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The organization of the *Pm* promoter of the TOL plasmid reflects the structure of its cognate activator protein XylS

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Abstract The toluate catabolic operon carried by the TOL plasmid pWW0 of *Pseudomonas putida* is positively regulated by the benzoate-responsive XylS protein which, when activated, stimulates transcription from the operon promoter *Pm*. In this study, the mode in which XylS effects the activity of the *Pm* promoter was examined in vivo by genetic analysis of both protein and promoter variants. Substitution of His31Asp/Ser32Pro,Leu113Pro,Phe214Leu/Glu215Asp/Arg216Pro or Thr312Pro, all predicted to disrupt the secondary structure, renders XylS inactive and unable to compete with the native protein for activation of *Pm*. In contrast, Pro substitutions at Ser64 or Gly160 had no or only minor effects on XylS activity, and a protein with residual capacity to activate *Pm* resulted when Glu11 was altered to Pro. None of a collection of truncated variants of XylS protein deleted for more than 10 terminal amino acids retained activity or were able to negatively interact the wild-type XylS protein. These data indicate that amino- and carboxy-terminal portions of the protein sequence depend on each other for the maintenance of their functional structure, rather than being arranged as independent domains. *Pm* promoter derivatives, in which the two half-sites of the direct repeat sequence that confers responsiveness to XylS were separated and/or inverted by one-half or one complete DNA helix turn, lost responsiveness in vivo. These results favour a model for XylS-mediated activation of *Pm* in which a tandem dimer is the protein form that binds the *Pm* promoter.

Key words TOL plasmid · XylS Domain interactions Directed repeats

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Introduction

Binding of regulatory proteins to specific DNA sequences is a critical step in transcriptional regulation of gene expression (Collado-Vides et al. 1991). With a few exceptions, bacterial promoters subject to positive or negative control contain palindromic or quasi-palindromic DNA sequences, which are located upstream of the transcription initiation site and bind cognate protein dimers or tetramers. In most cases, the regulators recognize such sequences around a pseudo-symmetry axis (Pabo and Sauer 1984; Schleif 1988; Travers 1989). However, there are examples known of prokaryotic promoters in which the *cis*-acting sequences that confer responsiveness to transcriptional regulators are not arranged as inverted repeats but rather as direct repeats (Brunelle and Schleif 1989; Meada and Mizuno 1990; Raibaud 1989).

The *Pm* promoter of the TOL (toluene degradation) plasmid pWW0 of *Pseudomonas putida* drives the expression of the *meta* operon of the system in response to benzoate effectors (Inouye et al. 1984, 1987a; Ramos et al. 1987). Extensive genetic evidence indicates that transcriptional activation occurs through the binding of the regulatory protein XylS (36 KDa, 321 amino acids) to a directly repeated 15 bp sequence (*Om*), which overlaps by a few bases with the −35 region of the RNA polymerase binding site (Fig. 1). Since the 15 bp *Om* half-sites are separated by 6 bp, equivalent bases are separated by 21 bp (i.e. two helix turns) and therefore face the same side of the DNA helix (Kessler et al. 1993). In this paper, we examine the effect on promoter activity of altering *Pm* geometry and producing XylS truncated proteins spanning different portions of the polypeptide. Our results support the notion that XylS binds to *Om* through the formation of a tandem dimer.

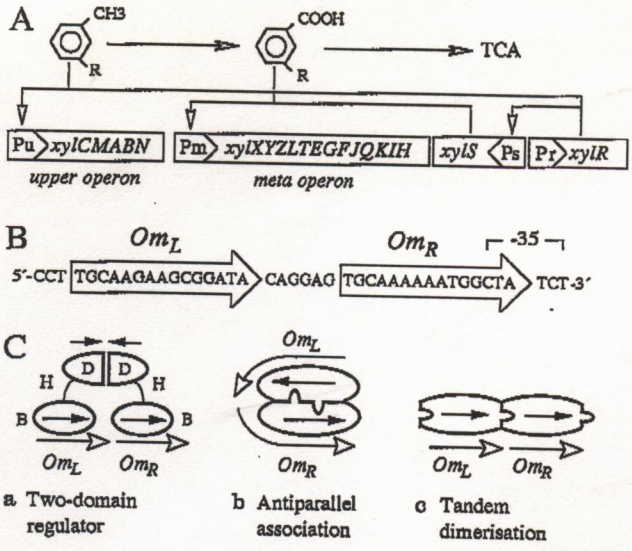

Fig. 1A–C The *Pm*-XylS regulatory circuit and models for *Pm* activation. **A** Regulation of *Pm* by XylS in the TOL plasmid. In the presence of TOL pathway substrates (i.e. *m*-xylene), *Pu* and *Ps* promoters are activated by XylR. Subsequently, an excess of XylS protein or XylS bound to its effectors (i.e., 3-methyl benzoate, 3MB) activates *Pm*, the final result of TOL activities being the mineralization of the aromatic substrates down to citric acid cycle intermediates (TCA). **B** Organization of the XylS binding sites at the *Pm* promoter of TOL plasmid. The *Pm* promoter contains two direct repeats (*Om* region), which include a left half-site (Om_L) and a right half-site (Om_R) required for XylS-mediated responsiveness to substituted benzoates (Kessler et al. 1993). *Om* overlaps the −35 hexamer of the promoter, so that bound XylS presumably interacts with RNA polymerase to initiate transcription into the first structural gene of the *meta*-operon, *xylX*. **C** Models for recognition of direct repeats of DNA by regulatory proteins. The three possible ways in which DNA-binding proteins may interact with direct repeats are summarized in the drawing. The only way to make the presence of a single dimerization/oligomerization domain (D) in the protein compatible with the recognition of a direct repeat is to assume that the single dimerisation domain may rotate through a highly flexible hinge (H) domain, as shown in model **a**, so that binding of the DNA-binding domain (B) occurs assymetrically. In contrast, if the protein does not have a flexible structure separating the domains, recognition of the other direct repeat requires that the protein have two distinct dimerization sites. Depending on the arrangement of such sites, the two monomers may associate in an anti-parallel fashion, as shown in model **b**, or as tandem dimers (model **c**). See text for further discussion

Materials and methods

Strains, media and general techniques

Relevant strains and plasmids used in this work are listed in Table 1. Solid and liquid LB media (Sambrook et al. 1989) were supplemented, when required, with 200 μg/ml ampicillin (Ap), 50–100 μg/ml streptomycin (Sm), 50 μg/ml rifampicin (Rif), 50–100 μg/ml kanamycin (Km), 7 μg/ml tetracycline (Tc) or 30 μg/ml chloramphenicol (Cm). Recombinant DNA methods were carried out according to published protocols (Sambrook et al. 1989). Pre-determined base changes were introduced into the *Pm* promoter cloned in pBK17 (Kessler et al. 1992) either through site-directed mutagenesis (Kunkel et al. 1987) or insertion of synthetic DNA linkers, and were confirmed by DNA sequencing. β-Galactosidase (β-Gal) levels were determined in cells permeabilized with chloroform and sodium dodecyl sulphate (SDS) according to Miller (1972).

Generation of mutant and truncated XylS derivatives

Vectors pBKT7-0 and pBKT7-1 (Fig. 2) were purposely designed for obtaining collections of XylS mutants and truncated proteins as convenient for further analysis in vivo. Both vectors contain origins of replication from pBR322 plasmid and M13 phage (from pCG2; Myers et al. 1985), a T7 polymerase promoter sequence (Tabor and Richardson 1985); and a *bla* gene as selection determinant. They differ, however, in the nucleotide sequence of the short polylinker downstream of the T7 promoter, which in the case of pBKT7-0 permits the synthesis of truncated XylS polypeptides (see legend to Fig. 2 for explanation). The coding sequence of *xylS* was cloned downstream of the T7 promoter in pBKT7-1 as an *Eco*RI-*Hin*dIII fragment from pVLT43 (de Lorenzo et al. 1993) to provide *xylS* with the translation initiation region (TIR) of the *ner* gene of Mu phage (Zaballos et al. 1987), thereby improving its translation efficiency. The resulting plasmid, pBKT7-1-XylS, was used as the template to generate single-stranded DNA for site-directed mutagenesis (Kunkel et al. 1987).

A subset of mutations introduced throughout the polylinker sequence produced *Bam*HI sites. These new sites were placed within the reading frame of the *xylS* structural gene such that they resulted in all cases in one Pro substitution and, occasionally, also in other changes in the 1-2 preceding amino acids (see the Results). These same *Bam*HI sites were used to produce truncated derivatives of *xylS*:*Eco*RI-*Bam*HI or *Bam*HI-*Hin*dIII restriction fragments from mutated pBKT7-1 XylS, spanning 5′- or 3′- terminal sequences of the *xylS* gene, respectively, were subcloned in equivalent sites of pBKT7-0 to allow synthesis of the corresponding truncated proteins under the control of the T7 promoter of the vector. Expression in vivo of the XylS mutant proteins and truncated polypeptides was confirmed by in vivo radioactive labelling of polypeptides obtained by T7 promoter-directed protein synthesis, as previously described (Tabor and Richardson 1985).

Construction and chromosomal integration of *Pm-lacZ* (*xylX′-′lacZ*) fusions

The *xylX′-′lacZ* reporter gene used to monitor *Pm* activity in vivo specifies a fusion protein encoding the 7 N-terminal amino acid residues of XylX, the product of the first gene of the TOL *meta*-operon and the *lacZ* gene (Harayama et al. 1991). For integration of *lacZ* fusions into the chromosome of *P. putida*, we used a transposon-based procedure described in Kessler et al. (1992). For this purpose, restriction fragments carrying wild-type (wt) or mutant *Pm* promoters were cloned in vector pBK17 and introduced by conjugation into a *P. putida* strain containing a mini-transposon in the chromosome, which provides homology to the sequences flanking the insertion site in the *lacZ* promoter probe vector pBK17 (Kessler et al. 1992). Double recombination of plasmid and transposon sequences results in the generation of a chromosomal *lacZ* fusion, which bears vector markers for selection (Kessler et al. 1992). The *Pm* mutations carried by the various pBK17 derivatives, which were finally integrated into the chromosome of *P. putida* KT2442 *hom.fg./xylRS*, are specified in Table 2. The *xylS* gene in these strains is carried by a second transposon inserted into the chromosome of *P. putida* and is expressed from its own XylR-dependent promoter *Ps* (Inouye et al. 1987b).

Activity and domain competition assays in vivo

To analyse the activity of the truncated XylS proteins described in this work (Figs. 3 and 4) and their ability to compete with wild-type

Table 1 Strains, plasmids and transposons (*Cm* Chloramphenicol, *Tc* tetracycline, *Km* kanamycin, *Sm* Streptomycin, *Sp* spectinomycin, *Ap* ampicillin, *wt* wild type)

	Genotype/phenotype/relevant characteristics	Reference
Escherichia coli strains:		
SM10 *λpir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu, Km^r, *λpir*	Simon et al. 1983
CC118	*Δ(ara-leu) Δlac galE galK phoA thi-1 rpsE rpoB argE*(am)*recA1*	Manoil and Beckwith 1984
CC118 *λpir*	CC118 lysogenized with *λpir* phage	Herrero et al. 1990
CC118 *supF*	CC118, mini-Tn*5*::*supF*, Cm^r	Kessler et al. 1992
CC118 *Pm-lacZ*	CC118, mini-Tn*5*::*Pm-lacZ*, Tc^r	This work
CC118 *Pm-lacZ/Pbla-xylS*	CC118, mini-Tn*5*::*Pm-lacZ* mini-Tn*5*::*Pbla-xylS*, Tc^r, Cm^r	This work
CC118 *Pm-lacZ/supF*	CC118, mini-Tn*5*::*Pm-lacZ* mini-Tn*5*::*supF*, Tc^r, Cm^r	This work
K38	*HfrC*, λ^+	Russel and Model 1984
Pseudomonas putida strains:		
KT2442 *hom.fg./xylRS*[a]	Prototrophic, Rif^r, Tn*10*::*hom.fg.*, mini-Tn*5*::*xylS/xylR*, Km^r, Hg^r	Kessler et al. 1992
KT2442 *PmΔ14/xylRS*	KT2442 *hom.fg./xylRS* but Km^s, Sm/Sp^r, *PmΔ14-lacZ* insert	Kessler et al. 1993
KT2442 *PmΔ16/xylRS*	KT2442 *hom.fg./xylRS* but Km^s, Sm/Sp^r, *PmΔ16-lacZ* insert	Kessler et al. 1993
KT2442 *Pm10/xylRS*	KT2442 *hom.fg./xylRS* but Km^s, Sm/Sp^r, *Pm10-lacZ* insert	This work
KT2442 *Pm11/xylRS*	KT2442 *hom.fg./xylRS* but Km^s, Sm/Sp^r, *Pm11-lacZ* insert	This work
KT2442 *PmL/xylRS*	KT2442 *hom.fg./xylRS* but Km^s, Sm/Sp^r, *PmL-lacZ* insert	Kessler et al. 1993
KT2442 *Pm invI/xylRS*	KT2442 *hom.fg./xylRS* but Km^s, Sm/Sp^r, *Pm invI-lacZ* insert	This study
KT2442 *Pm invII/xylRS*	KT2442 *hom.fg./xylRS* but Km^s, Sm/Sp^r, *Pm invII-lacZ* insert	This study
Plasmids:		
pBK17	Sm/Sp^r (am), *lacZ* (am) promoter probe vector	Kessler et al. 1992
pBK17-Δ14	pBK17 with *PmΔ14-lacZ* (am) fusion, wt *Pm* (Table 2)	Kessler et al. 1993
pBK17-Δ16	pBK17 with *Pm-lacZ* (am) fusion, *Pm* deletion *Δ16* (Table 2)	Kessler et al. 1993
pBK17-PmL	pBK17 with *PmL-lacZ* (am) fusion, variant *PmL* (Table 2)	Kessler et al. 1993
pUJ9	Ap^r, *lacZ* promoter probe vector	de Lorenzo et al. 1990
pUT/mini-Tn*5* Tc	Ap^r, Tc^r, mini-Tn*5* Tc delivery vector	de Lorenzo et al. 1990
pUT/mini-Tn*5* Cm	Ap^r, Cm^r, mini-Tn*5* Cm delivery vector	de Lorenzo et al. 1990
pNM185	Km^r, expression vector, source of *xylS* and *Pm* sequences	Mermod et al. 1986
pBKT7-1	Ap^r, T7 promoter expression vector (Fig. 2)	This work
pBKT7-0	Ap^r, T7-based expresssion vector for truncated proteins (Fig. 2)	This work
pBKT7-0-XylS	pBKT7-0 inserted with wt *xylS* gene as a 1.6 kb *Eco*RI-*Hin*dIII fragment from pVLT43 (de Lorenzo et al. 1993)	This work
pBKT7-1-XylS	pBKT7-1 inserted with wt *xylS* gene as a 1.6 kb *Eco*RI-*Hin*dIII fragment from pVLT43 (de Lorenzo et al. 1993)	This work
pGP1-2	Km^r, P_L-T7 RNApol, $cI857^+$	Tabor and Richardson 1985
Transposons:		
mini-Tn*5* *Pm-lacZ*	Tc^r, hybrid mini-Tn*5* Tc containing *Pm-lacZ* fusion	M. Herrero
mini-Tn*5* *Pbla-xylS*	Cm^r, hybrid mini-Tn*5* Cm containing *xylS* gene expressed through weak promoter *Pbla*	This work
mini-Tn*5* *supF*	Cm^r, hybrid mini-Tn*5* Cm containing $supF^+$ sequence	Kessler et al. 1992

[a] *hom.fg.* designates the presence in the chromosome of a region of homology to sequences flanking the insertion site in pBK17, thus permiting integration of plasmid inserts

XylS in vivo, we constructed two *recA E. coli* strains containing, in the chromosome, the specialized transposons mini-Tn*5* *Pm-lacZ* and mini-Tn*5* *Pbla-xylS*. To construct mini-Tn*5* *Pm-lacZ*, a 150 bp *Eco*RI-*Bam*HI restriction fragment containing *Pm* (Kessler et al. 1993) was cloned into the *lacZ* vector pUJ9 (de Lorenzo et al. 1990) and the resulting *Pm-xylX'-'lacZ* fusion was excised as a 4 kb *Not*I fragment and cloned in the transposon delivery plasmid pUT/mini-Tn*5* Tc (de Lorenzo et al. 1990). The *Pm-lacZ* containing transposon is organized such that the fusion is transcriptionally shielded upstream by the Tc-Ω element (Fellay et al. 1987) and downstream by a strong T7 terminator. This affords expression of the reporter gene virtually independently of the site of insertion. Transposon mini-Tn*5* *Pbla-xylS* was made by cloning in the vector pUC18Not (Herrero et al. 1990) a 1.5 kb *Bam*HI fragment containing the *xylS* gene of pNM185 (Mermod et al. 1986) downstream of a synthetic *bla* promoter (Su et al. 1992) cloned as a 78 bp *Eco*RI-*Bam*HI insert in the same vector. The unit was then excised as a 1.6 kb *Not*I fragment an cloned at the *Not*I site of pUT/mini-Tn*5* Cm (de Lorenzo et al. 1990) to form mini-Tn*5* *Pbla-xylS*. In this construction, expression of the regulator gene results from residual transcription from the *bla* promoter (Su et al. 1992) through the *Pr/Ps* divergent promoter region (Inouye et al. 1987b) into the *xylS* sequence, and it is therefore very weak.

To construct specialized strains to carry out activity and competition assays of XylS protein variants, the mini-transposons were inserted into the chromosome of *E. coli* CC118 or CC118 *supF* as described previously (Kessler et al. 1992). The resulting strains *E. coli* CC118 *Pm-lacZ*, *E.coli* CC118 *Pm-lacZ/Pbla-xylS* and *E.coli* CC118 *Pm-lacZ/supF* were subsequently transformed with pBKT7-derivatives, producing XylS variants. Since all the elements of the regulatory pathway that controls the expression of *Pm* in *P. putida* can be reproduced in *E. coli* (Inouye et al. 1987a; Michan et al.

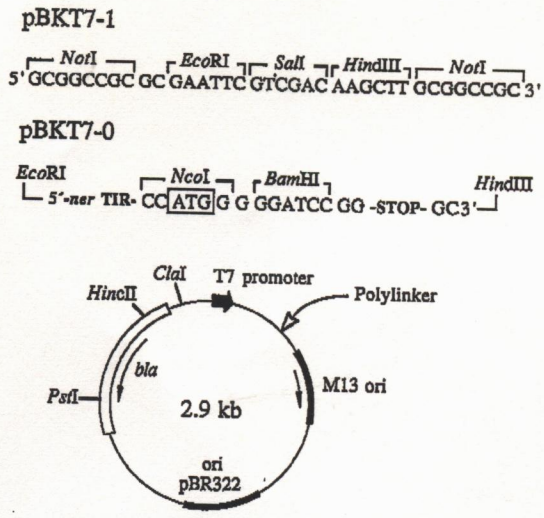

Fig. 2 Organization of pBKT7-1 and pBKT7-0 plasmid vectors used to generate mutant and truncated XylS proteins. The two vectors share the basic plasmid structure shown at the bottom of the Figure, which is a hybrid between plasmids pCG2 (Myers et al. 1985) and pT7-5 (Tabor and Richardson 1985). From pCG2, the two vectors inherit the *bla* gene (ampicillin resistance) and vegetative origins of replication of pBR322 and M13 phage, thus allowing isolation of the plasmids and their derivatives in both double- and single-stranded DNA forms. From pT7-5, they contain the promoter sequence recognized by the T7 phage RNA polymerase, which permits the specific radioactive labelling of proteins encoded by the DNA sequences cloned downstream of the T7 promoter (Tabor and Richardson 1985). As indicated on top of the Figure, the two vectors differ in the restriction sites in, and the organization of, their polylinkers. pBKT7-1 has a short sequence from p18Not (Herrero et al. 1990), which includes unique sites for *Eco*RI, *Sal*I and *Hin*dIII, flanked by *Not*I sites. pBKT7-0 has instead the very efficient TIR of the *ner* gene of Mu phage adapted to contain a single *Nco*I site, so that the ATG included in the recognition site for the endonuclease (framed in the figure) becomes the first codon of in-frame structural sequences. Furthermore, the polylinker of pBKT7-0 includes stop codons TGA derived from plasmid pAZe3ss (Zaballos et al. 1987) located, in the three possible reading frames, between the *Bam*HI and *Hin*dIII sites. Such an arrangement of sites permits the generation in vivo of N- or C-terminal truncated proteins by cloning the corresponding sequences as *Eco*RI-*Bam*HI (C-deletions) or *Bam*HI-*Hin*dIII (N-deletions) restriction fragments

1992a; Ramos et al. 1987, 1990b), the phenotypes of the XylS derivatives in the different assays were determined through measurement of the levels of β-Gal produced in each strain.

Results

Pm promoters with displaced and/or inverted *Om* half-sites are not responsive to XylS

To gain some insight into the mechanism by which XylS activates the *Pm* promoter, we examined the in vivo activity of a series of analogous *Pm-lacZ* fusions, varying only in the relative arrangement of the *Om* half-sites (Om_L and Om_R). For this purpose, we constructed isogenic *P. putida* strains carrying a single copy of the *xylS* gene and a collection of *Pm-lacZ* fusions with the *Om* sequence variants. This ensured that all the regulatory elements that control expression of the *meta*-operon from *Pm*, were present in near-native concentrations and stoichometry. The different strains were grown and induced with the XylS/*Pm* effector 3-methyl benzoate (3MB) and their β-Gal levels were measured as described in Materials and methods; results obtained are presented in Table 2. A partial offset (and minor separation) of the two *Om* half-sites, as is the case in the strain carrying *Pm* derivative *Pm10*, resulted in a sharp decrease of β-Gal levels in induced cells to 10% of the level exhibited by cells carrying the wild-type (wt) promoter *Pm Δ14*, while the basal, uninduced level was the same in both cases. This level of β-Gal in induced cells is in the range of that exhibited by cells carrying *Pm Δ16*, which is deleted for Om_L and retains only the proximal half-site Om_R. When the distance between Om_L and Om_R was increased by 10 bp, as in *Pm11*, which further separates the two half-sites while restoring their original phasing, all XylS-dependent regulation of *Pm* is lost and *lacZ* is expressed constitutively at an intermediate level. This probably results from the activation of a cryptic promoter rather than a change in transcription from *Pm* itself (Kessler et al. 1993).

Data on the inversion of the two *Om* half-sites are also shown in Table 2. To eliminate one source of variability, we used a derivative in which both half-sites have the sequence of the Om_L site. We previously showed that a *Pm* promoter derivative carrying two L sites, *PmL*, is not only active but is more efficiently regulated than the native promoter (Kessler et al. 1993). As indicated in Table 2, unlike *PmL*, promoter derivatives in which one of the two half-sites has been inverted are completely inactive, regardless of whether or not equivalent DNA surfaces are placed on the same (*Pm invII*) or on opposite helix sides (*Pm invI*). These data indicate that proper phasing, orientation and spacing of the directly repeated sequence is essential for XylS-dependent activity of *Pm*.

Permissive and non-permissive amino acid changes in *XylS* sequence

We wished to probe for the presence of permissive, potentially flexible domains in the XylS protein. For this purpose we generated eight site-directed mutations through the substitution of residues located in or nearby regions, previously identified (Ramos et al. 1990b; Zhou et al. 1990) as being critical for the different functions of the protein (namely, DNA binding, effector recognition and interactions with the polymerase; see Fig. 2 and Michan et al. 1992a), by Pro or Lys, accompanied in some cases by additional changes of one or two of the preceding amino acids (Fig. 3). Since the introduction of Pro residues within polypeptide secondary

Table 2 Responsiveness to activated XylS of mutant *Pm* promoters containing different arrangements of *Om* half-sites. (3MB 3-Methyl benzoate, 3MBA 3-methylbenzyl alcohol)

Om variants in Pm-lacZ fusions[a]		Uninduced[b]	+ 3MB/3MBA
PmΔ14 (wt)	L→ CAGGAG →R	22	2340
PmΔ16	AG →R	20	115
Pm10	L→ CACTAGTAG →R	22	320
Pm11	L→ CACTAGGGTACCCTAG →R	343	550
PmL	L→ CAGGAG →L	16	2780
Pm invI	L← CAGGAG →L	20	60
Pm invII	←L CAGGATCCGAG →L	2	42

[a] *Pm* derivatives containing diverse orientations of the *Om* half-sites were made either by site-directed mutagenesis in plasmid pBK17-Δ14 (Table 1) or by reconstructing the whole region with synthetic linkers adapted to restriction sites available in pBK17-Δ14. The heads on the arrows indicate the orientation relative to the DNA helix face and the direction of the equivalent L (5′-TGCAAGAAG-CGGATA-3′) and R (5′-TGCAAAAAATGGCTA-3′) half-*Om* sequences. Promoter variants with inverted half-sites (*Pm invI* and *Pm invII*) were made with the two half-*Om* elements adjusted to the L sequence, which seems to bind XylS with higher affinity than the R counterpart (Kessler et al. 1993). To place the distal Om_L element in the opposite direction from that of the proximal site in *Pm invI* and *Pm invII*, we synthesized a linker including the cognate inverted L sequence 5′-TATCCGCTTCTTGCA-3′ upstream of the proximal site as indicated. Each of the resulting pBK17 derivatives was recombined into the chromosome of *P. putida* KT2442 *hom.fg./xylRS* to generate an isogenic collection of strains each harbouring a single copy of a *xylX′-′lacZ* (*Pm-lacZ*) reporter downstream of each of the *Pm* variants indicated in the Table, together with the *xylR/xylS* genes, thus reproducing all the elements that control regulation of *Pm* in the TOL plasmid (Kessler et al. 1993). The boundaries of the *xylX′-′lacZ* fusions are the same in all cases

[b] To determine the XylS-mediated responsiveness of the *Pm* promoter variants shown in the Table, overnight cultures of each of the *P. putida* strains (Table 1) were diluted 1:200 and grown in LB medium at 30° C to OD_{600} of 0.4, after which they were induced with 4 mM 3MBA, an inducer of XylR, which activates expression of *xylS*, and 2 mM of the XylS effector 3MB. Cultures were grown for a further 5 h and their β-Gal levels determined. Values expressed in Miller units (Miller 1972) are the average of three different experiments

structures uses to be disruptive (Chou and Fasman 1978), we reasoned that Pro insertions at sites involved in the maintenance of domain structure would impair the activating phenotype of XylS, whereas changes in flexible regions connecting potential domains would not. We therefore constructed a series of pBKT7-1-XylS derivative plasmids encoding the mutant XylS varieties shown in Fig. 3. Production in vivo of the corresponding

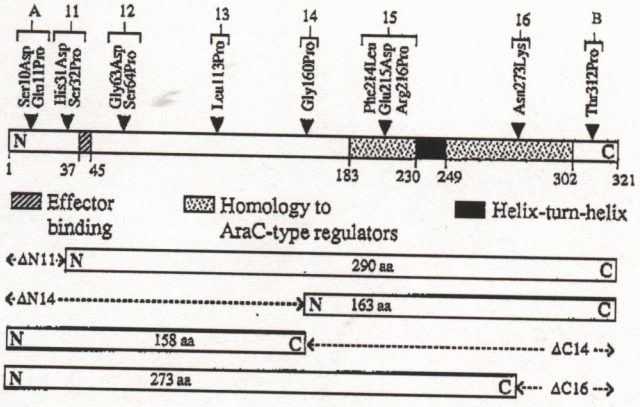

Fig. 3 Mutant and deletion derivatives of XylS used in activity and competition assays. The *long horizontal box* represents the whole XylS protein (321 amino acids) with its amino (N) and carboxyl (C) termini, the boundaries of regions within the primary sequence involved in distinct functions (Michan et al. 1992a) and the location of the changes introduced in the different mutants are indicated. The *numbers* or *letters* on top refer to the serial numbers given to the pBKT7-1-XylS derivatives encoding the corresponding XylS mutants (Table 1). The lower part of the Figure shows the lengths of the truncated XylS proteins produced after subcloning various portions of the *xylS* sequence in vector pBKT7-0, as explained in the text, as well as the serial number given to the cognate plasmids encoding them (ΔN, amino terminal deletions; ΔC, carboxyl terminal deletions)

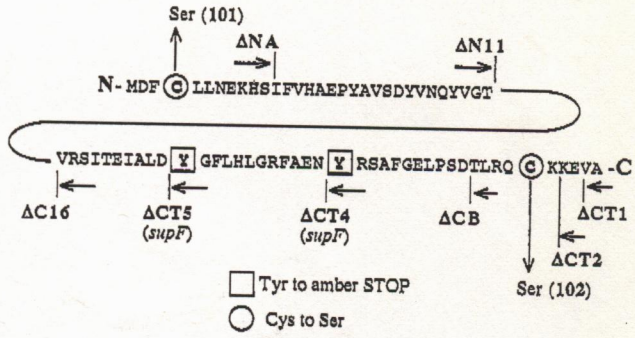

Fig. 4 Mutations in, and deletions of, the termini of the *xylS* gene. The Figure shows the amino acid sequences of the extremities of the wt XylS protein and the various small deletions and mutations made to assess the role of these termini in activation of *Pm*. The *vertical lines* indicate the extents of the amino (ΔN) and carboxyl (ΔC) deletions in the XylS sequence specified by the corresponding pBKT7-1 and pBKT7-0 derivatives. The *encircled* residues indicate the locations of single-site mutations introduced into the sequence by site-directed mutagenesis and the serial number of the corresponding vector plasmid containing each change. The two Tyr residues framed in a *squared box* were changed in the corresponding mutants to the *supF*$^+$-suppressible (amber) stop codon TAG. As indicated in the Figure, these mutant sequences give rise to truncated proteins XylSΔCT4 and XylSΔCT5 when expressed in non-suppressor strains, while they produce full length, wt XylS protein in *supF*$^+$ strains

proteins was confirmed in each case by selective radiolabelling of the polypeptides (not shown). pBKT7-1-XylS and its derivatives were transformed into *E. coli* strain CC118*Pm-lacZ*, which carries a chromosomal insertion with the *Pm-lacZ* reporter system as described in Methods; each strain was subjected to XylS-dependent induction of β-Gal with 3MB. Note that due to the multicopy state of the *xylS*$^+$ plasmid and the optimization of the TIR, readthough transcription from vector promoters sufficed to provide enough XylS to activate *Pm-lacZ* expression in a perfectly regulated fashion and it was therefore unnecessary further to express *xylS* or its derivatives from an additional T7 polymerase-encoding plasmid (not shown). As indicated in Table 3, XylS variants with the changes Gly63Asp/Ser64Pro and Gly160Pro retained a substantial capacity to activate *Pm*, as did the more conservative Asn273Lys substitution, while other variants exhibited no activity in vivo. The change Ser10Asp/Glu11Pro, located at the extreme amino-terminal portion of XylS, exhibited a greatly reduced, though measurable, activity.

Phenotypes conferred by truncated XylS proteins

Since the above mutagenesis suggested the presence of a potentially flexible region in the middle of the protein (Gly160), we decided to produce and analyse the amino (effector-binding) and carboxyl (DNA-binding) halves of XylS separately. The activity of such truncated polypeptides was investigated by testing in vivo whether truncated products could inhibit, the wt *xylS* (transdominance). The rationale for such a test is that if separate functional domains exist as distinct portions of the whole polypeptide, they could be produced independently while maintaining their native conformation. They could then inhibit the activity of the native protein by forming non-functional complexes with it. The mutation Gly160Pro (Fig. 3)

Table 3 Assays of activity and competition capacity of mutant and truncated XylS proteins, (wt wild type, 3MB 3-methyl benzoate)

Plasmid	Protein encoded	Activity[a]	Competition[b]
Controls:			
pBKT7-0	None	21	260
pBKT7-1-XylS	wt XylS	2400	n.d[c]
pBKT7-0-XylS	wt XylS	2490	n.d.
Mutants:			
pBKT7-A	Ser10Asp/Glu11Pro	111	312
pBKT7-11	His31Asp/Ser32Pro	12	282
pBKT7-12	Gly63Asp/Ser64Pro	2020	n.d.
pBKT7-13	Leu13Pro	12	277
pBKT7-14	Gly160Pro	1370	n.d.
pBKT7-15	Phe214Leu/Glu215Asp Arg216/Pro	35	385
pBKT7-16	Asn273Lys	2550	n.d.
pBKT7-B	Thr312Pro	26	233
pBKT7-101	Cys4Ser	2990	n.d.
pBKT7-102	Cys316Ser	2420	n.d.
Deletions:			
pBKT7-0-A	XylSΔNA	18	247
pBKT7-0-N11	XylSΔN11	13	256
pBKT7-0-N14	XylSΔN14	17	243
pBKT7-0-C14	XylSΔC14	21	259
pBKT7-0-C16	XylSΔC16	15	254
pBKT7-0-B	XylSΔCB	19	184
pBKT7-0-T1	XylSΔCT1	2590	n.d.
pBKT7-0-T2	XylSΔCT2	321	n.d.

[a] For activity assays, each of the plasmids specifying the XylS variants listed in the Table were transformed into *E. coli* CC118 *Pm-lacZ*, which carries a chromosomal insertion of a transcriptional fusion between the wt *Pm* promoter and a *xylX'-'lacZ* reporter (Table 1). Each transformant was grown at 30° C in LB medium with ampicillin to OD_{600} of 0.4, after which cultures received 2 mM of XylS effector 3 MB and were grown under the same conditions for a further 3 h. The β-Gal (Miller 1972) levels in each strain were determined. Note that, in the absence of T7 polymerase, the amount of XylS protein produced by pBKT7-1-XylS and pBKT7-0-XylS is sufficient to activate the chromosomal fusion 100-fold, due to efficient readthrough transcription of the gene from vector promoters.

[b] For competition assays, each of the plasmids listed was transformed into *E. coli* CC118 *Pm-lacZ/Pbla-xylS*. This strain has, in addition to the chromosomal *Pm-lacZ* fusion mentioned in [a], a hybrid transposon containing the *xylS* gene expressed through a weak *Pbla* promoter (Table 1). Each transformant was subjected to the same induction procedure as in [a]. Note that the amount of XylS protein encoded by mini-Tn*5* *Pbla-xylS* activates the chromosomal *Pm-lacZ* fusion about ten times less efficiently than when XylS is produced from the pBKT7 derivatives, thus suggesting that the plasmid-encoded proteins are produced in great excess relative to the transposon-encoded *xylS*.

[c] Not determined: mutant and truncated proteins which exhibited significant activity were not assayed in competition experiments

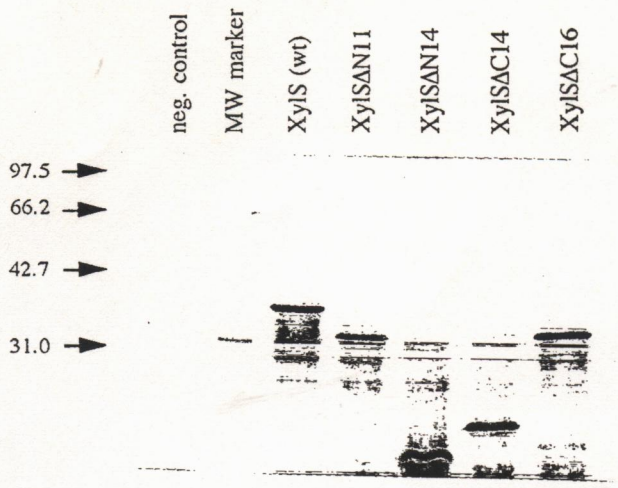

Fig. 5 Expression *in vivo* of truncated XylS derivatives. Plasmids pBKT7-0-XylS, pBKT7-0-N11, pBKT7-0-N14, pBKT7-0-C14, pBKT7-0-C16 encoding the truncated proteins indicated in the Figure were transformed into *E. coli* strain K38 (pGP1-2). The resulting strains were cultured to mid-exponential phase in the presence of Ap and Km. Plasmid-encoded proteins, whose genes are located downstream of the T7 promoter, were specifically radiolabelled as described in Tabor and Richardson (1985). A negative control with pBKT7-0 was also included. After running the samples on a 12% SDS-PAGE system, the gel was exposed to X-ray film. Molecular weight markers in kDa are specified on the left. Note distinct expression of each of the proteins

generated a new *Bam*HI site, which was used for separately subcloning the two halves of the *xylS* sequence in pBKT7-0 for production of the corresponding protein domains, namely XylSΔN14 for the amino-terminal fragment, and XylSΔC14 for the carboxy-terminal fragment (Fig. 3). Expression of each of the resulting truncated genes in vivo with the pBKT7-0 vector system (Fig. 2) was demonstrated by radiolabelling of plasmid-encoded proteins.

Visual inspection of the protein gels (Fig. 5) indicated that the truncated proteins are synthesized to the same level as the wt XylS, with no obvious sign of instability or proteolytic degradation (Fig. 5). However, neither the XylSΔC14 nor XylSΔN14 proteins encoded by the pBKT7-0 derivatives indicated in Table 3 could activate *Pm* in *E. coli* CC118 *Pm-lacZ*, and they also could not attenuate the in vivo activity of the wt XylS protein in *E. coli* CC118 *Pm-lacZ/Pbla-xylS* (Table 3). Since this lack of transdominance is observed under conditions in which expression of the plasmid-borne truncated protein greatly exceeds that of the chromosomally-encoded XylS (Table 3), the active structure either of the amino- or the carboxyl-terminal portion of XylS seems not to be maintained in the absence of the other (truncated XylS proteins found in other TOL plasmids were equally unable to activate the *Pm* promoter; Assinder et al. 1993).

To ascertain whether the position selected for splitting the protein into its two parts was particularly inappropriate for maintenance of the structure of corresponding domains, we constructed two additional truncated proteins, namely XylSΔN11 and XylSΔC16 (Fig. 3) which spanned in both directions a substantial portion of the sequence to each side of Gly160. For these truncated proteins, also, no transdominance over the wt *xylS* gene was observed (Table 3), further strengthening the notion that the terminal amino-acid carboxyl-portions of XylS are interdependent for the maintenance for their respective functional structures and activities.

Deletion analysis of the amino- and carboxy-termini of XylS

To examine in more detail the extent to which the integrity of the amino- and carboxyl-ends of the protein determines its overall function, we generated a number of smaller deletions at both ends of the *xylS* sequence (Fig. 4). As before, we transformed the corresponding pBKT7-0 derivatives into both *E. coli* CC118 *Pm-lacZ* and *E. coli* CC118 *Pm-lacZ/Pbla-xylS* to assay the activity of the truncated protein alone and to test its ability to compete with wt XylS (synthesis of the truncated products in vivo was confirmed, as before, by labelling of the proteins with the T7 system; data not shown). The results of the induction and competition experiments are presented in Table 3. Deletion of the eleven N-terminal amino acids rendered the protein both inactive and unable to compete with wt XylS. There was, however, a gradation in the phenotype endowed by the carboxyl-terminal deletions. Protein XylSΔCT1, which was deleted for just the two C-terminal amino acids had an activity comparable to that of the wt XylS protein. XylSΔCT2, which lacks the 4 C-terminal amino acids, had a much lower, but clearly measurable activity. Finally, XylSΔCB had no activity by itself, but it detectably competed out the activity of wt XylS protein on *Pm*.

To ensure that the phenotypes resulting from the deletions at the carboxy-terminus of XylS were not due to a lack of production of the protein in the assay conditions, we set up an experiment to eliminate all variables involved in the induction of *Pm-lacZ* except for the production of the truncated or wt XylS protein. For this purpose, we constructed *supF*-suppressible nonsense mutants in positions Tyr289 or Tyr301 and placed each of the plasmids encoding them in either $supF^0$ or $supF^+$ isogenic strains carrying *Pm-lacZ*. As shown in Table 4, under same conditions in which the wt protein was active, the truncated proteins were not. Furthermore, the truncated derivatives had only a minor influence on the activity of the wt XylS (Table 4), suggesting that the deletion severely affected maintenance of the overall protein structure.

Table 4 Transdominance of wild-type *xylS* gene over 3'-mutants encoding short carboxy-terminal deletions[a]. (3MB 3-Methylbenzoate, n.d. not determined)

	E. coli CC118 *Pm-lacZ*		*E. coli* CC118 *Pm-lacZ/Pbla-xylS*		*E. coli* CC118 *Pm-lacZ/supF*$^+$	
Plasmid	Unind.	+3MB	Unind.	+3MB	Unind.	+3MB
pBKT7-0	14	21	15	289	n.d.	n.d.
pBKT7-0-XylS	38	2490	n.d.	n.d.	33	2690
pBKT7-0-T4	22	34	14	216	24	2030
pBKT7-0-T5	30	23	14	218	32	1560

[a] Each of the plasmids listed was transformed in the *E. coli* CC118 strains carrying the chromosomal insertions specified in the column headings of the Table. The resulting strains were induced with 2 mM of XylS effector 3MB as described in Table 3; the resulting β-Gal (Miller 1972) levels produced by each strain under uninduced/induced conditions is shown. In the non-suppressor strains, pBKT7-0-T4 and pBKT7-0-T5 plasmids encode inactive carboxy-terminal truncated proteins XylSΔCT4 and XylSΔCT5 respectively (Fig. 4), while the same plasmids produce a mixture of functional and truncated XylS proteins in *supF*$^+$ strains, which activate the chromosomal *Pm-lacZ* fusion

The results with XylS proteins truncated or mutated at their amino- or carboxyl-termini raised the possibility that the extremities of the XylS polypeptide might be covalently linked through an S–S bridge between Cys4 and Cys316. To examine this, we altered each of the Cys residues to Ser; as shown in Table 3, neither of the mutants (encoded respectively by plasmids pBKT7-101 and pBKT7-102) showed any significant decrease in their capacity to induce β-Gal synthesis in the *E. coli* CC118*Pm-lacZ* strain.

Discussion

The geometry of the DNA sequences recognized by regulatory proteins determines the number and orientation of the monomers that bind them (Travers 1989). Therefore, models for the recognition of direct repeats predict distinct phenotypes resulting from offsetting, separation or inversion of the cognate operators (Lu et al. 1992). Furthermore, the different types of DNA-protein interaction involved are amenable to genetic scrutiny and provide some indications of the protein structure itself. When the DNA sequence recognized is a direct repeat, the binding domain of the protein must be asymmetric (Laudet and Stehelin 1992). Three somewhat mutually exclusive models can account for the effective recognition of direct repeats by transcriptional factors, either eukaryotic or prokaryotic (Fig. 1C). In model a (Fig. 1C), the protein may contain two domains (i.e. a dimerization domain and a DNA binding domain), which are connected by a flexible arm or hinge so that the DNA-binding moiety may rotate and/or reach out to bind either direct or inverted repeats. This seems to be the case in some eukaryotic systems such as the nuclear receptors for thyroid hormone or retinoic acid (Laudet and Stehelin 1992), as well as in the case of the XylS-like protein AraC (Carra and Schleif 1993; Bustos and Schleif 1993). Alternative model b (Fig. 1C) involves an antiparallel association of two monomers, which requires considerable bending of DNA around the dimer, as seems to be the case with the regulator MalT (Raibaud 1989). In model c (Fig. 1C), direct repeats are recognized by a tandem protein dimer. In this work we produce genetic data which favour the third model as the mode of activation of the *Pm* promoter by the XylS protein.

Model a (Fig. 1C) makes two very straightforward predictions. Firstly, the flexibility of the interdomain sequence H (hinge) of the regulator should permit some degree of permissiveness in the relative positions of the two *Om* half-sites. As shown in the data summarized in Table 2, we could find no configuration of Om_L and Om_R other than the native one that could sustain *Pm* activation by XylS. In this respect, *Pm* clearly differs from the homologous promoter P_{araBAD}, which tolerates the separation and the inversion of the two AraC half-binding sites (Carra and Schleif 1993). A second prediction is that truncated proteins containing either the operator-binding domain or the dimerization site would *trans*-dominate over (i.e. suppress) the activity of the complete protein. Since the dimerization domain and the DNA binding domain should be relatively independent, hyperexpression of truncated proteins containing either of them should prevent the native protein from engaging in productive interactions with the promoter. In the case of XylS, only very short carboxy-terminal deletions were found to retain some activity or to compete with the wt activator (Table 3), while other truncated proteins were inactive in the two in vivo assays. Therefore, it seems that restrictions in *Pm* geometry are paralleled by an inability to isolate from XylS independent domains that retain in vivo the conformation present in the native protein. Considered altogether, these results suggest that maintenance of the functional domain conformations of XylS involves peptide sequences mapping at distant positions within the protein sequence, rather than being determined by specific and relatively independent portions of the

protein as model a would predict. Again, this appears to be different from the case of AraC, where dimerization and DNA binding domains remain perfectly active even after physical separation (Menon and Lee 1990; Bustos and Schleif 1993).

While model b (Fig. 1) accounts for the recognition of an asymmetric site, it also makes predictions which are difficult to reconcile with experimental data obtained with XylS. The major problem is that DNA bending around two monomers arranged in an antiparallel fashion requires considerable spacing between the two repeated sequences. In the case of *Om*, we have previously shown that base changes at the extremes of each of the *Om* half-sites decrease *Pm* activity substantially (Kessler et al. 1993), suggesting that all of them are engaged in productive DNA-protein contacts. This leaves only 6 bp between the two half-sites, a distance that leaves little room to accomodate either totally or partially two protein subunits. Furthermore, model b predicts that introduction of a complete helix-turn of DNA at the intervening sequence between Om_L and Om_R should not significantly diminish the functionality of the binding site. As shown in Table 2, this is not the case, since insertion of 10 bp between Om_L and Om_R abolishes all XylS-dependent regulation of *Pm*.

Model c, i.e. tandem association of two monomers that form a functional dimer (Fig. 1C), seems to be compatible with all data generated in this and previous reports (Michan et al. 1992a, b). In this model, the functional domains (DNA binding, dimerization) within the three-dimensional structure of the XylS dimer are spatially invariant and correspond with equally invariant sites in *Pm*. Alteration of one *Om* half-site with respect to the other or alteration of a functional site in the protein would lead to loss of functional XylS-*Pm* contacts and hence loss of XylS-mediated regulation of *Pm*. Furthermore, analysis of mutant and truncated XylS proteins (see above) suggests that the structure of XylS is quite compact in the sense that disruption of one protein domain is not easily achieved without affecting the activity and even the structure of the other domains. Several communications have reported that single amino acid substitutions present throughout the sequence of XylS can alter effector specificity (Michan et al. 1992a; Ramos et al. 1987, 1990b), suggesting that protein segments participating in the binding of benzoate derivatives are distributed along the primary structure of the activator. Furthermore, interactions between domains can be deduced from intra-allelic dominance in double XylS mutants (Michan et al. 1992a), suggesting also that carboxy- and amino-terminal regions of XylS are not independent.

Finally, if the monomers form tandem dimers, the protein might generate polymers because of the unoccupied dimerization sites present at the end of the molecule. Although XylS polymerization has not been directly demonstrated, the protein does, unlike other members of the same AraC family of activators (Carra and Schleif 1993; Gallegos et al. 1993; Lauble et al. 1989; Lee and Schleif 1989; Lobell and Schleif 1990; Ramos et al. 1990a), have a strong tendency to form protein aggregates (de Lorenzo et al. 1993), a feature that has so far hindered its purification and biochemical analysis. In conclusion, the genetic data presented here for XylS-*Pm* support a model for protein-DNA interactions (namely model c of Fig. 1C), which predicts a quite strong structural interdependence between the amino- and carboxyl-ends of the XylS protein. In any case, these considerations assume that the form of XylS that binds DNA is a dimer. While this hypothesis explains all genetic data observed, it still awaits rigorous scrutiny in vitro.

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