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XylS–Pm Promoter Interactions through Two Helix–Turn–Helix Motifs: Identifying XylS Residues Important for DNA Binding and Activation

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The XylS protein is the positive transcription regulator of the TOL plasmid *meta*-cleavage pathway operon Pm. XylS belongs to the AraC family of transcriptional regulators and exhibits an N-terminal domain involved in effector recognition, and a C-terminal domain, made up of seven α-helices conforming two helix–turn–helix DNA-binding domains. α-Helix 3 and α-helix 6 are the recognition helices. In consonance with XylS structural organization, Pm exhibits a bipartite DNA-binding motif consisting of two boxes, called A and B, whose sequences are TGCA and GGNTA, respectively. This bipartite motif is repeated at the Pm promoter so that one of the XylS monomers binds to each of the repeats. An extensive series of genetic epistasis assays combining mutant Pm promoters and XylS single substitution mutant proteins revealed that α-helix 3 contacts A box nucleotides, whereas α-helix 6 residues contact B box nucleotides. In α-helix 3, Asn246 and Arg242 are involved in specific contacts with the TG dinucleotide at box A, whereas Arg296 and Glu299 contact the second G and T nucleotides at box B. On the basis of our results and of the three-dimensional model of the XylS C-terminal domain, we propose that Ser243, Glu249 and Lys250 in α-helix 3, and Asn299 and Arg302 in α-helix 6 contact the phosphate backbones. Alanine substitutions at the predicted phosphate backbone-contacting residues yielded mutants with low levels of activity, suggesting that XylS–Pm binding specificity not only involves specific amino acid–base interactions, but also relies on secondary DNA structure, which, although at another level, also comes into play. We propose a model in which a XylS dimer binds to the direct repeats in Pm in a head-to-tail conformation that allows the direct interaction of the XylS proximal subunit with the RNA polymerase sigma factor.

Keywords: XylS; AraC family; DNA contacts; transcriptional regulators; *Pseudomonas*

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Introduction

The AraC family of transcriptional regulators includes a large number of activators that regulate cellular processes involved in carbon metabolism, virulence and stress responses.[1] Many members of this family have a nonconserved domain involved in effector binding and dimerization processes,[1–6] and are characterized by significant homology over an ~100-amino-acid stretch containing two helix–turn–helix (HTH) motifs (Fig. 1a) that contact two adjacent major groove sections, as shown by Niland *et al.*[7] These regulators recognize 15- to 20-bp operator sequences at target promoters,[8–12] as documented by single-base substitutions of a large number of bases of the AraC target sequence and measurement of DNA binding to the mutant promoters by AraC.[8]

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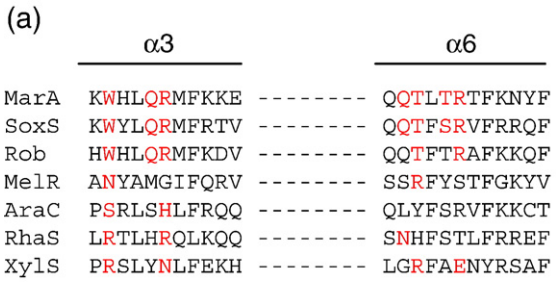
Abbreviations used: HTH, helix–turn–helix; 3MB, 3-methylbenzoate; RNAP, RNA polymerase; EMSA, electrophoretic mobility gel shift assay.

(a)



(b)

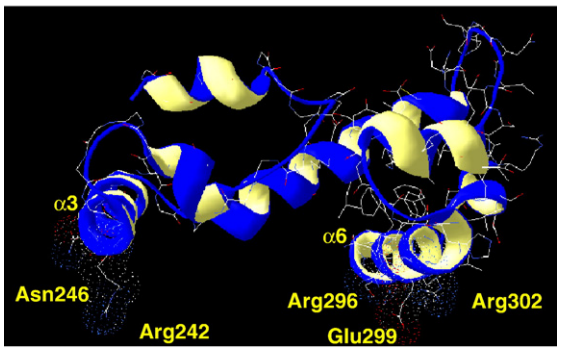


Fig. 1. (a) Partial alignment of members of the AraC family covering the recognition helices α3 and α6. Bases in red represent positions of base-specific contacts. (b) Structure-based model of the XylS DNA-binding domain. The XylS C-terminal domain was modeled on the structure of the MarA protein. Residues Arg242 and Asn246 in α3 and residues Arg296 and Arg302 in α6 are highlighted in yellow. In this work, we propose that these residues establish base-specific contacts with XylS binding sites at the Pm promoter.

The DNA-binding domain of AraC family members is predicted to adopt the same tertiary structure as MarA and Rob, that is, seven α-helices that fold forming two HTH DNA-binding motifs.[1,12,13] Despite the fact that AraC family members share great homology at the DNA-binding domain, the organization of the target DNA-binding sites at the different promoters and the specific activation mechanisms vary from one promoter to the other. For instance, while AraC and XylS seem to recognize direct repeats, with one of them overlapping the −35 hexamer recognized by RNA polymerase (RNAP),[8,9,14,15] MelR, RhaS and RhaR preferably recognize inverted repeats.[16–23] Hence, for any AraC family member, it is important to define the specific contacts established between the protein and the DNA as well as the relative orientation of the protein subunits.

The *Pseudomonas putida* XylS protein belongs to the AraC family of transcriptional regulators.[1,24,25] In response to the presence of benzoates, XylS activates expression from the Pm promoter that controls expression of the *meta*-cleavage pathway for the oxidative catabolism of benzoates and toluates present in *P. putida* TOL plasmid pWW0.[26] XylS is a 321-amino-acid protein with a molecular mass of 36 kDa. Like most AraC family members, XylS appears to consist of two domains: the N-terminal domain is involved in effector recognition and protein dimerization,[4,5,27] whereas the C-terminal domain seems to be responsible for DNA binding.[28–30] The XylS oligomeric state in solution has not been determined directly since this protein, like many AraC members, is difficult to study *in vitro* because of its intrinsic insolubility.[20,31–37] However, Ruíz *et al.*[4] found that the XylS N-terminal domain was able to dimerize, suggesting that XylS, like most AraC members, can form dimers in solution.[2,16,34,36]

The XylS C-terminal domain is able to bind and activate transcription from the Pm promoter independently of the presence of 3-methylbenzoate (3MB).[29,37] Genetic analyses established that XylS recognizes two 15-bp direct repeats (TGCA-N_6-GGNTA) extending from −69 to −55 and from −48 to −34. Furthermore, XylS binding-site architecture was defined as two repeats, each consisting of a 5′-box A (TGCA) and a 3′-box B (GGNTA). The arrangement of the two repeats is such that the proximal XylS binding site overlaps the RNAP binding site by 2 bp at −35.[14] This structural organization with two repeats and the overlapping of the −35 hexamer is reminiscent of the mechanism of activation mediated by AraC.[8,15,36]

In this study we used genetic analysis to identify important XylS residues for DNA recognition and binding, focusing on the orientation of XylS binding at its tandem sites. We used genetic epistasis experiments to identify XylS amino acids that make base-specific contacts at Pm. Several XylS mutant alleles were tested *in vitro* for their ability to bind Pm promoter DNA. Our results suggest that XylS binds Pm direct repeats in a head-to-tail organization, with α-helix 3 in the first HTH motif recognizing the A boxes, and α-helix 6 in the second HTH motif interacting with the B boxes. Both HTH motifs contribute to DNA binding and are critical to establishing contacts with target DNA at Pm.

Results

Alanine-scanning mutagenesis of the XylS HTH binding domains

The 108-amino-acid-long MarA protein was the first AraC family member whose high-resolution structure was determined.[12] It is composed of seven α-helices that fold in two HTH subdomains connected by a long α-helix.[12] Most members of the family are around 300 amino acids long and the alignment of over 1000 family members revealed that they exhibit high conservation at their DNA-binding domains.[1,24,27] Indeed, the 3-D structure of the DNA-binding domain of the Rob protein, another AraC family member, also revealed the above-described organization of seven α-helices with two of them acting as recognition helices to contact target DNA.[13] Based on the multiple alignment of AraC family proteins, residues Glu231 to His251 in XylS are predicted to form