The differential response of the *Pben* promoter of *Pseudomonas putida* mt-2 to
BenR and XylS prevents metabolic conflicts in *m*-xylene biodegradation

by

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1 ABSTRACT

2
3 *Pseudomonas putida* mt-2 encompasses two alternative and potentially conflicting routes for benzoate
4 metabolism, one *meta* pathway encoded by *xyl* genes of the pWW0 plasmid and mastered by the *Pm*
5 promoter and XylS, and one chromosomally-encoded *ortho* pathway initiated by *Pben* and the BenR
6 protein. Any cross-activation of *Pben* promoter by XylS ought to cause a metabolic conflict during the
7 degradation of *m*-xylene, because 3-methylbenzoate generated as an intermediate can be channelled
8 through the *ortho* pathway and produce toxic dead-end metabolites. The activation of *Pben* by XylS was
9 revisited using both reporter technology and tiling arrays targeted to the sequences of interest around
10 mRNA initiation of both *Pben* and *Pm* promoters. Analysis of supersensitive *luxCDABE* fusions,
11 inspection of *xylX* vs. *benA* transcripts and growth tests of *benR* mutants indicated that the natural
12 expression ranges of XylS under various conditions are insufficient to cause a significant cross-regulation
13 of *Pben* whether cells face endogenous or exogenous 3-methylbenzoate. This seems to stem from the
14 nature of the operators for binding either transcriptional factor, which in the case of the *Pben* promoter of
15 *P. putida* mt-2 appear to have evolved for avoiding a strong interaction with XylS.

18 INTRODUCTION

19
20 Biochemical pathways for metabolization of the vast array of chemical substrates found in nature often
21 encode an evolutionary record of the problems that bacteria had to overcome before reaching a workable
22 enzymatic and genetic solution (George and Hay, 2011). Examples include mutations in transcriptional
23 activators which alter effectors specificity expanding or narrowing the substrate range of the controlled
24 pathway (Pavel *et al.*, 1994; Jaspers *et al.*, 2000; Choi *et al.*, 2003), the modification of repressors
25 preventing operator binding for allowing constitutive expression of required enzymes (Arai *et al.*, 1999), or
26 the creation of promoter elements by insertions/deletions resulting in increased transcription of catabolic
27 genes (Kasak *et al.*, 1997). A recurrent instance of adaptations required for avoiding metabolic conflicts is
28 the inactivation of catabolic enzymes that produce toxic metabolites or result in misrouting of pathway
29 intermediates. In these cases, the loss of enzyme activity is readily accomplished by point mutations or
30 gene deletions (Gerischer and Ornston, 1995; Ju and Parales, 2010).

The TOL pathway, encoded by the archetypal plasmid pWW0, is probably the best characterized route for degradation of aromatics from a biochemical and genetic point of view (Ramos *et al.*, 1997). This system, which has been described in the soil bacterium *Pseudomonas putida* mt-2, allows mineralization of toluene and *m*-xylene and it is composed of two operons encoding an *upper* and a *lower* pathway. Through the *upper* pathway, the methyl group of toluene or *m*-xylene is sequentially oxidized to render benzoate (Bz) or 3-methylbenzoate (3MBz), respectively. Bz is then converted to catechol by either the *lower* route enzymes encoded by *xylXYZL* or by the products of the highly similar genes *benABCD* encoded in the chromosome (Fig. 1). Both gene clusters can be induced by Bz itself (Ramos *et al.*, 1997; Cowles *et al.*, 2000). Thereby generated catechol can be channelled towards either a *meta*-cleavage pathway by catechol 2,3-dioxygenase encoded by the *xylE* gene or in an *ortho*-fission route initiated by the chromosomally-encoded catechol 1,2-dioxygenase (Fig. 1). Instead, the transformation of 3MBz formed during *m*-xylene turnover into the corresponding 3-methylcatechol poses a metabolic conflict. This is because the action of catechol 1,2-dioxygenase over the methyl substituted ring-cleavage substrate generates 2-methyl-2-enelactone, a dead-end product (Schmidt *et al.*, 1985). In this undesirable scenario the activation of the unproductive chromosomally-encoded *ortho* cleavage route should be avoided to ensure that the generated methylcatechol is channeled in its entirety through the *meta* fission pathway that is proficient for methyl substituted substrates. Therefore, a finely tuned regulation able to sort out the expression of both cleavage pathways is a key requirement for a sound catabolic phenotype.

In *P. putida* mt-2 the regulation of Bz and 3MBz degradation routes relies on transcriptional regulators belonging to AraC-family, the so-called XylS and BenR activators. Members of this family present a characteristic modular structure. The conserved C-terminal domain includes two potential tetra-helical HTH DNA-binding motifs while the N-terminal domain is not well conserved and is involved in effector recognition and dimerization (Gallegos *et al.*, 1997). The pWW0-encoded XylS regulator mediates transcriptional activation of the *Pm* promoter driving the expression of the *meta* pathway in response to 3MBz and Bz as inducers. Likewise, BenR is able to trigger the activity of *Pben* promoter by recognition of Bz as effector, allowing the expression of the *benABCD-catA2* operon (Cowles *et al.*, 2000). Both regulators share about 60% amino acid identity through their full sequence and the similarity is even higher in the C-terminal DNA binding domain suggesting that cross-activation of their target promoters could take place. In fact the ability of BenR to activate the *Pm* promoter of TOL system in response to Bz has been recognized (Kessler *et al.*, 1994). However, the role of XylS as an activator of *Pben* has not

been entirely clarified. Earlier reports have suggested the occurrence of cross-activation of *Pben* by XylS in a different *P. putida* lineage, PRS2000 strain (Jeffrey *et al.*, 1992; Cowles *et al.*, 2000). A more recent publication has documented also this occurrence in *P. putida* mt-2 by using a plasmid harboring a *Pben-lacZ* fusion (Dominguez-Cuevas *et al.*, 2006). While the phenomenon may well happen, the question is how the corresponding parameters are set to avoid the predicted scenario of metabolic stress generated by the self-defeating activation of *ortho*-cleavage enzymes in the presence of 3MBz as described above.

In this work we have employed a suite of alternative techniques to clarify [i] whether cross-activation of *Pben* by XylS actually occurs in wild-type *P. putida* mt-2 cells exposed to endogenously produced and exogenously added 3MBz and [ii] if so, whether the parameters of the system under natural conditions lead to a metabolic conflict or instead, the same parameters are poised to avoid it. To this end we resorted to various configurations of supersensitive promoter fusions to *luxCDABE*, to transcriptomic analyses of *benA* and *xylX* under diverse conditions, and to growth tests of strains bearing different regulatory combinations. The results below reveal that while a degree of cross-activation of the *ortho*-cleavage pathway by XylS can be detected, its extent is kept low owing to changes in the *Pben* promoter that withhold the binding of the potential cross-regulator. This scenario provides a remarkable case of regulatory tinkering that facilitates recruitment of a catabolic pathway in a new host with a non-fully compatible metabolism.

RESULTS

Pben is only marginally activated by 3MBz in *P. putida* mt-2

In an attempt to examine the responses of the chromosomally-encoded *Pben* promoter and pWW0-borne *Pm* promoter in entirely comparable and super-sensitive conditions we constructed reporter plasmids pSEVA226-*Pb* and pSEVA226-*Pm*. These are constructs based in the very low-copy replicon RK2 of pSEVA226 (Silva-Rocha *et al.*, 2013) in which DNA fragments encompassing 500 bp (for *Pben*) and 80 bp (for *Pm*) regions of these promoters were cloned in front of a promoterless *luxCDABE* cassette (note that the RBSs are in both fusions those of the reporter operon; see Experimental procedures). Such segments bear the entire regulatory regions that bind, respectively BenR and XylS (Kessler *et al.*, 1994; Cowles *et al.*, 2000) and thus are expected to report faithfully the expression of either the *ortho* or the

meta pathway when introduced in strain *P. putida* mt-2. With these strains at hand, *P. putida* mt-2 (pSEVA226-*Pb*) and *P. putida* mt-2 (pSEVA226-*Pm*), we judged side by side the capacity and inducibility of each promoter in the presence of either Bz or 3MBz in a genetic background with wild-type levels of both transcriptional factors. To this end, each strain was pre-grown in M9 minimal medium with succinate as sole C source, diluted 1:20 in fresh media supplemented with 1 mM of either Bz or 3MBz and incubated as explained in Experimental procedures. Light emission was quantified in each of the cultures 4 h after the addition, the results being shown in Fig. 2. Perusal of the data reveals at least three aspects that are relevant for the sake of this study. First, the basal level of *Pben* is visibly lower than that of *Pm*. Note that in the pSEVA226-derived constructs there is a strong transcriptional terminator upstream of the cloned DNA, thereby ensuring that any luminescence of the reporter originates in the inserted promoter. Second, the *Pm-luxCDABE* fusion generates a level of luminescence with Bz that is markedly higher than that brought about by *Pben* under the same conditions. And, third, the data of Fig. 2 shows that 3MBz induces *Pm* to a degree that is $\geq$ 11-fold higher than the same parameter in *Pben*. From a mere phenomenological point of view, these results put forward that Bz induces both the *ortho* and the *meta* pathway but 3MBz seems to activate significantly only the pWW0-encoded route. Since these data somehow question the significance of previously reported experiments of cross-regulation between *XylS/Pm* vs *BenR/Pben* (Cowles *et al.*, 2000; Dominguez-Cuevas *et al.*, 2006), we revisited the issue with 3 entirely different experimental approaches which, as disclosed below, do not leave any reasonable doubt on the matter at stake.

Quantification of *benA* and *xylX* transcripts in response to aromatic inducers

In order to dissect the effect of Bz and 3MBz on expression of chromosomally encoded *benA* (driven by *Pben*) and the plasmid-borne *xylX* (transcribed by *Pm*) we inspected the corresponding transcripts by means of a customized tiling array of overlapping 50-mers that covered the entirety of the target genes (see Experimental procedures). Employing succinate-grown *P. putida* mt-2 cells as the reference, we exposed the cultures not only to exogenously added Bz and 3MBz but also to *m*-xylene and *o*-xylene. In the first case, 3MBz is produced endogenously through the conversion of *m*-xylene to the cognate carboxylic acid through the action of the *upper* TOL enzymes (Fig. 1). But note also that *m*-xylene suffices to overexpress *xylS* which in turn activates *Pm* even before 3MBz is formed (Ramos *et al.*, 1997). To sort this out we run a control with *o*-xylene, a non-metabolizable but otherwise efficient inducer of the *upper*

pathway and the *xyIS* gene (Abril *et al.*, 1989). This allowed assessing how much cross-activation of *Pben* by XylS can be attributed to the complex effector/regulator and how much to the mere overproduction of the protein. To follow the evolution of *xyIS* expression under such conditions, the tiling array included also sequences covering this gene. The results of the corresponding experiments are shown in Fig. 3. While the data match fairly well those of Fig. 2 generated with *luxCDABE* reporter fusions to *Pben* and *Pm*, the tiling array revealed details of the cross-talk that were not noticeable before. First, 3MBz endogenously produced from *m*-xylene is expectedly a good inducer of *xyIX* but it only triggers a quite limited expression of *benA* (note vertical axes scales of the panels). Such minor activation of *Pben* could be due to either cross-regulation by XylS or the consequence of a residual recognition of 3MBz as an effector of BenR (Silva-Rocha and de Lorenzo, 2012). Note that BenR levels remained low and invariant in all tested conditions (data not shown). A second detail exposed by the tiling array experiment was that overproduction of XylS by *o*-xylene altogether failed to cause any activation of *Pben* (Fig. 3). This is in contrast with *Pm*, which is indeed triggered by such increased levels of XylS even in the absence of any inducer. Finally, as anticipated by the *luxCDABE* fusions of Fig. 2, exogenously added Bz brings *xyIX* and *benA* to high levels while 3MBz does so efficiently only in the first case (again, note different scales of vertical axes). While the origin of the residual activation of *Pben* by 3MBz and *m*-xylene is addressed below, the phenomenological picture that emerges from the data of Fig. 3 is that Bz elicits the *ortho* and *meta* routes to approximately the same expression range. In contrast, 3MBz seems to predominantly bring forth the operation of the *meta* pathway. This state of affairs makes sense for avoiding the metabolic conflicts mentioned above, but say little on the mechanism(s) that allow such differential response of *Pben* and *Pm* to either unsubstituted or substituted Bz.

Natural XylS levels of P. putida mt-2 fail to cross-activate Pben

The data above leave the question open on what regulator activates what promoter in the presence of what inducer in the naturally occurring *P. putida* mt-2. Available literature indicates that [i] XylS recognizes both Bz and 3MBz as *bona fide* effectors (Ramos *et al.*, 1997), [ii] BenR is responsive to Bz but only marginally to 3MBz (Silva-Rocha and de Lorenzo, 2012) and [iii] BenR can activate *Pm* (Jeffrey *et al.*, 1992; Kessler *et al.*, 1994). Alas, the condition that completes the whole picture of interactions between the two regulatory systems (i.e. testing whether XylS can activate *Pben* in the absence of BenR) has not been unambiguously tested. In an effort to address this question with a gene dosage and stoichiometry

that faithfully reproduces the natural conditions, we generated transcriptional fusions of *Pben* and *Pm* to *luxCDABE* in a Tn7 mini-transposon vector (Choi *et al.*, 2005) that placed the whole reporter device in the same neutral site of the chromosome of a *P. putida* KT2440 (i.e, the pWW0-cured derivative strain) variant lacking the *benR* gene (see Experimental procedures). The resulting reporter strains were then used as recipients of either the wild-type pWW0 or plasmids pJB653 (Blatny *et al.*, 1997), pSEVA438 (Silva-Rocha *et al.*, 2013) and pVLT43 (de Lorenzo *et al.*, 1993), each of them capable of expressing XylS at increasingly higher levels (pJB653 < pSEVA438 < pVLT43). Note that high XylS concentrations lead to activation of *Pm* without any need for inducer view (Ramos *et al.*, 1997). The reporter output was then recorded for each of the strains added with Bz or 3MBz as before. In the case of strains bearing pVLT43, the cultures were amended or not with IPTG in order to bring about still higher intracellular levels of XylS. The data of Fig. 4a revealed that in the absence of BenR, the ordinary levels of XylS stemming from the pWW0 plasmid were unable to trigger any significant induction of *Pben* with either Bz or 3MBz. The same held true when XylS was supplied by a low-copy number plasmid (pJB653). A minimal induction was detected with the XylS levels produced by a medium-copy number plasmid (pSEVA438), but luminescence took off the background only when *xylS* expression was strongly stimulated by IPTG in the overexpressing plasmid pVLT43 (Fig. 4b; note the scales of the vertical axes). In contrast, the *Pm* promoter was always induced –albeit to very different levels by all combinations of XylS and inducers (Fig. 4c), including overproduction of the regulator in the absence of aromatics (Fig. 4d). Taken together, these data indicate that physiological concentrations of XylS in *P. putida* mt-2 do not grant any significant cross-regulation of the *ortho*-cleavage route-encoding genes. It is necessary to artificially increase such XylS levels (e.g. by using multicopy vectors or overexpressing plasmids) for causing a still moderate induction of *Pben* by this regulator. But even under such unlikely conditions the responses of *Pben* to XylS are 10-15 times weaker than *Pm* under the same dosage of the regulator. In contrast, the presence of BenR affords a strong induction of *Pben* by Bz (Fig. 2).

The results on *Pben* above deal with exogenously added 3MBz, but still they did not clarify the state of affairs when this effector is generated intracellularly as a consequence of the action of *upper* TOL enzymes on *m*-xylene. In order to check if increased levels of XylS naturally reached during *m*-xylene metabolism (leading to 3MBz production) are enough to cross-activate *Pben*, we re-examined the activity of this promoter (and *Pm* as a positive control) in the *P. putida* KT2440 *benR* strains bearing the pWW0 plasmid and engineered with the same *luxCDABE* fusions as before. Since *m*-xylene might cause a

degree of physiological stress that could interfere with reporter readout we took the extra precaution of testing also 3-methylbenzylalcohol (3MBA), a less-toxic substrate of the *upper* TOL pathway that is also a good XylR effector and yields 3MBz as a metabolic intermediate as well (Ramos *et al.*, 1997). The results shown in Fig. 5 revealed that neither *m*-xylene nor 3MBA were able to induce high enough XylS levels to trigger the *Pben* promoter in the strain that lacks *benR* gene. On the contrary, the physiological overexpression of XylS by either *upper* pathway inducer triggered induction of *Pm*. Note that these results clarify also the origin of the residual *Pben* activation by *m*-xylene in the wild-type *P. putida* mt-2 strain during tiling array experiments (Fig. 3, see above). That the *Pben* promoter remains silent in cells of *P. putida* KT2440 (pWW0) exposed to *upper* pathway inducers when BenR is lacking implies that in the *benR*⁺ wild-type context, the response of *Pben* to *m*-xylene necessarily stems from a residual recognition of the endogenously generated-3MBz as an effector of BenR.

Taken together, the data above indicated that while *Pben* can be activated by XylS under somewhat unusual circumstances, such activation is not significant under physiological conditions. Prediction is, therefore, that low-level XylS cannot replace the ordinary function of BenR. Yet, such a substitution could occur when XylS levels are artificially increased. The experiments below shed light on this conjecture.

Physiological levels of XylS do not restore growth on Bz of P. putida mt-2 lacking BenR

The experiments presented thus far indicate that *Pben* can be shown responsive to XylS *in vivo* by manipulating the intracellular pool of the regulator. The question is whether any possible cross-activation of this sort has metabolic consequences or the phenomenon is just the result of the engineered experimental setup. The simple experiment of Fig. 6 was made to answer this question. In this test, the growth on Bz of a control wild-type *P. putida* KT2440 without XylS was compared with that of a *benR* mutant bearing a plasmid encoding XylS in various configurations. The positive control (wild-type strain) grew rapidly in the medium with Bz, while the negative control (isogenic *benR* strain), as expected, failed to grow entirely. Then a *xylX::bla* variant of pWW0 was transferred to the *benR* strain. This plasmid bears an inactivated allele of the first gene of the *lower* TOL pathway that encodes a subunit of the toluate 1,2-dioxygenase that starts degradation of Bz / 3MBz through that route. pWW0-*xylX::bla* thus provides physiological levels of XylS but does not contribute to any possible metabolization of Bz, which has to go necessarily through the chromosomally-encoded *ortho* cleavage pathway driven by *Pben* promoter. As

shown in Fig. 6, such strain failed to grow at all on 5 mM Bz. The same happened when *P. putida benR* was transformed with low-copy number *xyIS*⁺ pJB653 that produces low levels of XylS. The situation changed however, when the concentration of the regulator was increased *in vivo* by either supplying the medium-copy number *xyIS*⁺ pSEVA438 or the *xyIS* expression plasmid pVLT43. In these cases, the *benR* strain could eventually resume growth on Bz after some prolonged lag phase, signifying that a functional replacement of *benR* by *xyIS* had taken place under such conditions. These results are fully consistent with those reported above with the *Pben* → *luxCDABE* and *Pm* → *luxCDABE* fusions. All the data thus coherently accredit that any possible cross-regulation of *Pben* by XylS lacks physiological significance in the original genetic architecture of strain *P. putida* mt-2.

DISCUSSION

Strain *P. putida* mt-2 provides a good example of the evolutionary roadmap that a bacterium had to go through during expansion of its metabolic repertoire, in this case from Bz to 3MBz. Although both molecules are chemically similar, their main sources in the environment are different. Bz is more abundant as a component of plant root exudates (Uren, 2007), while methylated benzoates are typically produced as side-biodegradation products of aromatic hydrocarbons like those often found in the volatile fraction of petroleum (BTEX compounds; Vandecasteele and Monot, 2008). In the case of *P. putida* mt-2 it is likely that a pre-existing Bz-degrader and plasmid-less bacterium received the *xyl* genes for metabolism of *m*-xylene through horizontal transfer of a conjugative plasmid. This scenario is supported by the fact that the *ortho* pathway at stake is found in every sequenced *P. putida* strain, suggesting that such metabolic activity is a core trait in this species. In contrast, the TOL pathway is an exceptional attribute found in just a few strains that suggest a more recent evolutionary origin. While non-substituted Bz can be channelled productively through both *ortho* and *meta* catabolic pathways, the routing of methylated aromatics into the chromosomally encoded intradiol route leads to methyl-substituted 4-carboxymethylbut-2-en-4-olides (trivially called *methyl-2-enelactones*) as dead-end metabolites (Fig. 1). Since the two catechol 1,2-dioxygenases encoded in *P. putida* mt-2 genome have activity on both catechol, 3-methylcatechol and 4-methylcatechol (Jimenez *et al.* 2014), it is plausible that a significant fraction of *m*- and *p*-xylene finish as dead-end metabolites. How has *P. putida* solved this problem? The results presented above expose the solution: under physiological conditions, Bz activates expression of both the *ortho* and the *meta* pathways genes, whereas methylated substrates trigger *only* the *meta* route,

leaving silent transcription of the chromosomal *ben* genes. Such a simple way out conflicts however, with some of the existing literature on the regulatory interplay between the *meta* and the *ortho* routes for degradation of Bz in *P. putida*. In particular, by using multicopy *Pben-lacZ* transcriptional fusions Cowles *et al.* (2000) and Dominguez-Cuevas *et al.* (2006) reported that XylS can induce the promoter of *benABCD-catA2* operon in response to Bz and 3MBz in two different strains of *P. putida*. If such a cross-induction had a physiological value, then growth of *P. putida* mt-2 in *m*-xylene or 3MBz would be hardly possible (Fig. 1). Our data above show, instead, that while XylS can indeed regulate *Pben* under some engineered conditions, the extent of the crosstalk is not significant for the wild-type strain in physiologically significant settings.

Cross-activation between AraC-type regulators besides BenR/XylS has been reported (Ledger *et al.*, 2002; Carl and Fetzner, 2005), indicating a degree of promiscuity in respect to operator binding specificities in this family of transcriptional factors. Such a cross-binding of activators to non-discriminating regulatory sequences surely plays a role in the evolution of novel catabolic routes, as it allows primal regulation of genes acquired by horizontal transfer (de Lorenzo and Perez-Martin, 1996). Once a regulatory device has been recruited, it has to be further finely-tuned into the indigenous transcriptional and metabolic network for avoiding detrimental effects on the host physiology (Cases and de Lorenzo, 2001). This evolutionary step implies additional genetic modifications in regulators and/or regulatory sequences to achieve more specific interactions. Our results suggest that such an adaptation has occurred during the acquisition of the TOL pathway by *P. putida*. This notion is strengthened upon examination of the regulatory sequences involved. In fact, inspection of the DNA upstream of the *Pben* and *Pm* promoters (Fig. 7) provides an explanation on how the respective BenR and XylS transcriptional factors ended up controlling differentially each of their cognate gene clusters. The two DNA sequences at the top of the alignment of Fig. 7 show the organization of the regulatory regions of the *Pm* and the *Pben* promoters (Kessler *et al.*, 1994). Note that *Pm* has two operator sequences for XylS, one partially overlapping the -35 promoter motif (*proximal* site or *Om-p*), and a second upstream site (*distal* or *Om-d*). Each operator includes two conserved boxes (A and B), the modification of which eliminates responsiveness to XylS (Kessler *et al.*, 1993). Perusal of the corresponding *Pben* sequences in *P. putida* KT2440 reveals the presence in this promoter of 3 out of the 4 boxes that are required for physiological XylS binding. In particular, the A box of *Om-d* has lost any similarity to the TGCA sequence. The reconstitution of a full TGCA box in the *Om-d* operator of *Pben* enhances BenR inducer sensitivity

particularly in response to 3MBz (Silva-Rocha and de Lorenzo, 2012), revealing the key role of the A box in productive operator recognition by this transcriptional regulator. It is plausible that the lack of XylS affinity for *Pben* caused by the missing A box in the *Om-d* operator can be compensated by overproducing the regulator –as repeatedly shown in this work, but leaves the promoter inactive under physiological levels of the factor. As shown also in Fig. 7, the modification of the distal A box that plausibly makes the *Pben* promoter unresponsive to XylS is shared by other *P. putida* strains, e.g. those named Idaho (Tao *et al.*, 2011) and B6-2 (Tang *et al.*, 2011). These strains use methyl substituted aromatics such as *m*-xylene and *p*-xylene (Cruden *et al.*, 1992) or (methylated) carbazoles, (methylated) dibenzothiophenes, and (methylated) benzothiophenes (Tang *et al.*, 2011) as carbon source. These methylaromatics are most likely metabolized through methylcatechols and therefore would run into the same metabolic conflict as with *P. putida* mt-2. In contrast, other strains not known to deal with substituted aromatics keep the regulatory region of their *Pben* promoters very similar to that of *Pm*. These alignments suggest that *Pben* promoters impaired to respond to XylS prevail in strains evolved to degrade methylaromatics. The adaptation that has permitted the coexistence of somewhat incompatible pathways for degradation of Bz and 3MBz seems therefore to rely on a discrete change in the regulatory region of *Pben* that makes it to remain responsive to BenR but impervious to XylS. In contrast, both regulators can activate *Pm*, although with very different kinetics and dynamic parameters (Kessler *et al.*, 1994; Silva-Rocha and de Lorenzo, 2012). Such a lose-to-win scenario exemplifies how intricate metabolic problems can be solved through an unsophisticated regulatory solution that alternates the appearance of divergent pathways rather than merging the two routes or inactivating trouble-making enzymes. Also, such a simple genetic tinkering suggests strategies for orthogonalization of new pathways implanted in a pre-existing metabolic chassis by engineering the regulation in a fashion that prevents misrouting or unproductive catalysis (Mampel *et al.*, 2013).

EXPERIMENTAL PROCEDURES

Bacterial strains, culture conditions and general DNA methods

All *P. putida* strains were derived from *P. putida* mt-2 (ATCC 33015) or *P. putida* KT2440 (Bayley *et al.*, 1977; Franklin *et al.*, 1981). *E. coli* strains were routinely grown in Luria-Bertani (LB) medium at 37 °C. *P. putida* strains were grown at 30 °C either in LB or in M9 minimal medium (Sambrook and Russell, 2001)

supplemented with citrate (0.3 %), succinate (0.3 %) or Bz (5 mM) as carbon source. Aromatic compounds used as inducers (Bz, 3MBz, 3MBA, *m*-xylene, *o*-xylene) were all purchased from Sigma-Aldrich at the highest purity grade available. Where appropriate, antibiotics were added to the medium at the following concentrations (μg/ml): kanamycin, 50; gentamycin, 10; ampicillin, 150; tetracycline, 20; and streptomycin, 50. Plasmids were transferred to *P. putida* derivatives by electroporation using a previously described protocol (Choi *et al.*, 2006). Mutant strain *P. putida* mt-2 (pWW0-*xyIX::bla*) was generated by disruptive homologous recombination as follows. An internal region of *xyIX* gene was amplified by PCR using Go Taq DNA polymerase (Promega) and oligonucleotides *xyIX*-int-FW (5'CAGATTCCCGAGAAGAACGA3') and *xyIX*-int-RV (5'GTTCTCGACCTGCACTTTCC3'). This fragment was cloned using the pCR2.1-TOPO system (Invitrogen Life Technologies) to generate pTOPO-Δ*xyIX* plasmid that can only replicate in *E. coli*. When transformed into *P. putida* mt-2 by electroporation, this plasmid was integrated in pWW0 by homologous recombination that disrupted the target sequence. Mutant derivatives were selected on LB agar containing ampicillin (750 μg/mL) and correct recombinational insertion was confirmed by PCR. The pWW0-*xyIX::bla* plasmid was transferred by bipartite conjugation on membrane filters (MFTM, 0.45 μm, Millipore) to a *P. putida* KT2440 derivative having a *benR* gene inactivated by mini-Tn5 insertion (Km^R) obtained from a genome-wide mutant library of *P. putida* (Duque *et al.*, 2007; Molina-Henares *et al.*, 2010). After 8 h incubation at 30 °C on LB agar, the conjugation mixture was placed on minimal media plates containing kanamycin and ampicillin to select transconjugants of *benR* mutant harboring the pWW0-*xyIX::bla* plasmid.

Construction of transcriptional fusions

The assayed promoters were cloned in two different *luxCDABE* reporter vectors. For transcriptional fusions to be located in chromosome we used the pUC18-mini-Tn7T-Gm-lux plasmid (Gm^R) designed as a single-copy integration system (Choi *et al.*, 2005). The *Pben* and *Pm* promoters were PCR amplified with Pfu DNA polymerase (Promega) using the primers pairs *SacI*-*Pben*-FW (5'ATGGAGCTCACCTG GTAGCTGCAAAAGGA3') / *Bam*HI-*Pben*-RV (5'GTCGGATCCGCCAGGGTCTCCCTTGTTAT3') and *SacI*-*Pm*-FW (5'ATGGAGCTCGGCGACGTTCAAGAAGTAT3') / *Bam*HI-*Pm*-RV (5'GTCGGATCCTTGTTCTGTTGCATAAAGCCTA3'), and genomic DNA from *P. putida* mt-2 strain as template. These primers introduced *SacI* and *Bam*HI restriction sites (underlined) at the 5' and 3' ends, respectively. The PCR products were gel purified, digested with *SacI*/*Bam*HI and ligated to the pUC18-mini-Tn7T-Gm-lux vector

previously cut with the same restriction enzymes. The resulting plasmids were sequenced to check integrity and transferred to *P. putida* by co-electroporation with the helper plasmid pUX-BF13 as described by Choi *et al.* (2005). Primers Pput-glmSUP (5'AGTCAGAGTTACGGAATTGTAGG3') / PTn7L (5'ATTAGCTTACGACGCTACACCC3') and Pput-glmSDN (5'TTACGTGGCCGTGCTAAAGGG3') / PTn7R (5'CACAGCATAACTGGACTGATTC3') were used to check Gm^R colonies for integration of the reporter construct into the natural Tn7 insertion site of *P. putida* (Choi *et al.*, 2005). For constructing transcriptional fusions in the low-copy and broad-host range pSEVA226 vector (RK2 replication origin, Km^R; Silva-Rocha *et al.*, 2013), the *Pben* promoter was PCR amplified with Pfu DNA polymerase (Promega) using the primers PBF (5'TGGATGAATTCGACAGTACCCTCC3') and PBR (5'GCGCGGATCCGCCAGGGTCTCCCTTG3'). This procedure introduced *EcoRI* and *BamHI* restriction sites (underlined) at the 5' and 3' ends, respectively, of the amplified promoter. The PCR product was gel purified, digested with *EcoRI*/*BamHI* and ligated to pSEVA226 digested with the same enzymes. The resulting plasmid was named pSEVA226-*Pb* and its DNA re-sequenced for ensuring integrity. The equivalent reporter plasmid for the *Pm* promoter (pSEVA226-*Pm*) is described elsewhere (Silva-Rocha *et al.*, 2011). Both reporter plasmids were transferred independently to *P. putida* mt-2 by tri-parental mating with a helper *E. coli* strain (de Lorenzo and Timmis, 1994).

Promoter activity assays

For analyses of promoter activity, the *P. putida* derivatives harboring the different *Pben*- and *Pm*-*luxCDABE* transcriptional fusions were inoculated into 2 mL of M9 minimal medium supplemented with citrate (0.3 %) as carbon source and allowed to grow for 16 h (overnight). The cells were then diluted 20 times in the same fresh minimal media added with 1 mM Bz or 3MBz. 200 µL aliquots of thereby diluted cells were placed in 96-well microplates (Optilux™, BD Falcon) and analyzed in a WallacVictor II 1420 Multilabel Counter (Perkin Elmer Inc.). The optical density at 600 nm (OD₆₀₀) and the bioluminescence were recorded at time intervals of 15 min. Promoter activities were determined as the ratio between absolute bioluminescence and cell density and are given in relative units (RU). The values reported represent the maximal activity reached after at least 3-4 h of incubation.

RNA isolation and tiling microarray experiments

1 For RNA extraction, *P. putida* mt-2 strain was precultured overnight in M9 medium with succinate, diluted
 2 100-fold in the same medium and grown to $OD_{600} = 0.3$. Samples were then exposed for 2 h to vapors of
 3 *m*-xylene or *o*-xylene released by a 50% dilution of these aromatics in dibutylphthalate (a non-effector) in
 4 flask for 2 h. Alternatively, soluble substrates Bz (5 mM) or 3MBz (5 mM) were added to the cultures once
 5 the growth had reached $OD_{600} \sim 0.3$. As a reference, the same cells were grown for 96 h at 30 °C in M9
 6 medium. Once grown, samples were transferred to 1/10 volume of ice-cold ethanol/phenol solution (5 %
 7 phenol in ethanol) for preventing RNA degradation and harvested by centrifugation (3.800 rpm for 15 min
 8 at 4 °C). After aspiration of supernatant, the pellets were frozen in liquid nitrogen and stored at -80 °C,
 9 until required. Total RNA was extracted by using miRNeasy Mini kit (Qiagen) with some modifications.
 10 The collected pellets were resuspended into 0.3 ml Tris-HCl (pH 7.5) containing 2 mg/mL lysozyme and
 11 incubated for 10 min at 37 °C. Then 0.1 mL of the lysate was used in order to extract total RNA according
 12 to the manufacturer's instructions. RNase-free DNase (Qiagen) treatment was performed during the
 13 isolation procedure to eliminate the residual DNA. The quality of the RNA was evaluated by using a model
 14 2100 bioanalyzer (Agilent Technologies Inc.). For transcriptomic analysis, a customized tiling microarray
 15 chip was used consisting of 60-mers oligonucleotide probes designed to be spaced every 10 bp and
 16 overlapping 50 nucleotides, covering the wholeness of target genes (Kim *et al.*, in preparation). Total RNA
 17 (20 µg) of each sample was retrotranscribed and aminoallyl-labeled using SuperScript Indirect cDNA
 18 Labeling System (Invitrogen) and 5-(3-aminoallyl)-2'deoxyuridine-5'-triphosphate (aa-dUTP, Ambion)
 19 following the manufacturer's instructions. To avoid antisense artifacts of second-strand cDNA during
 20 reverse transcription, actinomycin D was added after the denaturing step at 70°C to a final concentration
 21 of 6 µg/mL, as described (Perocchi *et al.*, 2007). For each sample, aminoallyl-labeled cDNA was
 22 resuspended in 0.1 M Na_2CO_3 (pH 9.0) and conjugated with either Cy3 or Hyper 5 Mono NHS Ester
 23 (CyTMDye Post-labelling Reactive Dye Pack, Amersham), following a dye-swap strategy. The samples
 24 were purified with Megaclear™ (Ambion) following the manufacturer's instructions. Cy3 and Hyper 5
 25 incorporation was measured in a Nanodrop spectrophotometer (Nanodrop Technologies Inc.).
 26 Preparation of probes and hybridizations were performed as described (Two-Color Microarray-Based
 27 Prokaryote Analysis, Agilent Technologies). The samples were placed on ice and quickly loaded onto
 28 arrays, hybridized at 65 °C for 17 h and then washed once in GE wash buffer 1 at room temperature (1
 29 min) and once in GE Wash Buffer 2 at 37 °C (1 min). Arrays were drained by centrifugation at 2000 rpm
 30 for 2 min. Images from Cy3 and Hyper5 channels were equilibrated and captured with a GenePix 4000B
 31 scanner (Axon) and spots quantified using GenPix software (Axon). After scanning, both datasets were

analyzed using the same methods: raw intensities were background-corrected by *normexp* method with an offset of 50 (Smyth and Speed, 2003). Background-corrected intensities were converted to log₂ scale and normalized by adjusting the quartiles of all replicates as described in Bolstad *et al.* (2003). After normalization, differential expression for every probe was calculated as log₂Ratios = log₂Intensity (experimental condition) - log₂Intensity (reference condition). Integrated Genome Browser (IGB; Nicol *et al.*, 2009) was used to represent the log₂Ratios of all probes along the particular regions.

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FIGURES

Figure 1. Genetic, regulatory and biochemical connections of 3-methylbenzoate (3MBz) catabolism in *P. putida* mt-2.

3MBz can be available either exogenously or generated intracellularly through biodegradation of *m*-xylene by the *upper* TOL pathway. The figure sketches both alternative metabolic itineraries involved in the further biodegradation of 3MBz, i.e. the *xyl* genes of the lower TOL operon (top), which are expressed from the *P_m* promoter activated by XylS, and the chromosomal *ben* genes (bottom) that are transcribed from promoter *P_{ben}* upon activation by the XylS homologue BenR. Both the *xyl* and the *ben* routes make 3MBz to converge towards methylcatechol, but then diverge at that point: this compound can be subject to extradiol cleavage by XylE or intradiol fission by CatA and CatA2. This last reaction originates the dead-end intermediate 2-methyl-2-enelactone. Note that the action of the chromosomally encoded benzoate 1,2-dioxygenase on 3-methylbenzoate may produce also a small fraction of 4-methylcatechol (Knackmuss *et al.*, 1976), which becomes efficiently converted by CatA/CatA2 into the equally toxic compound 4-methyl-2-enelactone (not shown). The metabolic conflict is exacerbated if the TOL substrate were *p*-xylene, as it is converted to 4-methylbenzoate, then exclusively to 4-methylcatechol by the *ben/xyl*-encoded enzymes and further to the same toxic enelactone by CatA / CatA2.

Figure 2. Activity of *Pben* and *Pm* promoters as measured by transcriptional *luxCDABE* fusions located in low-copy plasmids harbored by *P. putida* mt-2.

Strains bearing reporter plasmids pSEVA226-*Pb* and pSEVA226-*Pm* were pregrown overnight in M9 medium, diluted 1:20 in the same fresh medium, added with 1 mM of each of the aromatic inducers indicated or kept as untreated control (Nil), and light emission recorded after 4 h. Promoter activities in each condition are expressed as relative units (RU: luminiscence / OD₆₀₀). Error bars represent standard deviation of a minimum of 3 experiments. Nil, untreated condition

Figure 3. Transcriptomic profile of *xylS* (a) and the *Pm* (*xylX*) (b) and *Pben* (*benA*) (c) regions of *P. putida* mt-2.

The panels show the signals from a high-density tiling array hybridized with RNA extracted from *P. putida* exposed to *m*-xylene, *o*-xylene, Bz, 3MBz and succinate as indicated. The bars express relative level of each mRNA segment in respect to the reference condition (i.e. terminal growth of *P. putida* mt-2 on succinate, see Experimental procedures). The horizontal axes indicate the extent of the genomic region of interest covered by the subset of oligonucleotides of the tiling array. (a) Entire *xylS* gene. (b) 5' untranslated region and leading 50 bp of the *xylX* coding region. (c) 5' untranslated region and leading 35 bp of the *benA* coding region. The vertical axes correspond to the Log₂ of such a ratio. Note different scales of the Y values in each case.

Figure 4. Activity of *Pben* (a,b) and *Pm* (c,d) promoters measured by transcriptional *luxCDABE* fusions located in chromosome of a *P. putida* KT2440 *benR* mutant and entered with different *xyIS*-encoding plasmids.

Strains were grown overnight in M9 medium with 0.3 % citrate, diluted 1:20 in the same medium and either added with 1 mM of each of the inducers or kept untreated (Nil). Light emission was recorded after 3-4 h, on the basis of which promoter activities were determined for each condition, calculated as relative luminiscence units (RU). Error bars indicate standard deviations of a minimum of 3 experiments. In (a) and (c) each *xyIS*-encoding plasmid provides a different level of XylS: wild-type copy number (pWW0), low-copy number (pJB653) and medium-copy number (pSEVA438). In (b) and (d), the pVLT43 plasmid carries the *xyIS* gene with an improved translation initiation region and under the control of the IPTG-inducible *Ptac* promoter, thereby providing high intracellular levels of XylS upon induction.

Figure 5. Activity of *Pben* and *Pm* promoters measured by transcriptional *luxCDABE* fusions located in chromosome of a *P. putida* KT2440 *benR* mutant bearing the pWW0 plasmid.

Strains were grown overnight in M9 medium with 0.3 % citrate, diluted 1:20 in the same medium and added with 1 mM of 3-methylbenzyl alcohol (3MBA) or exposed to vapors of *m*-xylene released by a 50% dilution in dibutylphthalate. The untreated condition is also shown (Nil). Light emission was recorded after 3-4 h, on the basis of which promoter activities were determined for each condition as relative units (RU). Error bars indicate standard deviations of a minimum of 3 experiments.

Figure 6. Growth curves of a *P. putida* KT2440 *benR* mutant supplemented with different *xyIS*-encoding plasmids when cultured on Bz (5 mM) as the sole carbon source.

Cultures of each strain were pregrown on succinate 0.3% and diluted 100X in Bz-containing medium. The mean values of the OD₆₀₀ of duplicate cultures are shown.

Figure 7. Comparative analysis of the *Pben* promoter region in different *P. putida* strains.

The structure of the *Pm* promoter of the pWW0 plasmid is shown on top as a reference. The predicted distal (*Om-d*) and proximal (*Om-p*) operators for the cognate transcription factors are each formed by two conserved boxes (labeled as A and B) as indicated. Additional features such as the -10/-35 boxes and the predicted transcriptional start sites (+1) are shown.