JOURNAL OF BACTERIOLOGY, Aug. 1987, p. 3581–3586
0021-9193/87/083581-06$02.00/0

Vol. 169, No. 8

Activation of the *xylDLEGF* Promoter of the TOL Toluene-Xylene Degradation Pathway by Overproduction of the *xylS* Regulatory Gene Product

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Received 5 January 1987/Accepted 30 March 1987

The *xylS* regulatory gene of the *Pseudomonas putida* TOL plasmid (pWWO) has been cloned under the transcriptional control of the *Escherichia coli tac* promoter in a broad-host-range controlled-expression vector. Induction with isopropylthiogalactoside allowed overproduction and characterization of the *xylS* product by specific interaction with the TOL *meta*-cleavage pathway operator-promoter region (OP2) in vivo in *E. coli*. Examination of plasmid-specified polypeptides in *E. coli* maxicells led to identification of the *xylS* product as a 36-kilodalton polypeptide. The operator sequences required for *xylS* interactions lay upstream of the OP2 transcriptional start, and the *xylS* gene product recognized this region even in the absence of known coinducers.

The TOL plasmid pWWO confers upon *Pseudomonas putida* mt-2 the ability to utilize toluene and some related hydrocarbons and their alcohol, aldehyde, and carboxylic acid derivatives as carbon sources (17, 27). Pathway structural genes are organized into two operons (8), one responsible for conversion of the hydrocarbon substrates to their corresponding carboxylic acids (*xylCAB*, the upper pathway), the other encoding enzymes that further degrade these to tricarboxylic acids (*xylDLEGF*, the lower or *meta*-cleavage pathway). A proposed regulatory model (26) requires two TOL plasmid-encoded regulatory genes, *xylR* and *xylS*. The *xylR* gene product XylR, in the presence of pathway hydrocarbons or alcohols, stimulates expression of both operons; the *xylS* product XylS, in the presence of pathway carboxylic acids, induces expression of the lower-pathway genes only. Subsequently it has become apparent that whereas the *xylS* gene product is essential for induction of the lower pathway, the XylR protein alone (with coinducers) is insufficient for this. Stimulation of expression of the lower pathway genes by the *xylR* product occurs, however, in the presence of a functional *xylS* gene, implying some interaction of these regulatory factors (8, 13; unpublished). Both *xylR* (9, 22) and *xylS* (7, 12) are positively acting regulatory genes which have been mapped to the *Xho*I D fragment of plasmid pWWO (8, 13). The XylR product is a polypeptide of 67 kilodaltons (kDa) (15, 25). The XylS protein, as expected from nucleotide sequence analysis, should have a subunit molecular size of 36 kDa (25). Recently, Inouye et al. (16) cloned DNA fragments containing the *xylS* gene into a *tac* promoter expression vector and detected a 36-kDa polypeptide in the maxicell system. It was not established, however, in these experiments, that either the 36-kDa polypeptide or expression of the *xylS* gene was indeed under transcriptional control of the *tac* promoter.

We describe here the cloning of the *xylS* gene downstream of the inducible *tac* promoter in a broad-host-range vector, pMMB66HE (10), as well as its subsequent overexpression and identification of the *xylS* product by both visualization in an *Escherichia coli* maxicell system and by specific interaction of the *xylS* product with the TOL *meta*-cleavage pathway operator-promoter region in vivo.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and vectors used are listed in Table 1. Plasmids were introduced into *P. putida* KT2440 by mobilization from *E. coli* strains containing pLG221 as described previously (6).

DNA manipulations. DNA deletions were made with nuclease *Bal*31 essentially as described by Maniatis et al. (18). DNA fragments were reisolated from agarose gels by electrophoresis onto DEAE membranes (NA45; Schleicher and Schüll) and were eluted from the membranes by incubation in 50 mM Tris hydrochloride (pH 7.5)–1 M NaCl (1 to 2 h at 70°C). The eluate was extracted twice with buffered phenol and extensively with ether, and DNA was collected by precipitation with ethanol. Other methods of DNA manipulation and DNA sequencing have been described (25).

Other methods. Methods for enzyme assays, protein determinations, and maxicell preparations have been described previously (25).

Construction of broad-host-range promoter-probe vector. A broad-host-range promoter-probe vector was constructed by ligating a DNA fragment containing *xylE*, the TOL gene encoding catechol 2,3-oxygenase (C2, 3-O), without its promoter (C. M. Thomas, personal communication) downstream from the Km^r determinant of pKT240. Plasmid pKT240 was chosen since there is no transcription through the *Eco*RI and *Sst*I sites (2). After transformation of *E. coli* DH5, Ap^r Km^r colonies that became yellow after being sprayed with catechol solution (0.1 M) owing to accumulation of 2-hydroxymuconic semialdehyde (the product of C2, 3-O) were picked. The plasmid in one such isolate was designated pCF32 (Fig. 1).

Replacement of the small *Hin*dIII-*Eco*RI subfragment of pCF32 with a promoter sequence might result in either increased resistance to streptomycin or altered activity of C2,3-O, depending on the direction of transcription, with concomitant loss of resistance to kanamycin. Both activities

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TABLE 1. Bacterial strains, plasmids, and phages used

Strain, plasmid, or phage	Characteristics[a]	Source or reference
E. coli		
DH5	F⁻ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ⁻ *recA1 gyrA96 relA1* φ80dΔ*lacZM15*	11
JM101	*supE thi* (r_K^+ m_K^+) Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q} Δ*lacZM15*]	20
CSR603	F⁻ *phr-1 recA1 uvrA6*	23
P. putida KT2440	*hsd* r⁻ m⁺	1
Plasmids and phages		
pRK240	Ap^r Km^r RSF1010 derivative	2
pCF32	Ap^r KM^r pKT240 derivative, *xylE*	This study
pCF22	Ap^r pKT240 derivative, OP2, *xylDLE*	25
pOP2	Ap^r pCF32 derivative, OP2, *xylE*	This study
pOP2D1	Ap^r pOP2 deletion derivative	This study
pOP2D2	Ap^r pOP2 deletion derivative	This study
pCF20	Tc^r pRK2501 derivative, *xylR*$^+$ *xylS*$^+$	25
pBR322	Ap^r Tc^r	3
pRAS5	Tc^r pBR322 derivative, OP2, *xylE*$^+$	This study
pCF30	Ap^r pUC8 derivative, *xylS*$^+$	25
pMMB66HE	Ap^r RSF1010 derivative, *tac* promoter	10
pRAS1	Ap^r pMMB66HE derivative, *xylS*$^+$	This study
pRAS2	Ap^r pRAS1 deletion derivative, *xylS*$^+$	This study
pRAS4	Ap^r pRAS1 deletion derivative, *xylS*	This study
M13mp8		21
M13-OP2	M13mp8 derivative, OP2	This study

[a] Ap^r, Km^r, and Tc^r, resistance to ampicillin, kanamycin, and tetracycline, respectively.

were verified by cloning TOL promoters into pCF32 (manuscript in preparation).

RESULTS

Deletion analysis of the OP2 promoter region. The 400-base-pair (bp) *Pst*I fragment of pCF22 (25) containing the operator-promoter region (OP2) of the *xylDLEGF* operon was cloned into M13mp8 for DNA sequencing, and a clone containing OP2 sequences (14, 19) with transcription directed towards the *Hin*dIII site of the polylinker was retained and designated M13-OP2. The 400-bp *Eco*RI-*Hin*dIII fragment of M13-OP2 was subcloned into pCF32 to generate the recombinant plasmid pOP2, in which the *xylE* gene is positioned downstream of the OP2 promoter (Fig. 2).

Plasmid pOP2 was introduced into *P. putida* KT2440 and KT2440(pCF20). Cultures of these strains were grown in the presence and absence of *m*-toluate, and C2,3-O activities were determined. Both *xylS* and *xylR* are expressed from pCF20 (25) so that activation of the OP2 promoter should be expected in cells containing this plasmid in the presence of *m*-toluate. As shown in Table 2, the activity of C2,3-O was indeed induced in cells containing the plasmid pOP2 only in the presence of both plasmid pCF20 and *m*-toluate as an inducer.

Deletions in the 400-bp *Eco*RI-*Hin*dIII fragment containing the OP2 promoter region were generated in M13-OP2 by two methods: in the first a deletion of 60 bp was obtained by *Bal*31 nuclease treatment at the *Bam*HI site, and in the second a deletion of 82 bp was obtained by digestion with *Bam*HI and *Xba*I, filling in the recessed ends, and religation. In both cases, the *Eco*RI-*Hin*dIII fragment of the M13-OP2 deletion derivative was inserted into pCF32 to give plasmids pOP2D1 (first method) and pOP2D2 (second method) (Fig. 2).

The 82-bp deletion extending to the *Xba*I site in pOP2D2 resulted in the loss of DNA sequences required for the regulated function but not the basal-level activity of the OP2 promoter. The derivative with the smaller (60-bp) deletion retained a regulatable OP2 promoter (plasmid pOP2D1 in Fig. 2 and 3 and Table 2).

Plasmid pOP2D1 was a convenient source of a DNA fragment carrying the *xylE* gene under the control of the OP2 promoter. The 2.9-kilobase (kb) *Eco*RI-*Sma*I fragment of pOP2D1 was inserted between the *Eco*RI and blunt-ended *Pst*I site of pBR322 to create plasmid pRAS5 (Fig. 2), which was compatible with the controlled-expression vectorpM-

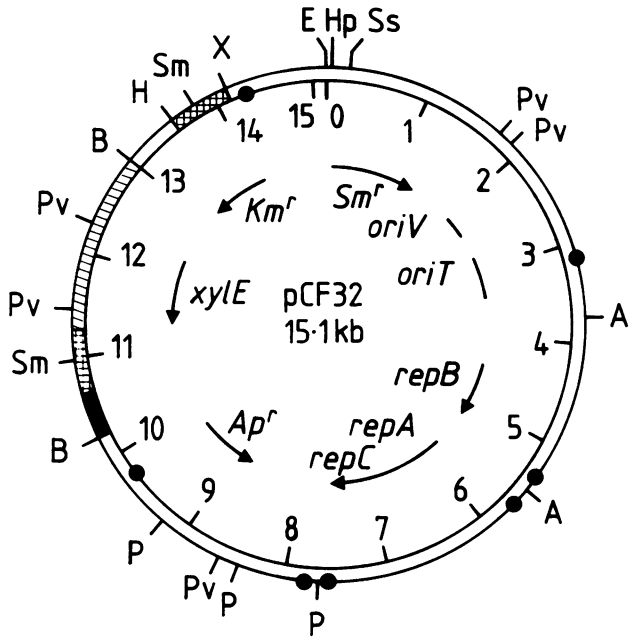

FIG. 1. Physical and genetic map of plasmid pCF32. The positions and directions of transcription of genes encoding C2,3-O (*xylE*), resistance to antibiotics (Km^r, kanamycin; Ap^r, ampicillin; Sm^r, streptomycin), and RSF1010 replication proteins (*repA*, *repB*, and *repC*) are indicated, as are the origins of vegetative replication (*oriV*) and conjugal transfer (*oriT*). Solid circles represent known promoters. Cleavage sites for enconucleases: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I; Pv, *Pvu*II; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I. The *xylE* gene is within a DNA segment bounded by *Bam*HI sites, obtained from plasmid pSRW40 (C. M. Thomas, personal communication). This fragment is composed of three fragments from different plasmids, a *Bam*HI-*Sal*I fragment of pWWO (coordinates 11.2 to 13; hatched area), an *Xho*I-*Hin*dIII fragment of pRK2501 (coordinates 10.7 to 11.2; crosshatched), and a *Hin*dIII-*Bam*HI fragment of pBR322 (coordinates 10.2 to 10.7; solid). Prior to insertion of this *Bam*HI fragment in pKT240, the *Hin*dIII site at the junction between the pRK250l and pBR322 segments was removed by cutting with *Hin*dIII, filling in the recessed ends, and religating. Crosshatching indicates a direct duplication.

MB66HE and its derivatives used in subsequent experiments.

Cloning of the *xylS* gene under *tac* promoter control. Plasmid pCF30 contains the entire *xylS* gene and, upstream of it, the 5′ end of the *xylR* gene transcribed in the opposite direction cloned in pUC8 (25). The 2.7-kb *Hin*dIII-*Eco*RI fragment of pCF30 was subcloned in pMMB66HE to generate the recombinant plasmid pRAS1 so that the *xylS* gene was expected to be under the transcriptional control of the *tac* promoter (Fig. 4).

In cells containing both pRAS1 and pRAS5, induction of C2,3-O activity, expressed from pRAS5, was expected to occur in the presence of *m*-toluate. This induction should be further increased by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) if the *xylS* gene in plasmid pRAS1 is indeed under *tac* promoter control. The results of such experiments showed that in cultures of strain DH5(pRAS1, pRAS5), C2,3-O was induced by *m*-toluate. However, this induction was only slightly increased by IPTG (Table 3). Since a likely explanation for this was interference from the *xylR* promoter, which directs transcription in the direction opposite that of the *tac* promoter (Fig. 4), deletions were generated at the *Hin*dIII site of pRAS1 with *Bal*31 nuclease. The shortened *Eco*RI–blunt-ended fragment was subsequently inserted between the *Eco*RI and *Sma*I sites of the vector pMMB66HE. Maps of two such deletion plasmids, pRAS2 and pRAS4, are presented in Fig. 4.

Plasmid pRAS4 did not express any function of the *xylS* gene, as expected. However, in the cells containing pRAS2, the activity of the *xylS* gene was markedly enhanced not only by *m*-toluate but also by IPTG alone, combined addition of IPTG and *m*-toluate having the greatest effect (Table 3). The difference in the ability of plasmids pRAS1 and pRAS2 to induce the OP2-driven *xylE* gene was best observed by following the initial kinetics of induction (Fig. 5).

Identification of the *xylS* gene product. Genetic and biochemical evidence outlined in the previous section indicated that in plasmid pRAS2 the *xylS* gene was under *tac* promoter control and that the concentration of *xylS* gene product in the cells containing this plasmid increased after derepression of *tac*. To visualize the XylS protein, plasmids pRAS1, pRAS2, and pRAS4 were introduced into the *E. coli* maxicell strain CSR603. Plasmid-specified proteins were labeled and separated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). As expected, a 37-kDa polypeptide was overproduced in the cells of CSR603(pRAS2) in the presence of IPTG, whereas no corresponding product was synthesized in large enough quantities for detection by autoradiography after SDS-PAGE in cells containing either pRAS1 or pRAS2 in the absence of IPTG or in cells containing pRAS1 in the presence of IPTG (Fig. 6). Cells containing pRAS4 did not produce a *xylS* product since the *Bal*31 deletion extended into the *xylS* gene (Fig. 4).

TABLE 2. C2,3-O activity in cell extracts of *P. putida* KT2440 and KT2440(pCF20) containing pOP2 deletion derivatives

Plasmid(s)	C2,3-O activity[a] (mU/mg of protein)	
	No inducer	*m*-Toluate
pOP2	220	180
pOP2, pCF20	110	920
pOP2D1, pCF20	50	910
pOP2D2, pCF20	220	150

[a] Assays were performed on extracts of cells harvested in the late exponential phase. Uninduced cultures were grown in LB broth, induced cultures in LB broth containing 5.0 mM *m*-toluate. All cultures contained carbenicillin (1 mg/ml) to select for pOP2 and its derivatives.

DISCUSSION

In the model describing the regulation of the TOL pathway, it has been postulated that the *xylS* gene product

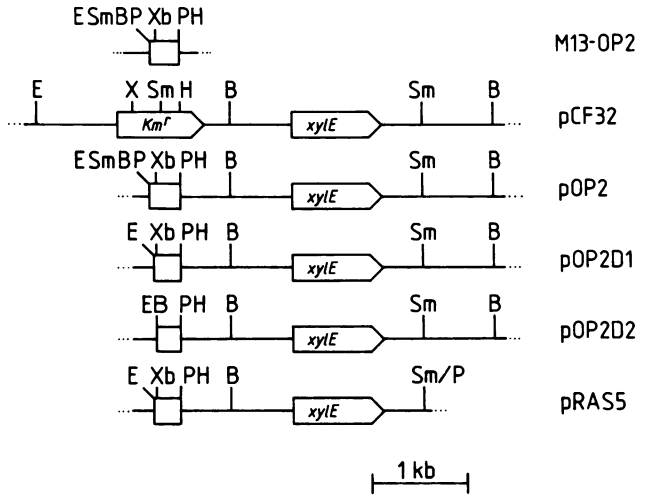

FIG. 2. Plasmids containing the operator-promoter regions (OP2) of the *xylDLEGF* operon and their derivatives. The 400-bp *Pst*I fragment containing OP2 sequences was cloned in M13mp8 and is represented by the box bounded by *Eco*RI and *Hin*dIII sites in M13-OP2. Below is shown the region of plasmid pCF32 encoding Km^r and the *xylE* genes, along with its derivatives containing the OP2 region and the two deletions thereof. Dots indicate the vector portion of the plasmids (which is pBR322 in plasmid pRAS5). Arrowheads indicate direction of transcription. Abbreviations: Xb, *Xba*I; Sm/P, fusion of *Sma*I and *Pst*I sites; others as in Fig. 1.

FIG. 3. Nucleotide sequence of the nontranscribed strand of the operator-promoter region OP2 (14), showing the first 140 bases from the *Eco*RI recognition sequence and the corresponding regions in deletion plasmids pOP2D1 and pOP2D2 (determined in this study). Extents of the deletions are indicated by contiguous dots. The start of transcription, identified by two groups (14, 19), is indicated (+, position 128). Upstream of this, presumed promoter sequences (19) are underlined. Restriction sites are overlined. An imperfect inverted repeat sequence is overlined by broken arrows.

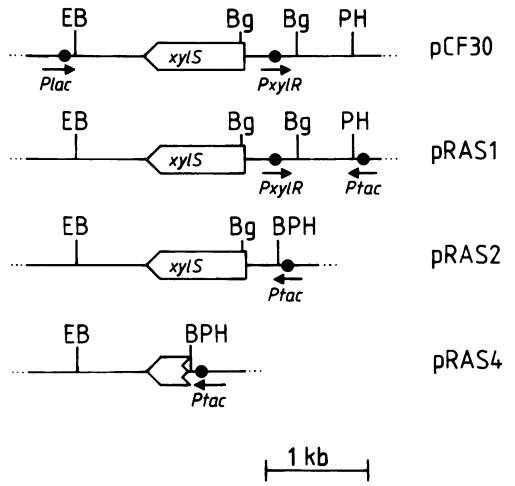

FIG. 4. Plasmids containing all or part of the *xylS* gene. Solid circles represent known promoters: *Plac*, the *lac* promoter of pUC8; *PxylR*, the *xylR* promoter (15); and *Ptac*, the *tac* promoter of pMMB66HE. Arrows indicate direction of transcription. Bg, *Bgl*II; other abbreviations as in Fig. 2. Plasmids pCF30 and pRAS1 contain a 2.7-kb *Hin*dIII-*Eco*RI fragment of pWWO which encodes *xylS* and the 5′ region of *xylR*. Plasmid pRAS2 is a deletion derivative of pRAS1 in which the 600 bp that encode the 5′ region of *xylR* have been removed. Plasmid pRAS4 carries a further deletion of about 600 bp which extends into the coding sequences of the *xylS* gene.

interacts with the DNA sequences known as the *meta*-pathway operator-promoter region (OP2), located upstream of the *xylDLEGF* operon (8, 13). We used a broad-host-range, promoter-probe vector to carry out a deletion analysis of this region. Sequences essential for induction by the *xylS* gene product were shown to lie within 60 bp upstream of the transcriptional start, which is believed to be at position 128 of the sequence shown in Fig. 3 (14, 19). Since other positive-acting regulatory proteins bind either upstream of the promoter they stimulate (for examples, see references 4 and 24) or upstream and overlapping with the promoter (for example, see reference 5), it is probable that XylS protein binds in the region 30 to 60 bp upstream of the transcriptional start. The most striking features in the nucleotide sequence of this region were (i) a string of six contiguous adenine residues (positions 83 to 88), (ii) a high frequency of purines in one strand (66%), particularly between positions 68 and 91 (78%), and (iii) an imperfect inverted repeat centered around the CT dinucleotide of the *Xba*I site. At least some of these features may be important for the binding of XylS protein. A test of this prediction, however, must await the results of site-directed mutagenesis of the OP2 region and purification of the XylS protein.

With this last goal in view, we attempted to overproduce the XylS protein in *E. coli* with the aid of a controlled-expression vector based on the *tac* promoter. Although induction of XylS activity by IPTG could be demonstrated in cells containing plasmid pRAS1, no polypeptide corresponding to XylS could be detected by SDS-PAGE. Removal of DNA sequences upstream of the *xylS* gene resulted in further overproduction of XylS protein, and in maxicells carrying plasmid pRAS4 a 36-kDa polypeptide could be detected by SDS-PAGE, a result also observed by Inouye et al. (16). Although the size of this polypeptide and the results of the in vivo induction experiments strongly suggest that it is indeed the *xylS* gene product, final verification will require

TABLE 3. C2,3-O activity of cell extracts of *E. coli* DH5(pRAS5) containing plasmid pMMB66HE or derivatives

Plasmid(s)	C2,3-O activity[a] (U/mg of protein)			
	No inducer	*m*-Toluate	IPTG	*m*-Toluate + IPTG
pRAS5	1.5	1.5	1.0	1.5
pRAS5, pMMB66HE	1.1	1.4	1.0	1.3
pRAS5, pRAS1	2.0	7.8	2.0	10.0
pRAS5, pRAS2	1.5	34.0	11.0	42.0
pRAS5, pRAS4	1.2	1.5	1.1	1.8

[a] Assays were performed on extracts of cells harvested in the late exponential phase. Uninduced cultures were grown in LB broth, induced cultures in LB broth containing 5.0 mM *m*-toluate, 0.1 mM IPTG, or both.

N-terminal amino acid sequence determination of the polypeptide.

By inserting the *xylS* gene under transcriptional control of the inducible *tac* promoter, it was possible to induce the synthesis of its product in the cell and test the importance of

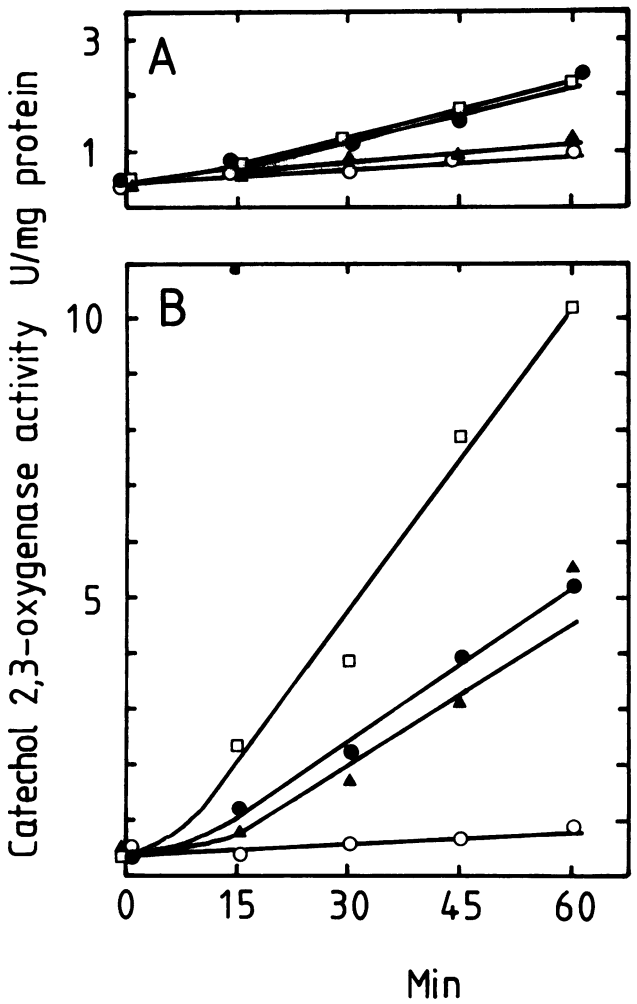

FIG. 5. Induction of C2,3-O activity expressed from pRAS5 in strains of *E. coli* DH5 also containing either (A) pRAS1 or (B) pRAS2. Cells were grown in LB broth to an OD_{650} of 0.2, and inducers were added at time zero. Samples were taken, and enzyme activities were determined. Symbols: ○, no inducer; ●, 5 mM *m*-toluate; ▲, 0.1 mM IPTG; □, 5 mM *m*-toluate plus 0.1 mM IPTG.

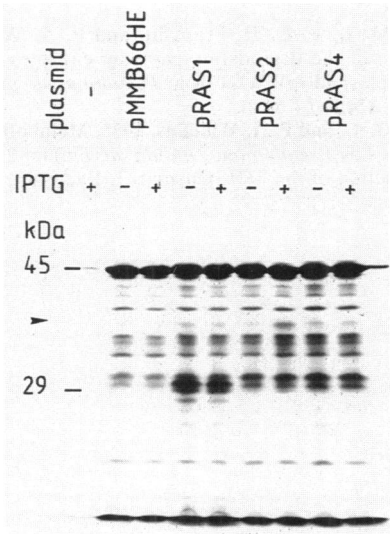

FIG. 6. Expression of the *xylS* gene inserted downstream of the *tac* promoter. Cells of the maxicell strain CSR603 containing the plasmids indicated grown in the absence (−) or presence (+) of 0.1 mM IPTG were UV irradiated and labeled with L-[^{35}S]methionine, and whole-cell lysates were analyzed by SDS-PAGE. The positions of the molecular weight standards ovalbumin (M_r 45,000) and carbonic anhydrase (M_r 29,000) are indicated on the left. The arrow marks the position of the *xylS* product.

the presence of *m*-toluate (coinducer of XylS protein) for XylS activity. The results (Table 3, Fig. 5) show that overexpression of the *xylS* gene resulted in activation of the *meta*-pathway promoter, even in the absence of cognate coinducers. To our knowledge, there is no indication that a normal metabolite, present in *E. coli* cells, can mimic *m*-toluate and activate the XylS protein. We therefore postulate that a portion of XylS molecules exist in an active configuration able to recognize the binding site on OP2 DNA and activate the OP2 promoter.

The system of XylS and the DNA of the OP2 region constitutes a convenient model for studying positive regulation of genes and DNA-protein interactions. The plasmids described in this work increase the versatility of this system.

ACKNOWLEDGMENTS

We thank S. R. Warne and C. M. Thomas for providing plasmid pSRW40 with the *xylE* gene on a *Bam*HI fragment, A. Bäckman for photography, E. Badger and E. Ivarsson for typing, and V. Shingler for critical reading of the manuscript.

This work was supported by grants from the Science and Engineering Research Council of Great Britain (SERC grant GR/C/59703), the Swedish National Research Council, and the Swedish Council for Technical Development.

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