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Communication

Identification of the Repressor Subdomain within the Signal Reception Module of the Prokaryotic Enhancer-binding Protein XylR of *Pseudomonas putida**

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In the presence of *m*-xylene, the protein XylR encoded by the TOL plasmid of *Pseudomonas putida*, activates the σ^{54}-dependent promoter *Pu*. Early activation stages involve the release of the intramolecular repression caused by the signal reception N-terminal (A domain) of XylR on the central module of the protein. A genetic approach has been followed to locate the specific segment within A domain of XylR that is directly responsible for its down-regulation in the absence of inducer, as compared to that involved in effector (*m*-xylene) binding. For this, a reporter *Escherichia coli* strain carrying a monocopy transcriptional fusion of *Pu* to *lacZ* was transformed with a collection of plasmids encoding equivalent truncated varieties of XylR, consisting of nested and internal deletions throughout the entire A domain. Examination of the resulting phenotypes allowed the assignment of the A domain region near the central activation domain, as the portion of the protein responsible for the specific repression of XylR activity in the absence of *m*-xylene.

Strains of the genus *Pseudomonas* harboring the TOL plasmid pWW0 can grow on toluene, *m*-xylene, and *p*-xylene as the only carbon source owing to the activity of a complex catabolic pathway (summarized in Fig. 1) that proceeds in two major biochemical steps (Nakazawa *et al.*, 1990). These are determined by two independent operons that become coordinately transcribed when bacteria face pathway substrates such as *m*-xylene (see Marqués and Ramos (1993) for a review). The main regulator of the system is the so-called XylR protein, a member of the family of prokaryotic enhancer-binding regulators that act in concert with the alternative σ factor σ^{54} (Morett and Segovia, 1993; North *et al.*, 1993). In the presence of xylenes, XylR bound to upstream sequences activates the *Pu* promoter of the *upper*-TOL operon, thus triggering expression of the corresponding catabolic genes (de Lorenzo *et al.*, 1991; Abril *et al.*, 1991). The very early chain of events that translates the presence of xylenes into activation of *Pu*, involve effector-mediated conversion of XylR into a transcriptionally competent form. For this, the inducer binds directly to the N-terminal, signal reception module of XylR termed the A domain (Delgado and Ramos, 1994; Fernández *et al.*, 1995; see Fig. 1) and triggers the release of the repression caused by this domain on the central portion of the protein that is involved in the contact and activation of the σ^{54}-containing RNA polymerase (Pérez-Martín and de Lorenzo, 1995). This notion is based on the observation that deleting the entire A domain of XylR gives rise to a truncated protein that activates constitutively *Pu* in the absence of aromatic effectors, both *in vivo* (Fernández *et al.*, 1995) and *in vitro* (Pérez-Martín and de Lorenzo, 1996). It seems, therefore, that the A domain of XylR has at least two functions: (*a*) recognition of the aromatic inducers and (*b*) intramolecular repression. Since these two are obviously connected, the question arises as whether discrete subdomains within the N-terminal module can be assigned to each of them.

To explore the presence and the location of specific portions within the A module of XylR that are accountable for the central domain repression, we chose a reporter system in which the effect of changes in the regulator could be related immediately to a distinct phenotype in activation of *Pu*. Since all transcriptional control elements can be faithfully reproduced in *Escherichia coli*, this reporter system employs a derivative of *E. coli* YMC10 that had been lysogenized with an specialized λ phage containing a transcriptional *Pu-lacZ* fusion (Pérez-Martín and de Lorenzo, 1995). This strain (*E. coli* λRS*Pu*) was transformed independently with each one of a collection of plasmids bearing truncated *xylR* alleles that differed only in A domain sequences (Fig. 1). These were generated with a polymerase chain reaction-based strategy (PCR)[1] described in the legends of Figs. 2 and 3. The activity of the truncated products expressed in *trans* was measured as the accumulation *in vivo* of β-galactosidase in the presence or absence of the XylR effector *m*-xylene. Simultaneously to each activity assay, we examined the level of expression of each XylR-derived protein through Western blot assays (Fernández *et al.*, 1995) to ensure that the proteins were produced at similar levels (not shown).

To have a preliminary indication on the portion of the A domain of XylR involved in intramolecular repression, we sought to divide the A domain (211 amino acids, Inouye *et al.* (1988) and Shingler *et al.* (1993); see Fig. 2) in 6 large segments, that were progressively deleted from the N terminus (Fig. 2). These deletions were generated by amplifying with PCR the sequences of interest with an adequate collection of primers, so that the resulting products were flanked by *Eco*RI and *Bam*HI sites as specified in the legend to Fig. 1. Transfer of the resulting DNA fragments to the specialized expression vector pPr (Pérez-Martín and de Lorenzo (1995); see legend to Fig. 2) provided a translation initiation sequence and a leading ATG for expression of the six *xylR* deletion alleles shown in Fig. 2. These were named, respectively, Δ30, Δ120, Δ150, Δ180, Δ210, and Δ226. The corresponding proteins were produced *in vivo* at levels comparable to those of the wild-type XylR with

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[1] The abbreviation used is: PCR, polymerase chain reaction.

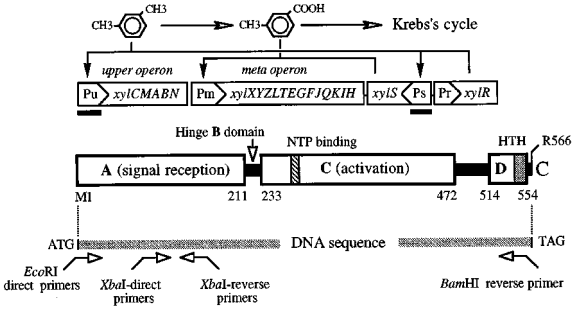


FIG. 1. **The TOL system of plasmid pWW0 and domain organization of XylR.** The TOL system for degradation of toluene and *m*-/*p*-xylene includes two gene clusters, the *upper*-operon and the *meta*-operon, as well as two regulatory genes, *xylS* and *xylR*, downstream of the *meta*-operon. The σ^{54}-dependent promoters *Pu* and *Ps* (*underlined*) are activated by the cognate activator XylR in the presence of *m*-xylene, while the *Pm* promoter is activated by XylS in the presence of benzoate or toluates. Functional domains of XylR are shown expanded, with an indication of the amino acid positions corresponding to the boundaries of each domain (Inouye *et al.*, 1988; Shingler, 1996). These include a signal reception N-terminal module (A domain), the central (C) activation domain, and the C-terminal segment (D domain) containing a helix-turn-helix (*HTH*) motif for DNA binding. The lower part of the figure sketches the strategy used to amplify with the polymerase chain reaction (PCR) specific DNA segments corresponding to different portions of the XylR protein for expression of truncated variants of the A domain. These include N-terminal deleted or internally truncated proteins (see legends to Figs. 2 and 3).

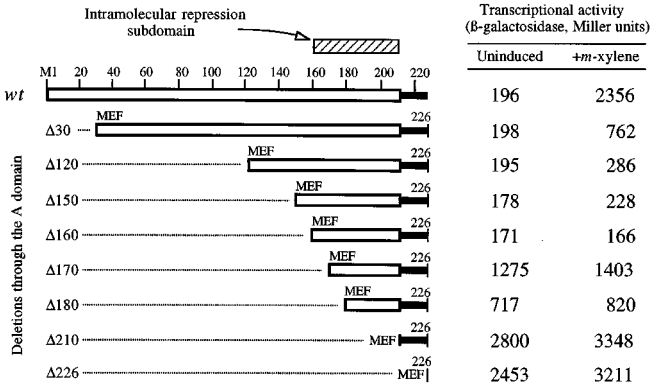
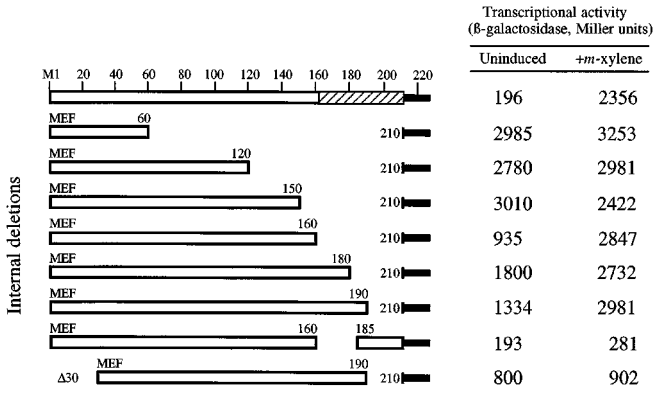


FIG. 2. **Effect of sequential N-terminal deletions through the A domain in the transcriptional activity of XylR.** The activity of the N-terminal truncated derivatives of the A module of XylR (211 amino acids, Fig. 1) indicated in the figure was monitored as accumulation of β-galactosidase in the *E. coli* strain λRS*Pu*, that carried a chromosomal *Pu-lacZ* fusion. For construction and expression of each of the truncated proteins, the following strategy was pursued. A DNA fragment containing the wild-type *xylR* sequence was subjected to a PCR reaction using as direct primers (Fig. 1) various oligonucleotides (33-mers) that attached an *Eco*RI site to the left of the site of deletion desired. For the reverse priming of the reaction, the same oligonucleotide was used in all cases, that generated a *Bam*HI site following the STOP codon of the *xylR* sequence, TAG. The amplified products were cloned as *Eco*RI-*Bam*HI fragments of different sizes at the same sites of vector pPr. This is a derivative of pCG1 (Myers *et al.*, 1987) in which the native *Pr* promoter of *xylR* within the TOL plasmid (Fig. 1) has been engineered in front of the same polylinker as pTrc99A (Amman *et al.*, 1988), that is led by an *Nco*I site overlapping a first structural ATG. This allowed all truncated proteins to be expressed through the very same native promoter and translation initiation regions as the wild-type *xylR*. The use of the direct *Eco*RI primers in the PCR reaction introduced in all cases amino acid residues EF (corresponding to 5′-GAA TTC-3′), next to the leading methionine of the truncated products. Replacement of the second (Ser) and third (Leu) amino acid residue of the wild-type XylR protein by EF had no effect on protein activity (not shown). For the experiment of the figure, each of the *E. coli* λRS*Pu* transformants were grown in LB medium (Miller, 1972) at 30 °C up to an A_{600} = 0.5, after which they were exposed, as indicated, to saturating vapors of *m*-xylene. Accumulation of β-galactosidase (Miller, 1972) was then measured after 5 h of induction. The figures for the reporter product are indicated with respect to the site of the deletions corresponding to each truncated regulator. The values shown are the average of 3 independent experiments carried out with duplicate samples. The approximate location of the repression subdomain suggested by the results is indicated on top.

the same expression system, as assessed by Western blot, and they were equally able to shut down expression of the *Pr* promoter of the *xylR* gene in an *in vivo* autorepression assay (Fernández *et al.* (1995); not shown). These experiments verified that the proteins were expressed at similar levels and that they were efficiently able to bind DNA. Intermediate deletions between positions 30 and 120 neither produced any detectable protein nor gave positive in an autorepression assay, and, thus, they were not further considered (not shown). The six productive deletions displayed very distinct phenotypes when the corresponding plasmids were transformed in the *Pu-lacZ E. coli* reporter strain (Fig. 2). While truncation of the 30 leading amino acids (XylRΔ30) gave rise to a protein with only a residual responsiveness to the XylR inducer, *m*-xylene (Fig. 2), subsequent removal of the protein segment up to position 120 (XylRΔ120) and beyond (XylRΔ150) gave rise to proteins unable to activate the *Pu* promoter to any significant extent. However, deletion of 30 additional amino acids (XylRΔ180) partially restored the ability of the protein to promote transcription (Fig. 2), although responsiveness to *m*-xylene was lost and the activity became constitutive. Deletion of 30 additional amino acids (XylRΔ210), which removed the A domain entirely, increased β-galactosidase accumulation to its highest level. Further deletions entering the Q-linker (XylRΔ226) did not result in an additional increase of promoter activity, while deletions beyond position 233, entering into the central C domain (Fig. 1), abolished any transcriptional activity of the resulting truncated proteins (not shown).

The results above confirmed the existence of a specific region within the A domain of XylR directly involved in intramolecular repression located between amino acid positions 150 and 210. To narrow down the location of the boundary, we made two additional deletions in 10-amino acid increments called XylRΔ160 and XylRΔ170. As shown in Fig. 2, while Δ160 remained inactive, Δ170 gave a substantial, but not full, constitutive activation of *Pu*, as compared to the maximum promoter activation caused by the deletion of the entire A domain (XylRΔ210, XylRΔ226). These results located the leftward limit of the repressor subdomain at around amino acid position 170, and, therefore, the whole functional module may span no more than 40 residues (or 60, if we also include some amino acids present at the hinge B domain, Fig. 1).

To verify the location of the repressor segment of the A domain suggested by the phenotypes caused by the N-terminal deletions, we constructed additional *xylR* alleles bearing sequential truncations of the A domain starting in position 210 (*i.e.* at the C-terminal end of the module) and spanning increasingly larger segments toward the N-terminal end (see legend of Fig. 3 for the procedure employed for their construction). As before, production *in vivo* of the predicted mutant proteins was verified through autorepression assays and Western blot of the corresponding cell extracts (not shown). The phenotypes endowed by each of the constructions listed in Fig. 3 were examined as before with the results summarized in Fig. 3. As expected, deletions spanning large portions of the A domain (Δ60/210, Δ120/210, and Δ150/210) originated truncated proteins that were fully constitutive. It was most remarkable, however, that deletions Δ160/210, Δ180/210, and Δ190/210 (that are totally or partially deleted of the repressor subdomain), although capable of activating transcription in the absence of inducer, maintained a significant degree of responsiveness to *m*-xylene. Such responsiveness was lost when the leading N terminus was deleted also (truncation Δ30-Δ190/210). This sug-



Transcriptional activity (ß-galactosidase, Miller units)	
Uninduced	+*m*-xylene
196	2356
2985	3253
2780	2981
3010	2422
935	2847
1800	2732
1334	2981
193	281
800	902

FIG. 3. **Phenotypes endowed by internal truncations of the A domain of XylR.** The ability of *xylR* alleles carrying the internal deletions within the A domain indicated in the figure was examined as for the sequential N-terminal deletions (Fig. 2). The strategy to generate the internally truncated proteins involved the production by PCR of two restriction fragments that were sequentially assembled in vector pPr (see legend to Fig. 2). The "left" restriction fragments (flanked by *Eco*RI-*Xba*I sites) were produced by amplifying the desired part of the A domain sequence with a direct *Eco*RI primer and a reverse *Xba*I primer (Fig. 1). The "right" restriction fragments were similarly produced with a direct *Xba*I primer, targeted to the site of interest within the sequence of the A domain, and a reverse *Bam*HI primer at the end of the *xylR* sequence (Fig. 1). Their assembly downstream of the *Pr* promoter in pPr gave rise to the truncated proteins under examination. Accumulation of *β*-galactosidase by each of the reporter strains, transformed with the corresponding plasmids, was examined as described in the legend to Fig. 2. The *hatched portion* of the A module shows the position of the repression subdomain.

gested that such a leading region is involved in effector recognition, lifting of intramolecular repression, or both. Since deletions Δ160/210, Δ180/210, and Δ190/210 are predicted to offset considerably the relative positioning of the remaining A sequence with respect to the central domain of the protein, the regulation by *m*-xylene retained by these proteins indicated that effector binding and associated changes in protein structure do not involve *per se* specific interdomain interactions. In addition, simultaneous deletion of the segment 190–210 and the leading 30 amino acids gave rise to a constitutive low activity regulator (Fig. 3), that may reflect the need of an intact N terminus for any responsiveness to the effector. Specific repression seems, therefore, to be due exclusively to the portion of the A domain encompassing positions 160 to 226. Furthermore, the fact that the internal deletion Δ160/185 (that lacks the leftmost boundary of the repressor subdomain with the rest of the protein) is virtually inactive (Fig. 3) suggested that the protein segments determining the response to effector recognition may be connected to each other close to positions 160–170. In fact, deletion of the zone around 170 seems to originate a protein that is inhibited unspecifically by the remainder of the A domain (see below). Interestingly, Delgado and Ramos (1994) found that a mutation in residue 172 made the protein to respond to a new effector (*m*-nitrotoluene), thus indicating that some direct or indirect determinants of ligand specificity may lie also around position 170.

The semiconstitutive phenotype of deletions Δ160/210, Δ180/210, and Δ190/210 (Fig. 3) indicated also that elimination of the repressor subdomain still affords a degree of down-regulation of XylR by the remainder of the A module. This phenomenon can be easily understood in light of the observations reported elsewhere (Fernández *et al.*, 1995) on the inactivation of XylR through substitution of its A domain by a bulky heterologous protein module. On this basis, it is very likely that although deletions Δ160/210, Δ180/210, and Δ190/210 have lost the specific region involved in intramolecular repression, they still retain a portion of the protein that inhibits its full activity in the absence of inducer by a mere physical hindrance of an activation surface and not because of an specific interdomain interaction. The same argument explains the phenotype of the deletion Δ160/185 (Fig. 3), that is virtually inactive *in vivo* in spite of having lost a protein segment that enters a region involved in specific intramolecular repression (see above). In this case, the lower activity of Δ160/185 (Fig. 3) as compared to Δ180 (Fig. 2) is the likely result of the steric hindrance caused by the remainder of the A domain present in the truncated protein.

Taken together, these results led to the conclusion that a protein segment as short at 40–50 amino acids fully accounts for the repression caused by the whole A domain on XylR in the absence of *m*-xylene. Our data are consistent also with the notion that only that portion of the A module maintains specific interactions with the central domain of the protein. In addition, the phenotypes originated by the internally truncated proteins (Fig. 3) suggest that the XylR portion involved in effector recognition may exist as a separate subdomain within the N-terminal module. Prediction of secondary structure within the region carried out with the PHD profile network method (Burkhard and Sander, 1993) indicated that amino acids 219 to 221 could be organized as a somewhat long (21 residues) α-helix. Interestingly, when compared to the library of protein crystal coordinates of the Brookhaven data bank, part of that α-helix and a few preceding residues (portion 204–222 of the A domain) bear significant resemblance to the region of the eukaryotic protein c-Fos (residues 168 to 186) that is involved in protein-protein interactions within a c-Fos/c-Jun/DNA co-crystal (Brookhaven ID code: 1FOS). This structural resemblance might be related to the specific interactions between the A domain and the central domain of XylR proposed to control the activity of the regulator (Pérez-Martín and de Lorenzo, 1995). On the contrary, no portion of the 160–220 region of XylR showed any structural similarity to the N-terminal domain of DctD, another member of the family of σ^{54}-dependent regulators whose activity is regulated through intramolecular repression as well (Gu *et al.*, 1994).

The A domain of XylR is the key component of this activator that endows specificity in the response to *m*-xylene (Delgado and Ramos, 1994; Shingler and Moore, 1994), so that direct effector binding is translated into release of intramolecular repression (Delgado *et al.*, 1995; Pérez-Martín and de Lorenzo, 1995). Although the precise mechanism by which this happens remains unsolved, the data presented in this work suggest that different portions of the A domain have specific roles in the process. It is possible that, similarly to what may happen to NtrC (Fiedler and Weiss, 1995), derepression could involve the dimerization of the N-terminal module in response to the reception of the activating signal. The different predictions raised by this hypothesis and other alternatives are currently under study in our laboratory.

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