Interactions of the XylS regulators with the C-terminal domain of the RNA polymerase α subunit influence the expression level from the cognate Pm promoter

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Abstract The *Pseudomonas putida meta*-cleavage operon encodes the enzymes for the catabolism of alkylbenzoates. Activation of *meta*-operon transcription is mediated by the XylS protein which, upon activation by effectors, binds two sites between −70 and −35 with respect to the main transcription initiation point at the Pm promoter. Two naturally occurring regulators, XylS and XylS1, that differ by only five amino acids, have been analyzed with regard to potential interactions of these positive regulators with the C-terminal domain of the α subunit of RNA polymerase (α-CTD). For these studies we expressed a derivative of α deprived of the entire C-terminal domain (α-Δ235) and found that expression from Pm with XylS or XylS1 was significantly decreased. To discern whether α-CTD activation depended on interactions with DNA and/or XylS proteins we tested a large collection of alanine substitutions within α-CTD. Most substitutions that had an effect on XylS and XylS1-dependent transcription were located in or adjacent to helix 1 and 4, which are known to be involved in α-CTD interactions with DNA. Two alanine substitutions in helix 3 (residues 287 and 291) identified a putative region of α-CTD/XylS regulator interactions.

Key words: TOL plasmid; RNA polymerase; C-terminal domain of the α subunit of RNA polymerase; XylS; Transcription

1. Introduction

The TOL plasmids of *Pseudomonas putida* encode the information for the catabolism of benzoate and alkylbenzoates through a *meta*-cleavage pathway, in which the benzoates are first oxidized to the corresponding catechols, which undergo *meta*-cleavage fission to yield a derivative of muconic acid semialdehyde, then further metabolized to Krebs cycle intermediates [1]. Two of these plasmids, pWW0 and pWW53, have been previously characterized in detail [2–4]. It has been shown that the pWW0 carries a single operon with all of the catabolic genes, and that the *xylS* gene encoding the regulator of the pathway is located 3′ of this and divergently transcribed [5]. Plasmid pWW53 carries two almost identical *meta*-operons and two copies of the *xylS* regulator: *xylS1* located 3′ of the so-called *meta*-operon 1; and *xylS3* located unlinked to the two *meta*-operons and *xylS1* [3]. The promoter region of the three *meta*-operons is very similar. In vivo assays with fusions of the corresponding Pm promoter to a promoterless *lacZ* gene revealed that the three regulators are able to mediate expression from each of the Pm promoters once they are activated by a cognate effector, i.e. 3-methyl benzoate. A detailed mutational analysis of Pm revealed that the XylS binding site at the Pm promoter involves two half-sites (5′-TGCAAGAAGCGGATA-3′ and 5′-TGCAAA-AAATGGCTAT-3′) located between −70 and −35 in the Pm promoter [6–8].

A feature of the three XylS regulators is that they share almost identical C-terminal domains where the bipartite DNA binding motifs of these proteins are located [9]. In contrast, some differences were noticed at the N-terminal end, the domain where effector interactions are thought to occur [10]. In fact, while XylS and XylS1 differ at only five amino acids in their N-terminal domains, the XylS3 protein is highly divergent from these two other regulators [11]. These divergences resulted in the following feature: while XylS and XylS1 recognized benzoate as an effector XylS3 did not, although all three regulators responded to 3-methyl benzoate [12]. Gallegos et al. [12] showed that although the effector profile of XylS and XylS1 is very similar, the induction level mediated from the Pm promoter with these regulators is markedly different, with XylS being able to mediate expression levels from Pm that were 6–10-fold higher than those mediated by XylS1. These differences are likely to be due to differences in transmission of the effector signal from the N- to the C-terminal domains (effector-mediated conformational change) or minor differences in DNA binding at the Pm promoter. Previous studies have shown that MarA, SoxS, Rob, AlkA and RhaS, which belong to the AraC/XylS family of transcriptional regulators, interact with the C-terminal domain of the RNA polymerase α subunit (α-CTD) [13–18], as is also the case with many other activators [19–23]; the most well char-

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Abbreviations: Tc[r], Km[r], Ap[r] resistance to tetracycline, kanamycin and ampicillin, respectively; α-CTD, the C-terminal domain of the RNA polymerase α subunit; α-Δ235, a derivative of the RNA polymerase α subunit deprived of the entire C-terminal domain; Pm, the promoter of the TOL plasmid *meta*-cleavage pathway operon

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acterized being CRP (see [24] for a review). However, to date no studies on potential interactions of XylS with the transcriptional machinery have been performed. This prompted us to analyze whether α-CTD was required for full activation by XylS and XylS1, and if so, whether any protein–protein contacts with the XylS regulators were required for α-CTD activation. We found that XylS-dependent transcription from Pm decreased upon overexpression of an α subunit derivative deleted of its entire C-terminal domain. Assays of strains overexpressing single alanine substitutions at every position within α-CTD identified a patch of residues that may be involved in interactions with XylS and XylS1 as well as a number of residues that most likely contact Pm DNA sequences.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli MC4100 (F′, *araD*139, Δ(*arg*F-*lac*) *rpsL* 150 [Str^r] relA1 *flbB* 5301 deoC1 ptsF25 rbsR) was used in all the assays. A series of compatible plasmids were used in this study: plasmid pERD100, which is a Tc^r low copy number vector bearing a Pm:′*lacZ* fusion [6]. Plasmid pLOW2 is a low copy number Km^R derivative of pACYC 177 [25]. Plasmid pLOW2::*xylS* and pLOW2::*xylS1* were generated by cloning a 1609 bp *Eco*RI–*Xba*I from pCMX2 and pCRAS1 [26] which bore the *xylS* and *xylS1* genes from pWW0 and pWW53, respectively. pREIIα [27] and pSE192 [14] are Ap^r plasmids encoding either the wild-type α, or a deletion of the entire C-terminal domain of α of *E. coli* (α-Δ235). It should be noted that deletion of the C-terminal one third of the α subunit, beyond residue 235 (intact α is composed of 329 amino acids) does not interfere with the assembly of core RNA polymerase or holoenzyme, and the mutant enzyme is enzymatically active [20,28,29]. A library of plasmids encoding alanine substitutions at every position within the C-terminal domain of α, constructed in the laboratories of R.L. Gourse and R.H. Ebright, and partially described in Gaal et al. [30] was kindly provided by Dr. Gourse.

2.2. β-Galactosidase assays

E. coli MC4100 was transformed with pERD100, pLOW2::*xylS* or pLOW2::*xylS1*, and either pREIIα (wild-type α), pSE192 (α-Δ235), or individual members of the α-CTD alanine substitution library. Bacteria were grown in LB medium overnight supplemented with 10 μg/ml Tc, 25 μg/ml Km, and 25 μg/ml Ap, then diluted 100-fold in the same medium but with 1 mM 3-methyl benzoate. β-Galactosidase activity was determined after 5 h. Each assay was repeated four times, and the data given are the average. The standard deviation in all cases was below 15% of the given average.

3. Results and discussion

3.1. Effect of expressing an α-CTD deletion derivative on Pm promoter activity

We first tested the expression from Pm by measuring β-galactosidase activity in *E. coli* MC4100 with pLOW2::*xylS* or pLOW2::*xylS1* and pERD100 over expressing either wild-type α (plasmid pREIIα, [26]) or the derivative deleted of the entire C-terminal domain, α-Δ235 (plasmid pSE192, [14]). The results obtained are shown in Table 1. It can be observed that upon expression of α-Δ235 the level of expression from Pm mediated by XylS or XylS1 decreased to about 50% of the wild-type level. These results indicated that truncation of α-CTD affected XylS-dependent transcription activation from Pm. This effect is similar to that reported for in vitro activation of transcription from the P_{COOF} promoter mediated by COOA, the co-sensing transcription activator from *Rhodospirillum rubrum* [31].

3.2. Effect of α-CTD alanine substitution derivatives on Pm promoter activity

The above results prompted us to analyze expression from Pm with XylS and XylS1 in combination with a library of point mutants in α-CTD. This series of assays was expected to reveal whether α-CTD activation depended on interactions with the Pm promoter DNA and/or XylS. This approach is based on an experimental setup in which most of the RNA polymerase pool contained a mutant subunit, and therefore, the assays aim at finding a dominant negative effect on transcription [32]. The results obtained are shown in Fig. 1A,B. It can be observed that alanine substitutions in the majority of residues in α-CTD had no significant effect on the expression level mediated by XylS or XylS1. In contrast, 11 point mutations in α-CTD affected the level of expression mediated by both XylS and XylS1, and an additional seven mutations had an effect on the induction level mediated by XylS1 but not by XylS (Fig. 1). The set of mutations that affected induction mediated by both regulators were alanine substitutions at positions 265, 268, 269, 273, 287, 291, 293, 296, 298, 299 and 306. A number of positions, i.e. 259, 260, 263, 289, 295, 297 and 303 had a modest effect on stimulation of transcription with XylS1 as a regulator, but did not affect transcription mediated by XylS. The structure of α-CTD has been resolved by Jeon et al. [33] and shown to be compactly folded and comprised of four α-helices (namely helix 1 (residues 264 to 273), helix 2 (residues 278 to 283), helix 3 (residues 286 to 292) and helix 4 (residues 297 to 309)) and two long loops. Chemical shift perturbation assays revealed that most residues in helix 1, the N-terminal half of helix 4, and the loop region between helices 3 and 4 in the structure of α-CTD are involved in the interaction with DNA [33]. A subset of residues in these regions of α-CTD have been shown by genetic and biochemical methods to be important for α-CTD interaction with UP element in different promoters [29,34–41]. Nine of the 11 mutations that significantly affected the level of expression from Pm mediated by both XylS and XylS1 were located in helix 1 (265, 268, 269, 273), helix 4 (298, 299, 306) and the loop between helix 3 and 4 (293, 296) and therefore are likely to interact with DNA. These residues are marked in red in the space-filling model of predicted α-CTD structure shown in Fig. 2. This set of residues form an extended patch, although not all the residues in the patch had an effect on the activation of transcription from Pm, suggesting that some but not all residues in this region are critical for DNA interaction. Our observations are in consonance with the identification of residues 262, 265 and 297 that play a major role in TyrR-dependent transcription from the *mtr* promoter [36], and the ternary complex of the Mar protein and RNA polymerase at the Pm promoter of bacteriophage Mu [39]. Among the residues we identified, the laboratory of Dr. Gourse has determined that

Table 1
Effect of α-CTD deletion on expression from the Pm promoter mediated by the XylS and XylS1 regulators

Regulator	β-Galactosidase activity (Miller units)	
	α-CTD	α-Δ235
XYLS	1000	615
XYLS1	150	70

E. coli MC4100 was transformed with pERD100, pLOW2::*xylS* or pLOW2::*xylS1*, and either pREIIα (α-CTD) or pSE192 (α-Δ235). β-Galactosidase was assayed as described in Section 2.

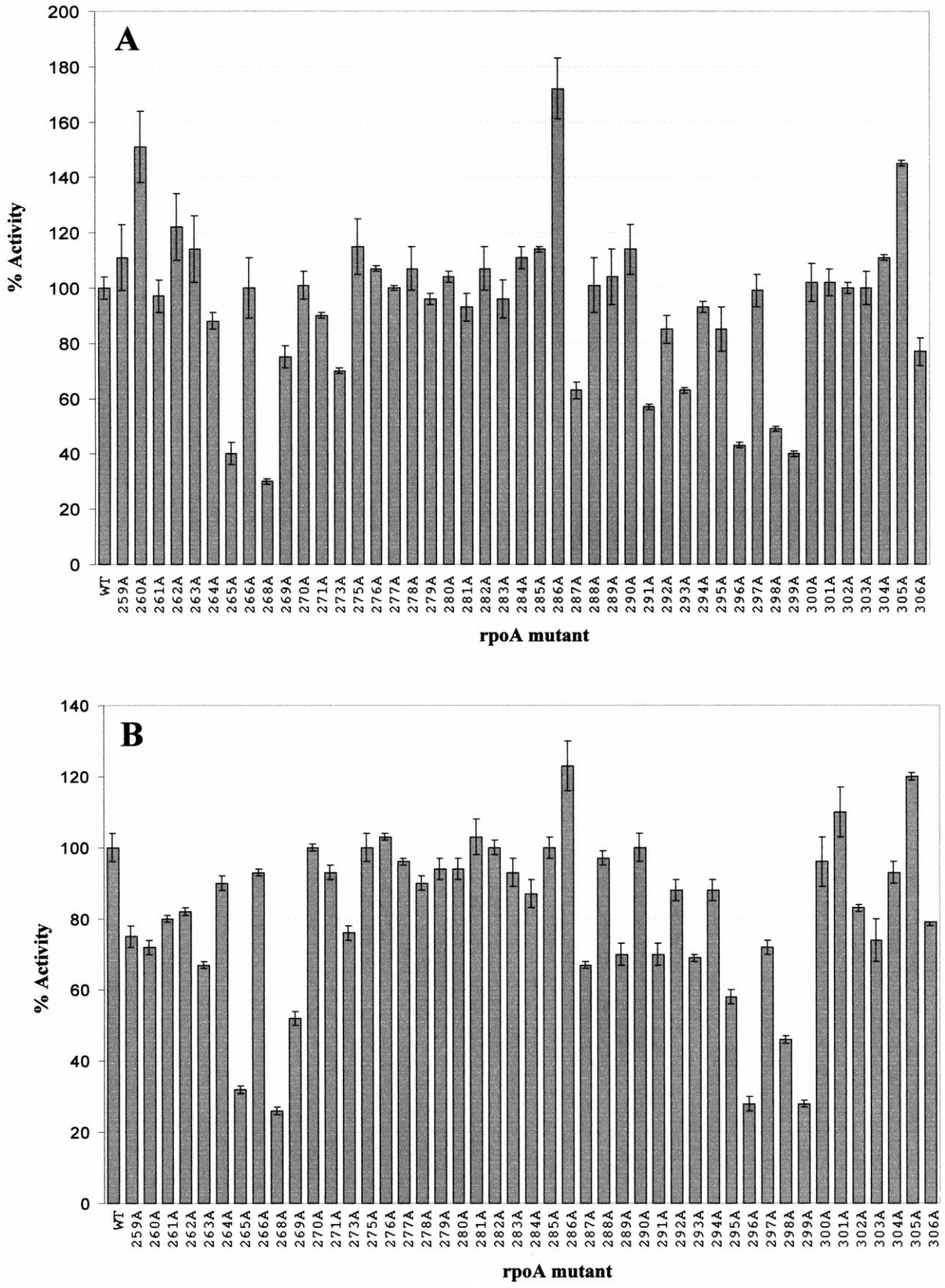

Fig. 1. Effect of single alanine substitutions within α-CTD on activation of transcription from Pm mediated by XylS (A) or XylS1 (B). Activities are expressed as percentage of the average activity measured from cells with plasmids encoding the corresponding α subunits. Values shown are the average of at least four independent assays. In addition to the data shown we also tested the effect of alanine substitutions from residue 255 to 258 and from residue 307 to 329. However, in all cases β-galactosidase activity ranged between 95 and 120% of the activity of the wild-type, values are not shown for figure quality.

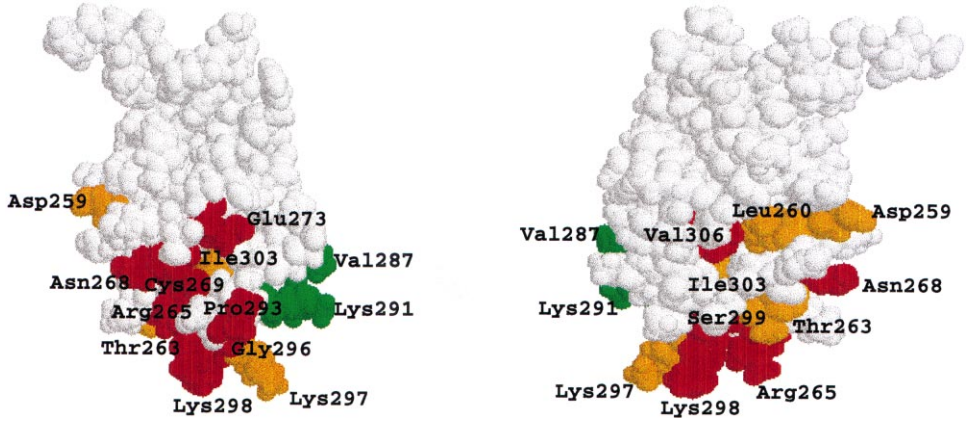

Fig. 2. Space-filling model of predicted α-CTD structure. The model was based on the atomic coordinates of Jeon et al. [33]. Colored residues are those identified as important at the Pm promoter. Red residues are those that may be important in interactions with DNA, in green are the residues that may be involved in interactions with XylS regulators, and in orange are those that in addition may be involved in interactions with DNA when the activator is XylS1. Residue numbers for some of the important residues are shown. The two models are related to one another by a 180° rotation around the vertical axis.

residues 265 and 298 are directly inserted into the minor groove of the DNA (personal communication). In agreement with this observation is that our results show a marked effect in transcription from Pm with the corresponding α subunits bearing alanine substitutions at positions 265 and 298 when overproduced. Of the seven point mutations that affected the level of transcription mediated by XylS1 but not by XylS, six (259, 260, 263, 295, 297 and 303) are located in or near regions shown to be involved in α-CTD interaction with UP elements [33], and therefore probably reflect a subtle difference in the α-CTD interaction with the two activators. It is possible that the weaker activator may weaken protein–protein interactions with α-CTD and therefore the role of interactions of α-CTD with DNA becomes more observable (see Fig. 1). The above series of results indicate that α-CTD is required for the full activation of the Pm promoter, and that at least some of the α-CTD-dependent activation involves interaction with DNA.

The other mutations that resulted in a decrease in activity, alanine substitutions at positions 287 and 291, could be the result of decreased interactions of α-CTD with XylS and XylS1 at the Pm promoter. A position important only for XylS1-mediated Pm transcription was 289, which is adjacent to residues 287 and 291. These three amino acids (287, 289, 291) are located in helix 3 and form a patch (marked in green in Fig. 2) that may represent a site of interaction between α-CTD, XylS and XylS1. Interestingly, residues 287 and 289 have been identified as part of the '287 determinant' of α-CTD that is required for cooperative interactions between α-CTD and CRP [42], supporting the conclusion that these residues may define a contact site with XylS and XylS1. In addition, residues 289 and 290 of α-CTD have been implicated in interactions with bacteriophage P2 Ogr protein [43]. Residues 289 and 290 of α-CTD could be involved in MetR interactions with α-CTD since MetR-dependent transcription from *metH* and *metE* was compromised with mutants of α-CTD exhibiting alanine at the above mentioned positions [37]. Therefore, our results suggest that the patch of residues 287–291 on the surface of α-CTD may define a site of interaction with the XylS and XylS1 activators.

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