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 α7-dependent tandem promoters, and the xylS1 and the xylS3 genes were also expressed from their α7-dependent promoters, called Ps2 and Ps3, respectively. In cells grown in the presence of α-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 α7-dependent, and the lack of effect on expression from the Ps2 α7-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 only 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 xylene, 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 alky chain of toluene and xylene to their corresponding carboxylic acids. The genes for the meta catabolic pathway encode the enzymes for oxidation of benzene and toluenes 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 (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.

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. α-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 pJBSKmD. It was obtained by digesting pJBSKmD with ScaI, 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 BamHI site of pJB3KmD.1. In pMT200, xylS3 is expressed from the Pm1 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'-TATCGTGATCTTCTGTGTTTGCCCTATGCC-3', which are partially complementary to the xylS3 sequence and contain a noncomplementary region to which a BamHI restriction site has been added. The PCR-amplified xylS3 gene was inserted at the BamHI site of pJBSKmD.1 and sequenced to check that it exhibited the known wild-type DNA sequence (3).

To construct pMT205, the 407-bp PstI fragment of pWW53-3514, which contains the Pm1 promoter from pWW53, was inserted at the PstI site of pEMBL9 to form pMT204. pMT205 consists of the translational fusion vector pMDI405 into which the EcoRI-HindIII fragment Pm1-containing Pm1 of pMT203 has been inserted between its EcoRI and HindIII sites.

pMT206 was constructed in the same manner as pMT205 except that the 381-bp PstI fragment of pWW53-3901, which contains the Pm2 promoter, was first inserted at the PstI site of pEMBL9 to form pMT204, and the EcoRI-HindIII fragment of pMT204 was then inserted in pMDI405.
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 A260. Primer extension analyses were done essentially as described by Williams and Mason (26). We used the single-stranded DNA primer 5′-ACGGATCTGGCTAAGGTCTTGC-3′ (complementary to the xylS mRNA), 5′-GCCACCTCGCACACCTCCATGG-3′ (complementary to the xylS1 mRNA), 5′-GGGTCACTGTTGAACTCCTCCTGG-3′ (complementary to the xylS3 mRNA), 5′-GGGTCACTGTTGAACTCCTCCTGG-3′ (complementary to the mRNAs originated from Pm1 and Pm2 promoters), and 5′-GGGGTCGGTGAACATCTCGCGTTGC-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 extension reactions were analyzed in a urea-polyacrylamide sequencing gel.

Nucleotide sequence accession numbers. The nucleotide sequences upstream from the first ATG of 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 PsrI 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-nt sequences recognized by the E-3′′ 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...
FIG. 2. Determination of the transcription initiation site of xylS3. (A) Total RNA was isolated from P. putida MT35(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 marked.

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 MT35 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).

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 σ70-dependent promoters were found on the xylS3 sequence.

The location and sequence of this promoter were different from those of the σ70-dependent promoter in xylSI 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 xylSI 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 xylSI in the pWW53 Ps1 or Ps2 promoter.

## 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

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<tr>
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* E. coli CC118 bearing plasmids pLR107 (Pm::lacZ), pMT205 (Pm1::lacZ), or pMT206 (Pm2::lacZ) plus pERD103 (XylS), pWW53-3508 (XylS1), pMT200 (XylS3 hyperproduced from Pm), 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 *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 +25 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

![Diagram A](image1.png)

![Diagram B](image2.png)

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 XylS regulator; ○ and ●, inactive and active forms, respectively, of the XylS3 regulator. The factor involved in the transcription of each promoter is indicated by ○ (σ70) or □ (σ54).
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 σ70-dependent tandem promoters, and xylS1 and xylS3 were also expressed at low levels from their corresponding σ70-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 σ70-dependent 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 σ70-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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