Activation of the *Pseudomonas* TOL Plasmid Upper Pathway Operon

IDENTIFICATION OF BINDING SITES FOR THE POSITIVE REGULATOR XylR AND FOR INTEGRATION HOST FACTOR PROTEIN

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Expression of the *Pseudomonas putida* TOL plasmid upper pathway operon requires a promoter that belongs to the -12/-24 class. Stimulation of transcription from this promoter is positively controlled by the effector-activated XylR protein and requires a form of RNA-polymerase holoenzyme containing the RpoN-encoded σ factor, σ^N*. XylR-dependent stimulation of transcription from the *Pseudomonas* TOL upper pathway promoter was examined using deletions, insertions, and in vivo dimethyl sulfate footprinting. Two upstream activator sequences were identified in the -160 (UAS1) and -130 (UAS2) regions. Deletion of these two regions abolished transcription activation, although conservation of the UAS2 element alone allowed limited transcription stimulation. Separation of UAS1 from UAS2 by half a turn or a full turn significantly reduced XylR stimulation of transcription from the upper pathway operon promoter. An inverted repeat ATTTGNCACAAAT (where N is any nucleoside), which most likely represented the XylR recognition sequence, was identified. Binding of XylR was observed in vivo in the absence of effector, but changes in the binding pattern were induced in the presence of m-methylbenzyl alcohol, a XylR effector. In vivo footprinting analysis revealed that changes in the methylation pattern of G and T also occurred in the -50 to -90 region, which is probably occupied by integration host factor (IHF) protein. IHF was required for maximal expression from the TOL upper pathway operon promoter in *Escherichia coli*. Separation of the IHF site from UAS2 by a full helix turn did not significantly affect stimulation of transcription, which is consistent with this region playing a conformational role, rather than a regulatory one, in promoter function.

The TOL plasmid pWW0 of *Pseudomonas putida* encodes the genetic information for the mineralization of toluene and xylenes (1). The genes of the catabolic pathway are organized in four transcriptional units. Two of these, the "upper" and the "meta" pathway operons, contain the xyl structural genes encoding the corresponding catabolic enzymes for the oxidations of toluene/xylenes via benzoate/toluates to amphi
tobic intermediates, whereas the two remaining genes, xylR and xysS, encode regulatory proteins. The XylS protein is involved in the stimulation of transcription of the meta cleavage pathway operon promoter (2-4), whereas the XylR protein, in concert with upper pathway effectors (e.g., toluene and m-methylbenzyl alcohol) and the specific RNA-polymerase σ factor RpoN (also called NtrA and σ^N*), trigger expression from the upper pathway operon promoter (Pu) (5-9). The XylR-activated promoters on the *Pseudomonas* TOL plasmid Ps and Pu belong to a specific promoter class characterized by conserved sequences in the -24 (5'-CTGGYAY-3') and -12 (5'-TTGCA-3') regions (10, 11), the expression of which requires the specific RNA-pol
ymerase σ factor RpoN (for a review, see Ref. 12).

Efficient transcription initiation from the -12/-24 promoters requires a specific additional activator protein, e.g., NtrC for glnAp2 in *Escherichia coli*, NifA for nif genes in *Klebsiella*, and DctD for dicarboxylic acid transport in *Rhizobium* (12). In a number of RpoN-dependent promoters, the binding sites for the specific regulators are located more than 100 bp from the transcription initiation point. These upstream activator sequences (UASs) are often inverted repeats that represent the target site for the activator protein and can be moved more than one kilobase away without losing their ability to mediate transcriptional activation (13-15). Recently, IHF protein was shown to stimulate transcriptional activation of nif promoters (16-18), and sequence alignments have suggested that a putative IHF-binding site may be located in several -12/-24 promoters (18, 19). For the best studied RpoN-dependent promoters, namely the nif promoters in *Klebsiella pneumoniae* and ntr promoters in *Enterobacteriaceae*, models have been proposed in which RpoN, as well as the activator protein, behave as DNA-binding proteins. Studies with the glnAp2 promoter have demonstrated that RNA-polymerase/RpoN binds to the promoter in the absence of its corresponding activator protein NtrC to form a closed complex (20-22), but interaction with the regulatory protein is then required for transcription initiation. The molecular mechanism of this protein interaction is as yet unknown (23, 24).

NifA, DctD, NtrC, and XylR activators for different -12/-24 promoters all exhibit a conserved central domain, but differ in their N-terminal region, which is involved in specific interactions with sensor proteins or effectors. The central

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†† The abbreviations used are: Pu, promoter for the upper pathway operon; Ps, promoter for the xylS gene; ΔPu, deleted Pu promoter; bp, base pair(s); IHF, integration host factor; UAS, upstream activator sequence; EGTA, ethylenebis(oxyethylenenitriilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
domain has been proposed as the region that interacts with RpoN/RNA-polymerase. This region harbors a putative helix-turn-helix motif characteristic of DNA-binding proteins and is the DNA-binding domain.

The model for remote stimulation of transcription in the -12/24 promoters proposes DNA loop formation to bring UAS-bound regulators close to the RpoN/RNA-polymerase promoter complex. The role of IHF-mediated DNA bending may be to facilitate DNA loop formation and, therefore, favor specific contacts between activator and polymerase (16-18, 23). However, since the UAS in some -12/24 promoters is not absolutely required for activation, the activator proteins may interact directly with the RpoN/RNA-polymerase promoter complex (26-28) in the absence of loop formation and IHF. In this study, we report the activation of the Pu promoter by the XylR protein. Deletions, insertions, and in vitro footprinting studies revealed three upstream regions, located between -106 and -144, between -144 and -200, and between -200 and -250, to be required for transcription activation. The two former regions seem to represent the Pu-XylR recognition sequences, whereas the latter one probably represents a target for IHF protein.

MATERIALS AND METHODS

Bacterial Strains—E. coli K-12 (recA, thi, tonA, supE, recA-); JM101 (supE, thi, Δ(lac-proA) F’ (traD26, proAB+, lacF, lacZAM15); T18 (supE, thi, Δ(lac-proA), F’ (proAB*, lacF, lacZAM15)); C332 (dut+ ung1, thi, relA1/pCJ105 (Cm’)) (29); and S90C (Δ(lac-pro), rasP) (30) and DBP101 (Δ(lac-pro), rasP, himD51-mini-tet) (30) were grown in LB at 30°C or in 2 x TY medium at 37°C (31). Media were supplemented when required with the following antibiotics: 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, 50 μg/ml kanamycin, 25 μg/ml streptomycin, and 10 μg/ml tetracycline.

Plasmids Used and Constructed in This Study—Plasmid isolation, transformation, and cleavage by restriction enzymes, agarose gel electrophoresis, and gene cloning were performed according to the methods described by Maniatis et al. (31) with minor modifications. Plasmids pTZ19 (Ap’, cloning vector) (Pharmacia LKB Biotechnology, Inc.), pMC1403 (Ap’, promoter probe) (32), pRD579 (Ap’, Fuc/lacZ) (6), pTS174 (Cm’, xylR+, P15 replicon) (7), pGC2 (Ap’, cloning vector) (29), and pHF450Km (33) have been described previously.

The following plasmids were constructed during the course of this study. pERD400 was constructed by cloning a 333-bp EcoRI-BamHI fragment carrying the Pu promoter from pRD579 between the EcoRI and BamHI sites of pTZ19. pERD401 contains the wild type Pu promoter fused to the lacZ gene in pMC1403. pERD402 contains a deleted Pu promoter fused to the lacZ gene in pMC1403. The end point of the deletion is located at -200 bp with respect to the main transcription initiation point in the Pu promoter. pERD403, pERD404, and pERD405 were similarly constructed, except that the end points of the deletions were located at -88, -40, and -25, respectively. The in-frame fusions were constructed by cloning the deleted promoters as blunt end-BamHI fragments between the unique Smal-BamHI sites of pMC1403 (see the Bal31 deletions below). Plasmids pERD410, pERD411, and pERD412 carry a deleted Pu promoter extending from -64, -106, and -144, respectively, fused to the promoterless lacZ gene in pMC1403 (see below for the detailed construction of these plasmids). Plasmid pERD414 carries a single point mutation (A → G at -63) in the Pu promoter.

5’ Deletions in the TOL Plasmid Upper Pathway Operon Promoter—Bal31 deletions in the Pu promoter in plasmid pERD400 were generated as described under “Materials and Methods.” The deleted promoters were obtained as Bal31-blunt end-BamHI fragments that were cloned between the Smal-BamHI sites of pMC1403, so that in-frame fusions to a promoterless lacZ gene were generated. Four groups of deletions that had lost between 50 and 200 bp were chosen, and one plasmid of each group from pERD402 through pERD405 was further characterized. To establish the exact end of the deletion in pERD402 through pERD405, the 100-300 bp EcoRI-BamHI fragments of pMC1403:Ap were subcloned in pTZ19 and sequenced. The accurate determination of the deletion established the ends for the four deletions at -200, -88, -40, and -25 bp with respect to the main transcription initiation point. To generate three additional deletions in the Pu promoter, an oligonucleotide site-directed mutagenesis approach was taken. Mutagenesis was carried out by using uracyl-enriched single-stranded DNA prepared from pERD406. This DNA was hybridized independently to three oligonucleotides that exhibited a single mismatch with respect to the wild type Pu sequence (5’-ACAAAGAAAAATCGATAATTTAGATG-3’, 5’-CCAGCGTCACAGACTCCAG-3’, 5’-CAGGTTGTATTGCCGATTGATG-3’) to generate A→
G, T → C, and G → T changes at -63, -108, and -147, respectively. These substitutions created single restriction sites for Clal, PstI, and NruI, respectively. After primer extension and transformation in E. coli 5K, mutants with a single nucleotide change at -63, -108, and -147 were identified by their acquisition of the new corresponding site. In order to generate deletions using the new sites, the pERD406 derivatives were cleaved with the restriction enzyme for the new site, and then the sticky ends of Clal and PstI were made blunt with Klenow enzyme and the four dNTPs and nuclease S1, respectively. After phenol extraction, DNA was cleaved with BamHl, and the deleted Pu promoters (blunt end-BamHl) were cloned between Smal-BamHl sites of pMC1403. These three ΔPu in pMC1403 were called pERD410 (deletion from -64), pERD411 (deletion from -106), and pERD412 (deletion from -144) (see Fig. 1).

β-Galactosidase levels arising from wild type Pu promoter and ΔPu promoters fused to lacZ in pMC1403 were estimated in E. coli 5K with or without the xylR gene in trans on plasmid pTS174 and also in the presence and in the absence of an effector for XylR protein, i.e. m-methylbenzyl alcohol. As expected, in the absence of XylR protein, no induction from Pu or ΔPu was observed, regardless of the presence of a XylR effector (Fig. 1). In the presence of XylR, m-methylbenzyl alcohol-dependent induction was only observed with the wild type promoter and with the deletion extending up to -200 bp from the main transcription initiation point (Fig. 1). The induced levels were about 10-fold higher than the basal level. The deletion extending up to -144 exhibited a low but repetitive increase in β-galactosidase with respect to its corresponding basal level, whereas no induction at all was observed with the wild type promoter and with the deletion extending up to -200 bp from the main transcription initiation point (Fig. 1). These results suggest that two DNA stretches in Pu, located between -106 and -144 and between -144 and -200 contain important elements for the full stimulation of transcription from Pu.

In Vivo Footprinting Analysis of Pu—The reactivity of guanine residues in the Pu promoters toward dimethyl sulfate was assayed in plasmid pRD579, a low copy number vector carrying a transcriptional fusion of Pu to a promoterless lacZ gene. DNA protection analysis was performed in E. coli bearing only pRD579 or bearing pRD579 and pTS174, a plasmid carrying the xylR gene, and in the presence and in the absence of the XylR protein effector m-methylbenzyl alcohol. Representative autoradiograms from the primer extension analysis are shown in Fig. 2. Fig. 3 shows the results of the densitometric analysis. The log ratio between the intensity of the band in the absence and in the presence of XylR are given, with negative values indicating hypermethylation and positive values indicating protection. There were three regions in which protection or hypermethylation of G was observed. In the top strand, the region furthest from the proposed RNA-polymerase binding site shows that G at -160 was hypermethylated in the absence of effector, but protected in the presence of m-methylbenzyl alcohol. In this region, the G located at -169 was hypermethylated both in the absence and in the presence of m-methylbenzyl alcohol (Figs. 2 and 3). In the -130 to -140 region, G located at -131 and -139 were protected and hypermethylated, regardless of the presence of effector. In conclusion, two regions, one around -160 and one around -130, clearly showed XylR-mediated protection. In the text below, we will refer to these regions as UAS1 and UAS2, respectively.

The primer extension strategy permitted detection of alterations in the in vivo methylation of the guanines in the region from -50 to -90. It was observed that G located at -54 and -70 were protected both in the presence and in the absence of m-methylbenzyl alcohol. An unexpected finding was that a T located at -84 apparently exhibited reactivity toward dimethyl sulfate attack and piperidine cleavage. This residue was protected in the presence of effector but hypermethylated in its absence (see Fig. 2). Other workers have also observed unexpected reactivity of A:T base pairs toward dimethyl sul fate (21, 35, 36). This effect could be due to methylation of the N-3 position of thymine, which is usually hidden in the DNA double helix (37); this may reflect a conformational change in DNA.

In the bottom strand, alterations in the -50 to -90, -130, and -160 regions were also observed. G located at -122, -128, and -130 were hypermethylated, regardless of the presence of effector. It is also worth noting that G at -144 and -146 were protected in the presence of effector. G at -161 and -166 were protected both in the absence and in the presence of effector, but this was very marked in the presence of m-methylbenzyl alcohol (see Fig. 3). The G located at -176 and
parison analysis (19) showed the presence of a putative binding site for IHF in the Pu region lying between -54 and -89 bp, where we observed alterations in the pattern of dimethyl sulfate methylation of G and T. A 100% match with a recently derived IHF consensus sequence (18, 37) was found by aligning the -55 to -67 region of Pu with the consensus one (Fig. 4). The IHF requirement for the expression of Pu was examined in E. coli strains proficient or deficient in IHF synthesis and carrying pERD401 and pTS174, both in the presence and in the absence of m-methylbenzyl alcohol, a XylR effector. In the absence of xylR, expression from Pu was low regardless of the presence or absence of the aromatic alcohol. In the presence of xylR and m-methylbenzyl alcohol, the expression level from Pu in the IHF mutant was about 25% of the level measured in the IHF-proficient strain.

To further assess the possible requirement of the -50 to -90 region in the activation of Pu, a single point mutation was generated within the IHF consensus sequence by replacing A at -63 with G, which created a single CiaI site. In addition, two other mutants were generated by inserting 2 extra bp (GC) in the open CiaI site with Klenow enzyme and the four dNTPs and by inserting 8 extra bp in an HpaI linker cloned in the filled CiaI site (CGGTTAAC). Moreover, a deletion extending from -64 to -111 was generated by using the PsI site at -106 and the CiaI site at -64 in mutant promoters. The promoters exhibiting a single point mutation, the 2 extra bp, the 8 extra bp, and a deletion of 47 bp were fused to lacZ in pMC1403, and the resulting plasmids were called pERD414, pERD415, pERD416, and pERD417, respectively.

The single point mutation introduced at -63 in the proposed IHF-binding site did not significantly alter the pattern of stimulation of transcription in IHF-deficient and IHF-proficient E. coli strains when compared with the wild type promoter in the same E. coli backgrounds (Table I), which is consistent with IHF still recognizing the mutated site. In contrast with the wild type promoter, no increase in b-galactosidase expression from the deleted promoter in pERD417 was observed regardless of the strain used, in the presence or absence of XylR and in the presence or absence of m-methylbenzyl alcohol (see 'Table I'). With the mutant promoters bearing 2 and 8 extra bp inserted into the IHF site (pERD415 and pERD416), the levels of expression in the IHF- background were similar to those determined for the wild type promoter in pERD401. However, in the IHF- background, maximal level of expression was about 20% of that determined for the wild type promoter in the same background, indicating the inserts disrupt IHF binding.

Separation of the Putative XylR Binding Sites—In order to separate the -130 UAS2 region from the -160 UAS1 region, a single NruI restriction site created at -144 was used to add 6 and 10 extra bps by introducing HpaI and NcoI linkers of 6 and 10mers, respectively. The mutant promoters were cloned in front of lacZ in pMC1403 to yield plasmids pERD419 and pERD420, respectively, b-Galactosidase levels in the presence

-178 were protected in the presence and hypermethylated in the absence of effector. Primer extension analysis also revealed that G at -92 was protected in the absence of m-methylbenzyl alcohol but hypermethylated in its presence. We also found that a T residue at -45 was hyperreactive in the presence of the effector. The T at -45 did not generate an extension termination product with DNA preparations from cells treated with dimethyl sulfate in the absence of the effector.

Expression of Pu in E. coli IHF Mutants—Sequence comp-
in the absence of a 10-mer NcoI linker. This generated a promoter with a 4-bp deletion (without linker) or with 6 extra bp (with the linker). The promoter exhibiting the NcoI site was digested with this enzyme, and the sticky ends were filled in with the Klenow enzyme and the four dNTPs so that the mutant promoter exhibited 10 bp between the -90 region and UAS1. The three mutant promoters exhibiting a 4-bp deletion or having 6 and 10 extra bp were fused to lacZ in pMC1403 to generate plasmids pERD421, pERD422, and pERD423.

β-Galactosidase activity was determined with and without XylR in trans and in the presence and the absence of m-methylbenzyl alcohol (Table II). Introduction of a full helix turn did not affect the level of β-galactosidase expression, but in contrast, shortening the distance by 4 bp or introducing half a turn (6 extra bp) clearly diminished expression of β-galactosidase (Table II), indicating that the UAS region must be correctly phased with downstream sequences to function efficiently.

### Discussion

In this study we investigated transcriptional activation from the TOL plasmid Pu promoter by generating deletions, point mutations, and small insertions, and by using in vivo dimethyl sulfate methylation.

The results of our Bal31 deletions suggest that full transcription activation of Pu is dependent on two DNA sequences located between -200 and -144 (UAS1) and -144 to -106 (UAS2) (Fig. 5). These regions have been previously suggested to be putatively involved in transcription activation, based on sequence homology between the Ps and Pu promoters (19). Comparison of these regions shows a highly conserved domain with a 5-bp inverted repeat in the -130 region 5′-ATTGGCT-CAAAAT-3′ (top strand) and a less well conserved inverted repeat in the -160 region 5′-ATTGGATCAAGG-3′ (bottom strand). The deletion of both binding motifs resulted in the complete loss of activation of transcription from Pu. Retention of UAS2 after deletion of UAS1 still allowed limited induction. Thus, as is the case for other -12/-24 promoters (e.g. glnAp2, nifH, and fdh), upstream activator sequences are required for the full and efficient activation of Pu.

Our in vivo footprinting experiments strongly support a
mechanism of remote activation of Pu promoter by XylR in which XylR binds the UAS1 and UAS2 regions but shows, in addition to the two above motifs, a third region of altered reactivity to dimethyl sulfate. This latter region, located from -50 to -90, showed no sequence homology with the other two (see Fig. 5). This could reflect changes in DNA conformation/protein binding related to the productive interaction of XylR with RNA-polymerase/RpoN complex during activation. In vitro footprinting with partially purified XylR protein has shown that the XylR protein binds only to the two motifs located beyond the -106 point of the Pu promoter (37b). The in vitro studies show a complex pattern of interaction of XylR with the UAS1 and UAS2 sequences. Thus, based on our in vivo data and on in vitro findings, we propose that the XylR protein recognizes and binds to these two UAS sequences (Fig. 5). This binding is independent of the availability of XylR effectors, although in the presence of effectors, changes in the binding pattern in the UAS1 motif were observed at positions -160 and -176 in the top and lower strands, respectively. Furthermore, changes in the intensity of protection or hypermethylation were also observed in the UAS1 motif (Figs. 2 and 3). In contrast with our observations in the UAS1 region, no obvious changes in the binding pattern or the intensity of protection or hypermethylation were observed in the UAS2 motif. It is, nonetheless, worth noting that G at -144 and -146 in the lower strand became protected in the presence of a XylR effector (Fig. 3). It is quite possible that the contacts in UAS2 were also altered; however, this region has few guanines, limiting our ability to detect changes. The significance of these changes needs to be further examined by in vitro footprinting experiments and in vivo footprinting with mutant promoters exhibiting point mutations in the bases putatively contacted by XylR in the presence and in the absence of effectors. The binding of effectors to the XylR protein may simply represent a small structural modification that slightly alters the interaction between XylR protein and DNA.

The interactions of XylR at UAS1 and UAS2 may not be symmetrical, as deduced from the fact that the inverted repeated sequences are positioned on opposite DNA strands. This pattern of nonsymmetrical interactions has also been observed in vitro (41).

The introduction of 6 bp between UAS1 and UAS2 or the separation of the activation sequences by a helix turn diminished stimulation of transcription from Pu. This suggests that either the XylR proteins do not bind to activator sequences or the XylR proteins bound to activator sequences are probably positioned at a definite distance; hence, cooperative interactions may be involved in stimulation of transcription.

Holte et al. (19) identified the -50 to -90 region of Pu as a putative binding site for IHF in the Pu promoter. In fact, the stretch between -60 and -70 exhibited a 100% match with a derived IHF consensus sequence (18, 38). In vitro footprinting experiments with purified IHF and Pu promoter have confirmed that the -54 to -90 region is contacted by IHF (41). Our in vivo studies of the top strand demonstrated G protection for G at -54 and -70. Protection occurred if XylR was present, but was independent of the presence of a XylR effector. In the bottom strand, it was observed that G at -92 was protected in the absence of m-methylenzyl alcohol, but hypermethylated in the presence of effector. An unexpected finding in vivo was the reactivity of T located at -84 in the top strand and -45 in the bottom strand toward dimethyl sulfate and piperidine cleavage. The T at -84 was protected in the presence of effector, but was hypermethylated in its absence. In contrast, the T at -45 was hypermethylated only in the presence of effector. Since the DNA sequence in this stretch is indeed very different from that of UAS1 and UAS2, we suggest that this region is recognized by IHF. The different reactivities of T in Pu in the presence and the absence of effector suggest the existence of DNA conformational changes, which are probably induced by the transcriptional complex (XylR + IHF + RNA-polymerase/RpoN).

In studies with E. coli deficient in IHF synthesis, we observed decreased stimulation of transcription from Pu. Furthermore, a deletion of the putative IHF-contacted sequences in plasmid pERD417 abolished activation from Pu (see Table 1), thus confirming a stimulatory role for this factor in transcription from Pu. This has also been observed for other promoters in which IHF seems to be involved (17, 39). Since separation of the IHF-binding site by a helix turn from UAS1 and UAS2 had no effect on transcription activation, we suggest that this piece of DNA plays a physical role rather than a regulatory one, which would require direct contact between IHF and XylR. The putative role of IHF may be to bend DNA and thus facilitate direct interaction between XylR and the RNA-polymerase/RpoN complex bound in the downstream promoter element. This type of interaction is in accordance with a model involving loop formation, as was recently found to be the case for the NtrC-activated RpoN-dependent glnAp2 promoter (23).

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