

Involvement of IHF Protein in Expression of the Ps Promoter of the *Pseudomonas putida* TOL Plasmid

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Received 26 May 1994/Accepted 16 February 1995

Regulation of the *xyl* gene operons of the *Pseudomonas putida* TOL plasmid is mediated by the products of the downstream clustered and divergently oriented *xylR* and *xylS* regulatory genes. The *xylR*-*xylS* intergenic region contains the *xylR* and *xylS* promoters Pr and Ps, respectively. A binding site for the XylR activator protein is located upstream of Ps and overlapping Pr. DNase I footprint experiments showed that one of these sites, which overlaps the recognition site for XylR activator, as well as an AT-rich region comprising the Ps promoter consensus were protected by integration host factor (IHF). IHF was found to act negatively in the *in vivo* activation of the Ps promoter, since the activity of a Ps promoter::*lacZ* fusion was elevated in an *Escherichia coli* mutant lacking IHF. In contrast, no alteration in the synthesis of XylR protein in the *E. coli* IHF-deficient mutant was detected.

The degradation of toluene and related aromatic compounds by *Pseudomonas putida* pWW0 is determined by four *xyl* gene operons on TOL plasmid pWW0 (3, 19). Two catabolic operons (upper and *meta* pathway operons, preceded by their cognate promoters Pu and Pm, respectively) contain the pathway structural genes, and two others contain the regulatory genes *xylR* and *xylS* (Fig. 1), which are adjacent but divergently transcribed from their respective promoters Pr and Ps. Transcription from the Pu and Ps promoters depends on the RNA polymerase sigma factor σ^{54} as well as XylR activator protein, which binds to recognition sequences (upstream activation sequences [UASs]) present in the Pu and Ps promoter upstream regions (1, 5, 9, 12, 13, 16). The current model for the activation of σ^{54} -dependent promoters suggests loop formation between upstream-bound activator and the promoter-bound RNA polymerase- σ^{54} complex to bring about transcriptional activation (21, 23). In the case of Pu, the DNA-bending protein integration host factor (IHF) (for a review, see reference 7) binds to a specific recognition site between the upstream XylR-target UAS and the promoter to provide the required loop. The promoter is not activated in *Escherichia coli* mutants lacking IHF (1, 2, 5). Like Pu, the *xylS* gene promoter Ps requires σ^{54} and XylR activator protein, in concert with a

specific effector, for activation. Ps forms part of a complex *xylR*-*xylS* intergenic region, with the XylR activator-binding site of Ps overlapping the *xylR* tandem Pr promoters (Fig. 2A and 4). Two potential IHF-binding sites have been identified, one with consensus sequences on both strands that overlaps the XylR-UAS/Pr promoter region, and another located close to the Ps promoter (13). Fragments containing these sites exhibited IHF binding in gel retardation assays, but IHF is not essential for activation of Ps in an *E. coli* background. Here we present data showing that IHF binds to recognition sites in the Ps promoter *in vitro* and that IHF negatively affects Ps activity.

IHF binds to two target sites in the *xylR*-*xylS* intergenic region. To identify IHF-binding sites, the region between the *StuI* and the *SphI* sites (Fig. 2A and 4) containing the Pr promoter was isolated as a 150-bp *EcoRI*-*SphI* fragment from plasmid pAH96 (13). End labeling was performed with [α -³²P]ATP and avian myeloblastosis virus reverse transcriptase. To test for binding of IHF in the Ps promoter region, a 370-bp fragment containing the region from the *BglII* site in the *xylS* gene (Fig. 2A) to the *SphI* site at -87 bp of Ps (Fig. 2A and 4) was synthesized by PCR with [γ -³²P]ATP-end-labeled M13 reverse primer, unlabeled universal primer, and, as the template, plasmid pAH94 (13), previously deleted between its

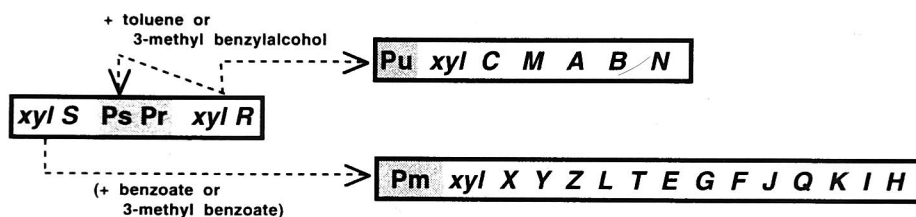


FIG. 1. Regulation of *xyl* gene operons. Operons are presented as boxes; shaded areas indicate promoter regions. Dashed arrows indicate induction by the XylR or XylS regulatory protein in concert with the respective aromatic effectors.

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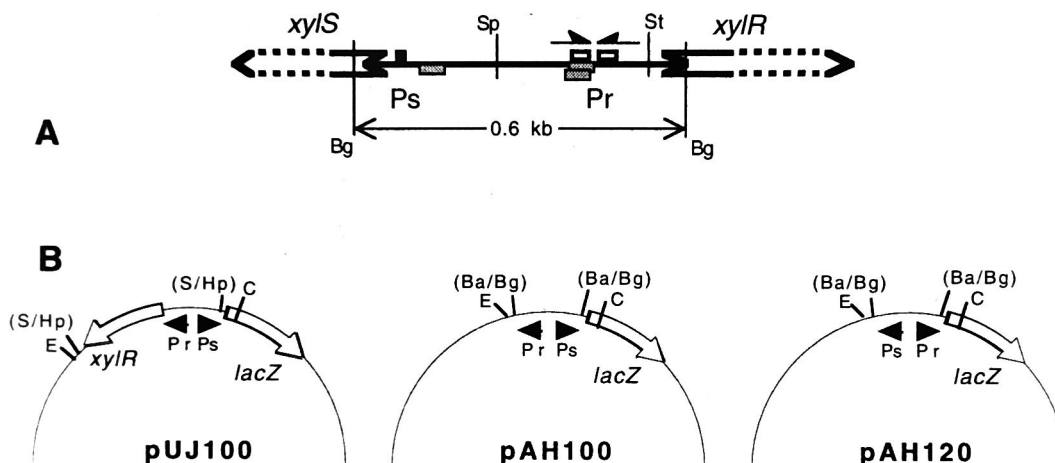


FIG. 2. *xylR-xylS* intergenic region. (A) The Ps and Pr promoters are indicated by a solid (Ps) and two open (Pr tandem promoters) boxes. The three shaded boxes represent the previously identified IHF consensus recognition sites. The inverted half arrows show the location of the invertedly repeated recognition motif of the XylR target UAS upstream of the Ps promoter. (B) Plasmid constructs carrying the Ps or Pr promoter:*lacZ* fusions described in the text. Solid arrowheads mark the promoter, and open arrows show the transcribed genes. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hpa*II; S, *Sma*I; Sp, *Sph*I; St, *Stu*I.

*Sph*I sites. DNase I footprint experiments were carried out as described by Mengeritsky et al. (20) with 0.5 ng of the labeled fragment and *E. coli* IHF protein at not less than 0.3 μ M.

Figure 3A shows protection from DNase I digestion of a region between -137 and -156 bp at all IHF concentrations tested (0.3 to 1.5 μ M). IHF may interact with a larger DNA region; however, this could not be determined since no corresponding digestion products beyond -156 bp are present in the control lane without IHF. Typically, the adjacent regions appear hypersensitive to DNase I digestion. The protected region corresponds to the previously identified IHF site, with good consensus sequences on both strands (13), which overlap at the same time the XylR activator recognition sequence and the Pr promoter consensus sequences (Fig. 4). In the Ps promoter-proximal region, IHF did not bind to the previously predicted imperfectly conserved consensus site between -35 and -47 bp (13), but rather to an adjacent region between -2 and -30 bp (Fig. 3B). This region does not exhibit a perfect IHF consensus site (10) but is typically AT-rich (8, 11) (Fig. 4). Specific protection of this sequence was observed with IHF concentrations of up to 1 μ M, while at higher IHF concentrations, protection became unspecific. In summary, we have identified two IHF-binding sites. The locations of both of these sites imply a negative role for IHF in the control of the Ps promoter.

IHF reduces the activity of the Ps promoter but does not affect the Pr promoter. To assess the role of IHF in the transcriptional activity of the Ps and Pr promoters, isogenic *E. coli* strains producing or lacking IHF protein were transformed with a set of *xyl* promoter-*lacZ* fusion plasmids based on the low-copy vector pJEL122 Δp^f (25). Plasmids pAH120 and pAH100 carry the *xylR-xylS* intergenic region on a 0.6-kb *Bgl*II fragment (Fig. 2A and B) in both possible orientations, providing the respective Pr:*lacZ* and Ps:*lacZ* promoter-reporter gene fusions (13). Construct pUJ100, like pAH100, carries the Ps promoter:*lacZ* fusion and, in addition, the entire *xylR* activator gene in its native configuration in *cis* (14) (Fig. 2B). High-copy-number plasmid pTS174 (15) bearing the *xylR* activator gene was used for activation of the Ps:*lacZ* fusion (on pAH100) in *trans*. The *E. coli* strains used were N99 (*supO*) (6) and K1299 (N99 Tn10-*himA* Δ 82, Tc^r; D. Friedman). Since activation of the Ps promoter was recently found to be inhib-

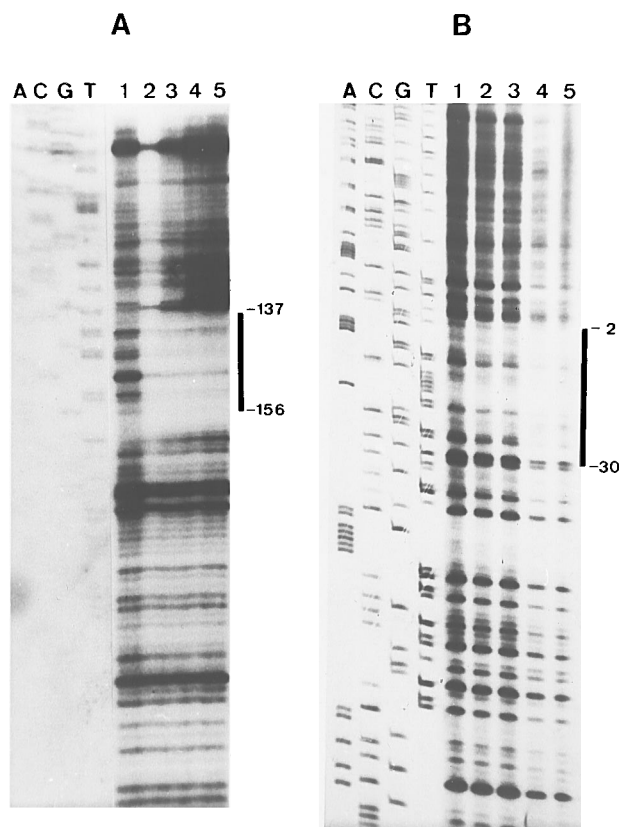


FIG. 3. Footprints of IHF on the *xylR-xylS* intergenic region containing the Pr and Ps promoters. Samples of end-labeled fragments were mixed with different amounts of IHF protein prior to DNase I digestion. The regions showing specific protection by IHF are indicated by vertical bars; all numberings refer to the transcriptional start from the Ps promoter. (A) Coding strand from the Pr promoter region. Lane 1, no IHF added; lane 2, 0.3 μ M IHF; lane 3, 0.6 μ M IHF; lane 4, 0.9 μ M IHF; lane 5, 1.5 μ M IHF. (B) Coding strand from the Ps promoter region. Lane 1, no IHF added; lane 2, 0.5 μ M IHF; lane 3, 1 μ M IHF; lane 4, 2.5 μ M IHF; lane 5, 5 μ M IHF.

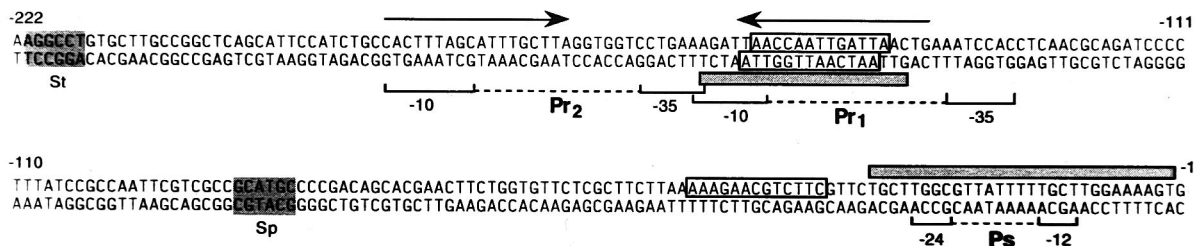


FIG. 4. Localization of protein-binding sites in the *xylR-xylS* intergenic region. Conserved nucleotides of the Pr tandem promoters (Pr₁+Pr₂, both with -35 and -10 bp consensus of σ^{70} -dependent standard promoters) and of the Ps promoter (-24 and -12 bp consensus of σ^{54} -dependent promoters) are marked by brackets below the sequence. The inverted repeat of the XylR binding site overlapping the Pr promoter is indicated by inverted arrows above the sequence. Potential consensus motifs of IHF-binding sites in the sequence are boxed. Shaded bars denote regions found to be protected by IHF in the DNase I footprint experiments. The *SphI* (Sp) and *StuI* (St) restriction sites are represented as shaded areas in the sequence. The numbering refers to the transcriptional start from the Ps promoter.

ited in the presence of LB broth in *P. putida* (18) and in the heterologous *E. coli* background (unpublished results), cultures to be assayed for β -galactosidase activity (22) were grown in M9 minimal medium (24) supplemented with micronutrient solution (0.25%, vol/vol) (4), succinate (0.8%, wt/vol), ampicillin (100 μ g/ml), and LB (24) at only 12.5% (vol/vol). For induction, 3-methylbenzyl alcohol (MBA) was added at 7.5 mM, and cultures were grown for 8 h at 30°C. Possible effects of the host mutations on plasmid copy numbers were excluded, since (i) no differences in cellular plasmid content were observed in the various host strains (data not shown) and (ii) comparable results were obtained when plasmid constructs were used and when the required activator gene and promoter fusion were in monocopy integrated into the chromosome (see below).

Table 1 summarizes the data obtained for the various promoter constructs in the wild-type strain N99 and IHF-deficient mutant strain K1299. IHF has no effect on the basal level of the Ps promoter (lines 1 and 2). The MBA-induced β -galactosidase activity of a plasmid-borne Ps::lacZ fusion (pAH100) was measured with the activator gene *xylR* present in *trans* on plasmid pTS174 (lines 3 and 4) or from construct pUJ100 with the *xylR* gene in *cis* (on the same construct, pUJ100; lines 5 and 6), and in both cases, higher β -galactosidase activity was found in the IHF-deficient mutant K1299 than in wild-type strain N99. The same result was obtained with the *E. coli* wild type and IHF mutant strains bearing *xylR*-Ps::lacZ

chromosomally integrated on a minitransposon (TnUT85; Table 1, lines 7 and 8). The activity of the Ps promoter was decreased to the level of activity seen in the wild type when plasmid pHX3-8 (17) carrying the IHF-encoding structural genes (*himA* and *hip*) was introduced into the IHF-deficient strain K1299 (line 9).

In a previous study (13), we assayed Ps promoter activation with plasmids pAH100 and pTS174 in cells grown in LB medium. More recently, LB was found to inhibit expression from the Ps but not from the Pr promoter (18), and in such experiments, we could never obtain as strong an activation of the Ps promoter as seen here with low-LB-content medium; the increase in Ps activity in the absence of IHF had thus previously been concealed.

Since IHF may affect XylR-mediated activation of the Ps promoter indirectly by altering the rate of synthesis of XylR activator, we examined expression of the *xylR* activator gene. The results show that IHF has no effect on transcription from the *xylR* tandem Pr promoters (Table 1, lines 10 and 11).

In conclusion, IHF acts differently in the regulation of two *xyl* gene operons of the *P. putida* TOL plasmid. While in the Pu promoter IHF was previously shown to bend the DNA so as to allow activation of Pu by the XylR activator, we have demonstrated here that in the Ps promoter IHF negatively affects promoter activity. The importance of the modulation of Ps activity by IHF remains to be investigated.

We are indebted to H. Nash and S. Goodman for generously providing us with strains and purified IHF protein and for kind advice. Ute Jakubzik contributed excellent technical help.

D.G. was supported by a Levi Eshkol grant from the National Council for Research and Development, Israel. K.N.T. gratefully acknowledges the generous support of the Fonds der Chemischen Industrie. This work was supported by a Lower Saxony-Israeli Cooperation grant funded by the Volkswagen Foundation and by a grant from the United States-Israel Binational Science Foundation. Part of this research was performed in the Irene and Davide Sala Laboratory for Molecular Genetics.

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TABLE 1. Activation of Ps and Pr promoters in isogenic *E. coli* wild-type and IHF-deficient mutant strains

Strain	IHF	Plasmids		β -Galactosidase activity (U)	
		Protein factor	Promoter fusion	-MBA	+MBA
N99	+	None	pAH100 (Ps::lacZ)	190	170
K1299	-	None	pAH100 (Ps::lacZ)	170	200
N99	+	pTS174 (<i>xylR</i>)	pAH100 (Ps::lacZ)	130	2,670
K1299	-	pTS174 (<i>xylR</i>)	pAH100 (Ps::lacZ)	160	4,540
N99	+	None	pUJ100 (<i>xylR</i> -Ps::lacZ)	300	1,200
K1299	-	None	pUJ100 (<i>xylR</i> -Ps::lacZ)	230	1,960
N99	+	None	TnUT85 (<i>xylR</i> -Ps::lacZ)	30	860
1299	-	None	TnUT85 (<i>xylR</i> -Ps::lacZ)	280	3,000
	-	pHX3-8 (IHF ⁺)	TnUT85 (<i>xylR</i> -Ps::lacZ)	30	970
N99	+	None	pAH120 (Pr::lacZ)	240	210
K1299	-	None	pAH120 (Pr::lacZ)	270	270

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