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Cross Talk between Catabolic Pathways in *Pseudomonas putida*: XylS-Dependent and -Independent Activation of the TOL *meta* Operon Requires the Same *cis*-Acting Sequences within the *Pm* Promoter

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The *Pm* promoter of the *meta* cleavage operon in the TOL (toluene degradation) plasmid pWW0 of *Pseudomonas putida* becomes activated by the plasmid-encoded XylS regulator in the presence of benzoate and certain substituted analogs such as 3-methylbenzoate. In the absence of XylS, *Pm* was still responsive to unsubstituted benzoate but with induction kinetics and a range of transcriptional activity which differed substantially from those for the XylS-mediated activation. XylS-independent induction by benzoate did not occur in a *rpoN* genetic background. *Pm* was also silent while cells were actively growing in rich medium. However, XylS-dependent transcription and XylS-independent transcription were initiated at the same nucleotide, as determined with primer extension mapping. Furthermore, a series of deletions and mutations at the *Pm* promoter sequence showed the same overall pattern of responsiveness to benzoate with and without XylS, thus providing genetic evidence that the same promoter structure is recognized and activated by at least two different regulators. One of them is XylS, while the other, provided by the host bacterium, could be related to the chromosome-encoded benzoate degradation pathway.

Strains of the genus *Pseudomonas* can grow on benzoate as the sole source of carbon through the activity of various catabolic operons, namely, the *ben*, *cat*, and *pca* gene clusters. The enzymes encoded by these chromosome-encoded operons convert benzoate into substrates of the Krebs cycle through catechol-*cis,cis*-muconate and β -ketoadipate intermediates (6, 23, 28). Unlike plain, unsubstituted benzoate, methylbenzoates are neither substrates nor inducers of this *ortho* cleavage pathway, and therefore, they cannot be metabolized through this route. Instead, 3- and 4-methylbenzoate can be degraded through the *meta* cleavage of the corresponding methylcatechol intermediate and the subsequent routing of the resulting hydroxymuconic semialdehydes into oxopentenoate derivatives (5, 22). This *meta* cleavage pathway may not be present in the normal genetic complement of *Pseudomonas* strains, but it is frequently added through the acquisition of a plasmid or transposon encoding additional catabolic operons (5, 6). In this way, *Pseudomonas* strains carrying an extra *meta* cleavage activity may, in principle, degrade benzoate through either of the two routes, the chromosome-encoded *ortho* pathway or the alternative *meta* pathway, though preferentially through the former (22, 24).

The promoter (*Pm* or *OP2*) which drives expression of the *meta* cleavage operon of the archetypal TOL plasmid pWW0 is activated by the cognate, TOL plasmid-encoded XylS regulator (18). This protein activates *Pm* in the presence of benzoate but also in response to a whole collection of structural analogs

which includes 3-methylbenzoate (18). XylS, which belongs to the AraC family of transcriptional activators (26), regulates *Pm* by binding to a distinct regulatory element termed *Om_L/Om_R*, which consists of a directly repeated sequence of 15 bp separated by 6 bp and overlapping with the potential -35 RNA polymerase recognition site (14) (see Fig. 1). Recent observations from different laboratories (1, 12, 13) have shown that, in the absence of XylS, the *Pm* promoter is still responsive to benzoate (but not to 3-methylbenzoate), perhaps by recruiting the regulator of the chromosomal *benABCD* pathway, BenR (12). Since *xylS* and *benR* do not share a high degree of homology, at least as determined by DNA-DNA hybridization (12), *Pm* would be a type of promoter which can be activated by either of two different regulators. However, an alternative explanation could be that the same DNA region which contains the *Pm* promoter elements recognized by XylS includes also a set of sequence motifs recognized by an alternative regulator.

In this article we address the question of whether XylS-dependent transcriptional activation of *Pm* by benzoate and XylS-independent transcriptional activation occur from the same promoter elements or from alternative overlapping structures. Our results show that the same core promoter structure can be activated by at least two different regulators and suggest that the *ortho* and the *meta* pathways for degradation of benzoate may have a significant level of regulatory cross talk.

XylS-dependent activation of *Pm* and XylS-independent activation are physiologically different. The XylS-dependent activation of *Pm* and XylS-independent activation were examined by using specialized reporter strains of *Pseudomonas putida* in which all elements relevant to the expression of *Pm* had been placed in the chromosome (13). The transcriptional/

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FIG. 1. DNA region of the TOL meta operon involved in XylS-independent induction of *Pm* by benzoate. The diagram summarizes the organization of the region preceding the reporter *xylX'*-*lacZ* gene fusion used to monitor *Pm* activity (Table 1). The *Pm*Δ1 promoter included a 1.2-kb restriction fragment bounded upstream by an *Eco*RI site (E) and downstream by a *Bam*HI site engineered next to the first seven codons of *xylX* (13, 14). The extents of the gross 5' deletions Δ13 and Δ14 are indicated at the top, while the different deletions and base changes present in each of the mutant *Pm* varieties, as described in reference 14, are indicated below. *Pm*Δ14 extends just 10 additional bp upstream of the next deletion, Δ15 (14). The sequence elements which determine the XylS-dependent activity of *Pm*—namely, the *Om_L* and *Om_R* direct repeats, the potential -10 and -35 hexamers of the promoter, and the transcription initiation site (9, 20)—are specified. The sequence at -35 clearly differs from the consensus hexamer, as is the case in many positively regulated systems (14, 20). The location of a 5'-GG-N₁₀-GC-3' element (i.e., a potential σ^{54} -dependent promoter) is indicated also.

translational *lacZ* gene fusion used as a reporter of *Pm* activity carried the first seven codons of the *xylX* gene sequence, the first gene of the TOL meta operon (6), and various lengths of the upstream region, in both wild-type and mutant varieties (Fig. 1). For integration of *lacZ* fusions into the chromosome of *P. putida*, a transposon-based procedure was employed (13). Restriction fragments carrying wild-type and mutant *Pm* promoter sequences were cloned in vector pBK17 (13) and introduced by conjugation into a *P. putida* strain containing a minitransposon in the chromosome that provides homology to the sequences flanking the insertion site in the *lacZ* promoter probe plasmid. Double recombination of plasmid and transposon sequences results in the generation of a chromosomal *lacZ* fusion (13). The *Pm* mutations and deletions carried by the various pBK17 derivatives were eventually integrated into the chromosome of *P. putida* (Fig. 1). The *xylS* gene, encoded by the hybrid transposon mini-Tn5 *xylR xylS* (7, 13), was inserted into the chromosome, the accompanying *xylR* gene being required for full expression of *xylS* (10). The induction patterns of *Pm* by benzoate in the presence and in the absence of *xylS* could then be compared.

Figure 2 shows the induction curves of β -galactosidase (β -Gal) (21) generated by isogenic *xylS*⁺ and *XylS*⁻ *P. putida* KT2442 *Pm-lacZ* strains in rich Luria-Bertani medium containing added benzoate (21). Since it was of particular interest to examine the induction pattern with respect to cell growth, the accumulation of β -Gal was plotted in each case against the optical density of the cultures. In neither case did benzoate addition have an influence on growth rate (data not shown). Figure 2 shows that induction followed quite different patterns for different strains, both in the range of β -Gal activity detected and in overall induction kinetics. Four to five hours after addition of the inducer, *XylS* presence raised the activity of the reporter *lacZ* fusion up to 4,000 Miller units. In this case (*xylS*⁺), the responsiveness of the promoter to the effector was

detected immediately after addition of benzoate (Fig. 2). On the contrary, *XylS*-independent responsiveness of *Pm* to benzoate was detected within a much more modest range, roughly 1 order of magnitude less than that for *XylS*⁺. Furthermore, in the absence of *XylS*, induction did not take place until cells had reached an advanced stage of growth, suggesting that under

FIG. 2. Kinetics of induction of the *Pm* promoter by benzoate in *xylS*⁺ and *XylS*⁻ *P. putida* strains. Isogenic *P. putida* KT2442 *Pm*Δ1-*lacZ* *xylS*⁺ *xylR*⁺ and *P. putida* KT2442 *Pm*Δ1-*lacZ* (*XylS*⁻) (13) were grown in Luria-Bertani medium (21) at 30°C and supplemented at the mid-exponential phase with 2 mM benzoate (arrows). Accumulation of β -Gal as the result of *Pm* promoter activity was monitored during the next 5 h. The data are averages for at least two separate assays (each of which was conducted with duplicate samples), the deviations of which did not exceed 15%. Note the different vertical scales of the two plots (0 to 4,000 versus 0 to 400) as well as the shape of the induction curve. Growth rate was not significantly affected in either case by the presence of the inducer in the medium. The *Pm-lacZ* fusion used in this induction experiment was that shown in Fig. 1 as *Pm*Δ1.

FIG. 3. XylS-independent induction of *Pm* by benzoate requires the RpoN product. The assays were made with a *Pm-lacZ* reporter fusion located on the chromosome (A) or on a low-copy-number plasmid (pGTK727) (B) in *P. putida*. In one case (A), isogenic strains *P. putida* KT2442 *Pm-lacZ* *rpoN*⁺ and *P. putida* KT2442 *Pm*Δ13-*lacZ* *RpoN*⁻ (the *Pm-lacZ* fusion used in both cases was that termed Δ13 in Fig. 1) were subjected to the induction procedure with benzoate specified in the text. In the other case (B), *P. putida* KT2442 *rpoN*⁺(pGTK727) and *P. putida* KT2442 *RpoN*⁻(pGTK727) were similarly induced with 5 mM benzoate and the β-Gal activity was monitored during the next 8 h. pGTK727 carries a fusion between the 90th codon of *xylX* and the *lacZ* sequence of pMLB1034 (8), which accounts for the lower range of activity of the plasmid-encoded fusion versus the chromosomal fusion (see the text).

these conditions the promoter undergoes some type of catabolite repression or growth phase-related control (Fig. 2).

To ensure that the induction observed was due to benzoate and not to other intermediate products of its metabolism by *P. putida*, the concentration of the aromatic effector present in the Luria-Bertani medium was monitored by analyzing samples of the culture by high-pressure liquid chromatography. The inducer concentration remained virtually unchanged at 2 mM until cells reached an optical density at 600 nm of 2.2 (i.e., following full *Pm* induction), after which benzoate quickly disappeared from the medium (data not shown), presumably having been consumed as a carbon source. This indicates that benzoate (and not one of its degradation products) was the authentic inducer of *Pm* activity.

XylS-independent activation of *Pm* requires *rpoN*. Since the induction kinetics of *Pm* in the absence of XylS resembled, to some extent, those of other σ⁵⁴-dependent promoters (2), the same reporter system was reproduced in an otherwise isogenic *rpoN*::Km mutant (16) lacking the gene which encodes that alternative sigma factor. In order to avoid incompatibility of antibiotic resistance markers, the reporter *Pm-lacZ* fusion was reconstructed within a mini-Tn5 Tc transposon vector (3) prior to its insertion into the chromosome of the *P. putida* *rpoN* strain. As shown in Fig. 3, the fusion had no significant activity in cells lacking σ⁵⁴. To rule out the possibility that the site of insertion of the mini-Tn5 Tc *Pm-lacZ* reporter transposon had an effect on the XylS-independent induction of *Pm*, the *Pm-lacZ* transcriptional fusion plasmid pGTK727 (derived from the broad-host-range, low-copy-number vector pGS72, [8]) was introduced into *rpoN*⁺ and *RpoN*⁻ isogenic *P. putida* KT2442 strains. Again, the data in Fig. 3 clearly show that the absence of σ⁵⁴ prevents any XylS-independent induction of *Pm* by benzoate.

The dependency of *Pm* activity on *rpoN* prompted an

FIG. 4. Identification of the benzoate-induced, XylS-independent transcription initiation site in the *Pm* promoter region. *P. putida* KT2442 *Pm*Δ1-*lacZ* and *P. putida* KT2442 *Pm*Δ1-*lacZ* *xylS*⁺ *xylR*⁺ were supplemented, at the early exponential phase, with either 2 mM benzoate or 2 mM 3-methylbenzoate (3MB) plus 4 mM 3-methylbenzyl alcohol (3 MBA) as indicated. Four hours after the addition, 2 ml of each culture was spun down and the RNA of the cells was extracted as described elsewhere (18). Forty micrograms of total RNA from each sample was then subjected to a primer extension assay (19) using a 5'-³²P-labelled *lacZ* reverse 24-mer, 5'-ATGTGCTGCAAGGCGAT TAAGTTG-3', which, upon extension with reverse transcriptase, generates a cDNA of a predicted size of 127 bp with respect to the canonical XylS-mediated transcription initiation site in *Pm* (9, 18, 20). The labelled products were resolved in a 7% polyacrylamide-8 M urea sequencing gel along with size markers (indicated in base pairs). Note the same transcription initiation site (Fig. 1) for benzoate inducers regardless of the presence or absence of *xylS*. The various intensities of the bands do not have a quantitative meaning. The band below the main extension product in the second sample (+XylR/S, 3MB+3MBA) may indicate some processing of the major transcript or a minor start site (20).

examination of the promoter region for potential recognition sites of the σ⁵⁴-containing form of RNA polymerase (17, 27). One of such sequences was found within the region spanning the direct repeats recognized by XylS (Fig. 1). While the sequence in question differed from the extended consensus proposed for this type of promoter (18, 27), it included the basic element 5'-GG-N₁₀-GC-3' (27). This region was subjected to further analysis to examine whether this potential σ⁵⁴-dependent promoter could account for the effects observed.

The initiation site of the XylS-independent transcript generated at *Pm* was identified. This was accomplished by using the same two strains employed in the experiment described in the legend to Fig. 2. *P. putida* KT2442 *Pm*Δ1-*lacZ* carries the *Pm-lacZ* fusion Δ1 (Fig. 1), but it is devoid of *xylS*, while *P. putida* KT2442 *Pm*Δ1-*lacZ* *xylS*⁺ *xylR*⁺ has, in addition, a chromosomal insertion of a transposon with *xylS* and the *xylS*-activating gene *xylR* (10, 13). Each of the two strains was grown and induced under the conditions specified in the legend to Fig. 4. Their total mRNA was then extracted and

TABLE 1. Activities of deletion and mutant *Pm* promoter variants^a

Promoter variant	% ^b	
	<i>xylS</i> ⁺ <i>xylR</i> ⁺	No <i>xylS</i>
<i>Pm</i> Δ1	100	100
<i>Pm</i> Δ13	100	100
<i>Pm</i> Δ14	100	100
<i>Pm</i> Δ15	25	28
<i>Pm</i> Δ16	5	8
<i>Pm</i> 4	24	22
<i>Pm</i> 5	10	8
<i>Pm</i> 6	12	6
<i>Pm</i> 7	94	93
<i>Pm</i> 8	10	11
<i>Pm</i> 54 ^c	85	227
<i>Pm</i> L	105	68

^a The DNA sequences preceding the reporter *xylX'*-*lacZ* fusions used to follow the effects of the various changes are shown in Fig. 1. Each *Pm-lacZ* fusion was integrated into the same location of the chromosome of *P. putida* KT2442 (13) so that resulting strains were totally isogenic except for the length or sequence of the *Pm* upstream region. The *xylS*⁺ *xylR*⁺ genetic background was constructed by inserting the hybrid transposon mini-Tn5 *xylS xylR* (13) into the chromosome of the same *Pseudomonas* strain prior to the introduction of the various *Pm-lacZ* fusions.

^b Relative to the value for the control wild-type *Pm* promoter, Δ13 (approximately 2,200 Miller units). Basal levels under uninduced conditions were 20 to 40 Miller units (ca. 1 to 3% of the value for the fully induced *Pm*Δ13 control promoter). The range of β-Gal activity for the *XylS*[−] strains was about 1 order of magnitude lower (basal, uninduced level of control *Pm*Δ13, 30 Miller units; level induced with 2 mM benzoate, 280 Miller units), although the activity is also shown as the percentage of the induced level. The data are averages for at least two independent experiments (each of which was conducted with duplicate samples), the deviations of which did not exceed 15%. Addition of 3-methylbenzyl alcohol plus 3-methylbenzoate, under the conditions used, had a lower net induction effect than the addition of unsubstituted benzoate, compared with the results in Fig. 2.

^c β-Gal activities for the *Pm*54-*lacZ* fusion: *xylS*⁺ background, 50 U (uninduced) versus 1,810 U (with 2 mM 3-methylbenzoate); *XylS*[−] background, 110 U (uninduced) versus 640 U (with 2 mM benzoate).

subjected to a primer extension assay (19). This revealed that the leading nucleotide of the mRNA was the very same regardless of the absence or presence of *XylS* (Fig. 4). Furthermore, the *Pm*Δ14 promoter was perfectly active in the *XylS*[−] strain (Table 1). Since *Pm*Δ14 contains just 10 additional bp upstream of the next deletion, Δ15 (Fig. 1), it lacks the whole DNA region upstream of the putative −12/−24 element (Fig. 1). This renders unlikely the possibility that the 5'-GG-N₁₀-GC-3' sequence behaves as an authentic σ⁵⁴-dependent promoter, because the known examples of this type require upstream activating sequences, which are target sites for cognate regulators, for their activity in vivo (17). Mutagenesis of the GG/GC sequences impaired the expression of the reporter gene (*Pm*5 and *Pm*8) (Fig. 1 and Table 1), but this result says little about the nature of the promoter, since this sequence might overlap a DNA region recognized by other regulators (see below).

***cis*-acting sequences within *Pm* which confer *XylS*-independent activation.** To identify the DNA sequences accounting for the responsiveness of *Pm* to benzoate in the absence of *XylS*, deletion and mutant derivatives (14) specified in Fig. 1 were used. For the *xylS*⁺ *xylR*⁺ strains, overnight cultures were diluted 1:200 and grown in Luria-Bertani medium at 30°C to an optical density at 600 nm of 0.3 to 0.4, and then 4 mM 3-methylbenzyl alcohol (an inducer of *XylR*, which activates expression of *xylS* [10]) and 2 mM 3-methylbenzoate were added. Cultures were grown for a further 5 h, and their β-Gal levels were examined (21). Cultures of *Pm-lacZ* strains devoid of *xylS* were treated similarly, but only 2 mM benzoate was

added as an inducer. Since 3-methylbenzoate cannot induce *Pm* in the absence of *XylS* (1), it was used as an effector in the *xylS*⁺ strain to clearly separate the *xylS*-dependent and *xylS*-independent effects. Table 1 summarizes the results obtained. Activities of the various deletion variants pinpointed the location of the upstream boundary of the sequence(s) involved in activation as approximately between −75 and −65 with respect to the transcription initiation site (−75 is the upstream boundary of the Δ14 deletion [14]). The sequences of the *Pm* region which remain in the shortest fully active deletion variant, Δ14, include an imperfect directly repeated sequence formerly identified as the *cis*-acting sequence which confers responsiveness to *XylS* (14). Deletions which entered as little as 2 bp into the repeats (*Pm*Δ15) decreased both *xylS*-dependent induction of the reporter gene by benzoate and *xylS*-independent induction. Furthermore, while various mutations through the repeated sequences lessened and even completely abolished β-Gal accumulation in response to benzoate, changes within the interrepeat intervening DNA sequence had no significant effect on *Pm* activity (Table 1). Therefore, the *XylS*-independent responses to benzoate of mutant promoters mimicked those in the *xylS*⁺ strain, though they showed different kinetics and ranges of activity. These data indicate that the very same promoter elements in *Pm* (namely, the *Om*_L/*Om*_R direct repeat [Fig. 1]) confer on the system the ability to be activated by *XylS* and also to be cross-induced by benzoate in the absence of *XylS*. This obviously should happen by recruiting a chromosome-borne regulator, perhaps of the same protein family, which unlike *XylS* (18), responds only to unsubstituted benzoate.

There were two promoters for which the *XylS*-dependent and -independent responses were different. One of them was the *Pm* L promoter (Fig. 1; Table 1). This mutant *Pm* has the two *Om* half-sites adjusted so that the imperfect wild-type direct repeat *Om*_L/*Om*_R has been changed to repeat twice the distal half-site, thus becoming *Om*_L/*Om*_L. As shown in Table 1, while this promoter responds perfectly to *XylS* in spite of the four base changes introduced within the sequence, its *XylS*-independent activation by benzoate decreased reproducibly by at least 30%. The second promoter was *Pm*54 (Table 1). This promoter contains two base changes, G→A and C→T, at the intervening region between the −35 and the −10 hexamers of the *Pm* promoter which predictably increase the flexibility of the region (11). These changes had little effect on *XylS*-mediated activity of *Pm*54, while they considerably augmented both the basal and the benzoate-induced levels of the promoter in the absence of the TOL-borne regulator (Table 1).

Cross-regulation of *Pm* by two activators? From the above results, it may be concluded that the same structural elements of the *Pm* promoter of the TOL plasmid which respond to *XylS* can respond also to at least one additional regulator which uses unsubstituted benzoate as an effector. Since the organization of the *cis*-acting DNA sequence involved in the activation, i.e., the *Om*_L/*Om*_R repeat, reflects the distribution of the functional domains of the regulator (15), it is possible that such an alternative activator may also belong to the same AraC family of proteins (26). Furthermore, *XylS*-independent activation of the system requires *rpoN*, while the promoter is not by itself σ⁵⁴ dependent (16, 25). This indicates that, similarly to the *xylS* gene (10, 25), the chromosome-borne regulator may form part of a regulatory cascade which includes at least one σ⁵⁴-dependent promoter.

Jeffrey et al. (12) found that *benR* mutants of *P. putida*, i.e., mutants lacking the regulator of the *benABCD* cluster for conversion of benzoate to catechol, did not show any *XylS*-unrelated activity of *Pm*. Furthermore, a DNA segment from

Pseudomonas aeruginosa which complemented the activity of the *benR* mutation in *P. putida* also restored XylS-independent expression of *Pm* (12). The same authors (12) provided indirect evidence that XylS restores the inducibility by benzoate of the *benABCD* genes in the absence of *benR*. These data suggested that BenR could actually be the regulator replacing XylS in the activation of *Pm*. However, the lack of DNA homology between the two regulatory genes *xylS* and *benR* (12), the ultimate dependency of the *Pm* promoter on *rpoN* for XylS-independent activation (see above), and the differences of the DNA sequences upstream of the *xylXYZ* and *benABC* regions in the known species (4) may argue against such a possibility. Furthermore, while *rpoN* mutants of *P. putida* grow well on benzoate as the sole carbon source, the same mutation decreases by at least two-thirds the growth rate of *P. putida* (TOL) on 3-methylbenzoate (16). This suggests that, unlike the activators of *Pm*, none of the activators of the *ben*, *cat*, and *pca* pathways is severely affected by the *rpoN* lesion. In any case, our results show that the cross-activation of *Pm* by transcriptional regulators alien to the TOL plasmid occurs through the recognition of the same *cis*-acting sequences and the same promoter elements within the region upstream of the *meta* TOL operon. Whether such cross talk plays an active role in routing certain aromatic compounds into an alternative *ortho* or *meta* cleavage pathway when both pathways coexist in the same cells deserves further studies.

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