Mutations Leading to Constitutive Expression from the TOL Plasmid meta-Cleavage Pathway Operon Are Located at the C-Terminal End of the Positive Regulator Protein XylS

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The XylS protein is the positive activator of the TOL plasmid meta-cleavage pathway operon for the metabolism of alkylbenzoates in Pseudomonas putida. The regulator stimulates transcription from the TOL meta pathway operon promoter (Pm) when activated by benzoate effectors or in the absence of effectors when overproduced. xylS mutant alleles that encode regulators which constitutively mediate expression from Pm were isolated and characterized. The mutant proteins all exhibit single amino acid substitutions adjacent to putative α-helix-turn-α-helix domains at their C-terminal ends. The XylS mutant proteins can still be partially activated by the usual and unusual benzoate effectors for the wild-type regulator and when activated stimulate higher levels of transcription from Pm.

Extensive genetic and molecular analyses of procaryotic regulatory systems have identified a number of positive regulators that promote transcription initiation after binding effectors. Examples include the Escherichia coli araC and mald gene products (14, 18) and the Pseudomonas xylS gene product (16). The positive regulator is a DNA-binding protein for which target binding sites exist upstream of the RNA polymerase-binding site of the regulated operon. The activation of positive regulators after binding effectors may lead to a conformational change in the regulator and to the conversion of a form inactive in terms of its ability to stimulate transcription to another form able to stimulate transcription from the regulated promoter or of a form with low affinity for its target to one with high affinity.

The TOL plasmid pWWO of Pseudomonas putida specifies enzymes for the oxidative catabolism of toluene and xylene and benzoate and toluates. Degradation of the aromatic carboxylic acids takes place through a meta-cleavage pathway. Genes encoding the TOL meta-cleavage pathway are grouped into a single operon (review reference 6) whose expression is positively regulated at the level of transcription by the xylS gene product (4, 7, 17). Transcription from the meta operon promoter (called Pm for promoter meta pathway [6] or OP2 [7]) is stimulated by benzoates that are metabolized through the meta pathway or by benzoate analogs that are gratuitous effectors (17). Transcription from Pm is also stimulated in the absence of benzene effectors by the hyperproduction of XylS protein (8, 15). The isolation of XylS mutants with altered effector specificities has provided genetic evidence for the direct binding of benzoate analogs to XylS (16, 17). In this article, we describe XylS protein mutants that constitutively switch on the meta operon and which contain mutations at their C-terminal ends, adjacent to putative α-helix-turn-α-helix domains likely to be involved in DNA binding.

MATERIALS AND METHODS

Strains, plasmids, and growth media. Escherichia coli SK (resK thr leu thi tonA supE λ*), E. coli CC118 [araD139 Δ(ara leu)7697 lacX74 phoA20 galE gaK thi rpsE rpmB argE(Am) recA1], and P. putida KT2442 (hsdM hsdR7 RifR) were described before (1).

The following plasmids have been previously described. pWWO is the archetypal TOL plasmid and encodes catabolic pathways for the metabolism of toluene and xylene and for benzoate and toluates in P. putida (19). pERD100 is an IncP plasmid that contains a translational fusion of the TOL meta-cleavage pathway operon promoter (Pm) to a promoterless lacZ gene and encodes tetracycline resistance (16). pKT231 is an IncQ plasmid that encodes resistance to kanamycin and streptomycin (2). The following plasmids were constructed during this study. pERD103 was constructed by cloning a 1.5-kilobase Sau3A fragment of the TOL plasmid carrying the xylS gene into the unique BamHI site of pKT231 and encodes resistance to kanamycin and streptomycin. Plasmid pJLR200 contains a transcriptional fusion of Pm to a promoterless tetracycline gene in pBR322, with the Ω interposon (13) inserted just upstream from Pm to prevent reading of the tet gene from a vector promoter.

Bacteria were grown on LB medium (11). When required, antibiotics were added at the following concentrations (micrograms per milliliter): ampicillin, 100; kanamycin, 25; rifampin, 50; streptomycin, 50; and tetracycline, 10.

Isolation and sequencing of xylS mutant alleles. xylS alleles encoding mutant regulators that constitutively switch on expression from the TOL meta-cleavage pathway operon promoter (Pm) were isolated as follows. Three milliliters of an E. coli CC118(pERD103) culture containing about 10⁸ cells per ml was harvested (10,000 × g for 2 min), suspended in 1 ml phosphate buffer (100 mM, pH 6.8), and incubated with 30 μg of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) for 60 min at 30°C. Cells were then harvested as described above, washed twice with LB medium, and suspended in 3 ml of LB medium (11). After 24 h, plasmid DNA was isolated and used to transform E. coli(pJLR200Q). Clones expressing TcR were directly selected in plates containing 10 μg of tetracycline per ml, 25 μg of kanamycin per ml, and 100 μg of ampicillin per ml. The putative mutations in the xylS gene were cloned in pTZ19 (20) and sequenced by the dideoxy chain termination method, using synthetic oligonucleotides.
that expanded at intervals of 200 nucleotides within the xylS gene sequence (9).

**Other methods.** Isolation of plasmid DNA, restriction endonuclease digestion, cloning, transformation, and matings were performed as previously described (1). β-Galactosidase was determined in permeabilized cells (17). Units are expressed as described by Miller (11).

**RESULTS AND DISCUSSION**

In our attempts to understand the mechanism of positive regulation of the TOL plasmid *meta*-cleavage pathway for the metabolism of alkylbenzoates in *P. putida*, we previously isolated XylS regulator mutants with altered effector specificities (16, 17); in the present study we isolated mutants that constitutively mediate transcription from Pm.

To isolate XylS mutants able to mediate constitutive transcription from Pm we used a modification of the strategy previously described to isolate XylS mutants with altered effector specificities (16, 17). Briefly, *E. coli* bearing the plasmid pERD103, which carries the xylS wild-type allele, was mutagenized with NTG. After mutagenesis, plasmid DNA was isolated and used to transform *E. coli* (pJLR200f). The latter strain is sensitive to tetracycline because the transcription of the tetracycline-resistant determinant in pJLR200Ω is under the control of the positively regulated Pm promoter. *E. coli* (pJLR200f) cells transformed with mutagenized pERD103 were directly spread on LB plates supplemented with ampicillin (the marker for pJLR200f), kanamycin (the marker for the mutagenized pERD103 plasmid), and tetracycline (to select clones expressing the tetracycline resistance determinant from the Pm promoter in pJLR200Ω). A single Ap' Km' Tc' clone from each mutagenesis was randomly chosen. Ten independent clones were maintained for further characterization. The pERD103-derived plasmid was then recovered from the transformant and used again to transform *E. coli* (pJLR200Ω) to confirm that the presence of the plasmid confers the tetracycline resistance phenotype. This was always the case, thus confirming that the expression of Tc' from pJLR200Ω was due to the presence of the compatible plasmid. The constitutive expression of the Pm promoter in pJLR200Ω in the presence of mutagenized pERD103 may have been due to the following: (i) the presence of xylS alleles which encode regulators that do not require effectors to stimulate transcription from Pm, (ii) XylS mutant proteins that exhibit increased stability and accumulate in the cell, (iii) mutations in the xylS gene promoter (Ps) that lead to hyperproduction of the XylS protein, (iv) mutations in the vector DNA that result in the reading of the xylS gene from vector promoters, and (v) mutations that lead to an increase in vector copy number. To rule out possible PsⅣ and PsⅤ, we cloned the 1.5-kilobase *BamHI* fragment from mutagenized pERD103 into the unique *BamHI* site of pKT231 and transformed it into *E. coli* (pJLR200Ω). All of the independently selected mutants conferred resistance to tetracycline, suggesting that the mutations lie in the xylS gene.

The xylS mutant alleles were sequenced. In half of the mutants a single mutation, consisting of a C→T change at position -56 with respect to the main transcription initiation point (8, 15), was found in the xylS promoter region. The other five mutants also exhibited single point mutations, but these were located in the coding region and most probably led to single amino acid substitutions at the protein level. Three mutants exhibited an A→T change in codon 274 (GAT→GTT), which should result in an Asp→Val change in the polypeptide chain. One mutant contained a single T→G change in codon 274 (GAT→GAG), resulting in an Asp→Glu change. Finally, one mutant contained a single G→T mutation in codon 229 (AGC→ATC), which should result in a Ser→Ile change. The three mutant types containing changes in the xylS coding sequence were designated mutant Val274, mutant Glu274, and mutant Ile229, respectively.

To determine the level of transcription from the Pm promoter mediated by mutant XylS proteins in the presence and absence of benzoate inducers, β-galactosidase levels were measured in *E. coli* bearing a translational fusion of Pm to the lacZ gene in plasmid pERD100 (16) and the mutant XylS allele in the compatible plasmid pKT231. The basal expressions (expression in the absence of benzoate inducers) from Pm with the XylS mutant Ile229 and mutant Val274 and mutant Glu274 were about 7-, 7-, and 15-fold higher, respectively, than with the wild-type XylS protein (Table 1).

### Table 1. Transcription from the TOL plasmid *meta*-cleavage pathway operon promoter (Pm) by the wild-type and mutant XylS regulators in the absence and presence of benzoates

<table>
<thead>
<tr>
<th>Effector</th>
<th>Wild type</th>
<th>Mutant Ile229</th>
<th>Mutant Glu274</th>
<th>Mutant Val274</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galactosidase (U)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>254</td>
<td>1,540</td>
<td>3,843</td>
<td>1,548</td>
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<tr>
<td>Benzoate</td>
<td>3,120</td>
<td>4,385</td>
<td>9,926</td>
<td>4,725</td>
</tr>
<tr>
<td>2-MB</td>
<td>1,802</td>
<td>7,678</td>
<td>5,545</td>
<td>4,469</td>
</tr>
<tr>
<td>3-MB</td>
<td>3,833</td>
<td>6,730</td>
<td>9,150</td>
<td>5,633</td>
</tr>
<tr>
<td>4-MB</td>
<td>974</td>
<td>8,174</td>
<td>10,159</td>
<td>4,775</td>
</tr>
<tr>
<td>2.5-DMB</td>
<td>277</td>
<td>5,852</td>
<td>8,380</td>
<td>3,343</td>
</tr>
<tr>
<td>2.5-DMB</td>
<td>353</td>
<td>7,056</td>
<td>8,555</td>
<td>5,154</td>
</tr>
<tr>
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<tr>
<td>3.5-DMB</td>
<td>196</td>
<td>4,063</td>
<td>5,480</td>
<td>1,461</td>
</tr>
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</table>

* E. coli (pERD100, pKT231::xylS or pKT231::xylS mutant alleles encoding the indicated mutant proteins) were grown overnight on LB medium supplemented with tetracycline and kanamycin. Cultures were then diluted 100-fold in the same medium with and without the indicated aromatics (1 mM). After 5 to 6 h β-galactosidase was measured. β-Galactosidase units are given as described by Miller (11). The values are averages of three to six independent determinations, with standard deviations in the range of 5 to 20% of the given values.

* Abbreviations: MB, methylbenzoate; DMB, dimethylbenzoate.
We have identified the XylS protein as a member of a family of positive regulators, which includes several regulators involved in carbon metabolism in E. coli (AraC, MelI, RhaS, and RhaR) and Erwinia spp. (AraC) and in pathogenesis in E. coli (Rns) and Yersinia spp. (VirF) (J. L. Ramos, F. Rojo, L. Zhou, and K. N. Timmis, Nucleic Acids Res., in press). These proteins exhibit greater homology in their C-terminal regions than in their N-terminal ends. On the basis of the properties of mutant E. coli AraC proteins and Pseudomonas XylS regulators, we have suggested that the nonconserved N-terminal end is specifically involved in effector binding whereas the C-terminal end is involved in DNA binding. The location of three mutations at the C-terminal end of the XylS protein that result in constitutive expression from the Pm promoter further support the notion that this region is involved in interactions with DNA. Furthermore, the fact that the mutant XylS proteins stimulated higher transcription levels from Pm when activated by benzoate effectors that did not activate the wild-type XylS protein suggests that an alteration in one domain affects interactions in the other. In this context it is worth noting that we previously isolated other XylS mutants that exhibited both altered effector specificities and altered transcriptional levels: mutant Arg256 and mutant Val288 are activated by 3,5-dichloro- and 4-methoxybenzoate, respectively, and exhibit a reduced induction capacity with some effectors, in contrast to the three mutants described here (16, 17). Mutant Leu88, although isolated on the basis of its activation by 2,4-dichlorobenzoate, stimulates high basal levels of transcription from Pm, i.e., in the absence of effectors (17). We suggested that the altered residue of this mutant protein and its surrounding amino acids were involved in signal transmission from the effector-binding pocket to the DNA-binding domain. Moreover, a mutant protein isolated as able to recognize 3-methoxybenzoate (mutant Arg41) leads to extremely high levels of transcription when hyperproduced, which indicates that residue 41 may also be involved in the transmission of a signal from the effector-binding pocket to the DNA-binding domain (16). These findings suggest that in the XylS protein, reciprocal interactions exist between the effector-binding pocket, the signal transmission region, and DNA-binding domains, since the mutational alterations of one functional domain may affect the functioning of another domain.

Two regions at the C terminus of the XylS protein, homologous to sequences found in other regulators and organized in α-helix-turn-α-helix domains (Fig. 1), were located by Mermod et al. (9). One of these domains, consisting of amino acids 233 to 253, is homologous to the α-helix-turn-α-helix domain of the lambda Cro protein and other regulators (12). In the best alignment of the E. coli AraC and XylS proteins, this region of XylS aligns well with the proposed DNA-binding domain of the AraC protein, which is probably formed by the stretch of amino acids between residues 196 and 215, with residues 208 and 212 apparently contacting regulatory ara DNA sequences (3). The other, less well conserved, domain, comprising amino acids 282 to 302, is found in the XylS family of regulators (Ramos et al., in press), sigma factors, and proteins that interact with DNA, such as the E. coli NusA protein (10). Interestingly, all three mutations in the XylS regulator which lead to constitutive expression from Pm are located adjacent to but outside these two domains. The substitution of Ile for mutant Ile229 has only an apparent effect on the local hydrophobicity of the region, but no apparent change in secondary structure was found in analyses performed with the algorithm of Garnier et al. (5). The Asp→Glu substitution at residue 274 has no effect on hydrophobicity, whereas the Asp→Val substitution at this point increases the hydrophobicity of the region. Analyses of the secondary structure of XylS with the algorithm of Garnier et al. (5) suggest that the replacement of Asp-274 by Glu or Val results in subtle changes in the predicted secondary structure; we presume that these subtle changes are responsible for the constitutive phenotype of the XylS mutants. Whether these changes affect the affinity of the XylS mutant proteins for their binding sites near the Pm promoter, or the oligomerization of XylS, or whether they have some other function remains to be determined by further genetic and biochemical analyses of Pm-XylS interactions.

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LITERATURE CITED