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Cross-Regulation of Toluene Monooxygenases by the Transcriptional Activators TbmR and TbuT

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The toluene-3-monooxygenase from *Burkholderia pickettii* PKO1 and the toluene/benzene-2-monooxygenase from *Burkholderia* (*Pseudomonas*) sp. strain JS150 are distinct enzymes which differ not only in catalytic specificity and substrate range but also in the arrangement and sequence of the genes within the operons that encode the enzymes, *tbuA1UBVA2C* and *tbmABCDEF*, respectively. In the present study, we examined the transcriptional activation of the *PtbuA1* and *PtbmA* promoters by their cognate regulators, TbuT and TbmR. TbmR and TbuT each exhibited activation of both *PtbmA* and *PtbuA1*, with toluene, benzene, and chlorobenzene serving as strong effectors. These results strongly suggest that TbmR is an NtrC-like regulator which is functionally homologous to TbuT, and they provide evidence for the evolutionary "recruitment" of the same or a similar type of regulator for both monooxygenase pathways.

In previous reports we have described the cloning, genetic organization, and sequencing of the operons encoding two toluene monooxygenases: the toluene-3-monooxygenase (Tbu) of *Burkholderia* (*Pseudomonas*) *pickettii* PKO1 (2, 20) and the toluene/benzene-2-monooxygenase (Tb2m) of *Burkholderia* (*Pseudomonas*) sp. strain JS150 (13). Our studies have shown that these operons, designated *tbuA1UBVA2C* and *tbmABCDEF*, respectively, are dissimilar with respect to (i) the arrangement of genes within the operon and (ii) the deduced amino acid sequences of the individual gene products. In this context, the *tbu* genes exhibit the highest degree of similarity (>60%) to genes of the identically organized *tmo* operon, which encodes the toluene-4-monooxygenase of *Pseudomonas mendocina* KR1 (28, 29). In contrast, the genes of the *tbm* operon were found to be most similar in their arrangement and sequence (>50%) to genes encoding phenol hydroxylases, including those of the *dmp* operon of *Pseudomonas* sp. strain CF600 (18), the *phe* operon of *Pseudomonas putida* BH (27), and the *phh* operon of *P. putida* P35X (17). On the basis of these observations, the monooxygenases studied to date appear to fall into two groups as shown in Fig. 1.

The differences in organization and content of the *tbu* and *tbm* operons depicted in Fig. 1 are mirrored also in their differing substrate ranges. Tbu is limited to unactivated aromatic hydrocarbons, such as benzene and toluene (20), whereas Tb2m shares with the toluene-*o*-monooxygenase of *Burkholderia* (*Pseudomonas*) *cepacia* G4 (23) the ability to oxidize phenols and cresols (13). The Tb2m enzyme is also active on carcinogenic chloro-substituted aromatic substrates such as chlorobenzenes, unlike the Tbu enzyme we reported previously and other monooxygenase pathways described by others (25).

Despite these differences, the expression of the *tbu* and *tbm* operons appears to be regulated in a similar manner (Fig. 2A and B). Each operon is positively regulated from a *trans*-acting locus, TbuT or TbmR, respectively, in the presence of toluene as an inducer (13, 20). In addition, putative σ^{54}-dependent −24, −12 promoter sequences, which require positive transcriptional regulation by activators of the NtrC family (14, 16), have been identified in the regions upstream of both the *tbuA1* (2) and *tbmA* (13) genes. These promoter sequences, which we have designated *PtbuA1* and *PtbmA*, are homologous to *Pu* and *Po*, which are the σ^{54}-dependent promoters for the *xylCMABN* (xylene methylmonooxygenase) operon of *P. putida* PaW1 (15) and the *dmpKLMNOP* (phenol hydroxylase) operon of *Pseudomonas* sp. strain CF600 (24), respectively (Fig. 2C). We have recently completed work (3) confirming that *PtbuA1* does function as the promoter for the *tbuA1UBVA2C* operon. We further showed that (i) the regulator TbuT is homologous to XylR and DmpR, the NtrC-like transcriptional activators of the *xyl* (11) and *dmp* (24) operons, respectively, and (ii) transcriptional activation by TbuT is dependent on the presence of distal *cis*-acting enhancer elements, or upstream activator sequences (UASs) homologous to those of the *xyl* (1, 10) and *dmp* (24) operons. When the sequence upstream of *PtbmA* was inspected, we found a palindromic region which was located in a similar position, relative to *PtbmA* (Fig. 2A), as the UAS for *PtbuA1* (Fig. 2B). The homology of this element to the UASs for the *PtbuA1*, *Pu*, and *Po* promoters (Fig. 2D) indicates that it may serve a similar function, i.e., as the binding site for an NtrC-like regulator that activates transcription from the *PtbmA* promoter. Given the role of TbmR as a regulator for the *tbm* operon, it can be inferred that TbmR may itself be the transcriptional activator of the *PtbmA* promoter, and, by extension, that it is an NtrC-like regulator analogous to TbuT.

In light of the similarity in the modes of regulation of the toluene-3-monooxygenase and the toluene/benzene-2-monooxygenase, it is apparent that there may be more shared traits among regulatory elements which control expression of the monooxygenases than would be expected from a comparison of structural genes for the two groups of monooxygenases, as shown in Fig. 1. To ascertain the uniqueness, or lack of it, for regulators and their interactions with promoters and UASs, we have compared both the specificities and the relative activities of TbuT and TbmR as regulators of the 3-monooxygenase and 2-monooxygenase pathways, respectively. For this purpose, we used transcriptional fusion analyses to (i) establish that the gene product of the *tbm* regulatory locus, TbmR, activates transcription from the *tbmA* promoter region, (ii) compare the effector specificities of the two regulatory proteins TbmR and

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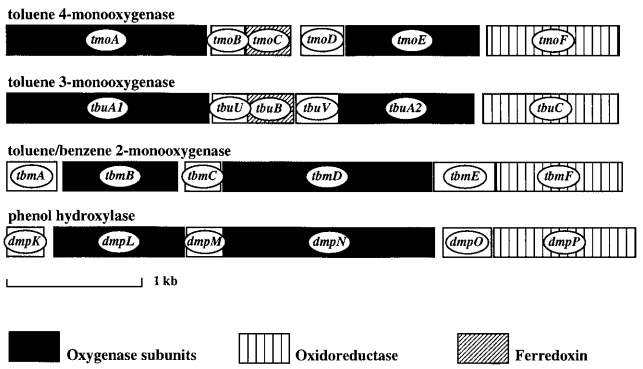

FIG. 1. Gene organization of the toluene-4-monooxygenase operon from *P. mendocina* KR1 (28, 29), the toluene-3-monooxygenase operon from *B. pickettii* PKO1 (2), the toluene/benzene-2-monooxygenase operon from *Burkholderia* (*Pseudomonas*) sp. strain JS150 (13), and the phenol hydroxylase operon from *Pseudomonas* sp. strain CF600 (18). The deduced biochemical functions of polypeptides encoded by the genes are represented by the shading of boxes as indicated by the key.

TbuT, and (iii) determine whether these regulators are able to functionally replace one another in the cross-activation of the other's cognate promoter, as has been previously demonstrated by Fernandez et al. (6) for the XylR and DmpR regulatory gene products, which do not have similar effectors.

Bacterial strains and general methods. *Escherichia coli* DH5α(*endA1 hsdR17 supE44 thi-l gyrA96 relA1* 80d *lacZ*) (9) was used for general cloning procedures. *E. coli* MM294 (pRK2013) (7) was used in triparental matings for the mobilization of the plasmid pKRZ1 and its derivatives from *E. coli* DH5α into *Pseudomonas aeruginosa* PAO1. *P. aeruginosa* PAO1 served as the host strain for transcriptional fusion experiments. Cultivation of strains, selection for antibiotic resistance markers, DNA manipulations, and β-galactosidase assays were carried out as described previously (3). Statistical analysis of the data was performed with the SAS System for Personal Computers, release 6.04 (22). The level of significance was an α value of 0.05 except where otherwise specified.

Plasmid and strain construction. Transcriptional fusions of the *tbmA* and *tbuA1* promoters were constructed by using the *lacZ* reporter plasmid pKRZ1 (21). The *tbmA* promoter region was isolated by using PCR to amplify a 468-bp fragment which extended from the *Xho*I restriction site 428 bp upstream of the translational start site to a site 40 bp into the coding region for *tbmA*. The template was pGJ10 (12), a pBluescriptKS+ derivative which contains a 4.0-kb *Not*I fragment from pRO2016 (13) that encompasses the target sequence. The oligonucleotides 5′-agttCTCGAGAATTGTGTTTCCTCTTCG-3′ and 5′-ggcgccCGGGCATCTCGTTGGAGG-3′ were used as the left and right primers, respectively (capitalized nucleotides correspond to the target sequence). The fragment thus amplified was digested with *Xho*I and *Sma*I and was religated with *Sal*I/*Sma*I-digested pKRZ1 so as to insert the *tbmA* promoter region in the correct orientation upstream of the promoterless *lacZ* gene of the vector. The resulting plasmid, p*PtbmA*, was transformed into *E. coli* DH5α and was then transferred to *P. aeruginosa* PAO1 by triparental mating (3). The similarly constructed PAO1 strain containing the pKRZ1-*tbuA1* promoter fusion, designated p*PtbuAI*, and the control strain, which contained only the vector pKRZ1, have been described previously (3).

In order to examine *trans*-activation of the *tbmA* and *tbuA1* promoters by TbmR or TbuT, analogous 3.1-kb *Eco*RI/*Pvu*II restriction fragments containing the regulatory loci were ligated with *Eco*RI/*Eco*RV-digested pRO1614 (19). The *tbmR* locus was derived from plasmid pRO2369 (13), while the *tbuT* locus was cloned from a pRO1614 derivative containing the 4.1-kb *Eco*RI fragment from plasmid pRO1966 (20). The resulting plasmids, designated pRO1614:*tbmR* and pRO1614:*tbuT*, were each transformed into *E. coli* DH5α. Plasmid DNA from each of the strains was then isolated and used to transform PAO1(pKRZ1), PAO1(p*PtbmA*), and PAO1(p*PtbuA1*) by electroporation at 2.1 kV (26).

Transcriptional activation of *PtbmA* by TbmR and TbuT. The results of experiments conducted with PAO1(p*PtbmA*, pRO1614:*tbmR*) (Fig. 3A) show that the transcriptional activity of toluene-grown cells, 158 Miller units, was almost 50 times higher than that of cells grown in the absence of effector. In contrast, the activity of toluene-grown cells of a control strain, PAO1(p*PtbmA*, pRO1614), was less than 3 Miller units. These results therefore confirm that the *tbmA* promoter was transcriptionally activated from the *tbmR* locus in the presence of toluene as an effector. Other substrates for Tb2m (12), including benzene, chlorobenzene, and *o*- and *m*-cresols, produced statistically significant ($\alpha = 0.05$) levels of β-galactosidase activity in PAO1(p*PtbmA*, pRO1614:*tbmR*), while *p*-cresol did not. Benzene and chlorobenzene were moderately strong effectors, eliciting transcriptional responses 36 and 28%, respectively, of that observed for toluene. *o*-Cresol and *m*-cresol were very weak effectors, inducing transcription to levels only two to three times that of uninduced cells. Although phenol has been previously demonstrated to induce Tb2m activity from the *tbmR* locus (12) and can serve as a growth substrate for PAO1 containing the cloned Tb2m and TbmR genes (13), the transcriptional response of the *PtbmA* promoter to phenol was also very low (significant at an α value of 0.15) and was similar to its response to *o*-cresol and *m*-cresol. Other weak effectors (data not depicted) included biphenyl (7.2 ± 0.7 Miller units) and *o*-xylene (6.2 ± 0.02 Miller units). No significant induction was observed for either naphthalene or cyclohexane (<2 Miller units). Overall, these results indicated that the effector specificity of TbmR for activation of the *tbmA* promoter was rather narrow, the only strong effectors being monosubstituted or unsubstituted monoaromatic compounds which are substrates for Tb2m.

Expression from the *PtbmA* promoter region was also measured by using PAO1(p*PtbmA*, pRO1614:*tbuT*). The results of these experiments (Fig. 3A) show clearly that TbuT was able to cross-activate transcription and that the effector specificity was identical to that of TbmR. Given the homology of TbuT to NtrC-like activators, as well as the presence of a putative activator binding site (UAS) in the region upstream of *tbmA*, the similarity in the patterns of transcriptional activation by TbmR and TbuT provides strong evidence that TbmR is itself an NtrC-like activator which is functionally homologous to TbuT. Notably, the amount of β-galactosidase activity induced in the presence of TbuT was significantly less than that for TbmR. In view of previous work with XylR showing that the activation of transcription involves the recognition and binding of the activator to the UAS (1, 5), our results indicate that TbuT may be less efficient in its interaction with the *tbm* UAS than is TbmR, although we cannot exclude the possibility that the concentrations of TbmR are higher than those of TbuT in PAO1 (p*PtbmA*, pRO1614:*tbmR*) and PAO1(p*PtbmA*, pRO1614:*tbuT*).

Transcriptional activation of *PtbuA1* by TbmR and TbuT. In order to examine the regulation of the *PtbuA1* promoter by TbmR and TbuT, β-galactosidase experiments were conducted as before with PAO1(p*PtbuAI*, pRO1614:*tbmR*) and PAO1 (p*PtbuA1*, pRO1614:*tbuT*) (Fig. 3B). Activation by TbuT in the

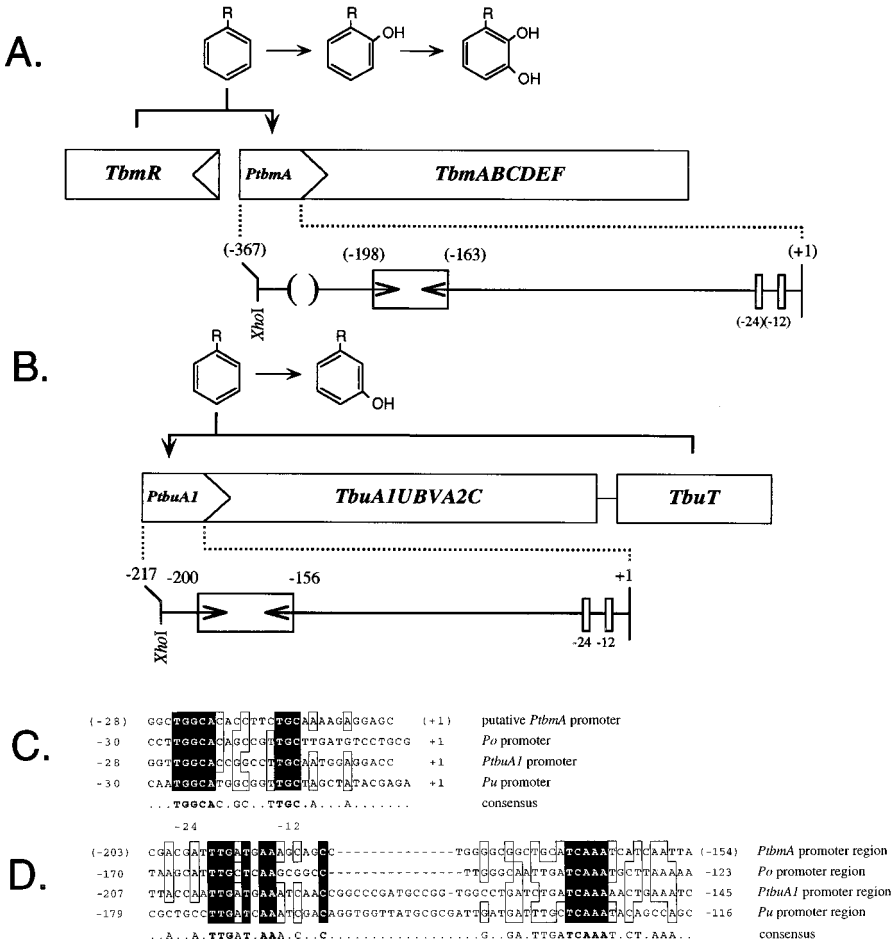

FIG. 2. (A) Regulation of the *tbmABCDEF* (toluene/benzene-2-monooxygenase) operon by TbmR (after Johnson [12] and Johnson and Olsen [13]). The operon is positively regulated from the divergently transcribed *tbmR* locus in the presence of toluene and is transcribed from the *PtbmA* promoter region in the direction indicated. The promoter region is expanded to illustrate the relative positions of (i) the −24, −12 sequence, *PtbmA*, which is homologous to other σ^{54}-dependent promoters (see Fig. 1), and (ii) a palindromic or inverse repeat region (shown as boxed arrows) which is homologous to other UASs (see Fig. 1). The *Xho*I restriction site corresponds to the upstream boundary of the *PtbmA* promoter region that was cloned for transcriptional fusion analyses (see text). Positions of the illustrated loci relative to a putative start site (based on the −24, −12 sequence) are given in parentheses. (B) Regulation of the *tbuA1UBVA2C* (toluene-3-monooxygenase) operon by TbuT (after Byrne et al. [2], Byrne and Olsen [3], and Olsen et al. [20]). The operon is transcriptionally activated by TbuT in the presence of toluene and is transcribed from the *PtbuA1* promoter in the direction indicated (3). The promoter region is expanded to illustrate the relative positions of (i) the transcriptional start site, (ii) the −24, −12 promoter sequence for the σ^{54}-dependent promoter, *PtbuA1*, and (iii) a palindromic region (shown as boxed arrows) which has been tentatively identified as a UAS. The *Xho*I restriction site corresponds to the upstream boundary of the *PtbuA1* promoter region that was cloned for transcriptional fusion analyses, as previously described (3). (C) DNA sequence alignment of the promoter sequence of *PtbmA* with the sequences of the σ^{54}-dependent promoters *Po* (24), *PtbuA1* (3), and *Pu* (15). (D) DNA sequence alignment of the palindromic region upstream of *tbmA* with palindromic regions containing the proposed DmpR-binding site upstream of *Po* (24), the proposed TbuT-binding site upstream of *PtbuA1* (3), and the XylR-binding site upstream of *Pu* (1, 5). Nucleotide sequence alignments in panels C and D were constructed with the PILEUP program from the Genetics Computer Group (8). Nucleotide sequence identities among any three of the four sequences are boxed. Nucleotides conserved in all four 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 *PtbmA*, *Po*, *PtbuA1*, and *Pu* sequences are displayed below each alignment. Gaps, indicated by dashes, were introduced to maximize homology. Nucleotide positions for the *PtbmA* promoter region are relative to a putative transcriptional start site (based on the −24, −12 sequence), and are provided in parentheses for purposes of comparison.

presence of appropriate effectors was observed, as described previously (3), as was cross-activation by TbmR. Consequently, chlorobenzene, which is not a substrate for the toluene-3-monooxygenase, regulated by TbuT, was a relatively strong effector for its induction. In general, the effector specificities of TbmR and TbuT were similar to those observed with the *tbmA* promoter, which is not surprising, given that the specificity of effector binding for NtrC-like activators is conferred by the activator itself and not by the promoter sequence (4, 25). However, the level of expression from the *PtbuA1* promoter was found to be significantly lower than that from the *PtbmA* promoter, possibly as the result of differences in the UAS and/or promoter sequences for the two promoter regions. In addition, while TbmR was previously shown to activate the *PtbmA* promoter to a greater extent than TbuT, there was no significant difference in their abilities to activate *PtbuA1*. Since it is likely that the relative effectiveness of the two activators would not vary on the basis of differences in promoter sequences, but would depend greatly on their ability to bind to the UASs, it is probable that the dissimilarity in the nucleotide sequences between the UASs from the *PtbmA* and *PtbuA1* promoter regions (Fig. 2D) is at least in part responsible for these observations.

Conclusions. In summary, the results of the present study indicate that although the toluene/benzene-2-monooxygenase and toluene-3-monooxygenase represent genetically and cata-

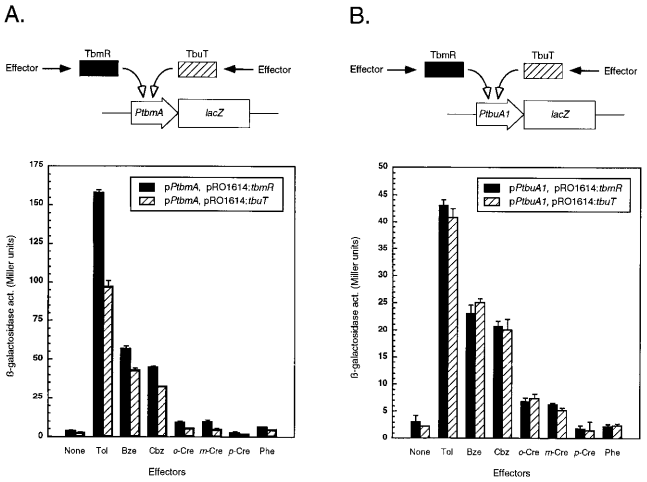

FIG. 3. Activation by TbmR and TbuT of the *PtbmA* promoter (A) and the *PtbuA1* promoter (B). The drawings at the top (not to scale) illustrate the structure of the transcriptional fusion plasmids p*PtbmA* and p*PtbuA1*, as well as the transcriptional activation of the promoters by TbmR or TbuT, which were provided in *trans* from plasmids pRO1614:*tbmR* and pRO1614:*tbuT*, respectively. The direction of transcription is indicated. For these experiments, PAO1 strains containing the designated plasmids were grown in the presence of effectors and assayed for β-galactosidase activity as described in the text. Abbreviations for effectors are as follows: Tol, toluene; Bze, benzene; Cbz, chlorobenzene; *o*-Cre, *o*-cresol; *m*-Cre, *m*-cresol; *p*-Cre, *p*-cresol; Phe, phenol. The results are the averages of triplicate determinations from two independent experiments. Error bars represent standard deviations. The data were not corrected for background β-galactosidase activity, which consistently amounted to less than 1.8 Miller units, as measured by using the control strains PAO1(pKRZ1, pRO1614:*tbmR*) and PAO1(pKRZ1, pRO1614:*tbuT*).

lytically distinct oxygenases, expression of these enzymes is controlled by similar regulators with the same effector range. Our findings can be distinguished from those of Fernandez et al. (6), who demonstrated cross-activation of the xylene monooxygenase and phenol hydroxylase pathways by regulators (DmpR and XylR) with differing effector ranges. However, the results of the present study are not inconsistent with their evidence for the evolutionary "recruitment" of regulatory genes by catabolic gene clusters, with the toluene/benzene-2-monooxygenase and toluene-3-monooxygenase operons possibly providing an example of distinct pathways acquiring the same or a similar type of regulator.

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