

m-Xylene-Responsive *Pu*-*PnifH* Hybrid σ^{54} Promoters That Overcome Physiological Control in *Pseudomonas putida* KT2442

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The sequences surrounding the $-12/-24$ motif of the *m*-xylene-responsive σ^{54} promoter *Pu* of the *Pseudomonas putida* TOL plasmid pWW0 were replaced by various DNA segments of the same size recruited from *PnifH* σ^{54} promoter variants known to have various degrees of efficacy and affinity for σ^{54} -RNA polymerase (RNAP). In order to have an accurate comparison of the output in vivo of each of the hybrids, the resulting promoters were recombined at the same location of the chromosome of *P. putida* KT2442 with a tailored vector system. The promoters included the upstream activation sequence (UAS) for the cognate regulator of the TOL system (XylR) fused to the $-12/-24$ region of the wild-type *PnifH* and its higher σ^{54} -RNAP affinity variants *PnifH049* and *PnifH319*. As a control, the downstream region of the *glnAp2* promoter (lacking integration host factor) was fused to the XylR UAS as well. When the induction patterns of the corresponding *lacZ* fusion strains were compared in vivo, we observed that promoters bearing the RNAP binding site of *PnifH049* and *PnifH319* were not silenced during exponential growth, as is distinctly the case for the wild-type *Pu* promoter or for the *Pu*-*PnifH* variant. Taken together, our results indicate that the promoter sequence(s) spanning the $-12/-24$ region of *Pu* dictates the coupling of promoter output to growth conditions.

Pseudomonas putida strains harboring the TOL plasmid pWW0 are able to grow on toluene, *m*-xylene, and *p*-xylene as the only carbon source because of a highly regulated pathway which renders benzoate or toluate from these aromatic substrates into Krebs cycle intermediates (49, 53). Expression of the upper TOL operon for bioconversion of toluene, *m*-xylene, and *p*-xylene into the corresponding carboxylic acids is driven by the σ^{54} -dependent promoter *Pu* (27) (Fig. 1). In the presence of suitable aromatic effectors (e.g., *m*-xylene), this promoter is activated at a distance by the XylR protein, a member of the prokaryotic enhancer binding protein family of transcriptional regulators (32, 64), with the assistance of integration host factor (IHF), which facilitates the appropriate *Pu* geometry (21) and helps the recruitment of the RNA polymerase to the promoter (2, 8, 38) (Fig. 1).

Pu activity in vivo not only requires the presence of XylR effectors in the medium, it is also strongly dependent on the metabolic status of the cell. An excess of certain carbon sources (16, 29) or rapid growth in rich medium inhibits the activity of the promoter in vivo even if the aromatic inducer is present in the culture (15, 20, 22, 23, 31, 40, 53). At least four distinct elements appear to take part in such down-regulation. First, the presence of glucose and other carbohydrates (29) inhibits *Pu* activity through a process which involves the *ptsN* gene, encoding the IIA^{Ntr} protein of the phosphoenolpyruvate-sugar phosphotransferase system (16, 48). Second, rapid growth in rich medium (for instance, Luria-Bertani [LB] medium) restrains the performance or activity of the σ^{54} protein (15). This is revealed by the fact that overproducing σ^{54} largely relieves physiological inhibition of *Pu*. Third, intracellular concentrations of the alarmone (p)p-

pGpp, a molecule involved in the stringent response (17), have a moderate stimulatory effect on the activity of *Pu* in vivo and in vitro (11). This outcome is mechanistically relevant, as ppGpp appears to regulate sigma factor competition for the scarce core RNAP during stationary phase (33), and it favors the entry of σ^{54} into the enzyme under conditions of amino acid starvation (37). Finally, *Pu* (Fig. 1) is entirely dependent on IHF (7), which binds its target site only during stationary phase in the *Pu* promoter (61). Other factors (for example, TurA) bind *Pu* as well, entering additional environmental inputs, such as responsiveness to low temperatures (51).

Perhaps because of all the somewhat redundant mechanisms mentioned above, it has been impossible so far to isolate a single *P. putida* mutant in which *Pu* could be altogether free of physiological control. But is the contrary true as well (i.e., can *Pu* variants devoid of metabolic coregulation be generated)? Previous reports have shown that changes in the promoter $-12/-24$ sequences lead to variations in promoter performance in vitro and in vivo (18, 19, 55, 62, 63). In this work, we created a number of hybrid promoters between the upstream activation sequence (UAS) and IHF sequence of *Pu* and the σ^{54} -RNAP binding regions from *PnifH049* and *PnifH319* promoters of *Klebsiella pneumoniae* (two variants of *PnifH* bearing nucleotide changes that increase the activity of the promoter) (5, 50, 55) or from the *glnAp2* promoter of *Escherichia coli*, which is independent of IHF for its activation (44). Our data show that *Pu* variants with such $-12/-24$ regions abolish growth phase-dependent control of transcription, suggesting that physiological regulation of this promoter largely reflects the engagement of RNAP with the $-12/-24$ motif.

MATERIALS AND METHODS

Strains and general procedures. The *E. coli* and *P. putida* strains and plasmids used in this work are listed in Table 1. The sequences and schemes of the hybrid promoters used are drawn in Fig. 2. The strains *E. coli* CC118 *supF* (35) and *E.*

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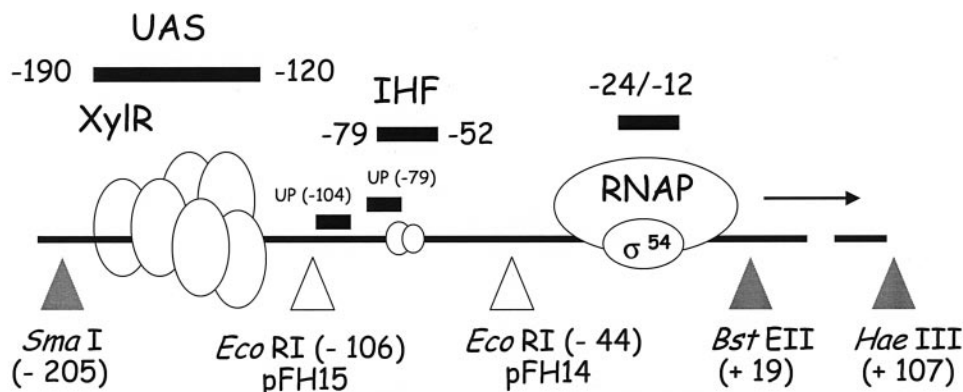


FIG. 1. Organization of the σ^{54} promoter *Pu* of *P. putida* plasmid pWW0. The distribution of relevant DNA sequences and their coordinates in respect to the transcription initiation site, as well as some important restriction sites, is shown. The region includes the UAS for XylR, the $-12/-24$ region recognized by σ^{54} -RNAP, the IHF binding site located within the intervening region, and the adjacent UP-like elements (38) for docking of the σ^{54} -RNAP. Protein sizes are symbolic. The upstream nucleoprotein complex may contain six or seven monomers of the regulator. The locations of the new EcoRI sites in pFH14 (-44) and pFH15 (-106) are indicated as well.

coli HB101(pRK600) were used as the host for pBK16 derivatives (Table 1) and as the helper for *oriT*-mediated mobilization, respectively. Hybrid promoters were mobilized into strain *P. putida* KT2442 *hom. fg. xylRS* (35) and integrated in its chromosome as explained below. The strain derived from *P. putida* KT2442 *hom.*

fg. xylRS but bearing the wild-type promoter fusion *Pu-lacZ* was named *P. putida* SF05X. Equivalent *P. putida* strains bearing other fusions were designated *P. putida* MR05X (*Pu-PnifH-lacZ*), *P. putida* SF02X (*Pu-PnifH319-lacZ*), *P. putida* MR02X (*Pu-PnifH049-lacZ*), and *P. putida* SF03X *Pu-PglAp2-lacZ*.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype, phenotype, or relevant characteristics	Reference
<i>E. coli</i>		
CC118	$\Delta(ara-leu) araD \Delta lacX74 galE galK phoA thi-1 rpsE rpoB argE(Am) recA1$	39
CC118 <i>supF</i>	CC118 mini-Tn5 $Cm^r supF$	35
HB101	$Sm^r recA thi pro leu hsd R^- M^+$	3
<i>P. putida</i>		
KT2442	Prototrophic; Rif ^r	28
KT2442 <i>hom. fg. xylR/xylS</i>	KT2442 $Km^r Hg^r$ mini-Tn10 <i>hom. fg. mini-Tn5 Hg xylRS</i>	35
SF05X	SF05X KT2442 <i>hom. fg. xylR/S</i> $\Omega Sm/Sp Pu-lacZ$	26
MR05X	MR05X KT2442 <i>hom. fg. xylR/S</i> $\Omega Sm/Sp Pu/PnifH-lacZ$	This work
SF02X	KT2442 <i>hom. fg. xylRS</i> $\Omega Sm/Sp Pu-PnifH319-lacZ$	This work
MR02X	KT2442 <i>hom. fg. xylRS</i> $\Omega Sm/Sp Pu-PnifH049-lacZ$	This work
SF03X	KT2442 <i>hom. fg. xylRS</i> $\Omega Sm/Sp Pu-PglAp2-lacZ$	This work
Plasmid		
pRK600	$Cm^r ColE1 oriV RK2 mob^+ tra^+$	35
pJES366	$Ap^r K. pneumoniae PnifH319$	55
pJES366-SF4	pJES366 with a new EcoRI site at position -44 of <i>PnifH319</i> sequence	This work
pBK16	$Sm^r Sp^r lacZ$ transcriptional fusion vector; <i>supF</i> -suppressible codons in <i>aada</i> and <i>lacZ</i>	35
pBK16 <i>Pu</i>	<i>Pu-lacZ</i> delivery vector; pBK16 inserted with region -211 to $+107$ of <i>Pu</i>	This work
pMJ1	pBK16 inserted with a EcoRI-BamHI fragment spanning -44 to $+33$ of the <i>PnifH</i> promoter.	This work
pMJ2	<i>Pu-nifH-lacZ</i> delivery vector; pMJ1 inserted with an EcoRI sequence spanning -211 to -44 of <i>Pu</i> from pFH14 plasmid	This work
pMB1	$Ap^r K. pneumoniae PnifH-lacZ$	6
pEZ9	$Ap^r pUC18$ inserted with a 312-bp EcoRI-BamHI fragment spanning -211 to $+107$ of <i>Pu</i>	21
pRF1	pBK16 inserted with fragment EcoRI-BamHI spanning -44 to $+33$ of the <i>PnifH049</i> promoter	This work
pRF2	<i>Pu-nifH049-lacZ</i> delivery vector; pRF1 inserted with EcoRI fragment spanning -211 to -44 of <i>Pu</i> from pFH14	This work
pFH44A	pBK16 inserted with EcoRI-BamHI fragment spanning -44 to $+33$ of <i>PnifH319</i> excised from pJES366-SF4	This work
pCG2 <i>Pu</i>	$Ap^r ori ColE1 ori M13$; phagemid vector pCG2 (43) inserted with EcoRI-BamHI fragment spanning -211 to $+107$ of <i>Pu</i> from pEZ9	This work
pFH14	pCG2 <i>Pu</i> with a new EcoRI site at -44 of the <i>Pu</i> promoter sequence	This work
pFH15	pCG2 <i>Pu</i> with a new EcoRI site at -106 of the <i>Pu</i> promoter sequence	26
pFH44	<i>Pu-nifH319-lacZ</i> delivery vector; pFH44A inserted with an EcoRI fragment spanning -211 to -44 of <i>Pu</i>	This work
pWC88049	As pMB1, but <i>PnifH049-lacZ</i>	5
pFH43	<i>Pu-PglAp2-lacZ</i> delivery vector; pFH43A with an EcoRI insert spanning -211 to -106 of the <i>Pu</i> promoter from pFH15	This work
pEZ9-PCR	pEZ9 with the sequence of 142-bp EcoRI-BstEII segment replaced by the equivalent sequence (-106 to $+19$) from <i>glnAp2</i> of <i>E. coli</i> .	This work
pFH43A	pBK16 inserted with fragment EcoRI-BamHI of 240 bp from pEZ9-PCR	This work
pTE103	$Ap^r pUC8$ derivative with a strong T7 terminator; vector for supercoiled templates earmarked for transcription	24
pEZ10	pTE103 inserted with EcoRI-BamHI fragment spanning -208 to $+93$ of <i>Pu</i>	46
pTE103 <i>Pu/PnifH</i>	pTE103 inserted with region -205 to $+35$ of the <i>Pu-PnifH</i> hybrid	This work
pTE103 <i>Pu/PnifH319</i>	pTE103 inserted with region -205 to $+35$ of the <i>Pu-PnifH319</i> hybrid	This work
pTE103 <i>Pu/PnifH049</i>	pTE103 inserted with region -205 to $+35$ of the <i>Pu-PnifH049</i> hybrid	This work
pTE103 <i>Pu/glnAp2</i>	pTE103 inserted with region -205 to $+76$ of the <i>Pu-PglAp2</i> hybrid	This work

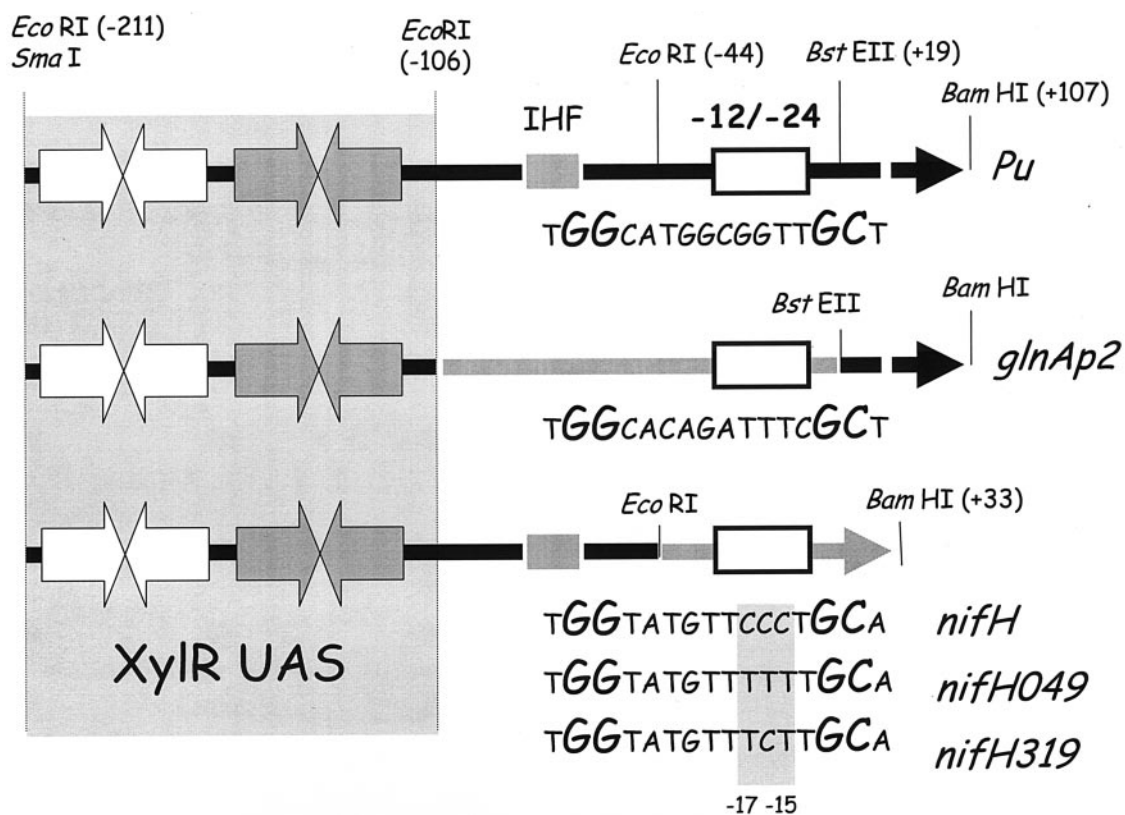


FIG. 2. Schematic representation of *Pu* variants and hybrid promoters. The reference wild-type *Pu* promoter is shown on top, along with an indication of functionally important segments and the restriction sites engineered for constructing the variants. A blowup of the sequence of the $-12/-24$ region spanning the essential GG-GC nucleotides is also shown. The other promoters (hybrids of *Pu* with various segments of *PglAp₂*, *PnifH*, *PnifH049*, and *PnifH319*) are displayed with the reference UAS sequence (two quasipalindromic binding sites), which is shared by all of them, and a grey tone code to trace each of the segments to the correct donor of the sequence. Note the nucleotide changes within positions $-17/-15$ of promoters with σ^{54} -RNAP binding variants *PnifH049* and *PnifH319*.

Recombinant DNA techniques were carried out according to published protocols (54).

Plasmid construction. The *lacZ* plasmid pBK16 (35) was used as the vector for the assembly of the different hybrid promoters. This mobilizable plasmid bears a *supF*-suppressible resistance to the streptomycin-spectinomycin gene (*aadR*) and also a *supF*-suppressible promoterless *lacZ* gene, in front of which the promoter of interest is cloned. These features confer host *supF*⁺ *E. coli* Δ *lac* strains (such as *E. coli* CC118 *supF*) resistance to streptomycin and *lacZ*⁺ phenotypes but make the plasmid unstable in *supF*^o strains. The *Pu-lacZ* delivery plasmid pBK16*Pu* was constructed by cloning the 312-bp EcoRI/BamHI fragment from pEZ9 spanning the *Pu* promoter from the TOL plasmid pWW0 (21). Plasmids bearing hybrids between the *Pu* promoter and downstream *PnifH* variants or *glnAp₂* were made as follows. First, *Pu* was cloned as a 312-bp EcoRI/BamHI fragment from pEZ9 into the site-directed mutagenesis vector pCG2 (43), yielding pCG2 *Pu*. Novel EcoRI sites were entered at positions -44 and -106 of the *Pu* promoter sequence (36), yielding plasmids pFH14 and pFH15, respectively (Fig. 2). The source of the *PnifH* σ^{54} -RNAP binding region was pMB1 (5). This plasmid was subject to PCR with oligonucleotides 10 (5'-ATGAATTCACAGG CACGGCT-3') and 11 (5'-GACGGGATCCATGGTGACTTCT-3'). This amplified an 88-bp EcoRI/BamHI DNA segment spanning the -44 to $+33$ region of *PnifH*, thus including the $-24/-12$ motif of the promoter. This segment was cloned in pBK16, producing plasmid pMJ1. The EcoRI insert of pFH14 (spanning coordinates -207 to -44 of *Pu*, including the UAS and the IHF binding site) was then ligated to EcoRI-digested pMJ1, and the correctly oriented insert gave rise to the *Pu-PnifH-lacZ* delivery plasmid pMJ2 (Fig. 2). The source of the $-12/-24$ region of *PnifH319* was plasmid pJES366, which bears a 320-bp EcoRI/BamHI insert spanning the whole *PnifH* region of *K. pneumoniae* with two C-to-T changes at positions -15 and -17 (Fig. 2), which increase affinity for σ^{54} -RNAP, all cloned in a pTZ18R vector (55). Site-directed mutagenesis (36) of this plasmid with the oligonucleotide SF4 (5'-ATAAGAATGAATTCACAGGCAC GGC-3') generated a new EcoRI site in position -44 , producing the plasmid

pJE336-SF4. The 88-bp EcoRI/BamHI fragment of pJE336-SF4 spanning coordinates -44 to $+33$ was then excised and cloned in the corresponding sites of pBK16, yielding pFH44A. The EcoRI insert of pFH14 (spanning coordinates -207 to -44 of *Pu*) was then ligated to the EcoRI-digested pFH44A, and the correctly oriented insert gave rise to the *Pu-PnifH319-lacZ* delivery plasmid pFH44 (Fig. 2). Similarly, a DNA segment spanning coordinates -44 to $+33$ of the *PnifH* variant *PnifH049*, in which three C-to-T changes at positions -15 to -17 had been made for increased affinity to σ^{54} -RNAP, was generated from plasmid pWC88049 (5). This segment was assembled as before in pBK16, along with the 168-bp EcoRI insert of pFH14, giving rise to the *Pu-PnifH049-lacZ* delivery plasmid pRF2. Finally, the UAS-less region -106 to $+19$ of the *glnAp₂* promoter of *E. coli* was amplified from genomic DNA with oligonucleotides *gln1* (5'-CCCCCGAATTC AACATTCAGATCGTGGTGC-3') and *gln2* (3'-AAAT GCCGCTGTGCCGGTTTCCACTGGCCCC-5'). The product was cloned as a 142-bp EcoRI-BstEII fragment in the equivalent sites of pEZ9 (yielding pEZ9-PCR) and recloned in pBK16 as a 240-bp EcoRI/BamHI insert, generating pFH43A. This plasmid was digested with EcoRI and ligated to the 100-bp EcoRI fragment of pFH15 (which spanned the UAS for XylR in *Pu*) (Fig. 2). The correctly oriented insert gave rise to the *Pu-glnAp₂* delivery plasmid pFH43 (Fig. 2). The plasmids used in the transcription assays (Table 1) were produced by cloning the promoter-bearing inserts of interest in vector pTE103, which adds a strong T7 terminator downstream of the promoter under study (24). All cloned inserts and DNA fragments were verified through automated DNA sequencing in an Applied Biosystems device.

Growth and induction conditions. Bacteria were grown at 30°C in either rich LB medium (41) or M9 minimal medium (54) amended with 0.2% Casamino Acids and 0.2% glucose, as indicated. When required, the media were supplemented with 150 μ g of ampicillin/ml, 50 μ g of streptomycin/ml, 50 μ g of spectinomycin/ml, 30 μ g of chloramphenicol/ml, or 50 μ g of kanamycin/ml. Promoter activity in vivo was monitored in all cases by assaying the accumulation of β -galactosidase in cells permeabilized with chloroform and sodium dodecyl sul-

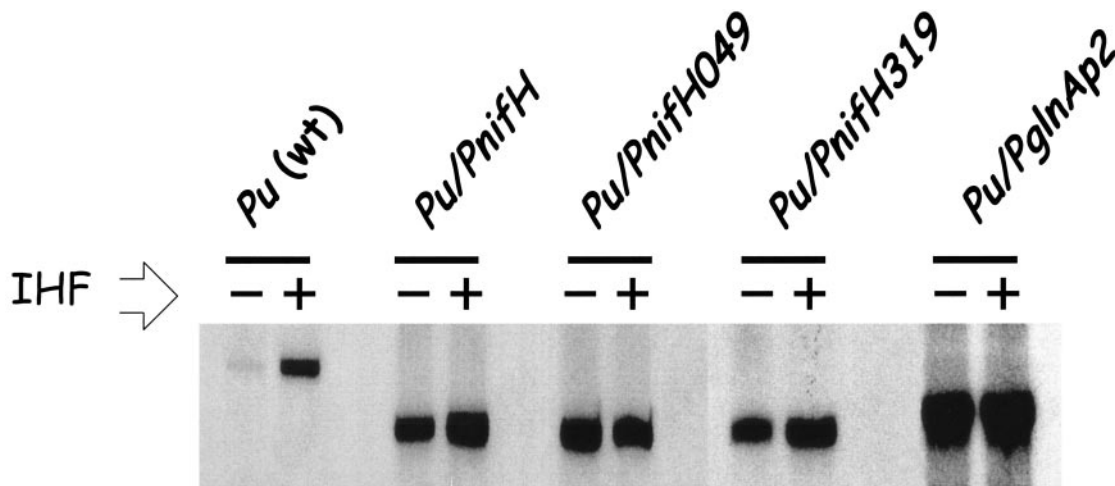


FIG. 3. Formation of transcriptionally open complexes by *Pu* variants. The results of single-round transcription assays with 5 nM (each) supercoiled pTE103 derivatives inserted with the *Pu* hybrids (Table 1) are indicated in each case. The experiment was run as explained in Materials and Methods with 100 nM XylR Δ A, 25 nM core RNAP, 100 nM σ^{54} , and, where indicated (+), 25 nM IHF. The reactions were initiated by addition of the four nucleoside triphosphates in the presence of heparin. Under these conditions, *Pu* produces a transcript of 394 nucleotides (nt), *Pu-PnifH* and its derivatives produce a transcript of 336 nt, and *Pu-PglnAp2* produces a transcript of 377 nt.

fate, as described by Miller (41), under the conditions specified in each case. The β -galactosidase activity values represent the averages of at least three independent experiments in duplicate samples; standard deviations were <15%. Where indicated, the cultures were exposed to saturating vapors of the *upper* TOL pathway inducer *m*-xylene (1).

Proteins and protein techniques. Purified factor σ^{54} and core RNAP from *E. coli* were the kind gift of B. Magasanik. Purified IHF protein was obtained from H. Nash. XylR Δ A was purified to apparent homogeneity by metalloaffinity of the His-tagged protein, as described by Pérez-Martín and de Lorenzo (46).

In vitro transcription assays. Plasmids used in the transcription assays (Table 1) were prepared with the QIAGEN (Valencia, Calif.) plasmid purification system. Transcription assays were performed following previously published procedures (18). Supercoiled DNA templates were used at 5 nM concentration; 50- μ l reaction mixtures were set at 37°C in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 0.1 mM bovine serum albumin, 10 mM dithiothreitol, and 1 mM EDTA. Unless otherwise indicated, each DNA template was premixed with 25 nM core RNAP, 100 nM σ^{54} , 25 nM IHF, and 100 nM XylR Δ A. The DNA templates and the proteins were incubated at 37°C with 4 mM ATP for 20 min to allow open-complex formation. Transcription was then initiated by adding a mixture of ATP, CTP, GTP (400 μ M each), and UTP (5 mM; 3,000/mmol). In single-round experiments, heparin (0.1 mg/ml) was added along with the nucleoside triphosphate mixture to prevent reinitiation. After the mixtures were incubated for 10 min at 37°C, the reactions were stopped with equal volumes of a solution containing 50 mM EDTA, 350 mM NaCl, and 0.5 mg of carrier tRNA/ml. The mRNA was then extracted, precipitated with ethanol, electrophoresed on a denaturing 7 M urea-4% polyacrylamide gel, and visualized by autoradiography.

Mobilization and recombination of hybrid promoters in single-gene dosage.

To generate *P. putida* strains harboring a monocopy fusion of the hybrid promoter *Pu-PnifH*, *Pu-PnifH319*, *Pu-PnifH049*, or *Pu-PglnAp2*, or the wild-type *Pu* promoter to *lacZ*, *E. coli* CC118 *supF* harboring the pBK16*Pu* (*Pu-lacZ*), pMJ2 (*Pu-PnifH-lacZ*), pRF2 (*Pu-PnifH049-lacZ*), pFH44 (*Pu-PnifH319-lacZ*), or pFH43 (*Pu-PglnAp2-lacZ*) plasmid was mobilized into the *P. putida* target strain KT2442 *hom.fg. xylRS* by tripartite mating using a filter technique with *E. coli* HB101 (pRK600) as the helper strain (28). After 8 h of incubation at 30°C on LB plates, the cells were washed with 10 mM MgSO₄ and plated on M9 citrate medium in the presence of streptomycin. The streptomycin-resistant exconjugants that arose by cointegration of the hybrid fusions (Fig. 3) were regrown and screened for kanamycin-sensitive blue colonies in medium with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). This phenotype is the necessary result of a double homologous recombination between the plasmid and the *P. putida* chromosome bearing the *hom.fg.* segment, as described by Kessler et al. (35) (Fig. 4).

RESULTS AND DISCUSSION

Organization of hybrid promoters and validation of their functionality in vitro.

The salient features of the promoters under scrutiny in this work are depicted in Fig. 2. They all share the binding sites for XylR (the UAS and adjacent sequences -205 to -106 of the wild-type *Pu* promoter) placed at identical distances upstream from the -12/-24 motif that is recognized by the σ^{54} -RNAP. The first hybrid promoter (*Pu-PnifH*) bears the whole *Pu* upstream region (coordinates -205 to -44) spanning its native UAS and an IHF binding site fused to the -44 to +33 sequence of the *PnifH* promoter, which provides the -12/-24 region. In its natural context, *PnifH* is also stimulated by IHF, and this effect was more apparent when the DNA template was linear (30). Since the distances between the UAS, IHF, and -12/-24 motif are retained in *Pu-PnifH* as in *Pu*, one can safely assume that the functional architecture of the hybrid promoter is kept as well, and thus that the only significant change affects the σ^{54} -RNAP binding site. Two other hybrid promoters (*Pu-PnifH049* and *Pu-PnifH319*) are identical to *Pu-PnifH* except for the sequences in the -15/-17 region within the σ^{54} -RNAP binding site. The nucleotides -17 and -15 of σ^{54} -dependent promoters are involved in modulating the recognition and binding of the polymerase to the wider -12/-24 region (5, 6). The wild-type *PnifH* has a CCC in -17/-15, while *PnifH319* and *PnifH049* have TCT and TTT sequences, respectively, at the same positions (5, 45, 55) (Fig. 2). Both *PnifH* variants possess superior promoter strength (30, 42, 55) and, unlike wild-type *PnifH*, they do not need IHF for transcription in vitro with either supercoiled or linear DNA (55). The last hybrid promoter constructed, *Pu-PglnAp2*, was the result of replacing the region -106 to +19 of *Pu* (which includes the IHF site and the -12/-24 region in this promoter) with the same coordinates of *glnAp2*. It is known that *glnAp2* lacks an IHF site and thus is totally independent of IHF for the initiation of transcription

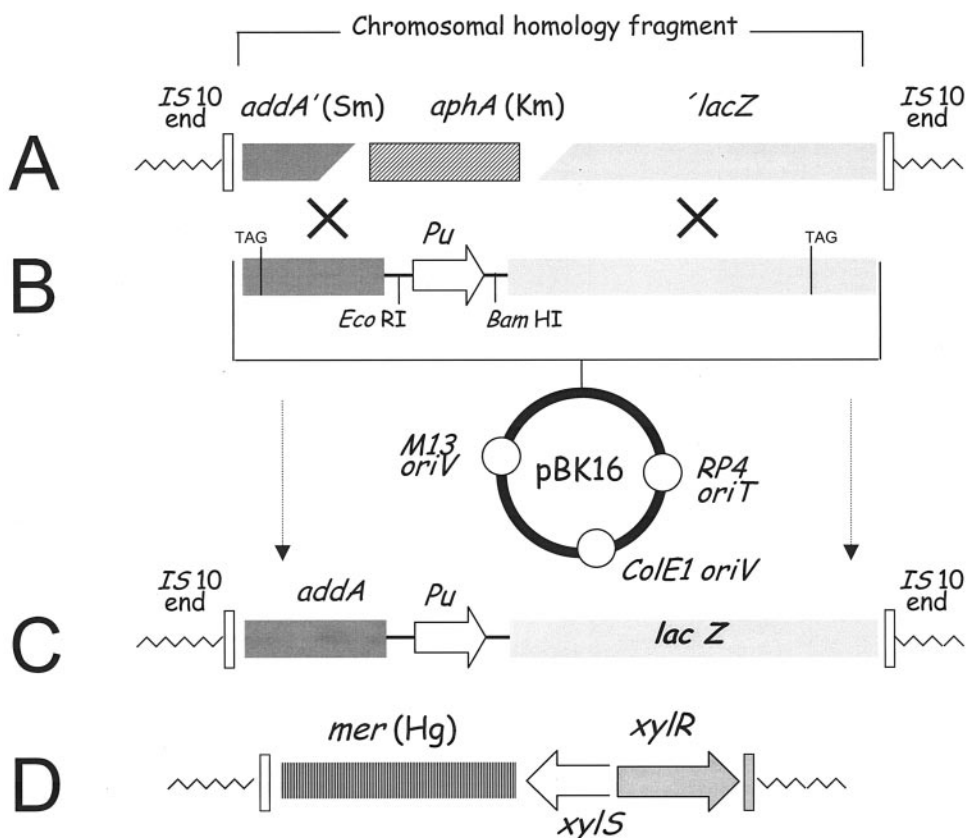


FIG. 4. Integration of *Pu* and its hybrid variants into the chromosome of *P. putida* KT2442 *hom. fg.* (A) The so-called homology fragment is stably inserted into the chromosome of *P. putida* KT2442 by means of a hybrid mini-Tn10 transposon (35). This fragment contains a selectable kanamycin resistance gene (*aphA*) flanked by an N-terminally truncated *addA* gene (streptomycin-spectinomycin resistance) and a divergently oriented and also N-truncated *lacZ* gene. These DNA segments provide homology to cognate sequences in the *lacZ* transcriptional fusion vector pBK16 (B) containing the promoter of interest. RP4 *oriT*-mediated mobilization of pBK16 derivatives into *P. putida* KT2442 *hom. fg.* (which harbors the homology fragment) allows double recombination, leading to the transfer of the promoter into the chromosome. The double crossover is selected by streptomycin-spectinomycin resistance and blue color on X-Gal and further confirmed by the loss of kanamycin resistance (C). This event is facilitated by the presence of amber codons (TAG) at the ends of the *addA* and *lacZ* genes of pBK16. Some functionally important elements of the system are indicated. The resulting *lacZ* fusion is transcriptionally shielded upstream by an Ω streptomycin-spectinomycin interposon and downstream by a strong T7 terminator (not shown). (D) Scheme of the second insert borne by the strain *P. putida* KT2442 *hom. fg. xylRS*. This is a mini-Tn5 Hg vector with a ~ 2.5 -kb segment of the pWW0 plasmid encoding the two regulators of the TOL system, *xylR* and *xylS*, in its natural divergently transcribed configuration.

(44) in either supercoiled or linear templates (10). Furthermore, the sequence $-17/-15$ (TTT) of *glnAp₂* matches those present in strong σ^{54} promoters (4, 5, 42, 62).

In order to examine whether hybrid promoters were functional, we performed single-round in vitro transcription assays using as supercoiled DNA templates plasmids bearing each of the promoters cloned in vector pTE103 (see Materials and Methods) (Table 1) under conditions described previously (9, 46). The activator protein included in these assays was XylR Δ A, a variant of the wild-type XylR with its N-terminal module (i.e., its A domain) deleted. This variant is constitutively active and can thus promote transcription in the absence of any aromatic inducer (25, 46, 47). As shown in Fig. 3, all promoters were able to initiate transcription, although their relative efficiencies varied. As expected, *Pu* activity was absolutely dependent on IHF, a feature that is completely preserved in vivo (7). This may be due to the indispensable need for IHF in *Pu* for recruitment of σ^{54} -RNAP to the promoter (2, 9, 61). Unlike *Pu*, the *Pu-PnifH* hybrid could form a significant

amount of transcript in the absence of IHF, which was further stimulated by addition of the factor (however, dependence on IHF did increase on a linear template [data not shown]). Similarly, *Pu-PnifH049* and *Pu-PnifH319* initiated transcription efficiently on both supercoiled and linear templates independently of IHF, although the factor had a detectable effect in *Pu-PnifH319* (Fig. 3 and data not shown). *Pu-PglnAp₂* also worked well in a fashion completely independent of IHF. These in vitro assays validated the capacities of the various hybrid constructs to bear transcription with the minimal set of components that suffice to activate *Pu* (46). However, since the assay system included mostly purified components from *E. coli*, the relative amounts of transcripts under various conditions cannot be simply projected onto the situation in vivo in *P. putida*. We thus examined this issue in the native context, as explained below.

Setup of a dependent genetic system to follow the activity of the *Pu* promoter in *P. putida*. In order to ensure the maintenance of the regulatory elements acting on *Pu* in its native gene

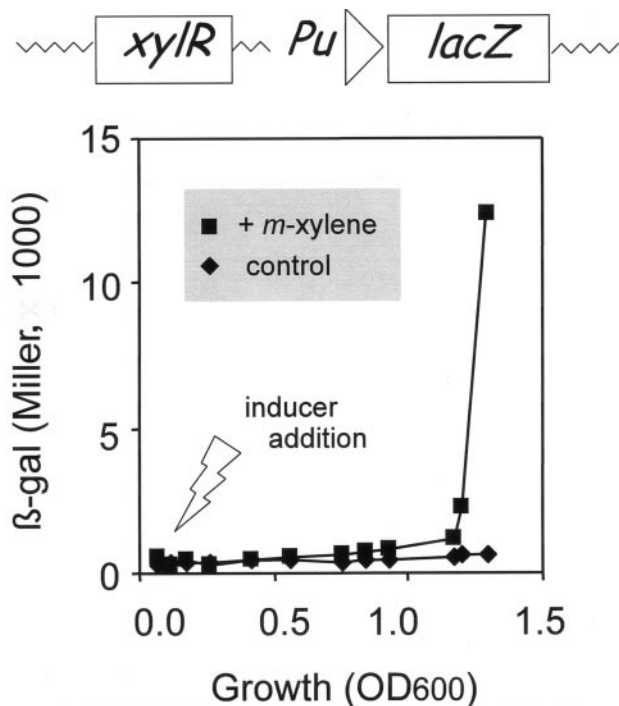


FIG. 5. Evolution of *Pu* activity during growth in rich medium. *P. putida* SF05X cells bearing all elements required for *Pu* regulation assembled in the chromosome by the procedure explained in the text (sketched on top) were grown overnight at 30°C in complete LB medium, diluted to an optical density at 600 nm (OD_{600}) of ~ 0.05 , and re-grown under the same conditions in the presence or absence of saturating vapors of *m*-xylene. β -Galactosidase levels were followed during growth as shown. Note that the promoter remained fully inhibited (as reflected by β -Gal output) until the cultures entered stationary phase.

dose and stoichiometry, we employed a genetic system tailored for site-specific integration of *lacZ* fusions into the chromosome of *P. putida* (35). This procedure (sketched in Fig. 4) allows a faithful comparison of the transcriptional output of any given *Pu* promoter variant in vivo. The basis of this system is the presence in the target chromosome of a DNA fragment that provides sequence homology to the regions flanking the *lacZ* fusion assembled in the delivery vector pBK16 (Table 1). This system ensures that all promoters are inserted at the same chromosomal location, in the same orientation, and shielded from external transcriptional flows by an upstream Ω element and a downstream T7 terminator so that their activities can be exactly compared. To examine whether this setup allowed the reproduction of the physiological control phenomenon of *Pu* in a fashion amenable to genetic scrutiny, we mobilized plasmid pBK16*Pu* toward *P. putida* strain KT2442 *hom. fg. xylRS*, which bears in its chromosome insertions of the above-mentioned homology fragment, as well as a DNA segment encoding both XylR and XylS proteins (Fig. 4D). Chromosomal recombination of the *Pu-lacZ* fusion of pBK16*Pu* gave rise to strain *P. putida* SF05X, which bears all the regulatory constituents of *Pu* in monocopy. To validate the use of this strain as a reference, we ran the experiment shown in Fig. 5, in which β -galactosidase accumulation of *P. putida* SF05X (*Pu-lacZ xylR*⁺) was followed during the growth curve in rich medium in the presence or absence of saturating *m*-xylene vapors. As shown in Fig. 5, even when *P. putida* SF05X was induced since

early in culture, *Pu* remained basically silent until the cells reached an optical density at 600 nm of ~ 1.2 , when a distinct burst of transcription occurred. These data validated the use of the chromosomal integration system of Fig. 4 as the preferred tool to judge the effect of replacing the σ^{54} -RNAP binding region of *Pu* with others from various origins.

Physiological control of *Pu* promoter variants with diverse $-12/-24$ regions. In order to study the physiological regulation of the *Pu* hybrids with RNAP binding sequences recruited from *PnifH* (and its variants) and *glnAp₂*, we constructed *P. putida* strains completely identical to *P. putida* SF05X (*Pu-lacZ xylR*⁺) except for the region downstream of the UAS for XylR (Fig. 2). For this, we mobilized plasmids pMJ2 (*Pu-PnifH-lacZ*), pRF2 (*Pu-PnifH049-lacZ*), pFH44 (*Pu-PnifH319-lacZ*), and pFH43 (*Pu-PglnAp₂-lacZ*) toward *P. putida* KT2442 *hom. fg. xylRS* and forced the chromosomal recombination of the *lacZ* fusions as before. These operations resulted in the strains *P. putida* MR05X (*Pu-PnifH-lacZ xylR*⁺), *P. putida* MR02X (*Pu-PnifH049-lacZ xylR*⁺), *P. putida* SF02X (*Pu-PnifH319-lacZ xylR*⁺), and SF03X (*Pu-PglnAp₂-lacZ xylR*⁺). Every strain was then subjected to an analysis of β -galactosidase accumulation during growth in the presence or absence of *m*-xylene vapors as described above.

The results in Fig. 6 show that each promoter behaved in a different way in respect to both the induction pattern and the strength of transcription. The fusion between the UAS and the IHF region of *Pu* followed by the $-12/-24$ sequence of the wild-type *PnifH* promoter (Fig. 6a) acted in vivo in a fashion nearly identical to that of the original *Pu* promoter (Fig. 5), i.e., there was an evident repression of transcription during fast growth, followed by a timely boost of activity at the onset of stationary phase. The induction pattern for *Pu-PnifH* could largely reflect, as is the case for *Pu* (61), the growth phase-dependent occupation of the IHF site and the ensuing recruitment of the polymerase to the $-12/-24$ region (38).

Given the sequence divergence between the $-44/+33$ regions of *Pu* and *PnifH* (except the actual $-12/-24$ motif), the fact that silencing is preserved in the *Pu-PnifH* hybrid rules out any influence of extra factors binding that region in a sequence-specific manner. Otherwise, this result says nothing about the role of the $-12/-24$ region in physiological control. In contrast, comparison of the induction profiles of *Pu-PnifH* (Fig. 6a) with those of *Pu-PnifH049* (Fig. 6b) and *Pu-PnifH319* (Fig. 6c) was informative. As mentioned above, the $-12/-24$ regions of *Pu-PnifH049* and *Pu-PnifH319* differ from that of the wild-type *PnifH* by only a number of bases within the $-15/-17$ coordinates that appear to increase their binding to the holoenzyme and facilitate the formation of an open complex in vitro (5, 55). These two aspects are problematical to separate in vivo for any given sequence, because higher affinity does not translate automatically into superior activity (62, 63). We prefer the operative term “engagement” to describe the combination of affinity and ease of open-complex formation that is inherent in every $-12/-24$ region variant.

The one outstanding aspect of Fig. 6b is the lack of any significant inhibition of *lacZ* production at any growth stage. Unlike *Pu* (Fig. 5) and *Pu-PnifH*, the hybrid *Pu-PnifH049* appears to respond to *m*-xylene as soon as the cells are exposed to the inducer. β -Galactosidase accumulation then follows a steady increase that is perhaps greater at late growth stages

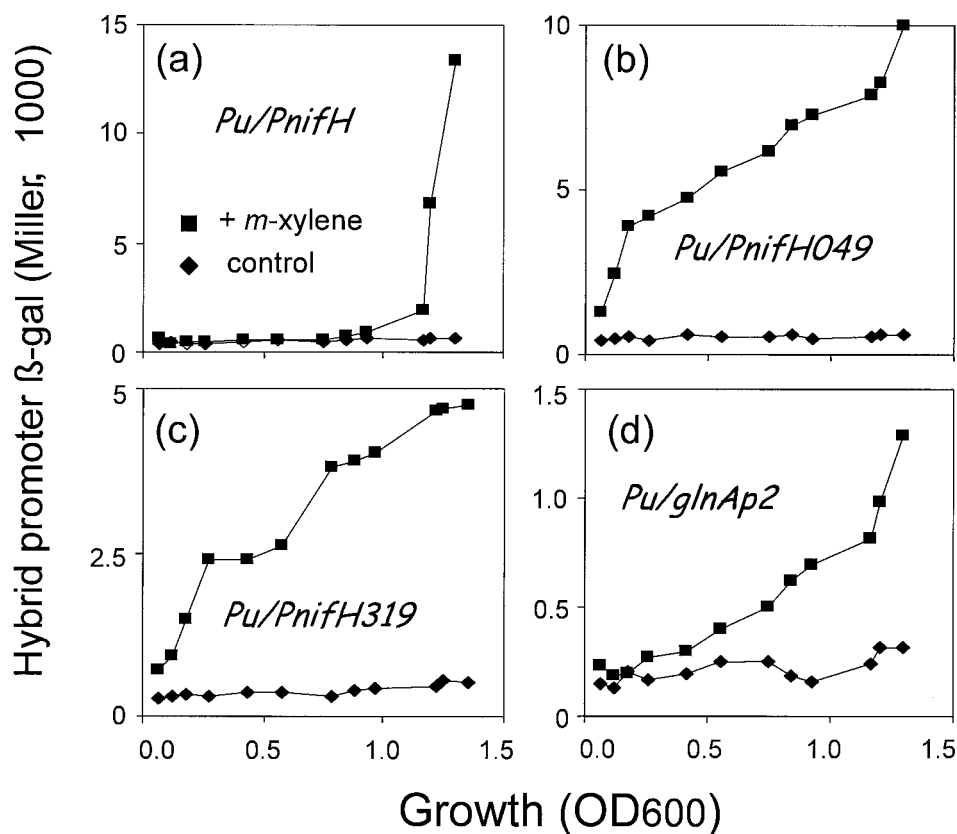


FIG. 6. In vivo performance of *Pu* variants. *P. putida* KT2442 *hom. fg. xylRS* strain derivatives bearing the chromosomal fusion *Pu-PnifH-lacZ* (a), *Pu-PnifH049-lacZ* (b), *Pu-PnifH319-lacZ* (c), or *Pu-PglAp₂-lacZ* (d) were grown in LB medium at 30°C and assayed for β -galactosidase activity (expressed in Miller units). The growth rates of all the strains were indistinguishable under all conditions tested. OD₆₀₀, optical density at 600 nm.

(Fig. 6b). In other words, the *Pu-PnifH049* hybrid seems to be relieved from any physiological down-regulation while reaching β -galactosidase levels in the same range as those of the reference promoters *Pu* (Fig. 5) and *Pu-PnifH* (Fig. 6a). Since the only difference between *Pu-PnifH* and *Pu-PnifH049* is 3 bases within the $-12/-24$ region that affect the affinity and the quality of the interaction with σ^{54} -RNAP, we argue that physiological control of *Pu* may reflect the binding of the enzyme, the only step which can be regulated in vivo for any fixed DNA sequence. This picture is reinforced by the behavior of the related hybrid promoter *Pu-PnifH319* (Fig. 6c), which also appears to be free of physiological down-regulation while exhibiting somewhat lower transcriptional activity on the whole. It thus appears that carrying such improved $-12/-24$ sequences accounts completely for the release of any silencing of *Pu* during rapid growth.

Finally, we examined the induction pattern of the hybrid promoter between the UAS of *Pu* and the rest of the DNA sequence, all the way to the $-12/-24$ motif, from the naturally IHF-less promoter *glnAp₂* (Fig. 2). As mentioned before, this promoter forms a stable complex in vitro with the σ^{54} -RNAP of *E. coli* (44). On this basis, we examined the inducibility of the hybrid *Pu-glnAp₂* (Fig. 2) in the same system employed in vivo before. It should be noted that the range of the transcriptional outputs in this case (Fig. 6d) is within much lower β -galactosidase activities. This is not unexpected, since the extensive sequence exchange between *Pu* and *glnAp₂* (Fig. 2) may

flaw the geometry of the promoter, which, however, keeps the same distances and phasing between the UAS and the $-12/-24$ region as all the other promoters tested. While comparing absolute activities is, for that reason, not informative, *Pu-glnAp₂* still shows a revealing induction profile (Fig. 6d). This consists of a rapid (but relatively low) response to *m*-xylene from early in growth, which appears to be increased at the onset of stationary phase.

C source (glucose) inhibition of *Pu* promoter variants. As mentioned above, the phenomenon that we refer to as physiological control of *Pu* is the result of processing various environmental conditions. One of them is the presence in the medium of some carbon sources (in particular, glucose), which down-regulates *Pu* output in a fashion phenomenologically similar to catabolic repression but mechanistically quite different (52). In fact, it has been possible to distinguish the effect of glucose on *Pu* from other growth phase-related inputs either genetically (14) or by using a chemostat that fixes growth rates (22, 23).

Interestingly, *Pu* inhibition brought about by C sources is specifically caused by carbohydrates metabolized through the Entner-Doudoroff pathway, such as glucose or gluconate, whereas organic acids, such as citrate or succinate, lack this negative influence (16, 29). To examine whether the relief of *Pu* silencing under rapid growth in rich medium brought about by altering the σ^{54} -RNAP binding site could also overcome down-regulation by glucose, we ran the experiment shown in Fig. 7.

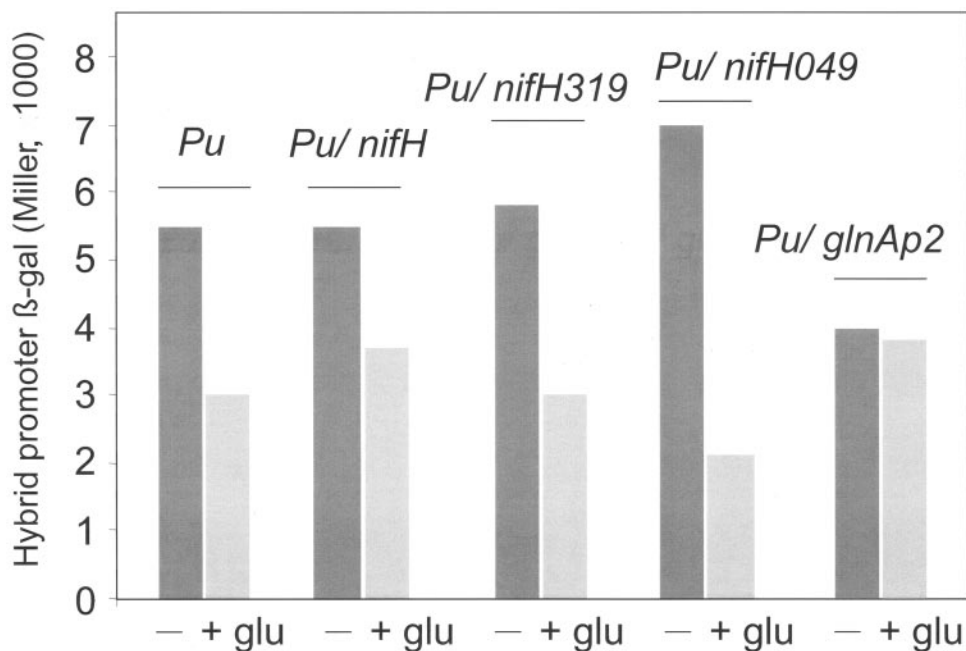


FIG. 7. Glucose repression of transcriptional activities of *Pu* variants. *P. putida* KT2442 *hom. fg. xylRS* strain derivatives' *lacZ* fusions to the promoters indicated on top of the diagram bars were grown for 15 h at 30°C in M9 medium supplemented with 0.2% Casamino Acids to equal growth rates with (+) and without (-) 0.2% glucose (glu) in the presence of saturating vapors of *m*-xylene.

In this case, induction experiments were done under conditions in which the growth rates of all strains were identical because of the addition to the minimal medium of an excess of amino acids (16). Any difference in *lacZ* could thus be traced to the presence or absence of glucose in the medium. In this case, *xylR*⁺ *P. putida* strains bearing *Pu-lacZ*, *Pu-PnifH-lacZ*, *Pu-PnifH049-lacZ*, *Pu-PnifH319-lacZ*, or *Pu-PglnAp₂-lacZ* fusions were induced with *m*-xylene for 15 h, and the accumulation of β-galactosidase was recorded. As shown in Fig. 7, the control *Pu-lacZ* fusion and its *Pu-PnifH* counterpart, which are subject to intense physiological inhibition (Fig. 5 and 6a), behaved in the standard manner in this assay, namely, 30 to 60% reduction of transcriptional output in the presence of glucose (16). On the other hand, the hybrids *Pu-PnifH049* and *Pu-PnifH319* kept, and even went beyond (40 to 70% reduction), the standard response to the carbohydrate, despite being altogether free of growth phase inhibition (Fig. 6). Finally, the *Pu-PglnAp₂* fusion was basically blind to the presence of glucose in the medium. These results reveal the independence of the carbon inhibition of *Pu* in respect to the mechanism(s) which releases the growth phase-dependent regulation (14).

Conclusion. *Pu* activity in vivo is not just dependent on the regulator-promoter pair which suffices to cause transcription in vitro but also on the overall metabolic and energy status (12, 13, 53, 57). Such a physiological check of the *Pu* promoter probably involves a number of mechanisms (8, 11, 13, 16, 29, 31). One aspect is the role of the sequence -12/-24 in such physiological control of *Pu*. It has been known for a long time that changes in the sequence bound by σ^{54} -RNAP alter the performance of other σ^{54} promoters in vivo and in vitro (18, 19, 42, 55). In this context, the main piece of information reported in this article is that the silencing of the *Pu* promoter of the TOL plasmid when cells grow exponentially in rich me-

dium (15) can be defeated by exchanging the native -12/-24 region for an equivalent sequence of promoters known to have a different degree of engagement with the polymerase.

How does this notion fit with the rest of the data available for *Pu*? This question finds a suitable context by comparing results from *Pu* itself with those of the similar (but not identical) σ^{54} promoter *Po*, which drives the expression of a pathway for degradation of dimethyl phenols when the cognate activator (akin to XylR), named DmpR, binds the corresponding aromatic effectors (37, 56-60). Growth phase control of *Po* has been attributed to the ability of the alarmone ppGpp to facilitate the access of σ^{54} to the core RNAP during sigma factor competition at stationary phase (33, 37). Such competition is critical in the case of σ^{54} , given that only a few molecules of the factor (~80) are present in the cell at any growth stage (34). In this context, it is possible that σ^{54} promoters with a better -12/-24 region can function even at the low concentrations of σ^{54} -RNAP available prior to stationary phase. In these instances (as seems to be the case for the *Pu-PnifH049* and *Pu-PnifH319* variants), the promoter may not be subject to any physiological inhibition, but it is active throughout the growth curve. However, it should be noted that (as shown in Fig. 7) defeating the down-regulation of *Pu* due to growth phase effects does not imply the same for the C source control of the same promoters. The mechanisms behind these phenomena will be the subject of future investigations.

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