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Mutational analysis of the highly conserved C-terminal residues of the XylS protein, a member of the AraC family of transcriptional regulators

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Abstract The XylS protein of the TOL plasmid of *Pseudomonas putida* belongs to the so-called AraC/XylS family of regulators, that includes more than 100 different bacterial proteins. A conserved stretch of about 100 amino acids is present at the C-terminal end. This conserved region is believed to contain seven α-helices, including two helix-turn-helix (HTH) DNA binding motifs (α_2-T-α_3 and α_5-Tα-$_6$), connected by a linker α-helix (α_4), and two flanking α-helices (α_1 and α_7). The second HTH motif is the region with the highest homology in the proteins of the family, with certain residues showing almost 90% identity. We have constructed XylS single mutants in the most conserved residues and have analysed their ability to stimulate transcription from its cognate promoter, Pm, fused to *'lacZ*. The analysis revealed that mutations in the α_5-helix conserved residues had little effect on the XylS transcriptional activity, whereas the distribution of polarity in the α_6-helix was important for the activity. The strongest effect of the mutations was observed in conserved residues located outside the DNA binding domain, namely, Gly-290 in the turn between the two helices, Pro-309 located downstream of α_6, and Leu-313, in the small last helix α_7, that seems to play an important role in the activation of RNA-polymerase. Our analysis shows that conservation of amino acids in the family reflects structural requirements rather than functionality in specific DNA interactions.

Key words: Benzoate; AraC/XylS family; DNA binding domain; Transcriptional activator; *Pseudomonas*

1. Introduction

The growth of *Pseudomonas putida* (pWW0) on alkylbenzoates requires the expression of the *meta*-cleavage pathway operon of the TOL plasmid, which is mediated by XylS activated by the presence of a benzoate effector (for a review see [1]). XylS is a 321 amino acid protein belonging to the so-called AraC/XylS family of regulators. This family includes more than 100 different bacterial proteins that participate in the stimulation of several cell processes such as carbon metabolism, pathogenesis or in the response to alkylating agents [2–4]. Pm is the target promoter of XylS, and drives the transcription of an operon with more than 13 genes. Genetic studies have shown that the regulator binds as a dimer to a 35 bp region of the promoter [5–11].

Alignments with proteins of the AraC/XylS family have allowed the definition of two domains in these proteins. The N-terminal domain is not conserved and it is involved in interactions with effectors and dimerisation [4,11–16]. In the C-terminal end, a conserved stretch of about 100 amino acids is present, which includes the DNA binding domain. This region of high homology is probably also involved in interactions with RNA polymerase [15–20]. The consensus for the family in the C-terminal region is: (I/V)--(I/V)A---G-(F/Y)-----F---F(R/K)---G--P, where - is any amino acid (Fig. 1).

MarA is one of the shortest proteins of the family, consisting of 129 residues which align with the C-terminal domain of AraC and XylS. Recently, co-crystallisation of MarA and its target DNA has revealed that the conserved region is composed of up to seven α-helices [21] with four helices (α_2, α_3, α_5 and α_6) forming two similar helix-turn-helix (HTH) DNA binding motifs, α_2-T-α_3 and α_5-T-α_6, connected by an α-helix linker, α_4. MarA binds as a monomer to adjacent DNA segments of the major groove, using the two recognition helices α_3 and α_6. It is worth noting that MarA is one of the few regulators of the family that lacks a dimerisation domain and that exists as a monomer in solution [22,23].

In AraC, the α_2-T-α_3 structure was considered a DNA binding domain since mutants in those two helices presented interactions with DNA and/or were impaired in promoter activation [17]. Recent genetic studies with this protein have shown that the second HTH motif may also contact DNA [24]. This is the only family of prokaryotic regulators described so far with a bipartite HTH motif, which could be related to the double-headed complex in the recent classification by Jones et al. [25]. The corresponding putative α-helices in XylS comprise residues 231–252 (α_2-T-α_3) and 282–305 (α_5-T-α_6) forming two HTH motifs (Fig. 1). We have previously shown that mutations in α_2 and α_3 of XylS produced regulators capable of activating the Pm promoter constitutively, i.e. in the absence of effector, suggesting a higher affinity of these mutant proteins for their target sequence in Pm [4,26,27]. The second HTH motif is the most conserved in the proteins of the family, with certain residues showing almost 90% identity [3]. Outside the α_6-helix, a proline residue (Pro-309 in XylS) is also conserved in more than 90% of the members of the family. The aim of this study is to genetically analyse the second HTH motif, defined by α_5 and α_6, mutating the most conserved residues. The functionality of the mutant regulators was assayed by determining activation of transcription by the Pm promoter. Results have shown that the most conserved residues in the family are involved in maintaining the protein structure required for its activity. The seventh helix at the C-terminal end of the regulators, whose se-

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Abbreviations: HTH, helix-turn-helix

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quence is poorly conserved in XylS, also seems to play an important role in activation.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli MC4100 [28] was grown at 30°C in Luria–Bertani medium supplemented, when required, with 100 μg/ml ampicillin, 25 μg/ml kanamycin or 25 μg/ml streptomycin.

The following plasmids have been described previously: pJLR107 (Pm::*lacZ*, Ap^R) [29]; pERD103 (pKT231::*xylS*, Km^R) [27]; pJB3KmD is an ampicillin-sensitive derivative of pJB3Km [30]. pERD802 is a pTZ18 (USB Corporation, USA) derivative containing a 1 kb *Nco*I/*Bam*HI fragment that includes the C-terminal end of *xylS*. pCM2 is a pSELECT derivative bearing a 1.7 kb *Bam*HI fragment containing the wild-type *xylS* gene cloned in the *Bam*HI site (C. Michán, personal communication).

The following plasmids were constructed in the course of this work: pERDX802 which is a pERD802 derivative bearing a silent mutation in the 266th codon of *xylS*, that creates a *Xho*I site. pCMX2 which is a pCM2 derivative bearing a silent mutation in the 266th codon of *xylS*, that creates a *Xho*I site. pJS5 which is a pJB3KmD derivative bearing the *xylS* wild-type gene cloned in the single *Bam*HI site against the P_{lac} promoter. pJS7 is pJS5 bearing a silent mutation in the 266th codon of *xylS* to create a *Xho*I site. pJB/* (where * refers to the amino acid change present in the *xylS* allele) were constructed by cloning a *Bam*HI fragment carrying each *xylS** mutant allele (see below) into the unique *Bam*HI site of pJB3KmD, against the P_{lac} promoter.

2.2. Construction of xylS mutant alleles by polymerase chain reaction (PCR)

Site-directed mutants of *xylS* were generated through overlapping PCR as described before [31]. The template DNA for the mutagenesis was pERDX802 and the internal oligonucleotides used for each mutant exhibited the required mismatches to render the desired amino acid change. The external oligonucleotides were the so-called M13 Universal and Reverse primers. The *Xho*I/*Bam*HI fragment of the wild-type *xylS* in pCMX2 was replaced by each one of the equivalent PCR fragments containing the mutated C-terminal region of *xylS*. After sequencing the mutated fragment to check for the absence of additional undesired mutations, each mutated *xylS* allele was subcloned in pJB3KmD to render pJB/* (see above). The following alleles were constructed in this work: *xylS*I282S, *xylS*I82E, *xylS*I282V, *xylS*I285E, *xylS*A286G, *xylS*G290A, *xylS*F291V, *xylS*F291Y, *xylS*F297Y, *xylS*F297V, *xylS*Y301S, *xylS*Y301F, *xylS*Y301V, *xylS*G306D, *xylS*P309E, *xylS*P309V, *xylS*P309H and *xylS*L313R.

2.3. DNA techniques

All DNA manipulations were carried out according to standard procedures [32].

2.4. β-Galactosidase assays

E. coli MC4100 bearing the wild-type and mutant *xylS* alleles in pJB3KmD plus the Pm::'*lacZ* fusion in pJLR107 were grown overnight in triplicate on Luria–Bertani medium containing the appropriate antibiotics. Cultures were diluted 100-fold in the same medium and grown at 30°C. After 1 h, each culture was divided in two and 1 mM of 3-methylbenzoate was added to one of them. β-Galactosidase activity was determined after 3 h of growth at 30°C, in permeabilised whole cells as described previously [33].

3. Results

3.1. Structure and conserved residues at the second DNA binding motif of XylS

We have revisited the alignment of the members of the XylS/AraC family of regulators based on the available 3D structure of MarA [21] and have found that two groups of proteins could be distinguished: 82% of the members of the family, including MarA and AraC, had a canonical turn of three or four amino acids between α_4 and α_5, whereas 18%, including the different versions of the XylS protein, presented a longer loop (six or seven amino acids) between these two helices. The distance between the first HTH motif and α_4 was three or four amino acids in both groups of proteins.

Fig. 2 shows amino acid residues of α_5 and α_6 in XylS on a planar α-helix wheel. α_5 is composed of eight amino acids, arranged as a hydrophobic face interrupted by two polar residues, and a negatively charged face with residues Glu-284 and Asp-288. α_6 is made up of 12 residues, organised in two clearly distinct halves. The solvent-inaccessible half is composed of five hydrophobic residues interrupted by the polar Tyr-301. The other half is strongly polar and includes the charged residues Arg-296, Glu-299 and Arg-302 (Fig. 2B). In MarA, the face of α_5 corresponding to the XylS α_5 hydrophobic half is oriented towards the inner side of the protein, i.e. α_4 and α_6, whereas the polar face is oriented towards the surface of the protein [21]. In α_6 of MarA, the helix region corresponding to the hydrophobic face of α_6 in XylS faces the inner side of the protein, particularly α_4, whereas the polar side is directed towards the major groove of the DNA.

Some residues in the α_5-T-α_6 are among the most conserved within the AraC/XylS family (Fig. 1). In XylS, these residues correspond to Ile-282, Ile-285 and Ala-286 within α_5; Gly-290 and Phe-291 within the turn between α_5 and α_6 and Phe-297 and Tyr-301 within α_6. After this helix, two other residues, which correspond to Gly-306 and Pro-309 in XylS, are highly conserved. Therefore, we became interested in producing XylS mutants in these conserved positions and determining their functionality in vivo.

3.2. Site-directed mutagenesis of selected conserved residues in XylS and analysis of the transcriptional activity of mutant proteins

In order to facilitate the construction of site-directed mutants in the second HTH motif of XylS, a mutant protein with a silent change in Gly-266 was generated, creating a single *Xho*I restriction site within the coding region. This allowed us to easily subclone each PCR-generated site-directed mutant to render a whole *xylS* mutant (see Section 2). The activity of the silent mutant protein was identical to that of the wild-type (not shown).

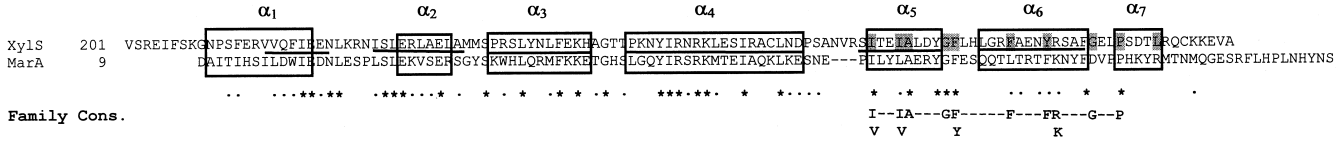

Fig. 1. Sequence alignment of the C-terminal region of XylS with MarA. An asterisk indicates amino acid identity and a dot marks homology between the two proteins. The consensus sequence in the second HTH region for the AraC/XylS family is indicated below the alignment. The α-helices determined according to the MarA structure [21] are enclosed in a box; the α-helices predicted by Gallegos et al. [3] are underlined. Residues mutagenised in this work are depicted in grey.

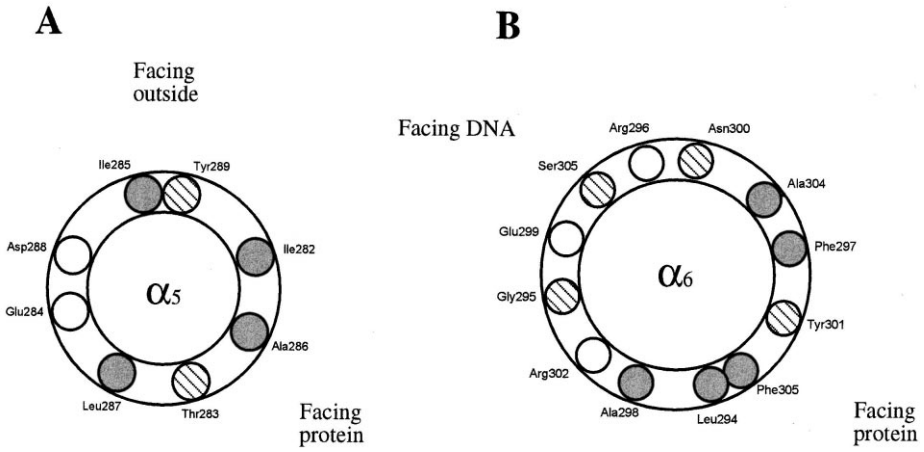

Fig. 2. Helical wheel presentation of the two α-helices of the second HTH motif of XylS. Shaded circles represent non-polar residues, striped circles polar residues, and white circles charged residues. The orientation of the helices towards DNA or the inner protein as predicted for the corresponding helices of MarA are indicated. A: α-helix 5; B: α-helix 6.

Table 1 shows the residues conserved among the members of the family and the changes introduced in XylS via site-directed mutagenesis in this study. In α_5, three residues, corresponding to positions 282, 285 and 286 of XylS, are especially conserved. At the aligned position of Ile-282, Gallegos et al. [3] found that in almost 98% of the members of the family, the residue was non-polar. For this reason, three changes were designed at this position: the conservative change to valine, the change to the neutral but polar serine, and the introduction of a negative charge in the change to glutamic acid. At the aligned position of residue 285, Gallegos et al. [3] found that 95% of the proteins exhibited a non-polar residue and that 5% of them exhibited a polar neutral substitution. A single mutant in which glutamic acid replaced Ile-285 was constructed. In the middle of α_5, alanine is present in 75% of the members at the position corresponding with residue 286 of XylS. A mutation to Gly was designed at this position. To determine the activity of mutant XylS proteins, we measured the expression from the Pm promoter fused to *'lacZ* in pJLR107 in the absence and in the presence of 3-methylbenzoate. Assays were carried out in *E. coli* MC4100 bearing the *xylS* mutant alleles cloned in the low copy number plasmid pJB3KmD.

Table 1
Conserved amino acid residues in the second HTH motif of XylS and their mutation

Residue	% Conservation	Second most frequent residue in the position	Change
Ile-282	42	Val	Val
			Ser
			Glu
Ile-285	58	Val	Glu
Ala-286	75	Ser	Gly
Gly-290	90	Phe	Ala
Phe-291	54	Tyr	Tyr
			Val
Phe-297	84	Leu	Tyr
			Val
Tyr-301	10	Phe[a]	Phe
			Val
			Ser
Gly-306	76	always polar	Asp
Pro-309	94	always non-polar	Glu
			Val
			His
Leu-313	11	Arg[b]	Arg

[a]This is the most represented residue at this position (70%).
[b]This is the most represented residue at this position (54%).

Fig. 3 shows the results obtained in the presence of effector. In α_5, the conservative change of Ile-282 to Val or to the polar amino acid Ser had little effect on the activity of the mutant protein (Fig. 3A). However, the substitution of Glu for Ile-282 completely abolished the activity of XylS. These results suggest that, although the actual residue present at that position is not crucial to maintain the structure of the helix, it is probably of major importance for the hydrophobic interactions with the rest of the molecule, since a change to a negatively charged amino acid abolished XylS activity. The same change, Glu for Ile, at position 285 of XylS had little effect on the activity of the protein, which was only reduced by 20% (Fig. 3A). In the planar projection of α_5 shown in Fig. 2A, Ile-282 is directly facing the inner core of the protein, whereas Ile-285 is located in a more external position of the helix. This could explain the minor effect of the last mutation on XylS activity. The conservative change Ala-286→Gly had no effect on the transcriptional activity of XylS.

Gly-290 and Phe-291 represent the first two amino acids of the turn between α_5 and α_6. Both the prediction by Gallegos et al. [3] and the structure deduced from MarA crystals place Gly-290 as the first amino acid in the turn [21]. Gly-290, one of the best conserved residues in the family, was replaced with Ala in XylS. At position 291 a Phe is present in XylS. This residue is conserved in 54% of the members, and a tyrosine is present in 32% of them. We replaced Phe-291 in XylS with Tyr, the next best represented residue, and Val, a non-polar amino acid with a shorter lateral chain. The conservative change of Ala for Gly at position 290 resulted in an inactive mutant (Fig. 3B). Substitution of the adjacent residue Phe-291 was relatively permissive. The change to the non-polar but smaller Val resulted in a mutant that conserved 50% of the maximal activity. The change to Tyr, that implied an increase in polarity with little change in the size of the residue, resulted in a mutant with a significantly higher activity than wild-type. This suggests that at this position, the size or spatial distribution of the residue, rather than polarity is important.

In the helix α_6 the two best conserved residues were those

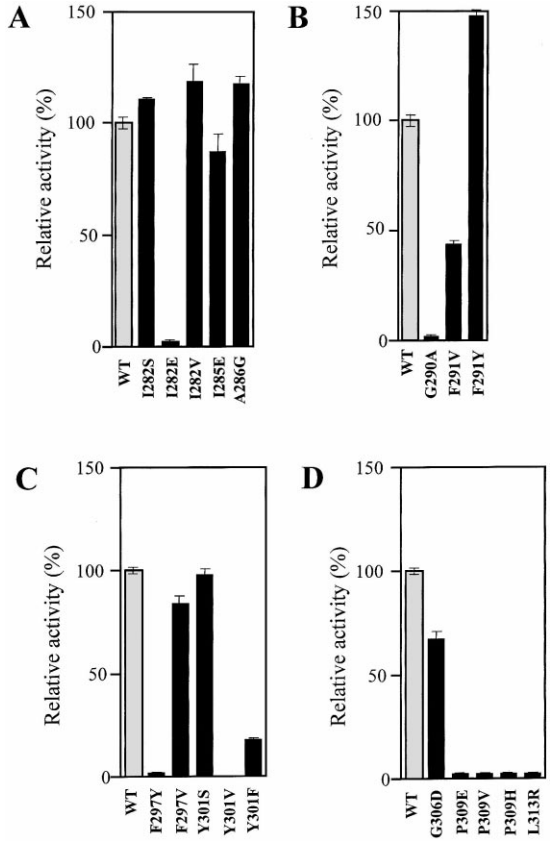

Fig. 3. β-Galactosidase activity of wild-type and mutant XylS proteins. Pm promoter-dependent activity was determined as described in Section 2 in *E. coli* MC4100 carrying pJLR107 and pJB3KmD bearing the different *xylS* mutant alleles, after 3 h induction with 3-methylbenzoate. A: Mutants in α_5; B: mutants in the turn between α_5 and α_6; C: mutants in α_6; D: mutants downstream of α_6. Activity is represented with respect to the β-galactosidase activity determined with the wild-type pair Pm/XylS, which corresponded to an average value of 8000 Miller units.

corresponding to positions 297 and 301 in XylS. In almost 84% of the family members, including XylS, the residue at position 297 is Phe. We replaced Phe-297 with Tyr and Val. At position 301 XylS exhibited a Tyr, but 70% of the family members had a Phe at this position and 10% of the members had a non-polar amino acid (Ile or Val). We replaced Tyr-301 with Phe, Val and Ser. The substitution of Phe-297 by the polar Tyr produced a complete loss of activity, contrasting with the insignificant effect observed when the change was to Val (Fig. 3C). A non-polar residue is present in the family at that position in 94% of the members. Fig. 2B shows that Phe-297 lies within the hydrophobic face of α_6. Here the lack of polarity seems to be important at this position, and the residue is probably involved in hydrophobic interactions with the core of the protein. Tyr-301 is the only polar amino acid present in that hydrophobic face (Fig. 2B). A change to either non-polar amino acids Val or Phe rendered a protein which was strongly impaired in the transcriptional activation of Pm, while a change to Ser had a very minor effect on the activity (Fig. 3C).

Downstream of α_6, further to the C-terminal end, residues 306 to 309 are proposed to form a turn, in which a Gly-306 is well conserved in the family. We replaced this residue in XylS with Asp. Residue 309 of XylS is a proline, as it is in the corresponding position of the alignment in 94% of the family members. We have replaced Pro-309 with Glu, Val or Hys. Finally, position 313 is a leucine in XylS, and it is only present in 11% of the members in the corresponding position. The most represented residue in this position was Arg (54%). Therefore, a mutant Leu-313→Arg was generated in XylS. The change of Gly-306 to the negatively charged Asp had no effect on the activity of the protein, which suggests that this amino acid is not crucial in the connection between α_6 and α_7. The uppermost conserved amino acid in the AraC/XylS family is the residue corresponding to Pro-309 in XylS. Any change in this position, either to negatively or positively charged, polar or non-polar amino acids gave a completely inactive protein, confirming the crucial role of this amino acid (Fig. 3D). Finally, the change of the poorly conserved Leu-313 to the highly conserved Arg rendered a mutant protein impaired in transcription activation (Fig. 3D).

For all the mutants studied above, the levels of Pm activity in the absence of effector were very low and similar to those obtained with the wild-type protein (not shown).

4. Discussion

The analysis of protein structures and sequences has established that a sequence homology greater than 25% between two proteins extending for 50 amino acids is sufficient to ensure a similar folding [34]. Given that members of the AraC/XylS family are transcriptional regulators and that the conserved region comprises almost 100 amino acids with an overall similarity greater than 20%, we can assume that the conserved region of these proteins presents similar structures. The determined crystal structure of the 129 residue MarA protein, one of the shortest members of the family, has shown that the structure prediction based on the analysis presented by Gallegos et al. [3] of more than 100 homologue proteins was essentially valid. The six anticipated α-helices forming two HTH motifs can be observed in the crystal, covering basically the predicted amino acid residues. Only the seventh short α-helix observed in MarA downstream of α_6 was not deduced from the family alignment.

The crystal structure of MarA bound to a target synthetic DNA indicated that the regulator binds DNA via two HTH motifs. This is in agreement with previous proposals of AraC and XylS recognising their target DNA sequences [7,24]. Analysis of the family alignment showed that the degree of sequence conservation at the first DNA-binding HTH motif (α_2-T-α_3) was low, with strong divergence in certain pairs of the sequences (Ramos, unpublished). This first HTH motif has been well characterised genetically as a DNA binding domain in several members of the family [4,10,11,14,15, 21,23,24,27,35]. The low degree of homology among the members of the family in this HTH could therefore reflect the diversity of DNA target sequences that the different regulators recognise. In contrast, the second HTH motif is highly conserved among the members of the family, which suggests a common role for this motif in all the regulators, such as contacting RNA polymerase, or interacting with the inner core of the protein [3].

There are some mutant regulators in the second HTH motif available. In MelR, substitution in α_6 of Ala and Asn for the non-conserved residues Ser-271 and Arg-272, respectively, produced mutants with a wild-type phenotype [35]. In AraC,

the analysis of mutations in α_6 (Gln-257→Ala, Ser-261→Ala and Val-264→Ile) and in the turn between α_5 and α_6 (Gly-253→Ser, Asp-256→Ala) revealed that, although certain mutants had lost the ability to bind DNA in vivo, the phenotype could not be attributed to this HTH constituting a DNA-binding motif [17]. However, genetic studies with combinations of mutant promoters and regulators have shown that this second HTH was indeed part of the DNA binding motif in AraC [24]. Genetic studies with mutant regulators have also been carried out with RhaS. Random mutagenesis of part of its α_6 and the residues in the turn between α_5 and α_6 showed that only changes in Ser-249 (located in the turn), Ser-251 and Ser-255, in positions 1 and 5 of α_6, respectively, were allowed. A detailed analysis of the mutants revealed that the loss of activity was due to the loss of specific DNA contacts only for the changes in Asp-250 and Asn-252, located in the turn and in α_6, respectively. Therefore, the phenotype of the other inactive mutants should be attributed either to the lack of interaction with DNA backbone, to the inability to stimulate transcription activation or to alterations in the protein conformation [36].

Within the α-helix structures, the most conserved amino acids in the family correspond to XylS Phe-297 and Ala-286, within α_6 and α_5, respectively. Non-polar residues can replace these amino acids without significant loss in the activity (Fig. 3A,C). A role for these residues could be to maintain hydrophobic interactions between these two helices or adjacent helices. In α_5, changes in Ile-282 and Ile-285 showed a high degree of permissiveness and only a drastic change of Ile-282 to Glu was able to abolish the activity. These results, together with the previous finding that changes of Val for Asp-288 in α_5 of XylS had no effect on its activity [4], suggest that the structure of this α-helix is not crucial for the activity of the protein.

The two amino acids of MarA corresponding to the most conserved residues in the family, Gly-89 and Pro-108, did not contact DNA. Interestingly, our results showed that any change in the corresponding residues in XylS, Gly-290 and Pro-309, was critical for protein activity (Fig. 3B,D). Gly-290 of XylS lies in the turn between α_5 and α_6 and probably maintaining the correct orientation between the two helices to allow proper α_6 contacts with DNA. Glycine is the most common residue of protein loops [37], especially in HTH structures, where it usually occupies the first position in the turn [38]. Results obtained with mutant XylSG290→A suggest that the relative orientation of α_5 and α_6 is of major importance for the activity of the protein. This is more striking with Pro-309 because any change at that position produced a complete loss of activity. Proline is known to force 'kinks' in helices and loops [39]. In XylS this residue is located after the recognition helix, probably positioning it with respect to the seventh small helix. This suggests that α_7 plays an important, though yet undefined, role in the activity of these proteins. This is further supported by the fact that a change in this helix, Leu-313→Arg, rendered an inactive regulator (Fig. 3D). As mentioned above, several members of the family present a longer loop between the connecting helix α_4 and the first helix of the HTH α_5. Coincidentally, in all these regulators, the α_7 sequence is not conserved. Instead, all but one of them have a non-polar residue in the position corresponding to the Arg residue present in 54% of the family members (Leu-313 in XylS) and lack the conserved Phe/Tyr in the preceding position (present in 72% of the members). This suggests a functional connection between the presence of a longer loop and a non-canonical sequence in α_7. In the crystal structure of MarA, α_7 falls in the proximity of the turn between α_4 and the second HTH structure, with the Arg residue oriented towards the turn. Therefore, the presence of an Arg at position 313 in XylS, close to the longer turn, would cause a steric hindrance, probably altering the protein conformation in this region. It is striking that changes in residues located downstream of the HTH motif, such as Pro-309 and Leu-313, can have such a strong influence on protein activity and it probably suggests an important function for this region of the protein.

Altogether, these results reflect a structural role for the most conserved amino acids within the family, rather than a role in direct interactions with DNA. The correct orientation of the different helices with respect to each other seem to be of major importance for maintaining the activity of the protein. Interestingly, available mutants in the structural helix α_4 which connects the two HTH motifs of XylS present two categories: inactive mutants, located in the first residue of the helix, Pro-256, and constitutive mutants, that lied in the last residue of the helix, Asp-274 [27]. Again, this reflects that the proper orientation of the different structures to each other is crucial in this family and that only a low degree of flexibility is allowed, probably due to the need of an accurate separation between the two HTH motifs to contact two major grooves in the DNA separated by a delimited distance. Further in vitro studies are needed to precisely define the role and interactions of these residues, especially that of α_7, in the maintenance of protein functionality.

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