

# Upstream binding sequences of the XylR activator protein and integration host factor in the *xylS* gene promoter region of the *Pseudomonas* TOL plasmid

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## ABSTRACT

The *xylR* and *xylS* genes, which encode the positive regulators of the TOL plasmid catabolic pathways, are adjacent genes on the TOL plasmid and are transcribed from divergent promoters. Transcription from the *xylS* gene promoter, Ps, is positively regulated by effector-activated XylR protein and requires the specific RNA polymerase sigma<sup>54</sup> subunit (RpoN). Deletions and point mutations in the Ps upstream region localized the site of XylR interaction to the region between –133 bp and –207 bp (with respect to the transcriptional start of the *xylS* messenger), which contains an inverted sequence repeat largely homologous to the motif recognised by XylR in the XylR-regulated 'upper' catabolic pathway promoter, Pu. Gel retardation experiments showed binding of IHF to the Ps promoter region. Corresponding sequences showing good homology to the IHF-binding consensus were identified close to the Ps Promoter (between –35 bp and –47 bp, Ps proximal site) and further upstream overlapping the XylR recognition sequence (Ps distal site). In the latter case IHF recognition motifs were found well conserved on both strands at nearly the same position (between –140 bp and –152 bp on the upper and between –141 bp and –153 bp on the lower strand). Expression from Ps, either under inducing or non-inducing conditions, was, however, only slightly influenced by the absence of IHF in an IHF-deficient mutant and thus activation of Ps, like that of other sigma<sup>54</sup>-dependent promoters which are rich in Ts, does not absolutely require IHF protein.

## INTRODUCTION

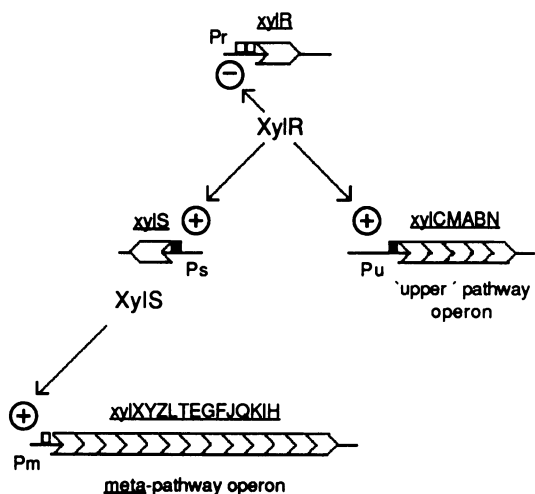
The *Pseudomonas putida* TOL plasmid contains four so-called *xyl* gene operons that contain the genetic information required for the degradation of toluene and related aromatic compounds. The 'upper' and *meta*-pathway operons, expressed from the Pu

and Pm promoters, respectively, comprise the *xyl* structural genes while the *xylR* and *xylS* genes, expressed from the Pr and Ps promoters, respectively, encode the regulatory proteins of the catabolic operons (1,2). *xyl* gene expression is controlled by a regulatory cascade that is depicted in Figure 1: XylR protein activated by pathway substrates (e.g. toluene, xylene, or 3-methyl benzylalcohol = 3MBA) and in concert with the RpoN sigma factor activates transcription from the Pu and Ps promoters. Hyperproduction of the XylS protein triggers transcription from the *meta*-pathway promoter Pm such that coordinated synthesis of the catabolic enzymes is achieved (3,4,5,6). The XylR protein when activating transcription from Ps represses its own synthesis from the Pr tandem promoters orientated away from the adjacent Ps promoter (7,4).

Both the Pu and Ps promoters belong to a specific class of promoters that is characterised by their dependence on a specific RNA polymerase sigma factor, sigma<sup>54</sup>, encoded by the *rpoN* gene. These promoters exhibit a well conserved consensus sequence, namely 5'-TGG-N<sub>9</sub>-TGCA-3', in the –12,-24 bp region (8,9,4,6). All promoters of this class require in addition a specific activator protein, which usually binds 100 bp or more upstream of its cognate promoter and which in its activated state (activated by an appropriate environmental stimulus) promotes transcription by interaction with the promoter-bound RNA polymerase / sigma<sup>54</sup> complex (10). Close proximity between upstream bound activator and the promoter site is believed to be brought about by DNA loop formation that often apparently is aided by the binding of IHF protein at sites between the promoter and upstream activator sequence (11).

Both Pu and Ps depend on sigma<sup>54</sup> and are stimulated by activated XylR protein. Activation of the XylR regulator is believed to occur through the binding of one of a large variety of activators (toluenes, benzylalcohols and related compounds; 12,13,7). Although a number of studies have been carried out on the Pu promoter (14,15,16,17), little has so far been reported on the Ps promoter. Here we present a functional analysis of the Ps-Pr promoter region with respect to XylR and IHF protein binding sites.

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**Figure 1.** Regulation of *xyl* gene operons. Black boxes denote sigma<sup>54</sup>-dependent -24, -12 type promoters, open boxes denote -35, -10 standard type promoters; coding regions (not drawn to scale) are presented as open arrows indicating the direction of transcription.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and growth conditions**

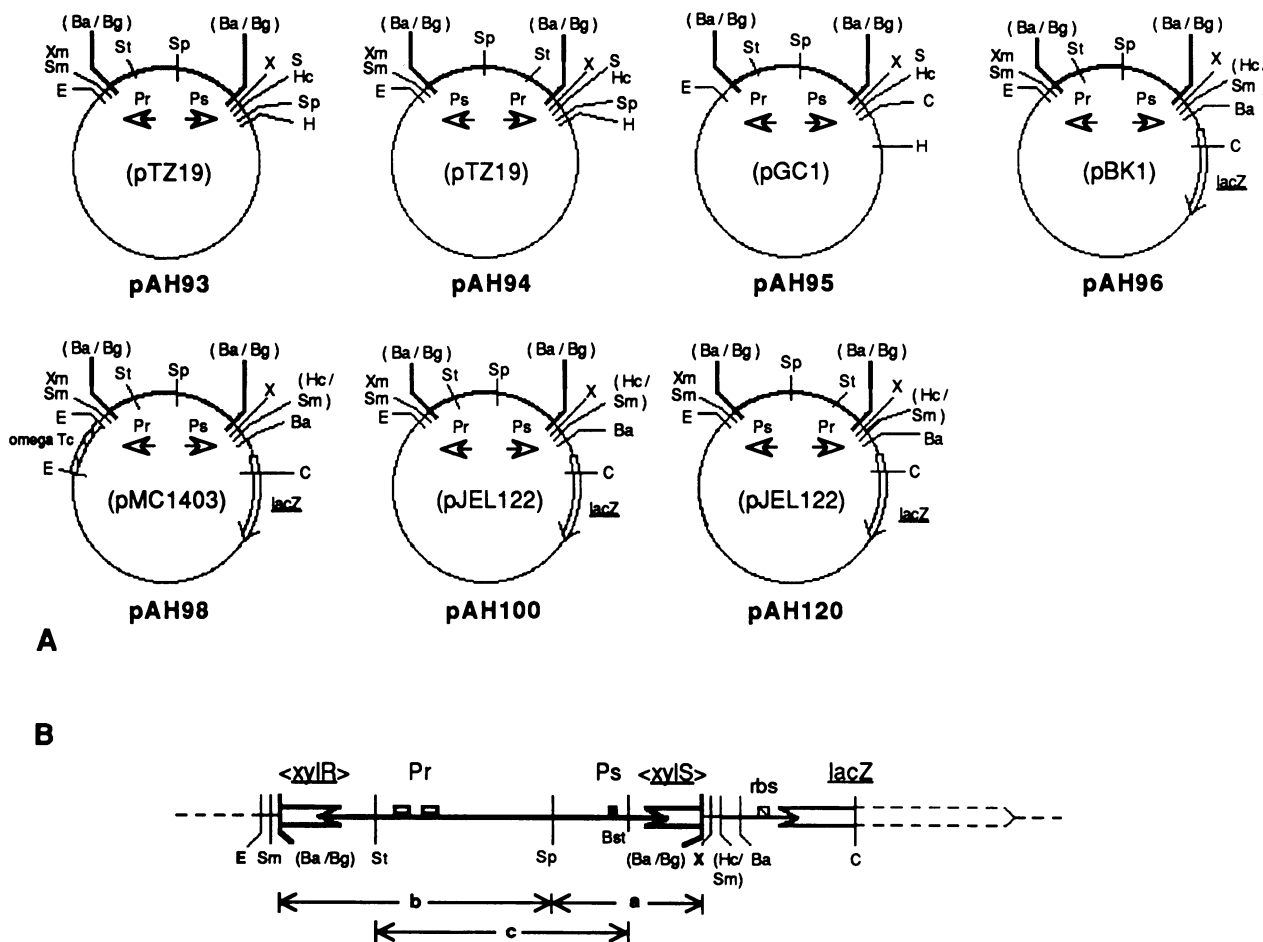
The *E.coli* strains and plasmids that were used in the course of this work are listed in Table 1. Relevant plasmid constructions are described in the **Results** section. Strains were routinely grown at 30°C in LB with the appropriate antibiotics added at the following concentrations (μg/ml): ampicillin (100), chloramphenicol (30), tetracycline (10), streptomycin (100), nalidixic acid (20).

**Enzyme assays**

Transcription from the Ps and Pr promoters was measured in *E.coli* strains ET8000, S90C or DPB101 carrying appropriate promoter-*lacZ* fusion constructs. β-galactosidase was measured according to Miller (18). Cultures were inoculated from uninduced LB precultures and subsequently grown, with and without 7.5 mM 3-methyl benzylalcohol for 16 hrs with vigorous agitation.

**DNA manipulations**

For transformation of cells, transduction (Mini-Mulac mutagenesis) and transfection (site-directed mutagenesis) standard methods were applied, and preparation of plasmid DNA,



**Figure 2.** A. Plasmid constructs. Vector names are given in brackets. For details of constructions see **Results** section in the text. B. Ps::*lacZ* fusion region on plasmids pAH96, pAH97, pAH98 and pAH100. Fragments a, b, and c, used in the gel retardation assay, are indicated. The 0.6 kb *Bg*III insert (marked as a bold line) derives from TOL plasmid pWVO, the small box preceding the *lacZ* gene in the figure denotes a strong ribosome binding site (rbs). Restriction sites: Ba=*Bam*HI, Bg=*Bg*III, Bst=*Bst*EII, C=*Cl*aI, E=*Eco*RI, H=*Hind*III, Hc=*Hinc*II, S=*Sal*I, Sm=*Sma*I, Sp=*Sph*I, St=*Stu*I, X=*Xba*I, Xm=*Xma*I.

restriction digests and separation of fragments from agarose gels were essentially carried out as described previously (19).

### Oligonucleotide site-directed mutagenesis

Point mutations were generated as described previously (20) using mutant oligonucleotides and single stranded Ps promoter DNA from plasmid pAH95. Plasmid pAH95 contains the Ps-Pr promoter region on an *EcoRI-XbaI* fragment from pAH93 cloned into the corresponding sites of plasmid pGC1 (21), a vector that yields high amounts of single stranded DNA. Mutant oligonucleotides were designed such that single base changes simultaneously introduced new restriction sites; mutations were thus easily verified by restriction analysis.

### Deletions in the Ps promoter

Deletions were generated by successive removal of nucleotides from the *StuI* site in the in the Ps promoter on plasmid pAH93 using exonucleaseIII / S1 nuclease (22). Deletions endpoints were determined by sequencing (23). Deletions from other restriction sites resulting from the mutagenesis or already existing ones, either blunt-ended or filled in with Klenow enzyme, were also created (see Figures 2 and 3).

### Gel mobility shift assay

End-labelled promoter fragments (0.1 to 10 ng, filled in with nucleotides using Klenow enzyme and <sup>32</sup>P dCTP and <sup>32</sup>P dATP

as the labelled nucleotides) and purified IHF protein (10 ng IHF) or IHF-buffer alone (without protein) were mixed in incubation buffer (10% (w/v) glycerol, 50mM KCl, 5mM Tris pH 7.5, 1mM EDTA, 2mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 100 µg/ml BSA) to give a final volume of 20 µl. Mixtures were incubated for 20 mins at 30°C. A 4% PAGE gel was prepared in TE Buffer (20mM Tris pH 7.5, 5mM EDTA) and the samples were run in the same TE buffer for 4 hs at 130 V in the cold (4°C). The following fragments were used: a) the Ps promoter region up to the *SphI* site at -87 bp (fragment a in Figure 2.B) on a 265 bp fragment with *XmaI* and *HindIII* ends (from *SphI*-deleted plasmid pAH94), b) a 325 bp *XmaI-HindIII* fragment derived from *SphI*-deleted plasmid pAH93, containing of the Ps promoter only the upstream region beyond the above *SphI* site as well as the complete Pr tandem promoter (fragment b in Figure 2.B), and c) the entire Ps-Pr promoter region present on a 260 bp *BstEII-StuI* fragment (fragment c in Figure 2.B) derived from plasmid pAH93.

## RESULTS

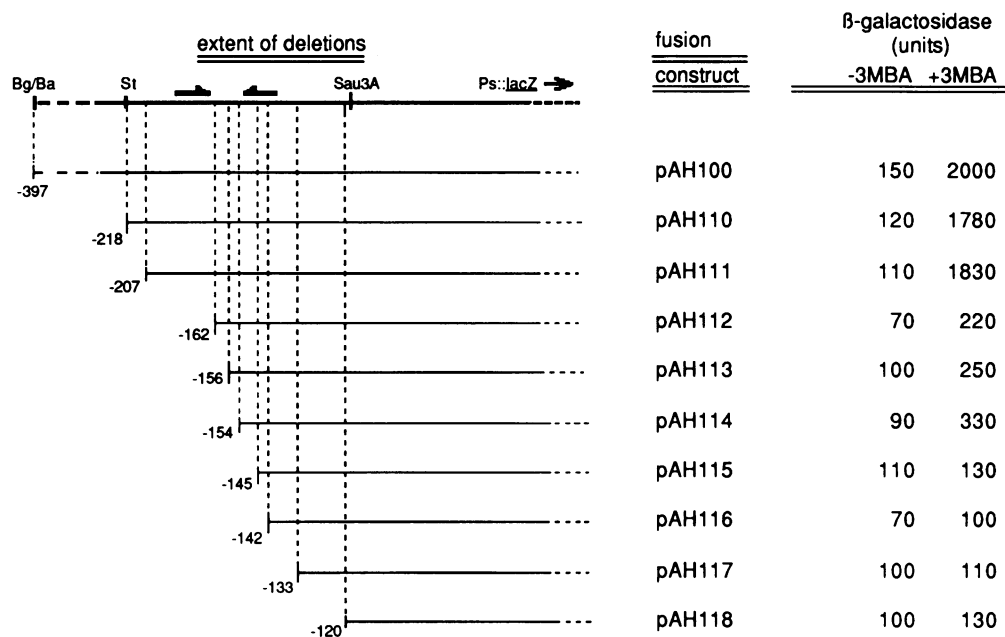
### Construction of transcriptional and translational fusions of Ps and Pr promoters to the *lacZ* reporter gene

A 0.6kb *Bg/II* fragment carrying the entire Ps and Pr promoter region as well as small portions of the *xylS* and *xylR* coding regions was excised from plasmid pRL38 (R.Leppik) and inserted

Table 1. Bacterial strains and plasmids

strain / plasmid	relevant characteristics	source / reference
strains		
<i>E. coli</i>		
ET8000	<i>rbs lacZ::IS1 gyrA hutC<sup>K</sup></i>	(38)
S90C	<i>(lac pro) rpsL</i>	(31)
DPB101	<i>(lac pro) rpsL himD451::mini-tet</i>	(31)
plasmids		
pTZ19	cloning / sequencing vector, M13 origin	Pharmacia
pGC1	cloning / sequencing vector, M13 origin	(21)
pBK1	high copy number <i>lacZ</i> fusion vector, M13 origin	B.Kessler
pMC14	03 intermediate copy number <i>lacZ</i> fusion vector	(25)
pJEL122	low copy number <i>lacZ</i> fusion vector	(24)
pRL38	<i>xylR-xylS</i> genes cloned into vector pBR322	R.Leppik
pTS174	<i>xylR</i> gene cloned into vector pACYC184	(39)
pAH90	Mini-Mulac translational <i>xylS::lacZ</i> fusion in plasmid pRL38	This work <sup>1)</sup>
pAH93	Ps-Pr promoter region cloned into pTZ19, < <i>xylS</i> > reading towards <i>XbaI</i> site	"
pAH94	as pAH93, but opposite orientation of insert, < <i>xylR</i> > reading towards <i>XbaI</i> site	"
pAH95	Ps-Pr promoter region cloned into pGC1, < <i>xylS</i> > reading towards <i>XbaI</i> site	"
pAH96	Ps-Pr promoter region cloned into pBK1, < <i>xylS</i> > reading towards vector <i>lacZ</i> gene, high copy number	"
pAH97	Ps:: <i>lacZ</i> fusion region from pAH96 cloned into vector pMC1403, intermediate copy number	"
pAH98	as pAH97, but omega(Tc <sup>r</sup> ) fragment cloned into <i>EcoRI</i> site upstream from insert	"
pAH100	Ps:: <i>lacZ</i> fusion region from pAH96 cloned into vector pJEL122, low copy number	"
pAH101	as pAH100, but A-T mutation at -134 bp (upstream from Ps promoter), new <i>EcoRV</i> site	"
pAH103	as pAH100, but T-C mutation at -141 bp (upstream from Ps promoter), new <i>BclI</i> site	"
pAH104	as pAH100, but T-C mutation at -145 bp (upstream from Ps promoter), new <i>ClaI</i> site	"
pAH106	as pAH100, but T-G mutation at -161 bp (upstream from Ps promoter), new <i>HpaII</i> site	"
pAH110	as pAH100, but deleted upstream from -218 bp (deleted upstream from <i>StuI</i> site)	"
pAH111	" -207 bp ( " <i>HpaII</i> site)	"
pAH112	" -162 bp ( " new <i>HpaII</i> site, see pAH106)	"
pAH113	" -156 bp (deleted by ExonucleaseIII/S1 nuclease treatment)	"
pAH114	" -154 bp ( " )	"
pAH115	" -145 bp (deleted upstream from new <i>ClaI</i> site, see pAH104)	"
pAH116	" -142 bp (deleted by ExonucleaseIII/S1 nuclease treatment)	"
pAH117	" -133 bp (deleted upstream from new <i>EcoRV</i> site, see pAH101)	"
pAH118	" -120 bp (deleted by ExonucleaseIII/S1 nuclease treatment)	"
pAH120	Pr:: <i>lacZ</i> fusion on low copy vector pJEL122	"

1) for details of construction see Results section in the text.



**Figure 3.** Activation of Ps promoter deletions. The inverted arrows indicate an inverted sequence repeat representing the site of interaction with XylR activator protein. The extents of the deletions are marked in the figure. Abbreviations used for restriction sites are the same as in Figure 2.

into the *Bam*HI site of the high copy sequencing vector pTZ19 to form pAH93 (Ps reading towards vector *Xba*I site, see Figure 2.A) and pAH94 (opposite orientation). In construct pAH93, the *xylS* reading frame reads into a stop codon located within the vector *Xba*I site directly adjacent to the vector *Bam*HI site.

In order to construct an appropriate promoter-*lacZ* fusion vector that would allow subcloning and measurement of the effects of the promoter upstream deletions, the Ps promoter fragment was excised from pAH93 as an *Eco*RI-*Hinc*II fragment and subcloned into the *Eco*RI-*Sma*I sites of fusion vector pBK1 (Figure 2.A), which contains the entire *lacZ* gene preceded by a strong ribosome binding site. However, due to its high copy number (about 80 per cell), this construct (pAH96) gives high uninduced basal levels of β-galactosidase. A 1.7 kb *Eco*RI-*Cla*I fragment carrying the promoter-*lacZ* fusion region was therefore subcloned into the corresponding sites of the fusion vector pJEL122 (24) (1 copy per cell), to form pAH100 (Figure 2.A and B). The same *Eco*RI-*Cla*I fragment was also subcloned into the corresponding sites of the intermediate copy number fusion vector pMC1403 (25), to form pAH97 (about 30 copies per cell); since pAH97 still exhibited high basal levels of β-galactosidase, a terminator-containing omega fragment (Tc<sup>r</sup>) (26) was inserted into the *Eco*RI site upstream of Ps on pAH97 in order to prevent potential readthrough from the vector. This latter Ps::*lacZ* fusion construct was called pAH98 (Figure 2.A).

As all the above constructs represent transcriptional fusions, a translational Ps *xylS*::*lacZ* fusion was constructed to confirm the validity of induction ratios of Ps as obtained from the transcriptional fusion pAH100. To this end, plasmid pRL38(Ap<sup>r</sup>) containing the complete *xylR*-*xylS* region was subjected to Mini-*Mulac*(Cm<sup>r</sup>) mutagenesis as described before (27), and Ap- and Cm-resistant blue clones (blue upon induction by 3MBA) were isolated that contain MiniMu inserted in *xylS* (no induction of Pm anymore) and thus provide the desired translational Ps::*lacZ* fusion. One such clone was named pAH90.

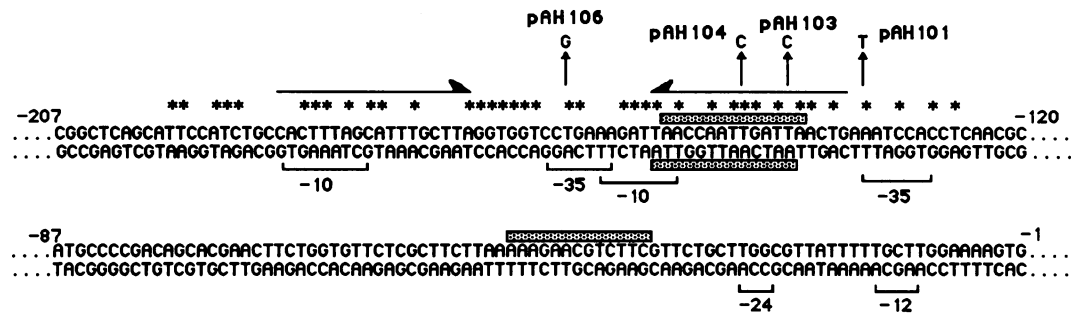
A Pr::*lacZ* transcriptional fusion plasmid was constructed as follows: from plasmid pAH94, which differs from pAH93 only by the orientation of the inserted *Bg*III fragment, an *Eco*RI-*Xba*I fragment carrying the insert was excised and cloned between the corresponding sites of pAH100 to form plasmid pAH120 (Figure 2.A). The resulting construct is identical to pAH100 except that the *Bg*III insert is inverted such that now the truncated *xylR* gene (instead of *xylS*) reads towards *lacZ*.

#### Deletions and point mutations in the upstream region of the *xylS* promoter

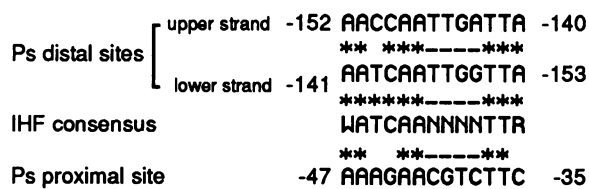
We have previously identified sequence homologies between Pu and Ps in the region from -120 to -210 bp upstream of the main transcription initiation points (15). In order to define the role of this region in the Ps promoter we generated a series of deletions from the *Stu*I site located at -218 bp. The wild type promoter in pAH100 was substituted by the deleted promoters, which were moved as *Eco*RI-*Xba*I fragments. β-galactosidase activity from wild type and deleted promoters was determined in *E. coli* ET8000 also bearing plasmid pTS174 (carrying the *xylR* gene) in the presence and absence of 3-methyl benzylalcohol. The extent of deletions as well as the corresponding β-galactosidase activities are shown in Figure 3.

In the wild type, construct pAH100 containing the complete Ps-Pr promoter region, an approximately 20-fold increase in transcription from Ps was obtained upon induction by the XylR-specific effector 3MBA. Similar induction levels were obtained with the translational *xylS*::*lacZ* fusion pAH90.

Deletion constructs containing Ps upstream sequences up to at least -207 bp, i.e. plasmids pAH111 and pAH110, are as well induced as the wild type; sequences beyond -207 bp are thus not required for promoter activation. Further deletions up to -162 bp, -156 bp or -154 bp (constructs pAH112, pAH113 and pAH114, respectively), greatly reduced promoter activity, though weak but significant activation (about 15% of induced wild type activity) was still observed. Further deletions to -145



**Figure 4.** Relevant regions of the Ps promoter region. The inverted arrows denote an inverted sequence repeat as the putative site of interaction with XylR protein; asterisks point out nucleotides that are conserved in the XylR-binding region of the Ps and Pu promoters; the two standard type (-10, -35 type) Pr promoters as well as the sigma<sup>54</sup> dependent (-12, -24 type) Ps promoter are marked out in the figure; the three dotted boxes indicate the IHF consensus sites.



**Figure 5.** Proposed IHF target sequences in Ps promoter proximal and distal regions aligned to the IHF consensus sequence (W=A or T, N=any nucleotide, and R=A or G; asterisks mark conserved nucleotides); all motifs presented read in direction 5' to 3'.

bp and -120 bp (constructs pAH115 to pAH118) completely abolished activation. In summary, the region between -133 bp and -154 bp contains a sequence that mediates a limited activation of Ps, whereas full promoter expression requires the presence of a longer sequence extending to -207 bp.

Within this region, a number of bases were changed by site-directed mutagenesis: the transitions A to T at -134 bp, T to C at -141 bp, T to C at -145 bp and T to G at -161 bp all created new restriction sites (*EcoRV*, *BclI*, *ClaI* and *HpaII*, respectively; Figure 4). With the corresponding mutant *Ps::lacZ* fusion constructs pAH101, pAH103 and pAH106, no obvious difference in the induction pattern with respect to the wild type was observed (Table 2). However, with the pAH104 (T -145→C) construct a significant reduction (about 50%) in basal and maximum levels was found, although the induction ratio ( $\beta$ -galactosidase activity in the presence of effector /  $\beta$ -galactosidase activity in the absence of effector) was similar to that in the wild type Ps promoter (see Discussion).

**Ps and Pr promoter activation in a mutant strain deficient in IHF protein**

In this paper we report two regions within the Pr/Ps-promoter region (Figures 4 and 5) that bind IHF protein. One of these, the Ps proximal site (between -35 bp and -47 bp), corresponds to a site that based on sequence homologies we had already predicted in an earlier report (15). The second region (the Ps distal site) in fact comprises two highly conserved IHF consensus sequences (for IHF consensus see refs. 28,29,30) present on the upper (8 out of 9 residues conserved) and on the lower strand (9 out of 9 residues conserved) differing in position by only one nucleotide. These latter (distal) sites are located further upstream

**Table 2.** Activation of mutant Ps promoter constructs

construct	characteristics	$\beta$ -galactosidase (units)	
		-3MBA	+3MBA
pAH100	wildtype Ps promoter region	150	2000
pAH101	A→T transition at -134 bp	140	1650
pAH103	T→C " -141 bp	120	1960
pAH104	T→C " -145 bp	90	880
pAH106	T→G " -161 bp	140	1980

$\beta$ -galactosidase activity from *Ps::lacZ* fusion constructs was measured in *E. coli* ET8000 harbouring pTS174 (*xylR*); 7.5mM 3MBA were added for induction; experimental details are given in the Materials and Methods section.

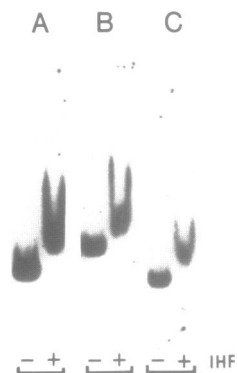
**Table 3.** Activation of Ps and Pr promoters in IHF proficient and IHF deficient strains

IHF fusion construct	characteristics	$\beta$ -galactosidase (units)	
		-MBA	+MBA
+ pAH100	<i>Ps::lacZ</i> fusion, 1 copy	190	2360
- "	"	290	1990
+ pAH104	as pAH100, but point mutation in XylR-binding site	70	820
- "	"	140	410
+ pAH98	<i>Ps::lacZ</i> fusion, 30 copies	510	4690
- "	"	1020	2460
+ pAH120	<i>Pr::lacZ</i> fusion, 1 copy	490	330
- "	"	450	330

Measurements of  $\beta$ -galactosidase activities were carried out as described in the Materials and Methods section; both the IHF-proficient strain (S90C, '+' in the table) and the IHF-deficient strain (DPB101, '-' in the table) carried in addition to the respective Ps promoter fusion construct plasmid pTS174 providing XylR activator protein.

within the region required for XylR-mediated activation of Ps, thereby also overlapping part of the Pr tandem promoters.

We therefore examined expression from both Ps and Pr promoters in isogenic IHF-minus and IHF-plus strains (bearing *xylR* on pTS174) with and without 3-methyl benzylalcohol (Table 3). The low copy plasmids pAH100 and its mutant derivative pAH104 were used to monitor activation of Ps in the IHF plus and minus backgrounds. pAH104 carries the point mutation that caused a decrease of Ps promoter activity. Our results show that a lack of IHF protein reduced activation of Ps by 3MBA/XylR



**Figure 6.** Gel retardation of promoter fragments by IHF protein. Identical incubation mixtures containing the respective fragments were incubated with IHF-free buffer ('-' samples) or with 10 ng IHF protein in the same buffer ('+' samples). For further details on incubation and electrophoresis conditions see **Material and Methods** section. Lanes A: Ps proximal promoter fragment a (containing Ps promoter); lanes B: Ps distal promoter fragment b (containing Pr tandem promoter); lanes C: Ps-Pr promoter fragment c (containing the entire Ps and Pr promoter region).

by 20–30%, which is by far not as much as the respective decrease observed for the Pu promoter (70% reduction; 14,17). Moreover, basal expression from Ps (i.e. in the absence of effector) is higher in the IHF minus background. This pattern of activation was similar for the wild type construct pAH100 and the mutant promoter pAH104, although activity levels obtained with pAH104 were in all cases by about 50% lower than those obtained with pAH100. To confirm the suggestion that IHF may be involved in 'repression' of Ps under non-inducing conditions we used a fusion in the intermediate copy number plasmid pAH98: here basal  $\beta$ -galactosidase levels in the absence of effector were 500 units in the IHF plus background, while they were twice as high in the IHF minus strain, which clearly reflects some involvement of IHF protein in Ps regulation (see **Discussion** section).

The *xyIR* gene promoter Pr is transcribed divergently from the adjacent Ps promoter. It has been reported that Pr is slightly repressed in the presence of inducer (4,7). This XylR/effector-mediated 'autoregulation' was observed for the Pr::lacZ fusion in pAH120 in both the IHF wild type and mutant background (Table 3). Therefore, IHF protein does not seem to be involved in the expression of the Pr promoter.

#### Gel retardation by IHF protein of fragments carrying distinct parts of the Ps-Pr promoter region

Since two putative regions of IHF binding were found in the Ps-Pr promoter region (in one case with the IHF consensus motif well conserved at almost the same position on both strands), the Ps-Pr promoter region was split into the two fragments described in **Materials and Methods** (see also Figure 2.B). One fragment (fragment a) contains the Ps promoter up to -87 bp and thus carries one IHF site (Ps proximal site), whereas the other (fragment b) extends from -87 bp further upstream and thereby includes the second (Ps distal) IHF binding region (with the IHF consensus on both strands) and the Pr tandem promoters. A fragment carrying both the entire Ps and Pr promoter regions (fragment c) was also tested. Figure 6 shows that both the Ps promoter fragment a (lanes A) and the Ps promoter upstream Pr promoter fragment b (lanes B) were retarded by added IHF

protein, as was fragment c bearing both these regions together (lanes C). Similar IHF-dependent shifts of the distinct Ps promoter fragments were also obtained with crude extracts from a strain bearing a plasmid with the IHF-encoding genes under the control of the IPTG-inducible *ptac* promoter: here fragment shifts were observed only with crude extracts prepared from IPTG-induced, i.e. IHF-producing cultures (data not shown).

## DISCUSSION

We reported earlier that XylR-mediated activation of both the Pu and Ps promoters requires promoter upstream sequences (15), in the case of Ps confined to a stretch between -118 bp (*Sau3A* site, Figure 3) and -218 bp (*StuI* site). Based on sequence homologies between the Pu and Ps promoter upstream sequences a small potential XylR-binding motif was deduced which appears twice in both promoters.

Recently, deletion and insertion studies on the Pu promoter (14,16) as well as complementing DNA-protection experiments (14,17) identified a rather large inverted sequence repeat between -127 bp and -172 bp upstream of the Pu promoter as the site of binding of the XylR activator protein, and this motif comprises the earlier suggested smaller motifs. A rather large inverted repeat sequence homologous to the region protected by XylR protein in the Pu promoter was also found in the Ps promoter between -136 bp and -187 bp (16) (Figure 4) comprising the region that contains the two previously identified smaller motifs. The results presented here suggest that the above proposed larger inverted repeat sequence motif represents the XylR-binding region within the Ps promoter: deletions further upstream that do not overlap the outlined motif (Figures 3 and 4) do not affect activation of Ps; removal of only the upper half of the inverted repeat reduces induction but does not abolish it entirely, whereas deletions that also remove partly or completely the downstream half of the repeat, totally prevent activation of the Ps promoter. Consistent with this was the finding, that from the 4 single point mutations generated in the Ps promoter only construct pAH104, where a conserved base within the inverted repeat region of Ps and Pu was changed, exhibited reduced basal and maximum levels of transcriptional activation of Ps. It has been reported for the Pu promoter that both *in vivo* (14) and *in vitro* (17) XylR binds to its recognition sequence to some extent also in the absence of effector. Therefore, a mutation in the XylR recognition motif is expected to diminish both basal and maximum levels of promoter activation, as it was observed here for mutant construct pAH104. A similar decrease of Ps activity is found when measuring transcription from Ps in a strain devoid of XylR protein (about 80 units of  $\beta$ -galactosidase, not shown). Mutations either of conserved bases just outside of the repeat regions (constructs pAH101 and pAH106) or of a non-conserved base within one arm of the repeat (pAH103) did not have any deleterious effect on Ps promoter activation. Thus, our data indicate that XylR activator protein recognizes the two halves of the proposed motif in the Ps promoter upstream region, as it does in the Pu promoter. Removal of only one half of the inverted repeat appears to allow recognition of the remaining half by XylR protein, thus permitting weak binding of XylR and a corresponding weak XylR-mediated induction of Ps. At present it is not clear whether the two halves of the inverted repeat are contacted by two subunits of one dimeric XylR molecule or by two individual XylR molecules. In the latter case, since binding to only one site permits only weak activation

whereas binding to both sides gives a much greater induction, cooperative binding of XylR would be likely.

We suggested previously the existence of potential IHF binding sites in the Pu and Ps promoters (15) and this has now been confirmed with the Pu promoter (14,17). In the present study we have identified by gel retardation assays two regions upstream of Ps that bind IHF and which exhibit good IHF consensus sequences: one site preceding the Ps promoter between -35 bp and -47 bp, we had already predicted in our previous report (15); two more sites were located further upstream at almost the same position but in opposite orientation on either of the opposing strands; these sites precede the Pr promoter and overlap part of the identified XylR-binding site (Figures 4 and 5). Since rather high concentrations (about 5 µg/ml) of IHF protein had to be applied both in our experiments and a previous study by de Lorenzo (17) and since the retarded bands displayed considerable smearing, we conclude that binding of IHF to the two regions in the Ps promoter region is rather weak. Such weak interaction of IHF with the Ps-Pr promoter region corresponds to the results obtained for the induction of Ps::lacZ and Pr::lacZ fusions in IHF-deficient and -proficient strains (Table 3). In IHF wild type strain S90C, under non-inducing conditions the relatively weak binding of IHF to the Ps distal site overlapping part of the XylR-binding motif may suffice to prevent uninduced XylR protein from binding and thus ensures complete 'repression' of Ps. Under inducing conditions, activated XylR protein binds strongly to its recognition sequence and is not notably affected by IHF which competes only weakly for part of the sequence. In the IHF-deficient strain DPB101, slightly impaired activation of Ps was observed. It may be assumed that IHF normally functions on the Ps proximal IHF-binding site by stabilizing bends or loop structures to favour good activation of Ps; lack of IHF thus could cause a small decrease of activation. It can be excluded that lack of IHF indirectly affects activation of Ps via decreased expression of XylR activator protein: the results for the Pr fusion construct pAH120 in Table 3 show that expression of the xylR gene promoter Pr is not at all impaired in the IHF-deficient strain. On the other hand, at the present stage it cannot be excluded that lack of IHF may indirectly alter transcription from Ps via reduced levels of DNA gyrase activity (31,32).

It appears that quite generally those sigma<sup>54</sup>-dependent promoters which, like Ps, are rich in Ts, do not absolutely require IHF protein for activation (37), probably because the required bend in the DNA is attained by the T-rich stretch of sequence itself. Our observations underline once more the notion that IHF exerts primarily structural rather than regulatory functions: while DNA-bending brought about by IHF protein often aids promoter activation as it is the case e.g. for the Pu, *KpnifH* or *Caulobacter* flagellar promoters (14,17,33,29), examples of negative effects of IHF on promoter activation under certain circumstances or on specific promoter activator-binding sites have also been described, e.g. for the *EcompC* and *ompB*, the *KpnifU*, or the *EcglHhp2* promoters (34,35,36,37) and here for the Ps promoter distal IHF sites overlapping the XylR-binding region. Thus, whereas XylR-mediated activation of both the Pu and Ps promoters is effected in exactly the same manner, the involvement of IHF protein apparently is of a different nature in the two promoters: in Pu (poor in Ts) IHF is absolutely essential for XylR-mediated induction; in Ps (rich in Ts) lack of IHF results only in slightly reduced activation of Ps upon induction and somewhat incomplete 'repression' of transcription under non-inducing conditions.

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