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Transcriptional Control of the Multiple Catabolic Pathways Encoded on the TOL Plasmid pWW53 of *Pseudomonas putida* MT53

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The TOL plasmid pWW53 encodes a catabolic pathway for the metabolism of toluene. It bears an upper-pathway operon for the oxidation of toluene to benzoate and a copy of the gene that encodes regulatory protein XylR. For metabolism of the aromatic carboxylic acid, it bears two functional homologous *meta*-pathway operons, together with two functional copies of the *xylS* regulatory gene (*xylS1* and *xylS3*). In cells growing in the absence of pathway substrates, no mRNA from upper- and *meta*-pathway operons were found; however, the *xylR* gene was expressed from two σ^{70}-dependent tandem promoters, and the *xylS1* and the *xylS3* genes were also expressed from their σ^{70}-dependent promoters, called Ps2 and Ps3, respectively. In cells grown in the presence of *o*-xylene, the XylR protein became active and stimulated transcription from the Pu promoter for the upper pathway. Expression from *xylS1* but not from *xylS3* was also stimulated by XylR; this was due to activation of transcription from the *xylS1* Ps1 promoter, which is σ^{54} dependent, and the lack of effect on expression from the Ps2 σ^{70}-dependent promoter. As a result of overexpression of the *xylS1* gene, the XylS1 protein was overproduced and activated transcription from Pm1 and Pm2. In cells growing on benzoate, the upper-pathway operon was not expressed, but both *meta* operons were expressed. Given that XylS1 but not XylS3 recognized benzoate as an effector, stimulation of transcription was found to be mediated by XylS1. This was confirmed with cloned *meta*-pathway promoters and regulators. When 3-methylbenzoate was present in the medium, both *meta* operons were also expressed and stimulation of transcription was mediated by both XylS1 and XylS3, which both recognized 3-methylbenzoate as an effector.

The TOL plasmids of *Pseudomonas* encode the information for the catabolism of toluene and xylenes, which is organized in two sets of operons conventionally referred to as the upper and *meta* operons (4, 21, 27). The genes of the upper operon encode the enzymes for oxidation of the lateral alkyl chain of toluene and xylenes to their corresponding carboxylic acids. The genes for the *meta* cleavage pathway encode the enzymes for oxidation of benzoate and toluates to Krebs cycle substrates. The 107-kb TOL plasmid pWW53 (Fig. 1) contains a single upper operon located between *meta*-1 and *meta*-2, two homologous but distinguishable *meta* operons (Fig. 1), both of which are functional (12, 20). pWW53 carries a single copy of the *xylR* regulatory gene, involved in the control of the upper operon, which is located downstream from the *meta*-1 operon. However, three copies of the *xylS* regulatory gene are located on pWW53, two of which encode positive regulator proteins for the *meta* operons. The gene designated *xylS1* is located downstream from the *meta*-1 operon and adjacent to *xylR*, whereas the gene designated *xylS3* is located between the two *meta* operons. The nonfunctional *xylS2* gene, located downstream from the *meta*-2 operon, is truncated because of the insertion of an insertion sequence element (3, 4).

Despite detailed knowledge of the organization of the catabolic operons in pWW53, little is known about the transcriptional control of this complex array of pathways and regulators (13). In the present study, we analyzed the in vivo transcription of the *xylR*, *xylS1*, and *xylS3* genes and of the upper and *meta* operons in *P. putida* MT53 bearing pWW53.

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MATERIALS AND METHODS

Culture conditions and strains. The strains and plasmids used in this study are described in Table 1. *P. putida* KT2440(pWW0) (6) and *P. putida* MT53 (12) were grown at 30°C on M9 salt-based minimal medium supplemented with trace elements (2) and with glucose (30 mM) as a carbon source. *o*-Xylene (2 mM), benzyl alcohol (5 mM), 3-methylbenzyl alcohol (5 mM), and 3-methylbenzoate (5 mM) were added as effectors. *Escherichia coli* cultures were grown on LB medium at 30°C. When required, the antibiotics ampicillin, kanamycin, and rifampin were added at 200, 50, and 20 μg/ml, respectively.

DNA techniques. DNA preparation, digestion with restriction enzymes, analysis by agarose gel electrophoresis, isolation of DNA fragments with Geneclean (Bio 101 Inc., Vista, Calif.), ligations, and sequencing reactions were done according to standard procedures (5, 25) or the manufacturer's recommendations.

Plasmids constructed in this study. Plasmids constructed previously and their relevant characteristics are described in Table 1. Plasmids constructed in this study are described below.

pJB3KmD.1 is a kanamycin-resistant and ampicillin-sensitive plasmid derived from pJB3KmD. It was obtained by digesting pJB3KmD with *Sca*I, which cuts into the β-lactamase-coding gene, and removing part of the gene by digestion with exonuclease *Bal* 31.

pMT200 and pMT201 were constructed by inserting the 1.3-kb fragment of pWW53-1001, which contains the *xylS3* gene of pWW53, in the *Bam*HI site of pJB3KmD.1. In pMT200, *xylS3* is expressed from the P_{lac} promoter; in pMT201, this gene is expressed from its own promoter. The 1.3-kb fragment of pWW53-1001 was amplified by PCR using the oligonucleotides 5′-TTCTATGGATCCCAGTGAGAAAGATCA-3′ and 5′-TATCTTGGATCCGTTGTTGCCCTTAGC-3′, which are partially complementary to the *xylS3* sequence and contain a noncomplementary region to which a *Bam*HI restriction site has been added. The PCR-amplified *xylS3* gene was inserted at the *Bam*HI site of pJB3KmD.1 and sequenced to check that it exhibited the known wild-type DNA sequence (3).

To construct pMT205, the 407-bp *Pst*I fragment of pWW53-3514, which contains the Pm1 promoter from pWW53, was inserted at the *Pst*I site of pEMBL9 (24) to form pMT203. pMT205 consists of the translational fusion vector pMD1405 into which the *Eco*RI-*Hin*dIII fragment Pm1-containing Pm1 of pMT203 has been inserted between its *Eco*RI and *Hin*dIII sites.

pMT206 was constructed in the same manner as pMT205 except that the 381-bp *Pst*I fragment of pWW53-3901, which contains the Pm2 promoter, was first inserted at the *Pst*I site of pEMBL9 to form pMT204, and the *Eco*RI-*Hin*dIII fragment of pMT204 was then inserted in pMD1405.

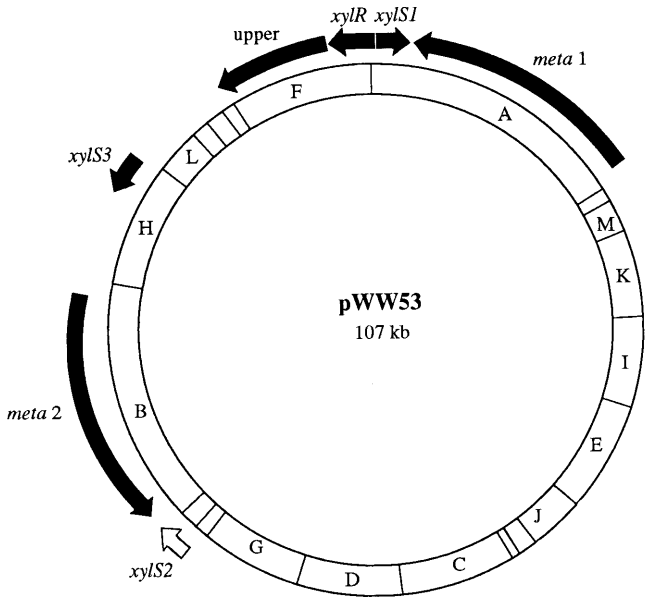

FIG. 1. Restriction map of the pWW53 TOL plasmid and genetic organization of the catabolic operons for toluene metabolism. This diagram, based on studies by Keil et al. (12), summarizes the known *Hin*dIII restriction sites, as well as the organization of the catabolic operons in pWW53.

RNA preparation, analysis, and primer extension. RNA was extracted by the guanidinium isothiocyanate-phenol method (25), and the RNA concentration was determined by measuring the A_{260}. Primer extension analyses were done essentially as described by Williams and Mason (26). We used the single-stranded DNA primers 5′-ACGGATCTGGCTGCTAAGGTCTTGC-3′ (complementary to the *xylR* mRNA), 5′-CGGAGACTGCATAGGGC-3′ (complementary to the *xylS1* mRNA), 5′-GCCCACCTCGCACACTCCATTG-3′ (complementary to the *xylS3* mRNA), 5′-GGGTCGGTGAACATCTCGCGTTGC-3′ (complementary to the mRNAs originated from Pm1 and Pm2 promoters), and 5′-GGCCAGCGTCACAGACTCCAGGCG-3′ (complementary to the mRNA originated from the Pu promoter). The extension reactions were done with avian myeloblastosis virus reverse transcriptase as described previously (7, 17). The products of the reverse transcriptase reactions were analyzed in a urea-polyacrylamide sequencing gel.

β-Galactosidase assays. *E. coli* CC118 harboring the Pm::′*lacZ* fusion in pMD1405 (Table 1), plus a compatible plasmid bearing a *xylS* allele, was grown overnight on LB medium containing the appropriate antibiotics. Then the cells were diluted 1:50 in the same medium supplemented or not with 1 mM benzoate or 3-methylbenzoate. After 6 h of incubation, the cells had reached the logarithmic phase (A_{660} of 1.2 to 1.5), and β-galactosidase activity was assayed in permeabilized whole cells (19).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to EMBL and assigned accession no. Y07775 for Pm1 and Y07776 for Pm2. The *xylS1* and *xylS3* sequences were reported previously (3); their accession numbers are L02356 and L02357, respectively.

RESULTS

The nucleotide sequences upstream from the first ATG of the *xylR*, *xylS1*, *xylS3*, and *xylU* (the first gene of the upper operon) regions of the pWW53 TOL plasmid had been determined previously (3, 14, 15). However, the nucleotide sequence upstream from the first gene in the two *meta* pathways had not been determined. In this study, we sequenced the *Pst*I fragments of 407 and 381 bp which contain the promoter region of the *meta*-1 operon, Pm1, and the promoter region of the *meta*-2 operon, Pm2, respectively. We also mapped the transcription initiation points of these promoters in *P. putida* MT53 cells growing in minimal medium in the absence and presence of effectors (*o*-xylene, benzyl alcohol, 3-methylbenzyl alcohol, and 3-methylbenzoate) to determine how transcriptional control is effected. The main transcription initiation points of *xylR*, *xylS1*, and *xylS3*, the upper operon, and the *meta*-1 and *meta*-2 operons were accurately mapped with reverse transcriptase techniques, and the amount of extended cDNA was used to reflect the actual mRNA concentration at a given moment.

Expression of the *xylR* gene. We detected two *xylR* transcripts of 187 and 215 nucleotides (nt) under all conditions tested, e.g., in the absence and in the presence of aromatics (benzyl alcohol, 3-methylbenzyl alcohol, and 3-methylbenzoate) (not shown). The corresponding transcription initiation points were mapped 121 and 140 bp, respectively, upstream from the first *xylR* ATG. Therefore, *xylR* in pWW53 is expressed constitutively from two tandem promoters, called Pr1 and Pr2, as is also the case for the well-characterized pWW0 plasmid (17, 18), although it should be noted that the relative strengths of the Pr1 and Pr2 promoters of pWW53 were about 20 times less than those of *xylR* of pWW0 (not shown).

Expression of the upper operon. In *P. putida* MT53(pWW53), a transcript of 133 nt was detected in RNA prepared from cells growing in the presence of *o*-xylene, benzyl alcohol, or 3-methylbenzyl alcohol but not in the absence of these aromatic compounds or in the presence of 3-methylbenzoate (not shown). The transcription initiation point of Pu in pWW53 was at the same position as in Pu in plasmid pWW0 (1, 9, 17), and it exhibits the characteristic −12 and −24 sequences recognized by the E-σ^{54} complex in Pu from pWW0 (18, 21).

Expression of the *xylS1* gene. *xylS1* was located adjacent to *xylR* but transcribed divergently. The *xylS1-xylR* unit in pWW53 was located between the upper and *meta*-1 operons (Fig. 1).

RNA was prepared from *P. putida* MT53(pWW53) growing

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
P. putida		
KT2440(pWW0)	Tol$^+$ 3MBz$^+$	6
MT53(pWW53)	Tol$^+$ 3MBz$^+$	12
E. coli CC118	Δ(*ara-leu*)*7697 araD139* Δ*lacX74 galK galE phoA20 thi rpsE rpoB argE*(Am) *recA1*	16
Plasmids		
pERD103	*xylS* from pWW0, Km^r, Sm^r, pRSF1010 replicon	This laboratory
pJB3KmD	Ap^r, Km^r	J. Blatny
pJB3KmD.1	Km^r	This work
pJLR107	Ap^r, Pm::′*lacZ* in pMD1405	24
pMD1405	Ap^r, ′*lacZ*, pBR replicon	M. Drummond
pMT200	P_{lac}::*xylS3* in pJB3KmD.1	This report
pMT201	*xylS3* in pJB3KmD.1	This report
pMT203	Ap^r, Pm1::′*lacZ* in pEMBL9	This report
pMT204	Ap^r, Pm2::′*lacZ* in pEMBL9	This report
pMT205	Ap^r, Pm1::′*lacZ* in pMD1405	This report
pMT206	Ap^r, Pm2::′*lacZ* in pMD1405	This report
pWW53-1001	*xylS3*, Ap^r, pBR replicon	3
pWW53-3508	*xylS1*, Sm^r, p15A replicon	12
pWW53-3514	*xylXYZ* from *meta*-1, Sm^r, p15A replicon	12
pWW53-3901	*xylXYZL* from *meta*-2, Sm^r, p15A replicon	20

[a] Ap^r, Km^r, and Sm^r denote resistance to ampicillin, kanamycin, and streptomycin, respectively; Tol$^+$ and 3MBz$^+$ denote ability to grow on toluene and 3-methylbenzoate, respectively, as sole C sources.

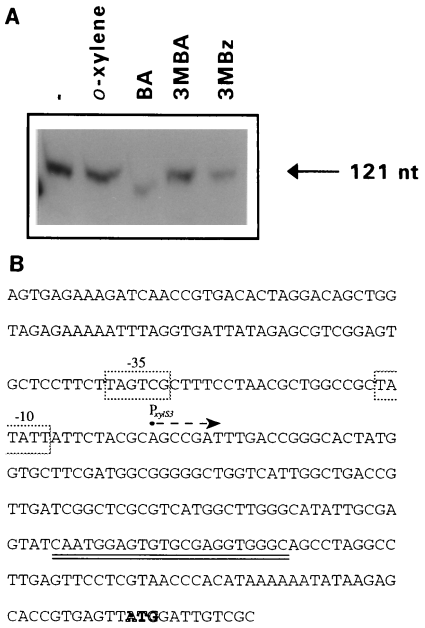

FIG. 2. Determination of the transcription initiation site of *xylS3*. (A) Total RNA was isolated from *P. putida* MT53(pWW53) cells growing on glucose (−) or glucose plus *o*-xylene, benzyl alcohol (BA), 3-methylbenzyl alcohol (3MBA), or 3-methylbenzoate (3MBz) and subjected to primer extension as described in Materials and Methods. The cDNA (121 nt) obtained after reverse transcription of 15 μg of total RNA with an oligonucleotide complementary to *xylS3* is marked. (B) DNA sequence of the *xylS3* promoter region. The transcription initiation point is indicated by a dot followed by an arrow which shows the direction of transcription; the −10 and −35 sequences are boxed; the first ATG of *xylS3* is boldfaced, and the complementary sequence of the oligonucleotide used for primer extension of *xylS3* is indicated by double underlining.

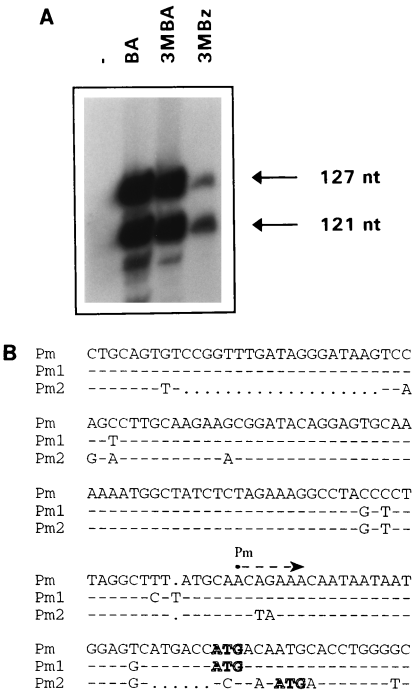

FIG. 3. Determination of the transcription initiation sites of pWW53 *meta*-1 and *meta*-2 operons. (A) Total RNA was isolated from *P. putida* MT53(pWW53) cells grown on glucose (−) or glucose plus benzyl alcohol (BA), 3-methylbenzyl alcohol (3MBA), or 3-methylbenzoate (3MBz) and subjected to primer extension as described in Materials and Methods. The figure shows the cDNAs (127 and 121 nt) obtained after reverse transcription of 10 μg of total RNA with an oligonucleotide complementary to *xylX* of *meta*-1 and *meta*-2. (B) Alignment of sequences of Pm from pWW0 and Pm1 and Pm2 from pWW53. The differences in Pm1 and Pm2 with respect to Pm are shown. The transcription initiation point is indicated by a dot followed by an arrow which shows the direction of transcription; the first ATG of *xylX* from pWW0 and *xylX* from *meta*-1 and *meta*-2 in pWW53 are boldfaced. The oligonucleotide used for primer extension of Pm1 and Pm2 was 5′-GGGTCGGTGAACATCTCGCGTTGC-3′. Dashes indicate sequence identity, and dots indicates gaps.

on glucose or glucose plus *o*-xylene, benzyl alcohol, 3-methylbenzyl alcohol, or 3-methylbenzoate as an effector. Under all conditions tested, we observed a 77-nt transcript expressed at a very low level (not shown); when *P. putida* MT53 was grown on *o*-xylene, benzyl alcohol, or 3-methylbenzyl alcohol, we detected another, 208-nt transcript (not shown). The transcription initiation points were mapped in the DNA sequence, and we established that *xylS1* in pWW53 was expressed from two promoters, Ps1 and Ps2. These promoters were homologous to the two promoters in pWW0 (7, 11, 22).

TABLE 2. Transcriptional activation of Pm from pWW0 and of Pm1 and Pm2 from pWW53 by XylS from pWW0 and by XylS1 and XylS3 from pWW53

Protein	Effector	β-Galactosidase activity (U)[a]		
		Pm	Pm1	Pm2
XylS	None	95	115	22
	Bz	16,141	17,123	3,730
	3MBz	19,110	19,024	3,494
XylS1	None	116	114	31
	Bz	629	693	122
	3MBz	1,630	2,368	261
XylS3	None	145	146	27
	Bz	196	195	43
	3MBz	15,158	16,592	3,583

[a] *E. coli* CC118 bearing plasmids pJLR107 (Pm::′*lacZ*), pMT205 (Pm1::′*lacZ*), or pMT206 (Pm2::′*lacZ*) plus pERD103 (XylS), pWW53-3508 (XylS1), pMT200 (XylS3 hyperproduced from P_{lac}), or pMT201 (XylS3 expressed at a low level) was grown on LB medium overnight with appropriate antibiotics. Then bacteria were diluted in the same medium in the absence or presence of 1 mM benzoate (Bz) or 3-methylbenzoate (3MBz), and β-galactosidase activity was determined after 6 h. The values are averages of three independent determinations with standard deviations below 15% of the given values.

Expression of the *xylS3* gene. A single mRNA for *xylS3* was found regardless of the growth conditions (glucose or glucose plus *o*-xylene, benzyl alcohol, 3-methylbenzyl alcohol, or 3-methylbenzoate) and primers used. The level of expression was also independent of the growth conditions. With the oligonucleotide shown in Fig. 2B, a 121-nt *xylS3* transcript was found (Fig. 2A). The transcription initiation point was mapped 178 bp upstream from the *xylS3* ATG, and sequences homologous to the −10 and −35 consensus sequences for σ^{70}-dependent promoters were found on the *xylS3* sequence.

The location and sequence of this promoter were different from those of the σ^{70}-dependent promoter in *xylS1* in pWW53 and *xylS* in pWW0. This was due to the fact that *xylS3* exhibited significant divergence in the sequence upstream from the start of the open reading frame (3).

Within the nonhomologous upstream region of *xylS3*, there were −12 and −24 sequences showing homology to the Ps1 promoter of *xylS* in pWW0 and *xylS1* in pWW53, but there were no sequences homologous to the upstream activation sequence recognized by XylR (8, 22). Therefore, *xylS3* seemed to be expressed constitutively from a single promoter which differed in sequence and position from *xylS* in pWW0 or *xylS1* in the pWW53 Ps1 or Ps2 promoter.

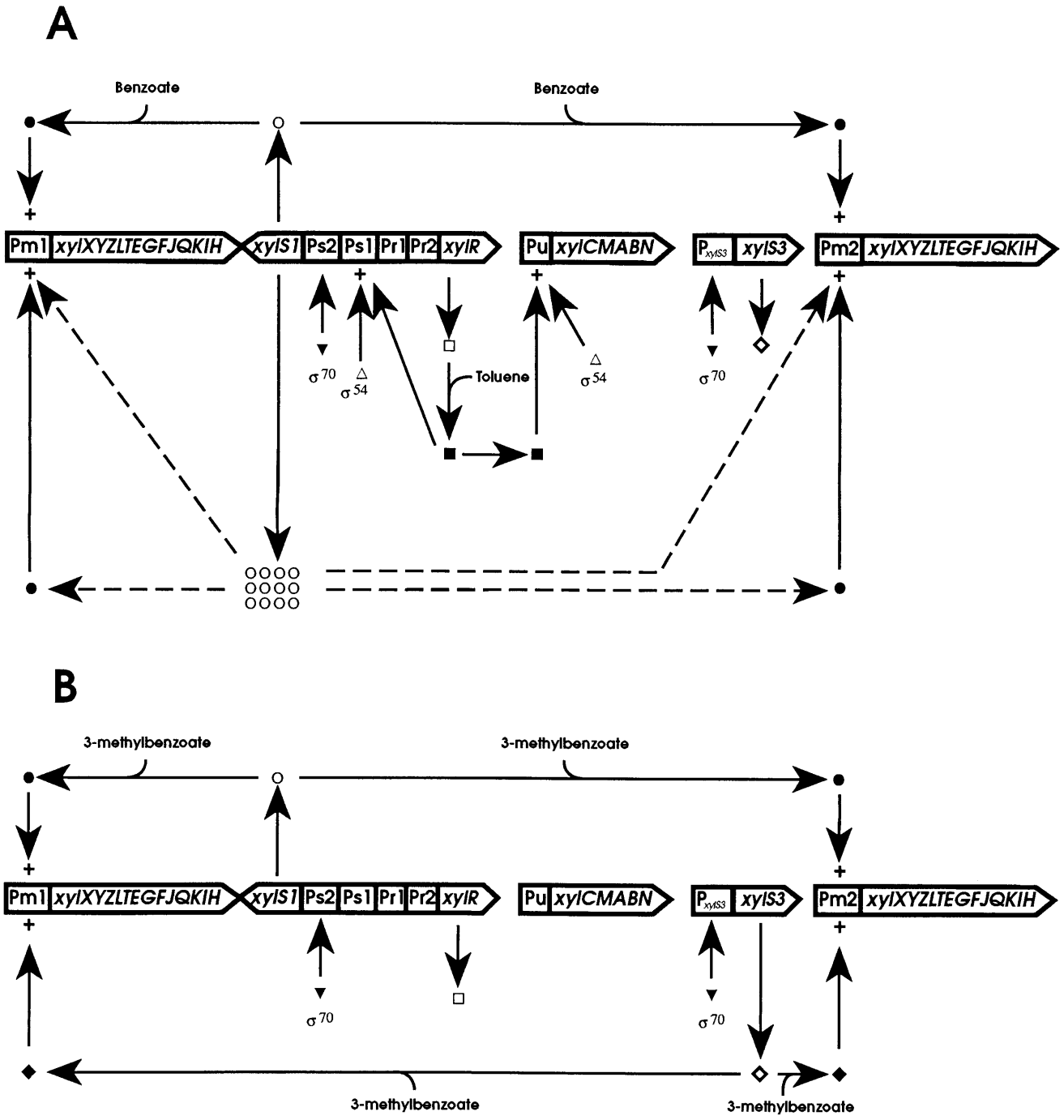

FIG. 4. Control of the catabolic pathways of the TOL plasmid pWW53. (A) Cells growing on toluene, benzoate, or 3-methylbenzoate. Genes and operons are shown by arrows that indicate the direction of transcription. +, positive stimulation of transcription negative control; □ and ■, inactive and active forms, respectively, of the XylR regulator; ○ and ●, inactive and active forms, respectively, of the XylS1 regulator; ◇ and ◆, inactive and active forms, respectively, of the XylS3 regulator. The σ factor involved in the transcription of each promoter is indicated by ▽ (σ^{70}) or △ (σ^{54}).

Expression of *meta*-1 and *meta*-2 operons. We detected two transcripts of 127 and 121 nt in RNA prepared from *P. putida* MT53(pWW53) growing in the presence of 3-methylbenzoate but not in its absence (Fig. 3A). A transcript of 127 nt was assigned to the mRNA originated from the *meta*-1 pathway promoter, referred to here as Pm1, and the 121-nt transcript was assigned to the mRNA originated from the *meta*-2 pathway promoter, Pm2. This decision was based on DNA sequence data. The Pm1 and Pm2 sequences were similar but not identical to each other and to the sequence of Pm in pWW0: Pm1 showed a 1-bp insertion at −5 with respect to Pm in pWW0 and Pm2 (Fig. 3B). Pm2 exhibited a deletion between +24 and +29 with respect to sequences of Pm in pWW0 and Pm1 in pWW53 (Fig. 3B). As a result, the transcript originated from Pm2 was 6 nt shorter than the Pm transcripts in pWW0. In the region from −15 to −17, Pm1 and Pm2 of pWW53

exhibited two point changes with respect to Pm in pWW0: T at −15 in Pm1 and Pm2 instead of C in Pm in pWW0, and G at −17 in Pm1 and Pm2 instead of C in Pm in pWW0 (Fig. 3B).

To confirm this assignment, we cloned Pm1 and Pm2 in pMD1405 to yield pMT205 and pMT206, respectively, and determined the transcription initiation point mediated by XylS from pWW0 with 3-methylbenzoate. The results confirmed the transcription initiation points for Pm1 and Pm2 (not shown).

Ramos et al. (22) showed that *P. putida*(pWW0) cells growing on upper-pathway substrates exhibited high levels of transcription of the *meta* operon. This was also the case for *P. putida*(pWW53), since the Pm1 and Pm2 transcripts were detected with RNA preparations from cells grown on benzyl alcohol, 3-methylbenzyl alcohol (Fig. 3A), or *o*-xylene (not shown). This reflects a cascade regulatory system in which XylR induces overexpression of *xylS1* (but not of *xylS3*), leading to transcription from Pm1 and Pm2 as a result of the hyperproduction of XylS1.

Effector profile of XylS1 and XylS3 proteins. The effector profile of the XylS1 and XylS3 proteins was determined in *E. coli*(pJLR107 [Pm::*lacZ*]) bearing the *xylS1* or *xylS3* allele. We determined the levels of β-galactosidase produced in response to the presence of benzoate and of 2-, 3-, and 4-methyl-, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-dimethyl-, 2-, 3-, and 4-fluoro-, 2-, 3-, and 4-chloro-, 2-, 3-, and 4-bromo-, 2-, 3-, and 4-iodo-, 2-, 3-, and 4-hydroxy-, and 2-, 3-, and 4-methoxybenzoate. XylS1 responded only to benzoate and benzoates substituted at position 3, e.g., 3-methyl-, 3-chloro-, 3-bromo-, and 2,3-dimethylbenzoate. XylS3 did not recognize benzoate as an effector, although Pm was activated in the presence of 3-methyl-, 2,3-dimethyl-, 3-chloro-, 3-bromo-, and 3-iodobenzoate (not shown).

Table 2 shows the β-galactosidase activities obtained with Pm from pWW0 and with Pm1 and Pm2 from pWW53 by XylS from pWW0 and by XylS1 and XylS3 from pWW53 in the absence and in the presence of benzoate and 3-methylbenzoate. XylS1 activated Pm1 and Pm2 in the presence of benzoate and 3-methylbenzoate, but XylS3 did so only in the presence of 3-methylbenzoate.

DISCUSSION

The presence of pWW53 on multiple copies of the *xylS* regulatory gene and a duplication of the *meta* operon makes the analysis of the regulation of the catabolic operons more complex than in the archetypal plasmid pWW0. The regulatory function of *xylR*, *xylS1*, and *xylS3* from pWW53 was demonstrated previously by the inducibility of the upper, *meta*-1, or *meta*-2 pathway enzymes in a *P. putida* strain carrying the cloned genes and operons (3, 12–15). In the present study, we analyzed in vivo transcription of the *xylR*, *xylS1*, and *xylS3* genes and of the upper and *meta* operons in *P. putida* MT53 (pWW53) under different growth conditions, and we mapped the transcription initiation points of the corresponding mRNAs in order to establish the corresponding promoters.

On the basis of this study, we propose a model for the regulation of expression of pWW53 catabolic operons (Fig. 4). Transcriptional control of the upper and *meta* pathways in *P. putida* MT53(pWW53) is influenced by the effector present in the medium: in *P. putida* MT53(pWW53) cells growing in the absence of both upper- and *meta*-pathway substrates, no mRNAs from the upper, *meta*-1, and *meta*-2 operons were found, although the *xylR* gene was expressed from two σ^{70}-dependent tandem promoters, and *xylS1* and *xylS3* were also expressed at low levels from their corresponding σ^{70}-dependent promoters.

When benzoic acid was added to the medium, the upper operon was not expressed; however, both *meta*-1 and *meta*-2 operons were expressed. Given that XylS1 but not XylS3 recognized benzoate as an effector, stimulation of transcription seemed to be mediated only by XylS1 (Fig. 4A). This was confirmed in *E. coli* with cloned *meta*-pathway promoters and regulators. When the aromatic carboxylic acid 3-methylbenzoate was added to the medium instead of benzoate, the upper operon was not expressed, whereas both *meta* operons were expressed at high levels. In this case, because XylS1 and XylS3 recognized 3-methylbenzoate as an effector, stimulation of transcription of the two *meta* operons was mediated by both proteins (Fig. 4B). It should be noted that the effector profiles of XylS1 and XylS3 were more restricted than that of the XylS protein from pWW0, given that the latter recognized a broad range of benzoates substituted at positions 2, 3, and 4 (23, 24), whereas XylS1 and XylS3 from pWW53 recognized only those benzoates substituted at position 3.

In cells growing on toluene or 3-methylbenzyl alcohol or in the presence of the nonmetabolizable aromatic hydrocarbon *o*-xylene, the XylR protein became active and stimulated transcription from the Pu promoter for the upper pathway. Overexpression of XylS1 but not of XylS3 was also stimulated by XylR. This was due not to an increase in transcription from the σ^{70} promoter of *xylS1* but to the stimulation of transcription of *xylS1* from a second promoter, called Ps1. Transcription from this XylR-dependent promoter did not affect transcription of the *xylS1* σ^{70}-dependent promoter Ps2 (Fig. 4A). As a result of expression of the *xylS1* gene from two promoters, *xylS1* mRNA was overproduced, and in turn the XylS1 protein is proposed to be also overproduced. The overproduced XylS1 stimulated transcription from the two *meta* operons of pWW53. Because this effect was also observed in *P. putida* MT53(pWW53) cells growing in the presence of *o*-xylene, a nonmetabolizable XylR effector, we suggest that in *P. putida*(pWW53) cells growing in the presence of aromatic hydrocarbons, expression of both *meta*-1 and *meta*-2 pathways is achieved via a cascade regulatory system in which the ultimate regulator is the effector-activated XylR protein.

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