JOURNAL OF BACTERIOLOGY, June 1994, p. 3171–3176
0021-9193/94/$04.00+0

Vol. 176, No. 11

Genetic Evidence that the XylS Regulator of the *Pseudomonas* TOL *meta* Operon Controls the *Pm* Promoter through Weak DNA-Protein Interactions

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Received 22 December 1993/Accepted 10 March 1994

The activation of the *Pm* promoter of the *meta* operon of the TOL plasmid of *Pseudomonas putida* by its cognate XylS activator protein in the presence and absence of benzoate inducers has been examined in specialized *Escherichia coli* strains carrying *Pm-lacZ* fusions and the *xylS* gene in different configurations in which all controlling elements are present in near native conditions and stoichometry. Expression of a chromosomal *Pm-xylX::lacZ* fusion was primarily dependent on the addition of an effector at a low *xylS* gene dosage, but such dependency decreased with increasing levels of the regulator, to the point that hyperproduced XylS could, in the absence of any aromatic effector, raise expression to a level 10^4-fold higher than normal basal levels. *Pm* activity never reached a defined saturation level within the range of intracellular concentrations permitted by the intrinsic solubility of the protein, thus suggesting a low degree of occupancy of the Om_R and Om_L (*Om* right and left half-sites, respectively) operator sequences by XylS. This was confirmed by transcription interference experiments, which indicated that the frequency of occupation of *Pm* by active XylS is low. This property permits a fine tuning of *Pm* activity in vivo through changes in intracellular XylS concentrations, as is predicted in current models to account for the coordinated regulation of TOL operons.

Pseudomonas putida strains containing the TOL plasmid pWW0 are able to metabolize toluene and xylenes and thereby utilize these hydrocarbons through benzoate and catechol intermediates as sole carbon sources (9, 23). Transcription of the TOL *meta* operon, which encodes the metabolism of toluate and benzoate to pyruvate and acetaldehyde, originates from the *Pm* promoter when it is activated by the XylS protein which has itself been activated by substituted benzoates (the substrates of the *meta* pathway) or by an excess of XylS protein without aromatic effectors (13, 21, 26) (see below). We have previously shown (15) that activation of *Pm* by XylS involves a 36-bp *cis*-acting DNA sequence within the promoter, which contains two 15-bp direct repeats (Om_R and Om_L, *Om* right and left half-sites, respectively) and extends a few bases into the −35 hexamer of the promoter (Fig. 1).

Induction of the *meta* operon by benzoate effectors is one of two mechanisms of activation of the *Pm* promoter. The presence of upper-pathway (toluene and xylenes to benzoate) substrates results in coordinated induction of both the upper operon and the *meta* operon. This occurs through upper-pathway substrate-effector activation of the upper-operon regulator XylR which, in turn, stimulates transcription of both the upper operon and the *xylS* gene, causing hyperproduction of the XylS protein, which then activates the *Pm* promoter in the absence of benzoate effectors (13, 21, 26). In this study we investigated the activation of *Pm* by varying intracellular concentrations of XylS. The results which we obtained suggest that *Pm* activity depends on oligomerization of XylS into an appropriate form for DNA binding, a process which is effector dependent and XylS concentration dependent.

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MATERIALS AND METHODS

Strains, plasmids, transposons, media, and general techniques. Relevant strains and constructions used in this work are listed in Table 1. Transposon vectors with different insertions were integrated into the chromosomes of target bacteria as previously described (10). For construction of pVLT24, which directs hyperproduction of XylS, an *Nco*I site overlapping the first ATG of the *xylS* structural gene (11) was generated by site-directed mutagenesis (16), and the resulting 1.3-kb *Nco*I-*Hin*dIII fragment was cloned into the corresponding sites of expression vector pTrc99A (1). Construction of pVLT43, a XylS-hyperproducing plasmid selectable by resistance to tetracycline has been described elsewhere (2). Transposon mini-Tn*10* *xylS*-Km, expressing *xylS* from a nonnative kanamycin resistance gene promoter, has been described elsewhere (14). Solid and liquid LB and NB media (19) were supplemented, when required, with 200 μg of ampicillin per ml, 50 μg of kanamycin per ml, 7 μg of tetracycline per ml, or 30 μg of chloramphenicol per ml. Recombinant DNA methods were carried out according to published protocols (19). β-Galactosidase (β-Gal) levels were determined in permeabilized cells by the method of Miller (22).

Construction and chromosomal integration of *Pm-lacZ* (*xylX::lacZ*) fusions. *lacZ* gene fusions used throughout this work as a reporter of *Pm* activity were derived from the sequence termed *PmΔ13* (23). This DNA segment can be excised as a 150-bp *Eco*RI-*Bam*HI restriction fragment and includes 100 bp upstream of the transcription initiation site (15) and the first seven codons of the *xylX* gene sequence, the first gene of the *meta* operon (8). The resulting *Pm-xylX::lacZ* fusion therefore specifies a hybrid protein containing the seven N-terminal amino acid residues of XylX. To monitor *Pm* promoter activity in monocopy gene dosage in *Escherichia coli*, the *lacZ* fusion *PmΔ13* was placed in the chromosomes of different *E. coli* strains by cloning *Pm* in vector pLC1 and

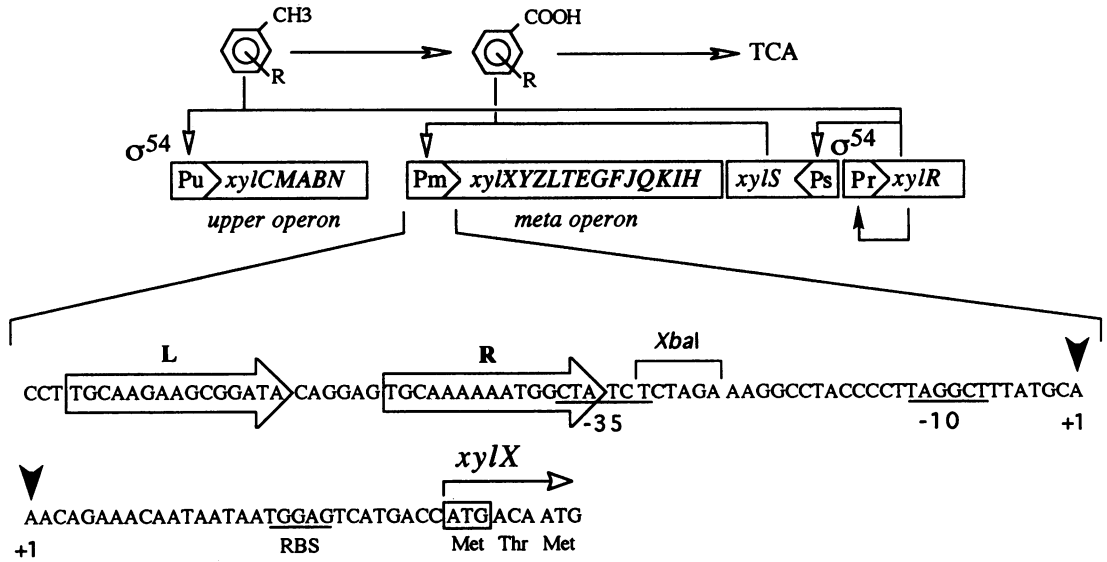

FIG. 1. Organization of *Pm* promoter of TOL plasmid. The upper scheme (not to scale) summarizes the current model for the regulatory network controlling transcription of the TOL genes of plasmid pWWO. In the presence of upper-pathway substrate inducers, such as *m*-xylene, the σ^{54}-dependent promoters *Pu* (upper operon) and *Ps* (promoter of the *xylS* gene) are activated by XylR. The σ^{70} promoter *Pm* (*meta* operon) is induced by the XylS regulator when this is activated by *meta* pathway substrates, such as *m*-toluate, or when it is present at high cellular concentrations following activation of *Ps* by XylR. XylR autoregulates, in part, its own transcription (represented as a solid arrow). The lower part of the figure represents an expansion of the *Pm* promoter region. The *Pm* promoter contains two direct repeats (*Om* region) which are required for XylS-mediated responsiveness to substituted benzoates and seem to be the site for the XylS interaction (15). The two *Om* half-sites are designated L (left) and R (right) as indicated with boxed arrows. Other details of the translation initiation region around *xylX* are shown also. *Om* overlaps the −35 hexamer of the promoter so that bound XylS presumably interacts with RNApol. TCA, tricarboxylic acids cycle; RBS, ribosome binding site.

recombining the resulting plasmids into a specialized λ lysogen as described elsewhere (3).

Transcription interference assays in vivo. Two types of in vivo transcription interference assays (5, 7, 17, 24), differing in the distance between the heterologous promoter and the XylS-binding site, were set up. In one case, a 74-bp *Pst*I-*Xba*I restriction fragment from the *Pm* promoter region carrying the XylS-binding site (*Om*) was cloned in pUC18Not (10). A *trp*::*lacZ* reporter gene fusion was subsequently introduced into the *Bam*HI site downstream of the *Om* insertion, and the whole unit was then cloned as a *Not*I fragment into the delivery plasmid pCNB5 (2), thereby generating a mini-Tn*5* *lacI*^{q} *Ptrc* *Om* *lacZ* transposon. In this mobile element, transcription of the reporter gene proceeds from the *Ptrc* promoter through the *Om* operator sequences and, therefore, might be diminished if a specific protein interacts with *Om*, thereby hindering the passage of RNA polymerase (RNApol) (5, 7, 17, 24). The mobile element was then transposed into the chromosome of *E. coli* SH252 Δ*lac* Δ*ara* as described elsewhere (4), and one of the exconjugants was used as a tester strain as explained in Results. In the other case, we constructed a consensus −10/−35 σ^{70}-dependent promoter sequence, overlapping with alternating *Om* half-sites, by synthesizing a 50-bp linker (see sequence in Fig. 3), which was cloned in front of a promoterless *lacZ* gene; further transferred as a *Not*I fragment to pUT/mini-Tn*5* Km (4), thus generating hybrid transposon mini-Tn*5* *XSB-lacZ*; and inserted into the chromosome of *E. coli* SH252 (Table 1).

RESULTS

XylS activates *Pm* promoter in a manner different from that of other members of the AraC family of regulators. Since RNApol does not form a closed complex with the *Pm* promoter in the absence of XylS (at least by the criterion of DNase I footprinting [data not shown]), it is possible that this activator favors this step during transcription initiation. XylS belongs to the AraC family of regulators (25); therefore, we considered the possibility of XylS functioning through the establishment of contacts with the α-subunit of RNApol, similar to those which seem to play an essential role in activation of promoters regulated by activators of the same family. RNApol with the mutation Lys-271 to Glu in the *rpoA* gene (encoding the α-subunit) fails to transcribe a number of promoters activated by the cognate regulators AraC and MelR (27), suggesting that protein-protein contacts important for promoter activation involve amino acid 271. To determine whether XylS interacts with RNApol in a similar manner, we lysogenized *rpoA341* of *E. coli* WAM105, along with its isogenic wild-type *rpoA*$^+$ strain *E. coli* WAM106 (Table 1), with the λ*Pm*Δ*13-lacZ* phage described above and transformed the strain with the *xylS*$^+$ plasmid pERD103. The results (Table 2) showed clearly that the amino acid change which abolishes responsiveness of RNApol to activation by AraC had no significant effect on *Pm* regulation and indicated differences in the AraC and XylS activation mechanisms.

XylS-binding site of *Pm* promoter is not saturable in vivo. To examine the degree to which XylS could activate *Pm* in the absence of benzoate effectors, we constructed an *E. coli* lysogen of a λ phage carrying the *Pm-lacZ* fusion of pLC1-Δ13 (Table 1). This produced the reporter strain *E. coli* CC118 λ*Pm*Δ*13*, which harbors a single *Pm-lacZ* fusion per cell. This strain showed negligible β-Gal levels when devoid of the *xylS* gene. Figure 2 shows that, depending on the level of *xylS* expression, the *Pm-lacZ* fusion expressed β-Gal levels ranging from about 100 Miller units, XylS was expressed from a weak

TABLE 1. Strains and plasmids

Strain plasmid, or transposon	Description or relevant properties	Reference or origin
E. coli strain		
CC118	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	20
CC118 λ*PmΔ13*	CC118, lysogenized with λ*PmΔ13lacZ*	This study
SH252	Δ(*ara-leu*) *lac*Δ*X74 araFGA*, Nal^r	R. F. Schleif
SH252 *Ptrc Om lacZ*	SH252, mini-Tn*5*::*Ptrc Om lacZ*, Km^r	This study
SH252 *XSB-lacZ*	SH252, mini-Tn*5*::*XSB-lacZ*, Km^r	This study
WAM106	F′ *araD139*, Δ(*argF-lac*) *U169* Δ(*his-gnd*) *thi rpsL150 gltSo flbB5301 relA1 deoC1 rbsR*	27
WAM105	WAM106 but *rpoA341*	27
WAM106λ*PmΔ13*	WAM106, lysogenized with λ*PmΔ13lacZ*	This study
WAM105λ*PmΔ13*	WAM105, lysogenized with λ*PmΔ13lacZ*	This study
Plasmids		
pUC18Not	pUC18 (28) derivative, MCS flanked by *Not*I sites Ap^r	10
pVLT31	Broad-host-range *Ptac-lacI*^{q} expression vector, Tc^r	2
pVLT43	pVLT31 inserted with *xylS* gene, Tc^r	2
pTrc99A	*Ptrc-lacI*^{q} expression vector, *oriV* pBR322, Ap^r	1
pVLT24	pTrc99A inserted with *xylS* gene, Ap^r	This study
pKT570	Broad-host-range plasmid, *xylR*$^+$/xylS$^+$, Sm^r	18
pERD103	Broad-host-range plasmid *xylS*$^+$, Km^r	29
pJMH16	*Plac*-based expression plasmid *rpoA*$^+$, Ap^r	27
pLC1	*lacZ* expression vector, Ap^r Cm^r	3
pLC1-Δ13	pLC1 with *PmΔ13-lacZ* fusion	This study
Transposons		
Mini-Tn*10 xylS*-Km	Mini-Tn*5* Hg containing *xylS*, Km^r Hg^r	14
Mini-Tn*5 lacI*^{q} *Ptrc Om lacZ*	Mini-Tn*5* Km containing *Ptrc Om lacZ*, Km^r *Om* inserted between *Ptrc* and *lacZ* reporter gene	This study
Mini-Tn*5 XSB-lacZ*	Mini-Tn*5* Km containing *XSB-lacZ* cassette, consensus promoter with alternating *Om* half-sites	This study

promoter in monocopy, up to >20,000 Miller units, when XylS was expressed from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible multicopy vector. In all cases, the reporter fusion was clearly responsive to variations in intracellular XylS concentrations obtained through the various expression systems employed. Furthermore, the levels of β-Gal systematically increased in the presence of effector 3-methyl benzoate (3MB), even under conditions in which the cytoplasm contained so much XylS protein that it precipitated as inclusion bodies, as is the case in bacteria containing pVLT24 and induced with IPTG (data not shown). However, the magnitude of induction by the addition of 3MB varied with the level of XylS expression in the bacteria: the addition of inducer 3MB increased the activity of the *Pm-lacZ* fusion by >100-fold when XylS was expressed moderately by plasmid pKT570 (Fig. 2), whereas only 1.5- to 2-fold increases were obtained when the regulator was hyperproduced from the IPTG-inducible pVLT24 plasmid (Fig. 2).

Since the induction experiments for which the results are shown in Fig. 2 were made with strains harboring only one *Pm-lacZ* fusion per cell, we concluded that the *Pm* promoter is very strong when fully induced but that full induction cannot generally be achieved because of the apparent lack of saturability of the system, even at the highest XylS concentrations in vivo. To rule out the possibility that this lack of saturability was due to the presence of additional XylS-binding sequences further upstream of the two direct repeats in the *lacZ* fusion used (15), we carried out similar experiments with an equiva-

TABLE 2. Regulation of *Pm* by XylS in *rpoA341* mutants[a]

E. coli strain (plasmid)	Genotype[b]	Mean β-Gal activity (Miller units)		Arabinose-chromate tolerance[c]
		Uninduced	Induced with 3MB	
WAM106λ*PmΔ13*	*rpoA* (wt) *Pm-lacZ*	18	16	−
WAM105λ*PmΔ13*	*rpoA341 Pm-lacZ*	44	42	+
WAM106λ*PmΔ13* (pERD103)	*rpoA* (wt) *Pm-lacZ* (*xylS*$^+$)	45	2,430	−
WAM105λ*PmΔ13* (pERD103)	*rpoA341 Pm-lacZ* (*xylS*$^+$)	65	2,180	+
WAM106λ*PmΔ13* (pERD103/pJMH16)	*rpoA* (wt) *Pm-lacZ* (*xylS*$^+$ *rpoA* [wt])	47	2,240	−
WAM105λ*PmΔ13* (pERD103/pJMH16)	*rpoA341 Pm-lacZ* (*xylS*$^+$ *rpoA* [wt])	30	2,170	(+)[d]

[a] The various *Pm-lacZ*-containing *E. coli* strains listed were either induced with the XylS effector 3MB under the same conditions as those given in the legend to Fig. 3 or not induced. The resulting levels of β-Gal reported are the mean values for two independent experiments.

[b] wt, wild type. Genotypes of plasmids are given in parentheses.

[c] To ascertain the retention of the *rpoA341* lesion in mutant cells, all strains were examined for tolerance to 0.02 mM chromate–0.1% arabinose, a phenotype endowed by the *rpoA341* mutation (27).

[d] *E. coli* WAM105λ*PmΔ13* (pERD103/pJMH16) was partially sensitive to arabinose and chromate, presumably because it is an *rpoA-rpoA341* diploid.

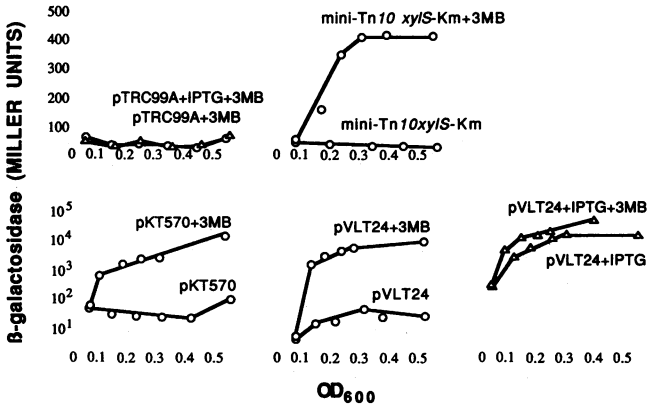

FIG. 2. *Pm* induction range at different XylS cytoplasmic concentrations in vivo. *E. coli* CC118 λ*PmΔ13* containing a chromosomal *Pm-lacZ* reporter fusion (see Materials and Methods) and either mini-Tn*10 xylS*-Km, which contains a constitutively expressed *xylS* gene (low level of expression), the low-copy-number *xylS*$^+$ plasmid pKT570 (18), or an IPTG-inducible, multicopy *xylS*$^+$ expression plasmid pVLT24. Control cells contained vector pTrc99A devoid of the *xylS* gene. Each strain was grown at 30°C in NB medium to an optical density at 600 nm (OD_{600}) of 0.1 before being supplemented, where indicated, with 2 mM 3MB and/or 50 μM IPTG. The levels of β-Gal obtained during subsequent growth are shown. Note that the vertical scale is linear for the upper plots and logarithmic for the lower plots. The induction conditions used are indicated.

lent strain harboring a 5′-deletion construct lacking all sequences upstream of the two direct repeats (except for 8 bp of the original sequence). Since this strain maintained the responsiveness of *Pm* to hyperproduced XylS (data not shown), we concluded that it was due exclusively to the sequence spanning the direct repeats.

Detection of XylS interactions with *Om* sequence of *Pm* in vivo. Since the in vivo data reported above suggested that only a small portion of the promoters might be interacting with XylS at a given time, we examined this notion with transcription interference assays. They are based on transcription termination caused by a protein bound to DNA downstream of an active promoter or inhibition of transcription initiation by competition with the RNApol-binding site (5, 7, 17, 24). In a first series of tests, we placed the XylS-binding site *Om* between a strong, IPTG-inducible *Ptrc* promoter and a reporter *lacZ* gene. This unit was engineered in monocopy in an *Δara* strain of *E. coli* to avoid interference by the analogous AraC protein (25). The results shown in Fig. 3a indicate that at the intracellular concentrations of the XylS protein achieved from the expression vector pVLT43 when induced with IPTG, expression of the reporter gene decreased consistently by about 30%. The presence of the XylS activator 3MB had only a minor effect on this level. This result validated the assay as a method to detect XylS-*Om* interactions in vivo but suggested also that these interactions are too weak to interfere strongly with transcription of the reporter gene. To rule out that this was due to an excessive distance between the promoter and the *Om* site, we constructed an additional transposon in which the −35 hexamer of a consensus σ^{70}-dependent promoter sequence was placed at the interrepeat region between the two *Om* half-sites. In this way, XylS binding in vivo to its cognate target would sterically hinder access of RNApol to the promoter, which is fused to a reporter *lacZ* gene. This transposon (mini-Tn*5 XBS-lacZ*) was introduced into the chromosome of *E. coli* SH252. The resulting strain was then transformed with

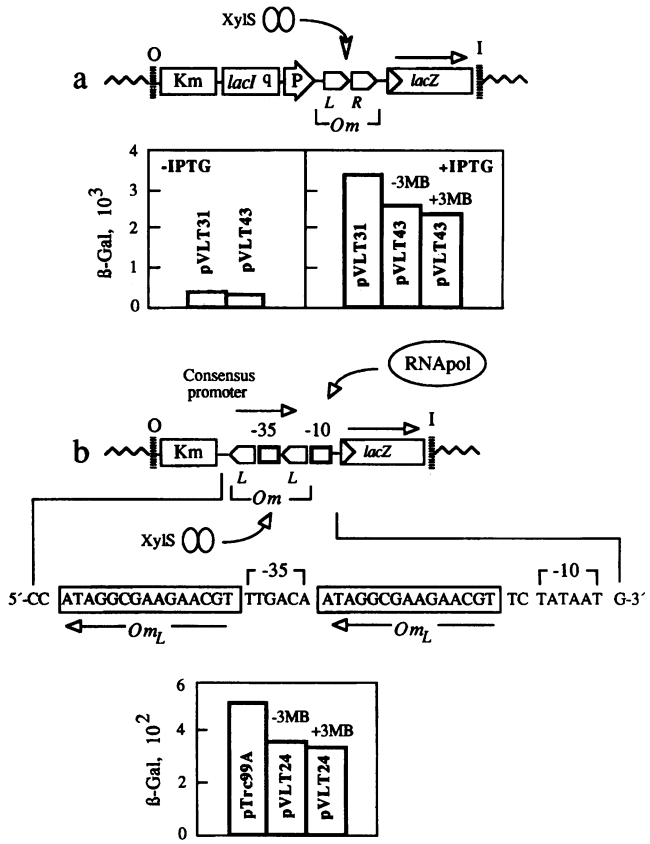

FIG. 3. Transcription interference assay to detect XylS-*Om* interactions in vivo. (a) *E. coli* SH252 *Ptrc Om lacZ*, the organization of which is schematically depicted at the top (Table 1), was transformed with the IPTG-inducible *xylS*$^+$ expression plasmid pVLT43 or with vector pVLT31 alone. An overnight culture of each strain was diluted 1:200 in LB medium and grown at 30°C to an optical density at 600 nm of 0.4, after which 50 μM IPTG and/or 2 mM 3MB was added as indicated. The bar diagram indicates the β-Gal levels reached 2 h after the inducer was added (b) *E. coli* SH252 *XBS-lacZ* (Table 1) carrying a chromosomal insert arranged as shown was transformed with the IPTG-inducible *xylS*$^+$ expression plasmid pVLT24 or with vector pTrc99A as a control. The resulting strains were treated as described in panel a, and their β-Gal levels were determined after 3 h. The drawing (not to scale) symbolizes the predicted competition between RNApol and XylS for binding overlapping DNA target sequences. The *Om* sequences used in this case included a repetition of two *Om* (left [L]) half-sites, which seems to improve effectiveness for XylS binding (15). In both panels the results shown are the averages for three separate experiments.

pVLT24 (*xylS*$^+$ placed under *Ptrc-lacI*^{q} [Table 1]), and β-Gal levels were determined in the presence and absence of XylS effector 3MB with or without IPTG (Fig. 3b). As in the former transcription interference assay and regardless of effector addition, XylS decreased the basal activity of the promoter by about 40% in relation to the β-Gal levels observed with a control strain devoid of XylS.

DISCUSSION

Although all genetic data indicate that XylS activates transcription from *Pm* by interacting directly with a specific operator *Om* which is arranged as a direct repeat (15), this has not yet been proved in vitro because of the tendency of the protein to form insoluble aggregates (2). In spite of this

obstacle, we have gained some insight into the mode of XylS-*Pm* interaction by engineering specialized *E. coli* reporter strains in which different elements of the regulatory circuit controlling *Pm* expression could be examined separately in genetic assays. Earlier communications (13, 21, 26) had reported that overproduction of XylS results in constitutive expression from the *Pm* promoter in the absence of aromatic inducers. Since this result has important implications for the coordinated regulation of the TOL pathway, we were interested in examining the phenomenon in more detail.

As shown in Fig. 2, the reporter fusion present in the chromosome of *E. coli* CC118 λ*Pm*Δ*13* was responsive over 4 orders of magnitude to variations in intracellular XylS concentrations, which were achieved with the various expression systems employed. Interestingly, increasing levels of XylS resulted in a decreasing dependency on inducer for expression, with virtual abolition of dependency when levels of XylS exceeded the intracellular solubility of the regulator. Since the induction experiments for which the results are shown in Fig. 2 were done with strains harboring only one *Pm-lacZ* fusion per cell, we conclude that while the *Pm* promoter is very strong (6) when fully induced, maximal activation of *Pm* can occur only at extremely high XylS concentrations in vivo, in fact, much higher than those existing under physiological conditions. This apparent lack of saturability of *Pm* was not due to the presence of cryptic XylS-binding sequences further upstream of the two direct repeats in the *lacZ* fusion used.

To test whether the responsiveness of *Pm* to a wide range of XylS levels was related to infrequent occupancy of *Om* by the activator protein, we employed additional genetic tests. Transcriptional interference assays (Fig. 3) indicated that XylS binding to the *Om* sequence in vivo could not inhibit by more than 30 to 40% the advance of the transcribing complex through XylS-binding sequences or the binding of RNApol to a consensus promoter overlapping *Om*. A plausible explanation of these data is that only a fraction of the target sequences is simultaneously occupied by XylS, and, hence, the effect of the protein is only a weak interference with the reporter system.

A corollary of these results is that the *Pm* promoter becomes maximally activated only at very high XylS concentrations, which probably exceed the intracellular solubility of the protein. Thus, a very wide range of expression levels can be obtained in vivo by changes in the concentration of the activator protein. This may be a useful evolutionary development to enable bacteria to adapt quickly to the presence of TOL pathway substrates: since expression of the *xylS* gene is itself controlled by the TOL upper-pathway regulator XylR (12), *Pm* can respond equally well either to low concentrations of XylS activated by *meta* (lower)-pathway substrates (benzoates) or to XylR-mediated increases of intracellular XylS induced by upper-pathway substrates (12).

What we describe operationally as infrequent occupancy of *Pm* by XylS may have different causes. They include (i) active XylS has a very short half-life, (ii) the protein-protein interactions involved in the formation of the XylS dimers which bind to the direct repeats of *Om* are weak, and (iii) *Om*-XylS interactions are weak. The fact that XylS seems to remain intact in a cell extract for some time (data not shown) suggests that cause (i) is not very probable. Since the addition of inducer is ineffective when the XylS protein is overproduced (Fig. 2), a combination of causes (ii) and (iii) would seem to be plausible. In any case, the features of XylS-*Pm* interactions described in this report, including the lack of effect on activation of *Pm* by XylS of a mutant α-subunit of RNApol, seem to differ significantly from those of other members of the AraC family of regulators to which XylS belongs (25).

ACKNOWLEDGMENTS

This work was supported by grant BIO92-1018-CO2-01 of the Spanish Comisión Interministerial de Ciencia y Tecnología (CICYT) to V.D., research contracts BIOT-CT91-0293 (BRIDGE Program) and BIO2-CT92-0084 (BIOTECH) of the European Union, the Program Acciones Integradas Hispano-Alemanas 1992/37A and 1993/118HA to V.D. and K.N.T., and a cooperative research agreement between the CIB and GBF. K.N.T. also acknowledges the support of the Fonds der Chemischen Industrie.

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