Residues 137 and 153 at the N Terminus of the XylS Protein Influence the Effector Profile of This Transcriptional Regulator and the σ Factor Used by RNA Polymerase to Stimulate Transcription from Its Cognate Promoter*

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The 321-residue XylS and XylS1 proteins, encoded by the pWW0 and pWW53 plasmids respectively, differ in only 5 residues at positions 4, 53, 90, 137, and 153. As a result, the effector profile of XylS is wider than that of XylS1, and XylS mediates higher levels of transcription from its cognate-regulatable promoter than does XylS1. We generated a series of XylS-pWW0 mutants and found that the single mutants Asp-137 → Glu and His-153 → Asn exhibited an activation pattern different from that of the wild-type regulator. In the double-mutant XylSD137E,H153N the effector profile for benzoates was similar to that of XylS1. This suggests that these two residues are crucial for effector recognition and regulator activation to stimulate transcription. XylS-dependent transcription from its cognate promoter is mediated by RNA polymerase with σ32 or σ70, whereas XylS1 uses RNA polymerase with σ32 or σ70. We also found that point mutations at positions 137 and 153 of XylS led RNA polymerase to mediate transcription with σ70 rather than with σ32, as demonstrated by primer extension analysis in a σ70-thermosensitive background proficient and deficient in σ32. This suggests that a positive transcriptional regulator can choose the RNA polymerase complex that mediates transcription from a given promoter.

Gene regulators respond to specific environmental, cellular, and other signals by stimulating or inhibiting transcription, translation, or some other event in gene expression so that the rate of synthesis of the gene product is appropriately modified (1, 2). Research efforts in our laboratory have focused on the regulation of the TOL plasmid-encoded catabolic pathway for the metabolism of benzoate and alkylbenzoates. Genes encoding the TOL meta-cleavage pathway in pWW0 are grouped into a single operon whose expression is positively regulated at the level of transcription by the xylS gene product, which is activated by benzoate effectors (for review, see Ref. 3). Another well studied TOL plasmid is pWW53, which bears two functional homologous meta-pathway operons, together with two functional copies of the xylS regulatory genes (xylS1 and xylS3) (4–7). XylS-pWW0 and XylS1-pWW53 differ in five amino acids, whereas XylS3 shows conserved sequence similarity with these two proteins only at their C-terminal end (6, 7), where there is a bipartite DNA binding domain made of two α-helix-turn-α-helix motifs (as in the MarA, Rob, and AraC proteins) (8–11). All three XylS isoforms are able to stimulate transcription from the Pmρ promoter (7).

We previously isolated XylS mutants with altered effector specificity (i.e. Arg-45 → Thr and Cys-41 → Gly) or impaired effector recognition (i.e. Arg-41 → Leu) (12, 13). These findings suggested that the amino-end part of XylS is involved in effector recognition and XylS activation. XylS1-pWW53 and XylS-pWW0 differ in only 5 amino acids (Cys-4, Cys-53, Gly-90, Asp-137, and His-153 in XylS versus Arg-4, Gly-53, Asp-90, Glu-137, and Asn-153 in XylS1), but despite this minor difference the latter exhibited a much narrower effector profile than XylS-pWW0 (7). In fact, XylS1-pWW53 recognizes only 3MB and 3CB as effectors, whereas XylS-pWW0 recognizes in addition other alkyl- and chlorobenzoates with substrates at positions 2 or 4 (14, 15). The difference in effector profile is consistent with the changes occurring at the N-terminal end of these regulators.

Both XylS and XylS1 mediate transcription with RpoH (σ32) in the early log phase (7, 16); however, XylS-pWW0-dependent transcription from Pm is subsequently mediated by RNA polymerase with σ32 (16), whereas in the case of XylS1 the σ factor used by RNA polymerase is σ70 (7). Therefore, the minor differences at the protein sequence level between the two XylS proteins also impose dependence on the σ used by RNA polymerase. In light of these effects we decided to introduce stepwise mutations in XylS-pWW0 to determine their effects on transcriptional activity of the mutants.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Culture Medium, Plasmids, and Phages—The bacterial strains were Escherichia coli MC4100 (F−, araD139 Δ[argF-lac] U169 rpsL150 (Strr) relA1 flbB5301 deoC1 ptsP25 rbsR), E. coli RH90 (MC4100 rpsS59: Tn10) (17), E. coli KY1429 (MC4100 rpoH6 [Am] zhf-50::Tn10) (18), E. coli P90A5e (thi lacZ4 argG75) (19), and E. coli UQ285 (thi lacZ4 argG75 rpoD285). This last strain has a short deletion at the rpoD gene so that σ32 is nonfunctional at 42 °C (19). We also used E. coli EE286, an rpoS mutant of UQ285 constructed after P1 transduction (20) of rpsS9::Tn10 into the UQ285 background (this strain). Bacteria were grown at 30 °C in Luria-Bertani medium supplemented, when required, with 100 μg/ml ampicillin, 25 μg/ml kanamycin, 50 μg/ml streptomycin, 30 μg/ml chloramphenicol, or 10 μg/ml tetracycline.

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1 The abbreviations used are: Pm, promoter for the TOL meta-cleavage pathway; CB, chlorobenzoate; MB, methylbenzoate; DMB, dimethylbenzoate.

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cline. Growth was determined turbidimetrically at 660 nm.

The previously constructed plasmids were used. pCMX2 is a tetracycline resistance derivative of pSELECT containing the entire xylS gene inserted into the BamHI site (21). pERD100 is an IncQ group plasmid that carries a fusion of Pm to a promoterless ‘lacZ gene and encodes resistance to tetracycline (22). Plasmid pLRL107 has a 401-base pair PstI fragment of the TOL plasmid containing the Pm promoter fused to a promoterless ‘lacZ gene in pMD1405 (pBR replicon) and encodes resistance to ampicillin (23). pLOW2 is a PACY177 derivative, low copy number cloning vector that encodes resistance to kanamycin (24).

DNA Techniques—DNA preparation, digestion with restriction endonucleases, and analysis by agarose gel electrophoresis, isolation of DNA fragments, ligations, transformations, transduction with P1 phage, and sequencing reactions were done according to standard procedures (25) or to the manufacturer’s recommendations.

Construction of xylS Mutants by PCR—The xylS mutants were generated by overlap extension PCR mutagenesis (26) with internal oligonucleotide primers that exhibited one mismatch with respect to the wild-type sequence. The external primers were 5′-GAGCTCTAGG-GATGCCTCAAGC-3′ and 5′-GGATTTTTGCTTATTGAACG-3′. After DNA amplification, the resulting DNA was digested with XhoI and BglII, and the 761-base pair XhoI-BglII xylS mutant fragments were inserted between the XhoI-BglII sites of pCMX2 to yield plasmids pCMX2::xylS* (the asterisk indicates that one or more of the amino acids in the wild-type protein has been changed). In a first round of mutagenesis we generated single mutants at positions 4, 53, 90, 137, 153, and 158 as indicated in Table I. These single mutants were used to construct the double mutants shown in Table I. All the xylS mutant alleles generated in this study were verified by DNA sequencing. Plasmids pCMX2::xylS* bearing the xylS* mutant alleles were digested with EcoRI and XhoI, and the 1609-base pair EcoRI-XhoI fragments, which contained the entire set of xylS* mutant alleles, were subcloned between EcoRI and XhoI sites of pLOW2 to generate plasmids pLAR1 through 18, which encoded the mutant XylS proteins shown in Table I.

β-Galactosidase Assays—E. coli bearing the wild-type xylS allele or xylS mutant alleles in pLARs (the x indicates a number between 1 and 18) plus pERD100 or pLRL107 were grown overnight in Luria-Bertani medium containing the appropriate antibiotics. Cultures were diluted 1:10 in 50 ml of the same medium supplemented or not with 1 mM of 3-chlorobenzoate. After 5 h of incubation, β-galactosidase activity was assayed in permeabilized whole cells as described previously (26).

Western Blot Analysis—Rabbit polyclonal antibodies against the XylS protein were produced and used to detect XylS proteins fractionated with SDS-PAGE gels with goat anti-rabbit antibodies conjugated to peroxidase according to the protocol described by Jones and Gregory (27).

RNA Preparation, Analysis, and Primer Extension—RNA was extracted with a modification of the guanidinium isothiocyanate-phenol method (16). The RNA concentration was determined by measuring A260. Hybridization of the 32P-labeled single-stranded DNA primer (105 cpm) complementary to the mRNA transcript produced with XylS and 153 as indicated in Table I. These single mutants were used to detect XylS proteins fractionated with SDS-PAGE gels with goat anti-rabbit antibodies conjugated to peroxidase according to the protocol described by Jones and Gregory (27).

RESULTS

Response of the XylS Mutant Regulators to Benzoates Substituted at Position 3 on the Aromatic Ring—The wild-type XylS regulator and the series of single point mutant regulators constructed by overlapping PCR mutagenesis were cloned in the low copy number plasmid pLOW2 as described above. The resulting plasmids were transformed in E. coli MC4100 (pERD100), and β-galactosidase activity in response to the addition of 3MB was determined (Table I). This benzoate analog was chosen in this first series of assays because it is the best effector for both XylS-pWW0 and XylS1-pWW53. The level of transcription mediated by the wild-type XylS-pWW0 increased about 40-fold with respect to the basal level, whereas the XylS1-pWW53 protein mediated only a 4-fold induction (Table I). Each of the single point mutants of XylS-pWW0 were also assayed, and the results showed little effect for the changes Arg, Cys-53, Glu or His-152 (Table I). The combination of either the Asp-137 Glu or His-153 mutation with Cys-4 → Arg, Cys-53 → Gly, and Gly-90 → Asp and a modest effect for the point mutants Asp-137 → Glu and His-153 → Asn (the activity decreased to about 50% that measured for the wild-type XylS-pWW0 protein) (Table I).

Given that the induction level mediated by XylS1 with 3MB was significantly lower than in the single mutants, we generated all possible double mutants. The level of induction of the double mutant XylS1D137E,H153N was similar to that of XylS1 (Table I). The combination of either the Asp-137 → Glu or His-153 → Asn mutation with Cys-4 → Arg resulted in levels of activity lower than that found with any of the single mutants. However, when Asp-137 → Glu or His-153 → Asn was combined with a mutation in position 53 or 90, the activity was similar to that seen with the Asp-137 → Glu or His-153 → Asn mutation alone (Table I).

This prompted us to analyze the combination of double mutant XylS1D137E,H153N with the rest of the mutations. The result was that these mutants behaved like the double mutant and exhibited an induction level similar to that determined with XylS1-pWW53 (Table I). These results, therefore, suggest that residues 137 and 155 in XylS-pWW0 play a key role in effector recognition and activation of this regulator.

To determine whether the effect of the mutations was specific for 3MB, we tested the level of induction with two other substituted benzoate analogs that are effectors of XylS-pWW0, 3CB and 3-bromobenzoate. In general, 3CB and 3-bromobenzoate induced less β-galactosidase activity than 3MB with any of the mutants, and the reduction in the induction level for each of the mutants with these effectors followed a pattern similar to the decreases observed with 3MB as the effector (Table I).

### Table I

| REGULATOR      | 3MB | 3CB | 3BrB |
|----------------|-----|-----|------|
| Wild-type XylS-pWW0 | 29  | 23  | 18   |
| C4R           | 32  | 14  | 10   |
| C53G          | 37  | 25  | 20   |
| G90D          | 36  | 17  | 12   |
| D137E         | 20  | 10  | 8    |
| H153N         | 20  | 10  | 8    |
| C4R+C53G      | 17  | 9   | 7    |
| C4R+G90D      | 14  | 6   | 5    |
| C4R+D137E     | 11  | 4   | 3    |
| C4R+H153N     | 9   | 4   | 3    |
| C53G+G90D     | 36  | 17  | 12   |
| C53G+D137E    | 25  | 10  | 8    |
| C53G+H153N    | 19  | 10  | 6    |
| G90D+D137E    | 21  | 8   | 6    |
| G90D+H153N    | 26  | 12  | 8    |
| D137E+H153N   | 5   | 2   | 2    |
| C4R+D137E+H153N | 3  | 2   | 2    |
| C53G+D137E+H153N | 5 | 2   | 2    |
| G90D+D137E+H153N | 3 | 2   | 2    |
| XylS1-pWW53   | 4   | 2   | 2    |
proteins, we performed Western blots with total protein from *E. coli* bearing the XylS-pWW0 mutant alleles and used a polyclonal antibody against the XylS protein. In all cases the amount of XylS mutant protein was similar (not shown), and therefore, differences in expression levels mediated by each mutant regulator from Pm cannot in principle be ascribed to altered stability of the mutant proteins.

**Effector Profile of the Mutant Regulators**—Based on the above results we concentrated our efforts on the characterization of the single XylS137E and XylSH153N mutants and the double mutant XylS137E,H153N. We tested the effector profile of the mutant regulators with all possible mono- and di-substituted methylbenzoates. Fig. 1 shows the results for the wild-type XylS-pWW0. The order of strength of inducibility was 3MB > 2,3DMB > B > 3,4DMB > 2MB > 4MB > 2,5DMB > 3,5DMB = 2,6DMB, with the last two dimethylbenzoates being considered non-effectors for XylS. Fig. 1 shows the induction profile of the single mutants XylS137E and XylS153N and the double mutant XylS137E,H153N. The two single mutants recognized most of the effectors, but as with the benzoates substituted at position 3, all mutants exhibited lower levels of induction than the wild type. In contrast, the double mutant XylS137E,H153N seemed not to respond to 3,4DMB, 2MB, or 4MB and exhibited an effector profile similar, if not identical, to that of the XylS1-pWW53 regulator (Fig. 1).

**Characterization of a Collection of XylS Mutants at Positions 137 and 153**—To further confirm the crucial role of residues 137 and 153 of XylS in effector recognition and regulator activation, we introduced many mutations as possible by random overlapping mutagenesis at positions 137 and 153 in the XylS protein. We sequenced about 20 putative mutants for each position and found 5 new mutants at position 137 (aspartic acid was replaced by glutamic acid, proline, serine, arginine, and leucine) and two new mutants at position 153 (histidine was replaced by glycine and aspartic acid) (the mutant proteins were shown to be stable as deduced from the level of protein made by the cells and revealed by Western blot immunodetection of XylS proteins). We determined β-galactosidase expression from Pm mediated by each mutant in response to a series of substituted benzoates. Replacement of aspartic acid 137 with arginine or aspartic acid resulted in mutants that showed no activity (Table II). These results suggest that residues 137 and 153 influence the ability of XylS to activate transcription from Pm.

**Residues at the N Terminus of XylS Influence the σ Factor**

*Used by RNA Polymerase to Stimulate Transcription from Pm*—in Vivo Evidence for Transcription from Pm Mediated by RNA Polymerase with σ70 by XylS-pWW0 Mutant Proteins—Marquès et al. (16) showed that 3MB induces the heat shock response upon addition to cell cultures and that RNA polymerase uses σ32 to mediate the XylS1- and XylS-dependent expression from Pm. Once the cultures reached the mid-log growth phase, RNA polymerase uses σ32 to mediate transcription from Pm in the case of XylS and σ70 in the case of XylS1. We tested whether transcription mediated by XylS mutants from a Pm: lacZ fusion followed the pattern of induction of the wild-type regulator when induction was assayed in a σ32-deficient background. The results obtained confirmed that expression from Pm in the mid-log growth phase mediated by XylS1 is σ32-independent, whereas transcription stimulation from Pm with the XylS regulator is dependent on σ70 (not shown). In contrast, in all three mutants we tested, the dependence on σ38 was lost. To further confirm that the level of β-galactosidase activity reflected the transcriptional activity from Pm, we determined the level of expression from Pm in different backgrounds by using σ70 variants proficient and deficient in the synthesis of σ38. The parental strain of these isogenic strains is *E. coli* P90A5c. This strain bearing the XylS or XylS1 alleles is able to transcribe the Pm promoter in response to the addition of 3MB regardless of the incubation temperature, as expected (Fig. 2A). *E. coli* UQ285 has a σ70 variant that is thermosensitive at...
Fig. 2. In vivo transcription from Pm mediated by XylS, XylS1, or XylSD137E in a α70, and α38-proficient strain and in a α70, thermostensitive background proficient and deficient in α38 synthesis at different temperatures. E. coli P90A5e (α70+, α38+) (panel A), E. coli UQ285 (α70ts, α38+) (panel B), and E. coli EEZ286 (α70ts, α38−) (panel C) bearing pJLR107 (Pm::lacZ) and pLAR1(XylS) (lanes 1-3), pLAR18 (XylS1) (lanes 7-9), or pLAR5 (XylSD137E) (lanes 4-6) were grown until the late exponential phase in the presence of 3MB at 30 °C. When the turbidity was ~1.2, a sample was withdrawn for RNA extraction (lanes 1, 4, and 7), then each culture was divided into two halves. One was kept as a control at 30 °C (lanes 2, 5, and 8), and the other was incubated at 42 °C (lanes 3, 6, and 9), and after 45 min RNA was extracted. Primer extension and separation of cDNA was done as described under “Experimental Procedures.”

42 °C. Because expression from Pm with XylS seems to be dependent on α38 whereas with XylS1 it seems to depend on α70, one would expect transcription from Pm to occur at 30 °C in UQ285 and at 42 °C with XylS and at 30 °C but not at 42 °C with XylS1. We tested transcription from Pm in XylS- and XylS1-proficient backgrounds in the absence and in the presence of 3MB. In these genetic backgrounds, no transcription from Pm took place in the absence of the effector (not shown). In the presence of 3MB, transcription occurred at 30 °C with either XylS or XylS1 (Fig. 2B), but at 42 °C transcription from Pm was seen only in the XylS-proficient background (Fig. 2B) (as a control of the correct functioning of the UQ285 strain, note that transcription from the α70 tandem Pr1 and Pr2 promoters occurred at 30 °C but not at 42 °C (not shown)). We then tested transcription stimulation from Pm at 30 °C and 42 °C with XylSD137E and XylSH153N. The results obtained were similar with both mutants and are shown for XylSD137E in Fig. 2B. XylSD137E behaved like XylS1 rather than like XylS; we interpret this result to indicate that XylSD137E facilitated transcription from Pm with α70 rather than with α38 in the logarithmic growth phase.

To further confirm the switch in σ factors in RNA polymerase to transcribe Pm depending on the regulator used, we constructed E. coli EEZ286, which in addition to the α70ts mutation has an inactive α38 allele. In this mutant background, transcription from Pm did not occur in the absence of 3MB; however, in the presence of the effector and in cultures incubated at 30 °C, transcription from Pm took place in the XylS1 background but not in the XylS background (Fig. 2C). Mutants XylSD137E and XylSH153N, in contrast with XylS, were able to stimulate transcription from Pm, as shown for XylSD137E in Fig. 2C.

DISCUSSION

XylS and XylS1 are 98.44% identical and differ in only 5 of 321 residues, all of which are located in the N-terminal end. The availability of these two different alleles of the regulator is of great interest, because these “minor” differences are clearly reflected in the regulator ability to induce transcription from Pm with different effectors and in the interactions with the transcriptional machinery. With regard to the kinetic properties, Gallegos et al. (7) first reported that the induction rate from Pm in the XylS-pWW0 regulator was about 6–10-fold as high as that in XylS1 and that XylS-pWW0 regulator had a wider effector profile than XylS1-pWW0. The introduction of stepwise mutations in XylS-pWW0 revealed that the single mutations with the greatest effect on the degree of transcriptional activation and the effector profile were those that involved residues 137 or 153. This was further confirmed when the effector profile for effectors in the double mutant XylSD137E-H153N were found to be similar to those of the XylS1-pWW53 regulator. This indicated that these residues may be involved in effector interactions.

The importance of the involvement of residues 137 and 153 in interactions with effectors was confirmed when random mutations at these positions resulted in point mutants (i.e. XylSD137R, XylSD137P, XylSH153G, and XylSH153D) that had lost the ability to recognize effectors. Recently the MarR regulator, which recognizes 2-hydroxybenzoate (salicylate) as an effector, has been crystallized with the aromatic carboxylic acid (28). In this regulator salicylate is bound in two sites, SalA and SalB; in both sites the salicylate carboxylate hydrogen bond is hydrogen-bonded to the positively charged lateral chain of arginine residues. The salicylate hydroxyl group is hydrogen-bound to the hydroxyl chain of a threonine at the SalA site and to the backbone carbonyl of an alanine at the SalB site. This indicates that substituents on the aromatic ring can establish interactions with different residues. On the basis of these results it is tempting to speculate that in XylS, histidine 153 might be involved in interactions with substituents on the benzoate aromatic ring, because mutations in this residue to uncharged or negatively charged amino acids resulted in loss of the ability to recognize benzoate derivatives. This in turn may be due to loss of interactions with the carboxyl group. The change His-153 → Asn resulted in a less efficient regulator, but the amide lateral chain of glutamine may allow certain interactions with benzoate derivatives.

Given the negative character of aspartic acid 137, this residue cannot be involved in interactions with the carboxyl chain of benzoate. Instead, residue aspartic acid 137 may be involved in other interactions with substituents or in the intramolecular signal transmission chain that leads to activation of the regulator. In fact, altering the charge of this residue influenced the ability of the regulator to recognize benzoates.

Our previous study revealed cysteine 41 as another amino
acid that may be part of the XylS effector pocket. The phenotype of mutants XyIS137R and XyIS153G regarding the lost ability to recognize benzoates is similar to the one we found when cysteine 41 was replaced with leucine (12). However, at position 41, certain mutations resulted in other phenotypic changes. For example, replacement of cysteine 41 with arginine resulted in a mutant that exhibited a semiconstitutive phenotype (i.e. it mediated a high level of transcription from Pm in the absence of effectors). Mutant XyIS1341G showed altered effector specificity; it did not recognize 4MB but retained the ability to recognize 3MB (12). That different mutations in positions 41, 137, and 153 give rise to different phenotypes is consistent with the different roles of residues 41, 137, and 153 in interactions with effectors or signal transmission to achieve the active form of this transcriptional regulator. However, whether any of these residues interacts with the effectors is still unknown. Details of these interactions await resolution of the three-dimensional structure of this regulator with and without the effector.

The XylS protein has so far been difficult to purify because of the intrinsic insolubility of the protein, a characteristic shared by many proteins of the XylS/AraC family of regulators (29–31). However, we recently solubilized the N-terminal end of XylS upon fusion to MalE and are now trying to obtain further insights into the structural characteristics of the N-terminal end of XylS.2

Two members of the XylS family, SoxS and MarA, which are about 110 amino acid long, are equivalent to the C terminus of the XylS family of proteins. This coincidence suggests that the C-terminal end in members of the XylS/AraC family is involved in DNA binding and probably in interactions with the transcriptional machinery. A study by Michán et al. (31) shows that although the N and C termini of XylS have specific functions, they are not independent, and they cross-talk. Their relationship is further supported by the present finding that mutations at the N terminus influence which σ factor the RNA polymerase chooses to stimulate transcription from Pm. In fact, the Pm requires RNA polymerase with either σ70 or σ38. Further studies with this system are expected to reveal the mechanisms by which subtle changes in XylS can influence transcription from Pm to such a significant degree.

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