juxtaposition. To explore this further, we examined the nucleotide sequence upstream of the *tbuA1* gene for features present in both the *upper* TOL operon promoter, *Pu*, and the similarly organized *dmp* operon promoter, *Po*. These features include (i) a σ^{54} (*rpoN*)-dependent −24, −12 promoter sequence, (ii) an inverted repeat believed to be the

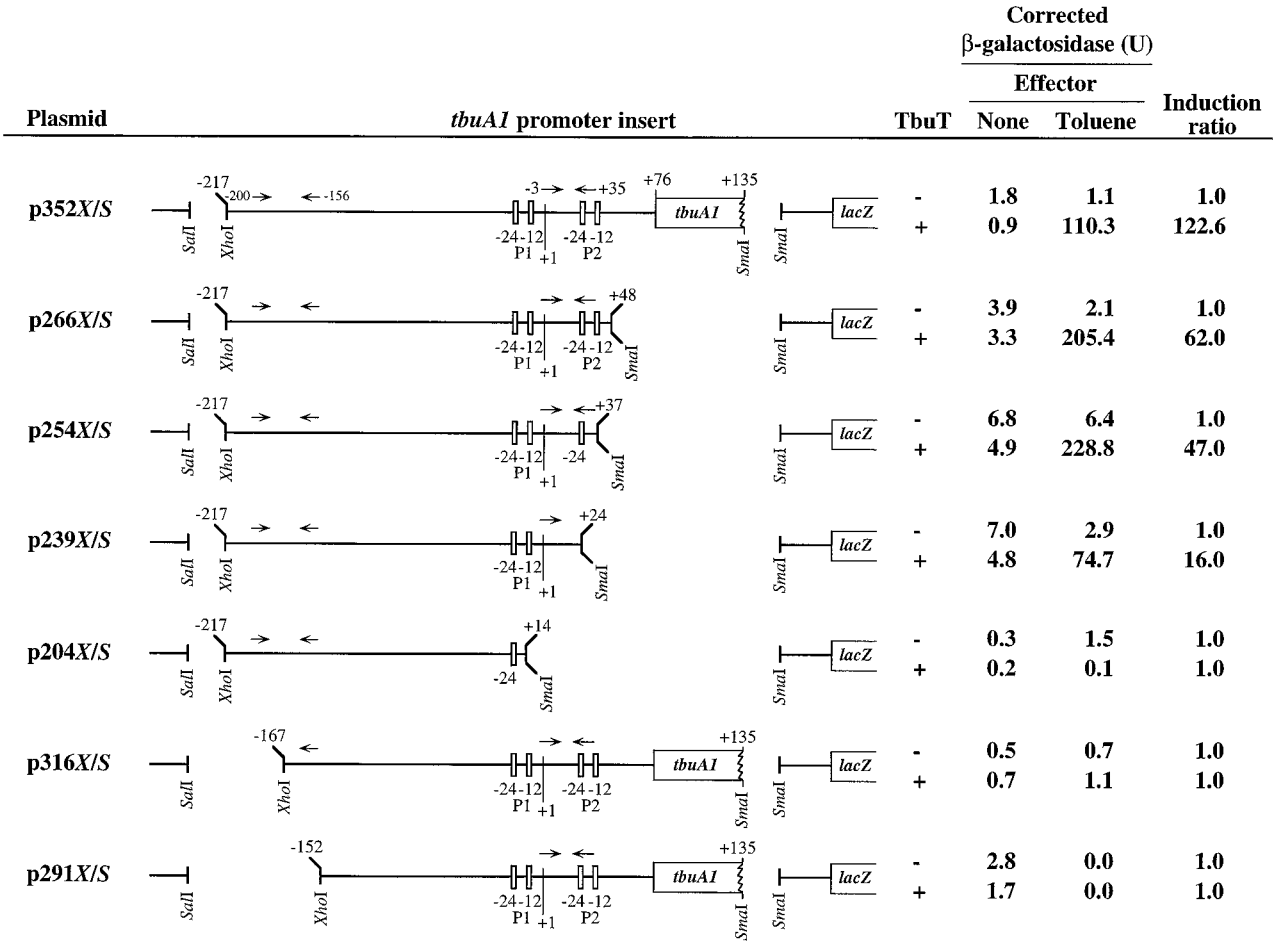

FIG. 2. Deletion analysis of the putative *tbuA1* promoter region. PCR-generated *Xho*I-*Sma*I fragments carrying portions of the putative *tbuA1* promoter region (as shown above) were cloned upstream of the *lacZ* gene (indicated by the open-ended rectangle labeled *lacZ*) of *Sal*I-*Sma*I-digested pKRZ1. The names of the resulting recombinant plasmids are given on the left. Refer to Fig. 1A for more information regarding the exact DNA sequences cloned within the depicted recombinant plasmids. Pertinent landmarks present within each cloned insert are shown. The solid arrows represent the two pairs of inverted repeats, one pair overlapping the P2 promoter sequence and the second pair, with homology to the XylR-binding sequence, immediately downstream of the *Xho*I site. The vertical bars indicate the locations of the −12 and −24 sequences of the putative promoters P1 and P2 (each labeled accordingly). The vertical line labeled +1 indicates the location of the toluene-3-monooxygenase transcriptional start site as determined by primer extension analysis (Fig. 3). The rectangle labeled *tbuA1* represents the location of the incomplete *tbuA1* open reading frame. The numbers above and at the ends of the inserts provide the locations of those landmarks relative to the transcriptional start site. To the right of each insert map are the corresponding β-galactosidase values obtained from *P. aeruginosa* PAO1 cells carrying the shown pKRZ1 derivative in *trans* with pRO1614 (−TbuT) or pRO1614::3.1-kb *tbuT* (+TbuT) and grown in the presence (Toluene) or absence (None) of effector. Test strains were grown at 37°C for 8 h in LB medium and exposed to saturating vapors of toluene. The results are the averages of at least three independent experiments, each conducted with duplicate samples. The corrected values (units) represent values obtained once the background levels of β-galactosidase synthesis obtained from *P. aeruginosa* PAO1(pKRZ1, pRO1614) or *P. aeruginosa* PAO1(pKRZ1, pRO1614::3.1-kb *tbuT*) have been subtracted. Induction ratio indicates the ratio of β-galactosidase observed from toluene-exposed cells versus cells grown without toluene.

regulator-binding site, and (iii) a sequence homologous to the binding site for integration host factor (IHF) (30, 46).

Our search for the invariant −24 GG, −12 GC sequence (11) upstream of *tbuA1* revealed two sequences, designated P1 and P2, 86 and 37 bp upstream from the *tbuA1* gene, respectively (Fig. 1A) (6). Comparisons of P1 and P2 with the −24, −12 promoter sequences from *Pu* and *Po* indicated that P1 had a greater degree of nucleotide sequence homology (Fig. 1B). Upstream of the P1 promoter sequence, a 59-bp DNA sequence with 62.7% identity to a region upstream of *Pu* containing the XylR-binding site (Fig. 1A and C) was also identified (10, 22). This 59-bp region contained an imperfect 13-bp inverted repeat which also showed sequence similarity with the inverted repeat comprising the putative DmpR-binding site upstream of *Po* (Fig. 1C) (46). Finally, a search for the presence of the core IHF-binding sequence (8) upstream of *tbuA1* revealed two sequences, each with two mismatches, overlapping the region homologous with the XylR-binding site.

Deletion analysis of the putative *tbuA1* promoter region. To assist in identifying the toluene-3-monooxygenase promoter(s) as well as elements which might influence promoter expression, we constructed a two-plasmid transcriptional fusion assay system. Seven DNA fragments containing portions of the putative *tbuA1* promoter region were fused to the promoterless *lacZ* gene on the broad-host-range plasmid pKRZ1 (42), yielding p352*X*/*S*, p316*X*/*S*, p291*X*/*S*, p266*X*/*S*, p254*X*/*S*, p239*X*/*S*, and p204*X*/*S* (Fig. 1A). To provide the necessary *trans*-activating function, the 3.1-kb *Eco*RI-*Pvu*II fragment containing the *tbuT* locus was cloned onto a second compatible plasmid, pRO1614 (35), yielding pRO1614::3.1-kb *tbuT*. The pKRZ1 derivatives were then mobilized into *P. aeruginosa* PAO1 strains carrying either pRO1614 or pRO1614::3.1-kb *tbuT*, and

expression was monitored by measuring β-galactosidase levels from cells grown in the presence or absence of the effector, toluene (Fig. 2).

Initially, to ascertain whether a promoter exists upstream of *tbuA1*, we constructed p352*X*/*S*, which contains the 352-bp DNA region shown in Fig. 1A. As expected, promoter activity was observed only from cells containing TbuT and grown in the presence of toluene (Fig. 2, p352*X*/*S*). To determine which promoter sequence P1 or P2, was responsible for the expression observed from p352*X*/*S*, p239*X*/*S* and p204*X*/*S* were constructed (Fig. 1A). Assays conducted with cells carrying these recombinant plasmids revealed that the primary toluene-responsive TbuT-dependent promoter was the P1 promoter. The 32.2% decrease in toluene- and TbuT-dependent promoter activity observed from cells carrying p239*X*/*S* was attributed to either the loss of the P2 promoter sequence or the disruption of a palindromic region overlapping P2. To distinguish between these possibilities, p254*X*/*S*, carrying the palindrome, P1 promoter sequence and an incomplete P2 promoter sequence, was constructed (Fig. 1A). Cells carrying p254*X*/*S* yielded more than two and three times the β-galactosidase levels observed from cells containing p352*X*/*S* and p239*X*/*S*, respectively, when grown in the presence of toluene and containing TbuT (Fig. 2, p254*X*/*S*). Comparable results were obtained from a similar recombinant plasmid, p266*X*/*S* (Fig. 2, p266*X*/*S*), whose insert included the entire P2 promoter sequence as well as a stretch of adenine residues immediately downstream of the inverted repeat (Fig. 1A). From these results, it was evident that the inverted repeat rather than the P2 promoter sequence affected promoter activity.

To assess the possible role of the palindrome, upstream of the P1 promoter sequence, on promoter activity, two more *tbuA1-lacZ* fusions, p316*X*/*S* and p291*X*/*S*, were constructed (Fig. 1A). As expected, loss of one (p316*X*/*S*) or both (p291*X*/*S*) arms of the palindrome resulted in total loss of β-galactosidase synthesis (Fig. 2, p316*X*/*S* and p291*X*/*S*), indicating that this region likely contains the TbuT-binding site.

Determination of the 5′ mRNA start of the toluene-3-monooxygenase operon transcript. To identify the in vivo transcriptional start of the toluene-3-monooxygenase operon transcript, primer extension analyses were performed with total RNA isolated from toluene-induced and uninduced *P. aeruginosa* PAO1 strains carrying pKRZ1 and p352*X*/*S* in the presence of *tbuT* in pRO1614. Two oligonucleotide primers (primer 1 and 2), located 40 bp downstream and 27 bp upstream of the *tbuA1* start codon, respectively, were used. As shown in Fig. 3, the analyses revealed a single toluene-induced transcript with RNA isolated from *P. aeruginosa* PAO1 carrying both p352*X*/*S* and pRO1614::3.1-kb *tbuT* and using primer 1. The same results were obtained for primer 2 (data not shown). Because of the compressions encountered when sequencing this region of DNA (Fig. 3, sequencing ladder), we were unable to determine whether the primer extension product corresponded to a transcriptional start site at either of two C residues located at 75 or 74 bp upstream of the ATG start codon. (For the purposes of clarity when discussing distances within the promoter region relative to the transcriptional start site, we are using the C residue located 75 bp upstream of the *tbuA1* gene as the transcriptional start site.) Nonetheless, these results place the start of the operon transcript at a position consistent with the initiation of transcription from the P1 promoter sequence. These findings also confirm earlier indications from the *tbuA1-lacZ* studies that the P2 promoter sequence is not a functional promoter and that transcription from the P1 promoter depends on the presence of the effector toluene. The P1 promoter sequence will be referred to as *PtbuA1*.

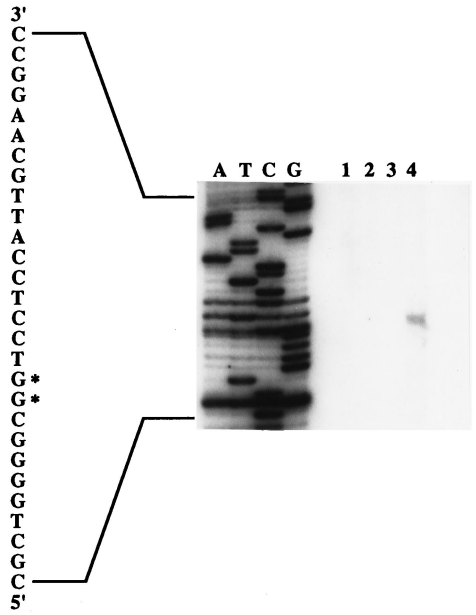

FIG. 3. Determination of the 5′ end of the toluene-3-monooxygenase operon transcript by primer extension analysis. RNA was isolated from *P. aeruginosa* PAO1(pKRZ1, pRO1614::3.1-kb *tbuT*) and *P. aeruginosa* PAO1(p352*X*/*S*, pRO1614::3.1-kb *tbuT*) grown in the absence (lanes 1 and 2, respectively) and presence (lanes 3 and 4, respectively) of toluene. A sequence ladder using the same primer and the pBluescript derivative containing the 352-bp *Xho*I-*Sma*I fragment is also shown (A, T, C, G). To the left, an expanded view of the nucleotide sequence surrounding the transcriptional start site (marked with asterisks) is shown. Refer to Results for further information regarding the two marked transcriptional start sites.

Identification of the *tbuT* gene. Nucleotide sequence analysis of the *tbuT*-encoding region (1,892-bp *Sst*II-*Sma*I fragment) within the 3.1-kb *Eco*RI-*Pvu*II fragment identified a 1,776-bp open reading frame encoding a putative protein with a predicted molecular mass of 65.2 kDa, 253 bp downstream of the stop codon of the *tbuC* gene. Determination of the 5′ end of *tbuT* was based on the identification of a putative ribosome-binding site (5′-GAGGAGA-3′) and the extensive homology of *tbuT* with genes from other organisms (as discussed later). Analysis of the sequence upstream of the putative ribosome-binding site revealed two palindromic regions (12- and 14-bp imperfect inverted repeats); however, no promoter sequences were evident. Downstream of *tbuT*, we identified a 16-bp imperfect inverted repeat which when transcribed could form a stem-loop structure (−21.7 kcal [ca. −90.8 kJ]/mol) that may serve as a potential transcriptional terminator. Similar to the toluene-3-monooxygenase structural genes (6), the *tbuT* coding region is G+C rich (65.4%) and accordingly displays a preferential use of codons with either a G or a C in the third position.

TbuT is a member of the NtrC family of transcriptional activators. Comparison of the deduced amino acid sequence of *tbuT* with translated nucleotide sequence entries in the GenBank database (release 93) showed extensive homology of TbuT with XylR, PhhR, PheR, and DmpR, all members of the NtrC family of transcriptional activators (for a review, see reference 45). DmpR, PhhR, and PheR regulate genes encoding similar phenol hydroxylases involved in the degradation of phenol and (methyl)phenols in *Pseudomonas* sp. strain CF600 (46), *P. putida* P35X (34), and *P. putida* BH (51), respectively, whereas XylR controls the expression of the toluene-xylene catabolic genes from the TOL plasmid of *P. putida* mt-2 (21).

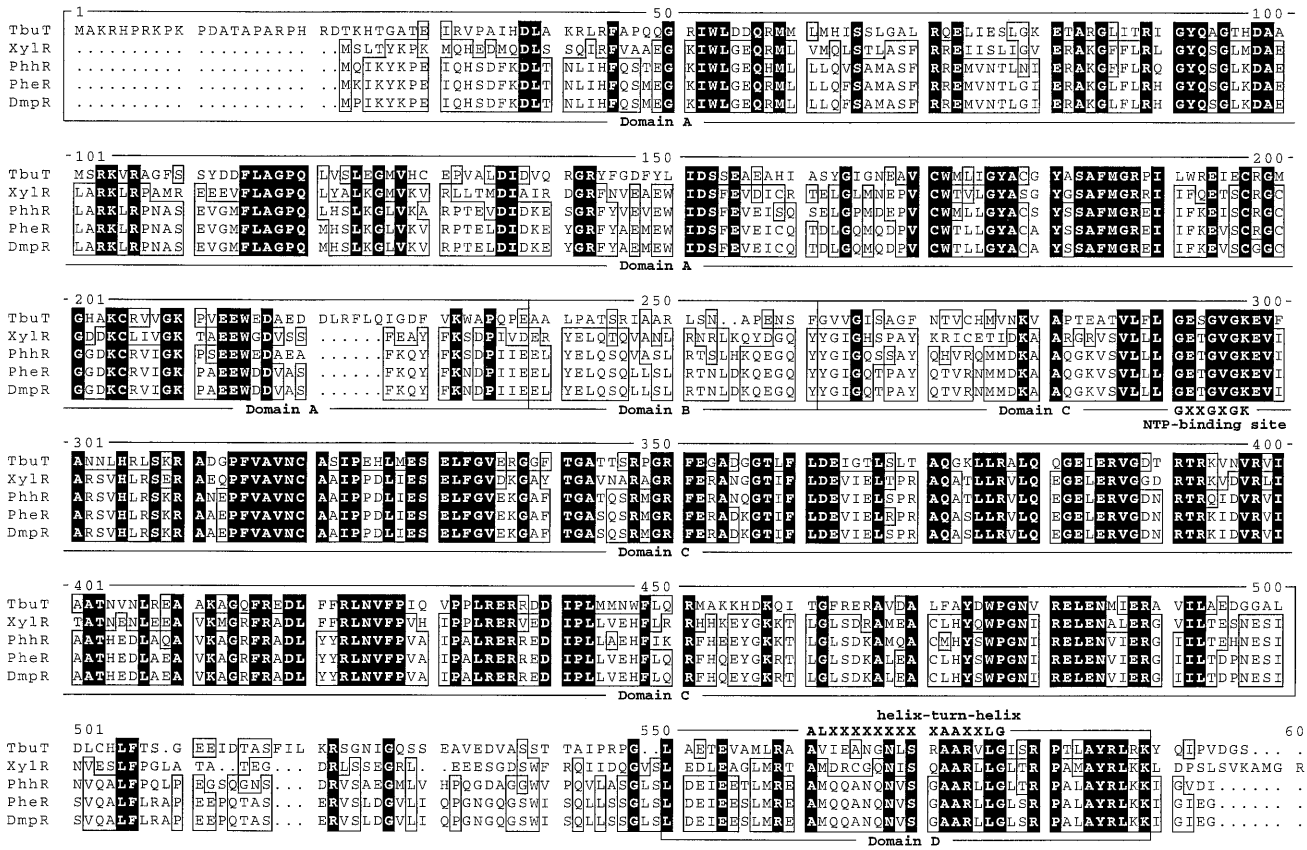

FIG. 4. Multiple sequence alignment of TbuT from *B. pickettii* PKO1, XylR from TOL plasmid from *P. putida* mt-2 (21), PheR from *P. putida* BH (GenBank accession number D63814 [51]), PhhR from *P. putida* P35X (34), and DmpR from *Pseudomonas* sp. strain CF600 (pVI150) (46). The amino acid residues conserved in all five sequences are indicated by white letters on a black background. Amino acid residues which are shared by three or four sequences are boxed. Gaps are represented by periods and were introduced to maximize the alignment. The locations and boundaries of the four domains (described in the text) are outlined and labeled accordingly. The locations of the putative nucleoside triphosphate (NTP)-binding site and helix-turn-helix motif along with their respective consensus sequences are also shown (32).

Pairwise comparisons between the overall amino acid sequences of these regulators revealed TbuT (592 amino acids) to be distinct, showing 47.6 to 48.1% identity with members of the group of phenol hydroxylase regulators, DmpR, PhhR, and PheR, and 45.0% identity with XylR. This was in contrast to the 85.5 to 99.5% identity among DmpR, PhhR, and PheR (each 563 amino acids). XylR (566 amino acids) had 66.8 to 67.9% identity with the phenol hydroxylase regulators.

Detailed examination of the regions of amino acid sequence homology shared by these peptides by multiple sequence alignment analysis, using PILEUP, revealed localized areas of identity corresponding to the functional domains characteristic of members of the NtrC family (Fig. 4). NtrC-like activators are modular in structure, composed of three functionally distinct domains: a carboxy-terminal domain (domain D) containing the site for DNA binding, a central domain (domain C) responsible for activation of transcription, and an amino-terminal domain (domain A) believed to be the target for specific regulatory signals (32). Domains A and C are joined by a flexible minor domain, referred to as domain B (Q-linker). A schematic of the modular organization of each of the five activators along with the percent identities obtained from pairwise comparisons of the amino acid sequence of each domain with the corresponding domain from TbuT is shown in Fig. 5.

Effector specificity of TbuT. Among members of the NtrC family, domain A is the most divergent since it acts as the signal reception module for specific chemical signals (32). In the case of XylR and DmpR, domain A appears to govern the effector specificity of the regulator by interacting directly with specific aromatic effectors (9, 37, 47). In view of the differences between the primary structure of the A domain of TbuT and those of XylR, DmpR, PhhR, and PheR, we set out to determine whether these variances were reflected in the effector specificity for TbuT. The responsiveness of TbuT to a specific effector was monitored by measuring β-galactosidase activity from *P. aeruginosa* PAO1 carrying p352*X*/*S* and pRO1614:: 3.1-kb *tbuT*, grown in the presence or absence of effector. The results from these experiments (Fig. 6) suggest the following TbuT-effector interactions: (i) an unsubstituted aromatic ring provides the contacts necessary to activate TbuT; (ii) an alkyl group ($-CH_3$ or $-CH_2CH_3$) or hydroxyl group at carbon 1 of benzene can be accommodated; (iii) alkyl substitution at carbon 2 or hydroxyl substitutions at carbons 2 and 3 of toluene is allowed (however, the closer the substitution is to the methyl group of toluene, the more efficient the activation of TbuT); and (iv) a $-CH_2OH$ or $-CHO$ group at carbon 1 of benzene can also be accommodated, albeit very poorly. Prompted by earlier observations regarding TCE induction of toluene- and TCE-oxidizing activity in *B. pickettii* PKO1 (29), we included TCE among the hydrocarbons tested and confirmed TCE's ability to promote significant levels of transcription from *PtbuA1* compared with the levels obtained from toluene.

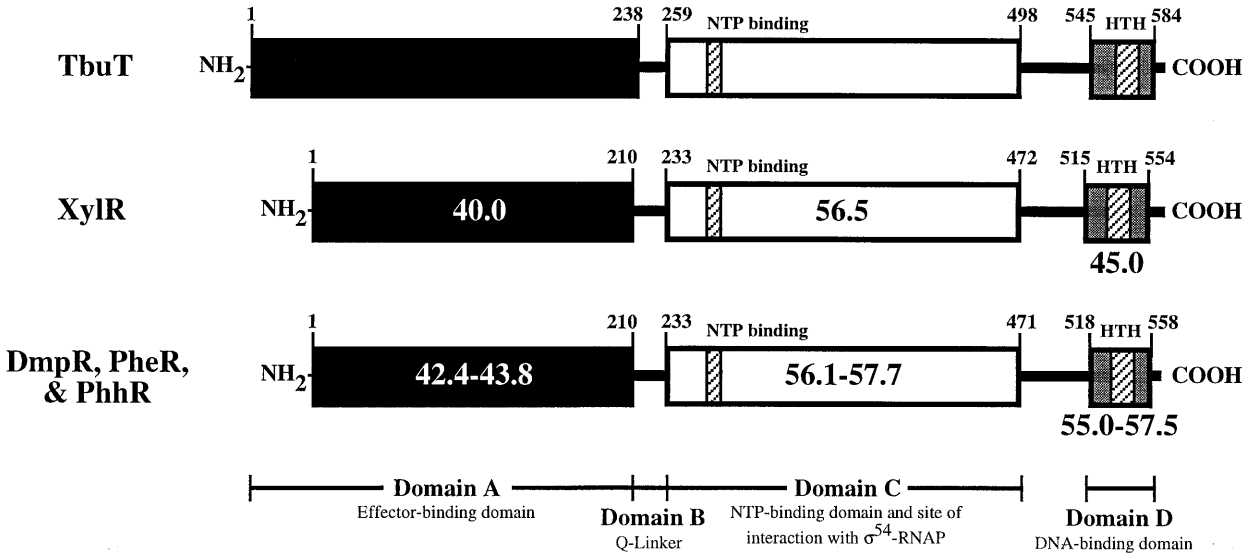

FIG. 5. Schematic illustration of the modular organization of the TbuT, XylR, PheR, PhhR, and DmpR proteins. Because of the significant identity among DmpR, PheR, and PhhR, their figures were combined. The rectangles delineate the locations and lengths of domains A, C, and D within each protein. The coordinates shown above each domain represent the locations of the first and last amino acid residues in domains A, C, and D. The smaller rectangles filled in with diagonal lines represent the locations of the nucleoside triphosphate (NTP)-binding site and helix-turn-helix motif (HTH). The identities and presumed functions of each of the domains are shown at the bottom. The percent identities obtained from pairwise comparisons of the amino acid sequence of each domain with the corresponding domain from TbuT are shown within the domains. In the case of domain D, these numbers are shown directly below the domain.

Readthrough transcription from the *tbuA1* promoter drives *tbuT* expression. Given the unusual arrangement of a regulatory gene downstream of the operon that it controls, and the notable absence of a promoter sequence immediately upstream of *tbuT*, we were interested in determining whether readthrough transcription from the *tbuA1* promoter would reach *tbuT*. To test this idea, the 5.5-kb *Xho*I fragment containing *PtbuA1*, the entire toluene-3-monooxygenase operon, and the first 375 nt of the *tbuT* gene was cloned upstream of the promoterless *lacZ* gene of pKRZ1. This fragment was cloned in both orientations; orientation I (designated p5.5*X*I-1) had the operon transcribed in the direction of the *lacZ* gene, whereas in the opposite orientation (designated p5.5*X*I-2), the operon was transcribed away from the *lacZ* gene (Fig. 7). Once again, expression from the operonic-*lacZ* fusions was monitored by measuring β-galactosidase levels in *P. aeruginosa*

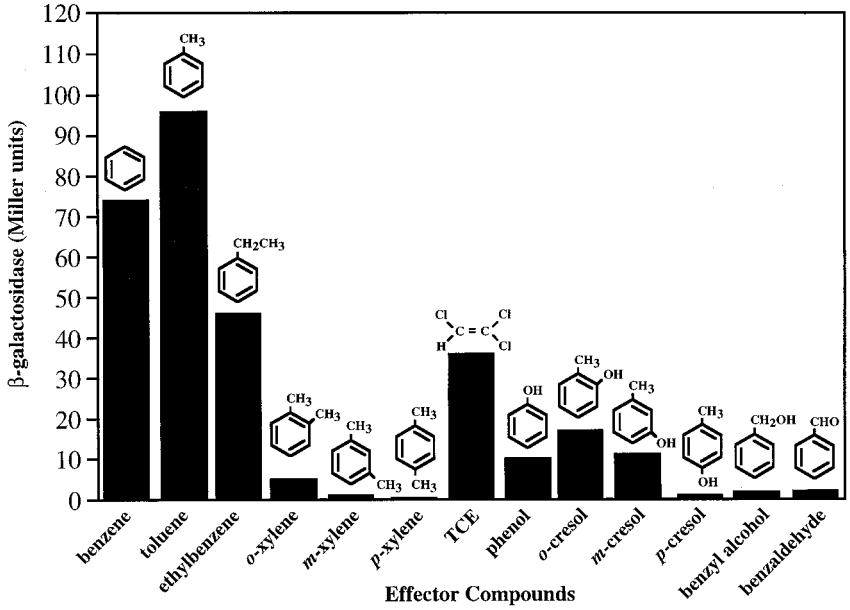

FIG. 6. Activation of the *PtbuA1* promoter by TbuT in response to the presence of hydrocarbons. *P. aeruginosa* PAO1 cells containing pRO1614::3.1-kb *tbuT* and the *PtbuA1-lacZ* transcriptional fusion plasmid p352*X*/*S* were grown and treated as described in Materials and Methods. The bar diagram represents the accumulation of β-galactosidase after 8 h of exposure of the cultures to the different effectors. Values are the averages of duplicate determinations from three independent experiments. The variability between triplicate values did not exceed 10%. The chemical structures of the tested compounds are shown above their respective bars.

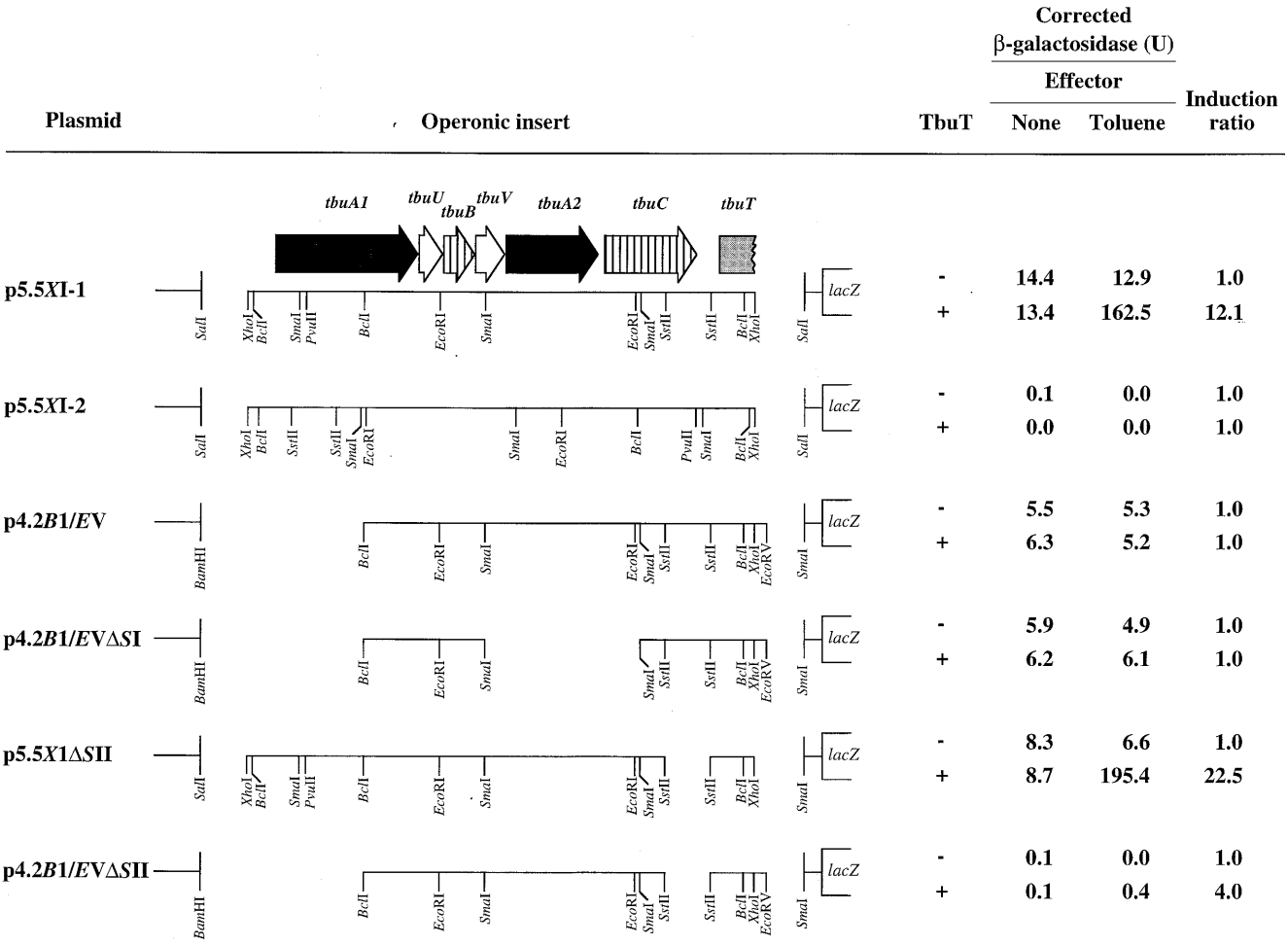

FIG. 7. Analysis of the influence of *PtbuA1* on the expression of *tbuT*. Restriction maps of the fragments cloned upstream of the *lacZ* gene of KRZ1 (indicated by the open-ended rectangle labeled *lacZ*) and used in this study to measure readthrough transcription from the *tbuA1* promoter into the *tbuT* gene are shown. The names of the resulting recombinant plasmids are given on the left. The locations and orientations of the *tbuA1*, *tbuU*, *tbuB*, *tbuV*, *tbuA2*, and *tbuC* genes are indicated by the arrows above the restriction maps. Also shown is the portion of the *tbuT* gene remaining on the cloned fragments (rectangle with frayed edge). To the right of each insert map are the corresponding β-galactosidase values obtained from *P. aeruginosa* PAO1 cells carrying the shown pKRZ1 derivative in *trans* with pRO1614 (−TbuT) or pRO1614::3.1-kb *tbuT*Δ0.5-kb *Sst*II (+TbuT) and grown in the presence (Toluene) or absence (None) of effector. Test strains were grown at 37°C for 8 h in LB medium and exposed to saturating vapors of toluene. The results are the averages of at least three independent experiments, each of which was conducted with duplicate samples. The corrected values (units) represent values obtained once the background levels of β-galactosidase synthesis obtained from *P. aeruginosa* PAO1(pKRZ1, pRO1614) or *P. aeruginosa* PAO1(pKRZ1, pRO1614::3.1-kb *tbuT*Δ0.5-kb *Sst*II) have been subtracted. Induction ratio indicates the ratio of β-galactosidase observed from toluene-exposed cells versus cells grown without toluene.

PAO1 in both the presence and absence of *tbuT* in pRO1614 and toluene. In these experiments, however, a different *tbuT*-containing pRO1614 derivative, designated pRO1614::3.1-kb *tbuT*Δ0.5-kb *Sst*II, was used. This pRO1614 derivative, in which the 0.5-kb *Sst*II fragment upstream of *tbuT* was deleted, was constructed to minimize the possibility of homologous recombination occurring between the operonic inserts cloned into pKRZ1 and the fragment containing *tbuT* cloned in pRO1614.

Assays performed with cells carrying both p5.5*X*I-1 and TbuT showed a 12-fold induction of β-galactosidase synthesis when the cells were exposed to toluene (Fig. 7). To determine whether this toluene-inducible TbuT-dependent activity was driven by *PtbuA1*, a new operonic construct with *PtbuA1* deleted was created. This was achieved by cloning the 4.2-kb *Bcl*I-*Eco*RV fragment into pKRZ1, resulting in the recombinant plasmid p4.2*B*I/*E*V. This construct produced low levels of β-galactosidase expression with no toluene-responsive TbuT-dependent induction (Fig. 7). These results indicate that in the presence of toluene, expression of *tbuT* is driven, at least in part, by readthrough transcription from the TbuT cognate promoter *PtbuA1*.

In addition to the inducible expression observed from the previous assays, low levels of β-galactosidase synthesis were also seen independent of the *PtbuA1* promoter, TbuT, or toluene (Fig. 7, p5.5*X*I-1 and p4.2*B*I/*E*V). To localize a possible origin of this promoter activity, an internal 1.7-kb *Sma*I fragment was deleted from p4.2*B*I/*E*V, yielding the recombinant plasmid p4.2*B*I/*E*VΔ*S*I. When this construct was tested, β-galactosidase levels remained the same as had been observed previously with p4.2*B*I/*E*V (Fig. 7). The deletion of a 0.5-kb *Sst*II fragment from p4.2*B*I/*E*V, yielding p4.2*B*I/*E*VΔ*S*II, however, resulted in a complete loss of β-galactosidase activity, pointing toward the possible existence of a weak promoter within this fragment that is unresponsive to TbuT. When the same *Sst*II fragment was deleted from p5.5*X*I-1, yielding p5.5*X*IΔ*S*2, β-galactosidase activity was observed from cells grown in the ab-

sence of toluene or not carrying TbuT; however, these levels were lower than those observed from p5.5*X*I-1. These results indicate that in addition to driving inducible *tbuT* expression in the presence of toluene, *PtbuA1* appears to promote significant low levels of *tbuT* expression in the absence of effector. Finally, no detectable levels of β-galactosidase synthesis were obtained from cells carrying p5.5*X*I-2, indicating that a promoter reading in the opposite direction to that of *PtbuA1* probably does not exist within the 5.5-kb *Xho*I fragment.

DISCUSSION

In this report we have described a unique regulatory scheme for the toluene-3-monooxygenase operon from *B. pickettii* PKO1 centered around the unusual role of the toluene-3-monooxygenase promoter, *PtbuA1*, in the expression of the cognate regulatory gene, *tbuT*. The results from the operonic-*lacZ* fusion studies revealed that the *PtbuA1* promoter was able to drive *tbuT* gene expression by readthrough transcription. As a consequence, toluene-induced activation of the *PtbuA1* promoter by TbuT leads to increased synthesis not only of toluene-3-monooxygenase but also of TbuT. Moreover, in the absence of anything that would limit readthrough transcription, the activation of *PtbuA1* would presumably result in continued elevated synthesis of TbuT as long as effector was present. This highly unusual relationship between regulatory gene and its cognate promoter differs significantly from what is described for the TbuT homologs XylR and DmpR. Both the *xylR* and *dmpR* genes are divergently transcribed from their cognate promoters, *Ps* (a second XylR-responsive σ^{54}-dependent promoter from the TOL plasmid) (30) and *Po* (46), respectively. In the case of *xylR*, this arrangement has permitted the aromatic effector-induced activation of the *Ps* promoter by XylR to be coupled with the simultaneous repression of *xylR* transcription from its own promoter, *Pr* (18, 20). Although less is known about the expression of the *dmpR* gene, the divergent organization of the *dmpR* promoter and *Po* similarly precludes readthrough from *Po* as a means of driving *dmpR* expression.

This genetic organization which allows for regulatory gene expression by readthrough transcription from its cognate promoter is novel. Although two similarly arranged regulons have been identified in *E. coli*, in both examples, transcriptional termination is used to curtail the amount of readthrough transcription reaching the regulatory gene. In the first case, the *kdpABC* operon, which encodes a K^+ transport complex, is followed by the *kdpDE* operon, encoding the sensor kinase/response regulator proteins (40, 55; for a review, see reference 3). Activation of the promoter upstream of the *kdpA* gene by the phosphorylated KdpE protein results in increased *kdpDE* expression by readthrough transcription. A significant fraction of the upstream transcripts, however, end in *kdpD* by an unknown mechanism of transcriptional termination. Similarly arranged, the *glnA* gene, encoding the glutamine synthetase, is directly followed by the *glnL* and *glnG* genes, which encode the sensor kinase/response regulator proteins controlling *glnA* expression (53; for a review, see reference 49). In this case, activation of the promoter upstream of *glnA* by the phosphorylated *glnG* gene product results in elevated expression of the *glnLG* genes via readthrough transcription, although in this instance, a rho-independent transcriptional terminator serves to block a significant portion of these transcripts.

Examination of the nucleotide sequence between *tbuC* and *tbuT* for any rho-independent terminators which could serve to block readthrough transcription revealed none. Interestingly though, a second putative TbuT-binding site overlapping the 3′ end of the *tbuC* gene was discovered. Deletion of DNA containing this second site from the pRO1614 derivative carrying *tbuT* resulted in increased levels of promoter activity from a *tbuA1-lacZ* fusion (7). These results suggest that this DNA region, possibly through the binding of TbuT in the absence of effector, may serve to attenuate readthrough transcription from *PtbuA1* through some unique mechanism.

In both the *kdpABCDE* and *glnALG* operons, there also exist weak promoters immediately upstream of the regulatory genes which provide the necessary basal levels of regulatory protein to activate transcription when inducing conditions are initially encountered (40, 53, 55). The ability to provide TbuT in *trans* from the *Eco*RI-*Pvu*II fragment used in this study suggests that this may be true in our system as well. Although there exists evidence to suggest that a vector promoter may also be responsible for *tbuT* expression from the pRO1614 constructs (4), the low levels of uninduced β-galactosidase activity obtained from the operonic-*lacZ* fusion studies also suggest the possible existence of a weak promoter within the *Sst*II fragment upstream of *tbuT*. Although a review of the nucleotide sequence within this area has not produced likely promoter sequences, we are continuing our work to define this putative promoter. More importantly, these results also indicate that in addition to driving *tbuT* expression under toluene-induced conditions, a leaky *PtbuA1* promoter appears to promote *tbuT* expression in the absence of effector, by readthrough transcription as well.

Central to our studies regarding the regulation of the toluene-3-monooxygenase operon has been our detailed characterization of the toluene-3-monooxygenase promoter, *PtbuA1*. Consistent with previous observations, we have shown that expression from *PtbuA1* is dependent on the presence of the regulatory protein, TbuT, and the aromatic effector toluene. Furthermore we have shown that *PtbuA1* expression is dependent on the alternative sigma factor, σ^{54}, by analyzing TbuT-mediated transcription from *PtbuA1* in both $RpoN^-$ and $RpoN^+$ backgrounds (7). The results from our deletion analyses suggest that activation of *PtbuA1* is dependent on a DNA region 170 bp upstream of the transcriptional start site. This region is part of a broader area of DNA showing extensive nucleotide sequence homology to the palindromic regions upstream of *Pu* and *Po*, to which in the case of *Pu*, the transcriptional activator XylR has been shown to bind (10). The sequences upstream of the *Pu* promoter recognized by XylR include the motif 5′-TTGANCAAATC-3′ (10). This sequence is present within each arm ([−200] 5′-TTGATGAAATC-3′ [−190] and [−156] 5′-TTGATCAGATC-3′ [−166]) of the 13-bp inverted repeat upstream of *PtbuA1*. Thus, as is the case with *Pu* and presumably *Po*, upstream palindromic sequences are required for activation of *PtbuA1* and likely act as the target site for activator binding. Furthermore, the similarities of the palindromic regions suggest that XylR and DmpR may be able to activate *PtbuA1* expression as has been observed with the cross-regulation of *Pu* and *Po* by both XylR and DmpR (14).

In a subset of σ^{54}-dependent promoters, an IHF-binding site is found between the activator-binding site and the −12, −24 promoter sequence (28). The binding of IHF to this site, presumably, facilitates the formation of a DNA loop which enables the upstream-bound activator protein and the promoter-bound RNA polymerase-σ^{54} holoenzyme to interact. As a result, the specificity and the activity of the promoter are thought to be enhanced. A functional IHF-binding site meeting these criteria is found in *Pu* (1, 10, 39). Upstream of *Po*, a putative IHF-binding site has also been identified; however, the location of this sequence is unusual, overlapping the putative DmpR-binding site (46). Nevertheless, maximal expres-

sion of *Po* has also been shown to be IHF dependent (50). In the case of *PtbuA1*, two putative IHF sites have also been identified, overlapping the putative TbuT-binding site. However, in view of the limited homology of these two sequences with the IHF consensus core sequence, it is unclear whether IHF plays a role in *PtbuA1* expression. The roles of sequence-directed and protein-assisted DNA bending have recently been examined in the other XylR-responsive σ^{54}-dependent promoter, *Ps*, from the TOL plasmid (38). *Ps* promoter activity was shown to be dependent on the combined effects of intrinsically curved DNA and HU protein-mediated DNA bending to achieve the promoter architecture required for transcription. Although IHF and HU proteins are homologs, HU protein does not bind to specific DNA sequences but prefers curved (52) or kinked (41) DNA or DNA containing several dA stretches. Since the *PtbuA1* promoter region contains several dA stretches, it is plausible that *PtbuA1* is similar to *Ps*, relying on both the intrinsic curvature of promoter DNA and a nonspecific DNA-bending protein, such as HU, to achieve the promoter architecture required for transcription.

A notable difference regarding the organization of the *PtbuA1*, *Pu*, and *Po* promoters is the presence of an inverted repeat downstream of *PtbuA1*. Unlike the *Pu* and *Po* transcriptional start sites, which are 29 and 18 bp upstream of the *xylC* and *dmpK* genes, respectively, the *PtbuA1* transcriptional start site is 74 bp upstream of *tbuA1*. Within this 74-bp region, we discovered a 15-bp inverted repeat which was absent downstream of *Pu* and *Po*. Data from the *PtbuA1-lacZ* fusions revealed that deletion of DNA containing one arm of the inverted repeat (p239*X*/*S*) resulted in a 32% decrease in TbuT- and toluene-dependent promoter activity. A smaller deletion in which the inverted repeat was preserved (p254*X*/*S*) surprisingly yielded more than three times the activity observed from p239*X*/*S*. Comparisons of the induction ratios from cells carrying TbuT with p352*X*/*S*, p266*X*/*S*, p254*X*/*S*, or p239*X*/*S* revealed that as progressive deletions were made distal to *PtbuA1*, the induction ratio steadily decreased. This trend appears to be the result of increasing promoter activities obtained from cells grown in the absence of toluene and likely reflects a leakiness of the promoter due to changes in the architecture of the promoter. It is, however, unclear whether the toluene- and TbuT-dependent promoter activities from these constructs also reflect a change in promoter geometry or suggest a more prominent role for the inverted repeat.

Our analysis of the regulator, TbuT, revealed strong homology to the NtrC class of transcriptional activators. The proteins of this family have been divided into domains based on the extensive studies of NtrC and NifA (13). Comparison of the domains of TbuT with those of XylR, DmpR, PhhR, and PheR revealed domain C to be the most highly conserved domain, exhibiting between 56.1 and 57.7% identity. This domain is believed to contain the site of interaction between the regulator and the RNA polymerase containing σ^{54}, the site for binding and hydrolysis of ATP (G-X_2-GXGK [Fig. 2]), and the oligomerization determinants necessary for transcription initiation. The next most highly conserved region is the carboxy-terminal domain D (45.0 to 57.5% identity), which contains the helix-turn-helix DNA-binding motif (AL-X_9-AA-X_2-LG) (32). The amino-terminal A domain, which in the case of XylR and DmpR appears to interact directly with aromatic effectors, is the least conserved of the three major domains for TbuT, exhibiting only 40.0 to 43.8% amino acid sequence identity with corresponding regions in XylR, PhhR, PheR, and DmpR. The A domain of TbuT is also 28 amino acids longer than its counterparts, with the first 20 amino acids encompassing a unique proline-rich region.

This divergence of the A domains prompted us to analyze the effector specificity of TbuT. These studies revealed that unlike DmpR, TbuT can be activated by toluene as well as phenols and cresols, whereas unlike XylR, TbuT does not respond well to the xylenes. These data support our assumption that the divergence of the amino acid sequence of domain A of TbuT has resulted in different abilities to accommodate and respond to various hydrocarbons. Among the hydrocarbons tested, TCE provided the most surprising result by promoting significant levels of transcription from *PtbuA1* compared with the levels obtained from toluene. To our knowledge, this is the first example of an isolated regulatory gene whose gene product responds to TCE. These results provide further evidence in support of earlier observations regarding TCE induction of toluene- and TCE-oxidizing activity in *B. pickettii* PKO1 (29).

ACKNOWLEDGMENTS

This research was supported by National Institute of Environmental Health Sciences Superfund research and educational grant ES-04911. DNA sequence analyses were supported in part by the General Clinical Research Center at the University of Michigan, funded by grant M01RR00042 from the National Center for Research Resources, National Institutes of Health.

We thank Ananda Chakrabarty for providing plasmid pKRZ1. The assistance of Deborah Jaworski in technical assistance with PCR is gratefully acknowledged.

REFERENCES

1. **Abril, M. A., M. Buck, and J. L. Ramos.** 1991. Activation of the *Pseudomonas* TOL plasmid *upper* pathway operon: identification of binding sites for the positive regulator XylR and for integration host factor protein. J. Biol. Chem. **266:**15832–15838.
2. **Abril, M. A., C. Michan, K. N. Timmis, and J. L. Ramos.** 1989. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol. **171:**6782–6790.
3. **Altendorf, K., P. Voelkner, and W. Puppe.** 1994. The sensor kinase KdpD and the response regulator KdpE control expression of the *kdpFABC* operon in *Escherichia coli*. Res. Microbiol. **145:**374–381.
4. **Brosius, J., R. L. Cate, and A. P. Perlmutter.** 1982. Precise location of two promoters for the beta-lactamase gene of pBR322: S1 mapping of ribonucleic acid isolated from *Escherichia coli* or synthesized in vitro. J. Biol. Chem. **257:**9205–9210.
5. **Byrne, A. M., J. J. Kukor, and R. H. Olsen.** 1993. Sequence analysis of the BTEX-degradative *tbuABC* operon of *Pseudomonas pickettii* PKO1 functional under oxygen limited conditions, abstr. K-81, p. 274. *In* Abstracts of the 93th General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.
6. **Byrne, A. M., J. J. Kukor, and R. H. Olsen.** 1995. Sequence analysis of the gene cluster encoding toluene-3-monooxygenase from *Pseudomonas pickettii* PKO1. Gene **154:**65–70.
7. **Byrne, A. M., and R. H. Olsen.** Unpublished data.
8. **Craig, N. L., and H. A. Nash.** 1984. *E. coli* integration host factor binds to specific sites in DNA. Cell **39:**707–716.
9. **Delgado, A., and J. L. Ramos.** 1994. Genetic evidence for activation of the positive transcriptional regulator XylR, a member of the NtrC family of regulators, by effector binding. J. Biol. Chem. **269:**8059–8062.
10. **de Lorenzo, V., M. Herrero, M. Metzke, and K. N. Timmis.** 1991. An upstream XylR- and IHF-induced nucleoprotein complex regulates the σ^{54}-dependent *Pu* promoter of TOL plasmid. EMBO J. **10:**1159–1167.
11. **Deretic, V., W. M. Konyecsni, C. D. Mohr, D. W. Martin, and N. S. Hibler.** 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technology **7:**1249–1254.
12. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
13. **Drummond, M., P. Whitty, and J. Wooton.** 1986. Sequence domain relationships of *ntrC* and *nifA* from *Klebsiella pneumoniae*: homologies to other regulatory proteins. EMBO J. **5:**441–447.
14. **Fernández, S., V. Shingler, and V. de Lorenzo.** 1994. Cross-regulation by XylR and DmpR activators of *Pseudomonas putida* suggests that transcriptional control of biodegradative operons evolves independently of catabolic genes. J. Bacteriol. **176:**5052–5058.
15. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
16. **Francesconi, S. C., A. C. Blake, and M. S. Shields.** 1995. Nucleotide se-

quence, organization, and regulation of the toluene ortho-monooxygenase operon of *Pseudomonas cepacia* G4 and its constitutive variants, abstr. K-198, p. 570. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
17. **Genetics Computer Group.** 1994. Program manual for the Wisconsin GCG package, version 8, September 1994. Genetics Computer Group, Madison, Wis.
18. **Gomada, M., S. Inouye, H. Imaishi, A. Nakazawa, and T. Nakazawa.** 1992. Analysis of an upstream regulatory sequence required for activation of the regulatory gene *xylS* in xylene metabolism directed by the TOL plasmid of *Pseudomonas putida*. Mol. Gen. Genet. **233:**419–426.
19. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–136. *In* D. M. Glover (ed.), The practical approach, vol. 1. DNA cloning. IRL Press, Ltd., Oxford.
20. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1987. Expression of the regulatory gene *xylS* on the TOL plasmid is positively controlled by the *xylR* gene product. Proc. Natl. Acad. Sci. USA **84:**5182–5186.
21. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1988. Nucleotide sequence of the regulatory gene *xylR* of the TOL plasmid from *Pseudomonas putida*. Gene **66:**301–306.
22. **Inouye, S. A., M. Gomada, U. M. X. Sangodkar, A. Nakazawa, and T. Nakazawa.** 1990. Upstream regulatory sequences for transcriptional activator XylR in the first operon of xylene metabolism of the TOL plasmid. J. Mol. Biol. **216:**251–260.
23. **Johnson, G. R.** 1995. Cloning and characterization of genes encoding toluene monooxygenases from *Pseudomonas* sp. strain JS150. Ph.D. dissertation. University of Michigan, Ann Arbor.
24. **Johnson, G. R., and R. H. Olsen.** 1995. Nucleotide sequence analysis of genes encoding a toluene/benzene-2-monooxygenase from *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. **61:**3336–3346.
25. **Kaphammer, B., J. J. Kukor, and R. H. Olsen.** 1990. Cloning and characterization of a novel toluene degradative pathway from *Pseudomonas pickettii* PKO1, abstr. K-145, p. 243. *In* Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990. American Society for Microbiology, Washington, D.C.
26. **Kukor, J. J., and R. H. Olsen.** 1991. Genetic organization and regulation of a *meta* cleavage pathway for catechols produced from catabolism from toluene, benzene, phenol, and cresols by *Pseudomonas pickettii* PKO1. J. Bacteriol. **173:**4587–4594.
27. **Kukor, J. J., and R. H. Olsen.** 1996. Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. Appl. Environ. Microbiol. **62:**1728–1740.
28. **Kustu, S., A. K. North, and D. S. Weiss.** 1991. Prokaryotic transcriptional enhancers and enhancer binding proteins. Trends Biochem. Sci. **16:**397–402.
29. **Leahy, J. G., A. M. Byrne, and R. H. Olsen.** 1996. Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. Appl. Environ. Microbiol. **62:**825–833.
30. **Marqués, S., and J. L. Ramos.** 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Mol. Microbiol. **9:**923–929.
31. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Morett, E., and L. Segovia.** 1993. The σ^{54} bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J. Bacteriol. **175:**6067–6074.
33. **Newman, L. M., and L. P. Wackett.** 1995. Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry **34:**14066–14076.
34. **Ng, L. C., C. L. Poh, and V. Shingler.** 1995. Aromatic effector activation of the NtrC-like transcriptional regulator PhhR limits the catabolic potential of the (methyl)phenol degradative pathway it controls. J. Bacteriol. **177:**1485–1490.
35. **Olsen, R. H., G. DeBusscher, and W. R. McCombie.** 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO1 chromosome. J. Bacteriol. **150:**60–69.
36. **Olsen, R. H., J. J. Kukor, and B. Kaphammer.** 1994. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J. Bacteriol. **176:**3749–3756.
37. **Pavel, H., M. Forsman, and V. Shingler.** 1994. An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of *Pseudomonas* sp. strain CF600 on *para*-substituted methylphenols. J. Bacteriol. **176:**7550–7557.
38. **Pérez-Martín, J., and V. de Lorenzo.** 1995. The σ^{54}-dependent promoter *Ps* of the TOL plasmid of *Pseudomonas putida* requires HU for transcriptional activation in vivo by XylR. J. Bacteriol. **177:**3758–3763.
39. **Pérez-Martín, J., K. N. Timmis, and V. de Lorenzo.** 1994. Co-regulation by bent DNA. J. Biol. Chem. **269:**22657–22662.
40. **Polarek, J. W., G. Williams, and W. Epstein.** 1992. The products of the *kdpDE* operon are required for expression of the Kdp ATPase of *Escherichia coli*. J. Bacteriol. **174:**2145–2151.
41. **Pontiggia, A., A. Negri, M. Baltrame, and M. E. Bianchi.** 1993. Protein HU binds specifically to kinked DNA. Microbiology **7:**343–350.
42. **Rothmel, R. K., D. L. Shinabarger, M. R. Parsek, T. L. Aldrich, and A. M. Chakrabarty.** 1991. Functional analysis of the *Pseudomonas putida* regulatory protein CatR: transcriptional studies and determination of the CatR DNA-binding site by hydroxyl-radical footprinting. J. Bacteriol. **173:**4717–4724.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
45. **Shingler, V.** 1996. Signal sensing by σ^{54}-dependent regulators: derepression as a control mechanism. Mol. Microbiol. **19:**409–416.
46. **Shingler, V., M. Bartilson, and T. Moore.** 1993. Cloning and nucleotide sequence of the gene encoding the positive regulator (DmpR) of the phenol catabolic pathway encoded by pVI150 and identification of DmpR as a member of the NtrC family of transcriptional activators. J. Bacteriol. **175:**1596–1604.
47. **Shingler, V., and T. Moore.** 1994. Sensing aromatic compounds by DmpR transcriptional activator of phenol-catabolizing *Pseudomonas* sp. strain CF600. J. Bacteriol. **176:**1555–1560.
48. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
49. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
50. **Sze, C. C., T. Moore, and V. Shingler.** 1996. Growth phase-dependent transcription of the σ^{54}-dependent Po promoter controlling the *Pseudomonas*-derived (methyl)phenol *dmp* operon of pVI150. J. Bacteriol. **178:**3727–3735.
51. **Takeo, M.** 1995. Unpublished data.
52. **Tanaka, I., K. Appelt, J. Dijk, S. White, and K. Wilson.** 1984. 3-A resolution structure of a protein with histone-like properties in prokaryotes. Nature (London) **310:**376–381.
53. **Ueno-Nishio, S., S. Mango, L. J. Reitzer, and B. Magasanik.** 1984. Identification and regulation of the *glnL* operator-promoter of the complex *glnALG* operon of *Escherichia coli*. J. Bacteriol. **160:**379–384.
54. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
55. **Walderhaug, M. O., J. W. Polarek, P. Voelkner, J. M. Daniel, J. E. Hesse, K. Altendorf, and W. Epstein.** 1992. KdpD and KdpE, proteins that control expression of the *kdpABC* operon, are members of the two-component sensor-effector class of regulators. J. Bacteriol. **174:**2152–2159.
56. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124:**7–13.
57. **Yen, K.-M., and M. R. Karl.** 1992. Identification of a new gene, *tmoF*, in the *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. J. Bacteriol. **174:**7253–7261.
58. **Yen, K.-M., M. R. Karl, L. M. Blatt, M. J. Simon, R. B. Winter, P. R. Fausset, H. S. Lu, A. A. Harcourt, and K. K. Chen.** 1991. Cloning and characterization of a *Pseudomonas mendocina* KR gene cluster encoding toluene-4-monooxygenase. J. Bacteriol. **173:**5315–5327.