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Insight box

Environmental bacteria with a history of exposure to chemical pollutants often carry metabolic operons that become transcribed when cells meet unusual recalcitrant and/or xenobiotic compounds. Prokaryotic transcriptional factors that recognize such molecules as effectors for activating cognate promoters become the starting point for development of biosensors useful for environmental monitoring or as components of synthetic biology circuits. Yet, the parameters that control naturally occurring regulatory nodes are frequently inadequate to meet the specifications that are needed for given genetic constructs. Predictions made by a simple model elaborated with available data guided us to exacerbate experimentally the activity of the *m*-xylene responsive and sigma-54 dependent promoter *Pu* of the soil bacterium *Pseudomonas putida* by genetic rewiring of the 3 key constituents of the control system. This opens the way to engineering better whole-cell sensors for small molecules.

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3 **Rationally rewiring the connectivity of the XylR/*Pu* regulatory node of the**
4 ***m*-xylene degradation pathway in *Pseudomonas putida***
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6 by
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1 **SUMMARY**
2

3 The XylR/*Pu* regulatory node of the *m*-xylene biodegradation pathway of *Pseudomonas putida* mt-2 is
4 one of the most intricate cases of processing internal and external cues into a single controlling element.
5 Despite this complexity, the performance of the regulatory system is determined *in vivo* only by the
6 occupation of *Pu* by *m*-xylene-activated XylR and σ^{54}-RNAP. The stoichiometry between these three
7 elements defines natural system boundaries that outline a specific functional space. This space can be
8 expanded artificially following different strategies that involve either the increase of XylR, σ^{54} or both
9 elements at the same time (each using a different inducer). In this work we have designed a new
10 regulatory architecture that drives the system to reach a maximum performance in response to one
11 single input. To this end, we first explored with a simple mathematical model whether the output of the
12 XylR/*Pu* node could be amended by increasing simultaneously of σ^{54} and XylR in response only to
13 natural inducers. The exacerbation of *Pu* activity *in vivo* was tested in strains bearing synthetic
14 transposons encoding *xylR* and *rpoN* (the σ^{54} coding gene) controlled also by *Pu*, thereby generating a
15 *P. putida* strain with the XylR/*Pu* output controlled by two intertwined feed forward loops (FFLs). The
16 lack of a negative feedback loop in the expression node makes *Pu* activity to reach its physiological
17 maximum in response to a single input. Only competition for cell resources might ultimately check the
18 upper activity limit of such a rewired *m*-xylene sensing device.
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1 **INTRODUCTION**
2
3 Regulatory networks have been revealed as complex webs of interacting molecular components, which
4 can adopt different conformations[1]. In fact, the shape and the strength of the network components
5 contribute to define and fine-tune the response[2,3]. Bacterial promoters are key elements of these
6 regulatory networks. They integrate physiological and environmental signals triggering gene
7 transcription demarcated by a specific functional space. This space is usually constrained by a number
8 of parameters defined during the evolution of the regulatory system[4]. A goal of Synthetic Biology is to
9 reprogram signal processing pathways by rearranging the regulatory nodes, to generate predictable and
10 beneficial applications[5]. Sometimes this entails strategies to amplify the output of specific regulatory
11 system by expanding its natural boundaries[6,7]. In general, the approaches employed to optimize the
12 output, rely on the introduction of exogenous modules in the cell that act as amplifiers[8,9]. Nevertheless,
13 our previous work revealed that it is possible to amplify the signal of a specific system without
14 introducing non-native elements, just by rewiring the host regulatory network[6]. Although amplification
15 devices can increase the response of a specific regulatory system, only by simultaneous removal of
16 extant physiological constrains it is possible to reach its maximum potential[4].
17
18 We have examined these questions using the *Pu* promoter of the environmental bacterium
19 *Pseudomonas putida* mt-2 as the preferred experimental system. This promoter is one of the most
20 intricate cases of processing internal and external cues into a single regulatory element[10-12]. *Pu* and the
21 various factors it interacts with belong to a complex metabolic and regulatory network that determines a
22 pathway for biodegradation of *m*-xylene borne by the TOL plasmid pWW0 of this bacterium[4,13]. This
23 pathway encompasses two catabolic operons, which are subject to a complex regulatory circuit that
24 involves the interplay between various transcription factors[14,15]. XylR is the main transcriptional regulator
25 that controls the system[15]. This regulator, in the presence of its natural inducers (*m*-xylene, 3-
26 methylbenzylalcohol) triggers the *Pu* and *Ps* promoters driving the expression of both, the catabolic
27 genes of the *upper* TOL operon and *xylS* respectively[16]. The activation of the *Ps* promoter not only
28 produces XylS, the second regulator of the system, but also leads to repression of *xylR* expression due
29 to the divergent disposition of *Ps* and the *xylR* promoter (P_R)[17]. XylR acts in concert with the RNA
30 polymerase (RNAP) containing the alternative sigma factor σ^{54} [15,18] sitting both at distant places of the
31 DNA sequence *Pu* promoter. With the assistance of the DNA-bending factor IHF (integration host

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1 factor[19, 20]) they form a tridimensional transcription initiation complex. Yet, the one sufficient condition for
2 full promoter performance *in vivo* is the complete occupation of *Pu* by *m*-xylene-activated XylR and σ^{54}-
3 RNAP[4]. Based on this fact we have demonstrated that is possible to increase the output of the system
4 enhancing the levels of both XylR and σ^{54}-RNAP individually or, in combination[4,6]. Nevertheless, the
5 approach that we reported involved two input signals: one to increase XylR amount and another one to
6 trigger the heterologous system responsible for the overexpression of σ^{54} RNA subunit. As this strategy
7 was of considerable interest for designing *e.g.* whole cell biosensors, and other heterologous expression
8 devices, we wondered if it was possible to re-design it and achieve the same optimized output in
9 response to one single signal. To this end, we first explored with a simple mathematical model whether
10 the output of the XylR/*Pu* device could be increased further by simultaneously increasing σ^{54} and XylR
11 levels in response to single TOL pathway inducers. For testing the predicted outcomes *in vivo,* we
12 constructed transposon vectors encoding *xylR* and *rpoN* (the σ^{54} coding gene) controlled by *Pu* and we
13 used them to generate a strain with the XylR/*Pu* node output controlled by two (positive) feed-forward
14 loops (FFLs). The results show that it is possible to magnify *Pu* output by implementing two intertwined
15 FFLs with *xylR* and *rpoN* which, by changing the stoichiometry between the key regulatory elements,
16 expand the extant functional boundaries of the system.
17
18 **RESULTS AND DISCUSSION**
19
20 **Rationale for expanding the functional boundaries of the XylR/*Pu* regulatory node**. Our previous
21 results proved that it is possible to expand natural functional space of the XylR/*Pu* system by changing
22 the boundaries imposed by the two elements that control *Pu*: σ^{54} and XylR[4]. The native XylR regulatory
23 scenario defines the limits of the *Pu* output by adjusting the XylR molecules number with a negative
24 feedback loop (NFL[17]). Also this output is limited by the defined number of σ^{54}-containing species in the
25 whole RNAP pool available for *Pu* binding (Fig. 1a). In previous works we followed different strategies to
26 increase the response of the system. Our first approach (Fig. 1b) focused on an induction-dependent
27 augmentation of XylR molecules by reshaping the *xylR* architecture and replacing the natural NFL
28 mediated by P_R promoter by a positive FFL generated by placing *xylR* under the control of *Pu*[6]. On the
29 other hand, we engineered the system to increase the number of σ^{54} RNA polymerase subunit
30 molecules by using a heterologous expression system dependent of an external inducer (Fig. 1c). Both
31 approaches led to a similar increase of the output of the system. Only by combining both strategies (Fig.

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1d) was possible to fill completely the potential functional space defined by these two elements. However, in these experiments[4] we used a salicylate-dependent heterologous expression system to overproduce the σ^{54} subunit of RNA polymerase, making necessary the use then of both external inputs (natural XylR inducers and salicylate) to lead to system to its optimal response. Besides, the requirement of another inducer for increasing σ^{54} levels could interfere with expression of other genes of the regulon[21]. On this basis, we set out to redesign the connectivity of the components shown in Fig. 1d to eliminate the need of an extra inducer -other than the specific effectors of XylR.

Optimized XylR/*P*u performance in response to a single input can be achieved with two intertwined positive FFLs. To explore the scenario mentioned before, we designed a circuit where *Pu* promoter was controlling not only the expression of *xylR* but also overexpression of σ^{54}. In this situation two FFLs cooperate to increase the amount of both XylR and σ^{54} upon induction of the system with e.g. *m*-xylene (Fig. 2c). In order to formalize this regulatory scheme we first simulated the performance of *Pu* after the induction of the system in two scenarios [i] *Pu* controls expression of *xylR* but σ^{54} levels are left constant (native levels, Fig. 2a,b) and, [ii] same *xylR* regulatory architecture but added with an extra copy of *rpoN* (encoding σ^{54} factor) controlled also by *Pu* (Fig. 2c,d). The readout of either architecture is *Pu* promoter activity (a parameter that can be measured, see below). As shown before, the first scenario predicts that addition of the aromatic inducer raises the XylR levels and therefore the output of the system (Fig. 2b[6]). The situation changes when an extra copy of *rpoN* controlled by *Pu* is introduced into the simulation (Fig. 2c). The model then predicts that the system output (i.e. transcriptional *Pu* activity) will be amplified because of two convergent effects: [i] the augmentation of the sigma factor after induction enlarges the share of σ^{54}-containing RNAP for *Pu* binding, thereby increasing its own expression and, [ii] there will be a further increase of XylR levels due to the strengthening of the *Pu* promoter. This arrangement generates two autonomous but linked positive feedback loops: one controlling the expression of *xylR* and another one enhancing the expression of σ^{54}, both triggered and sustained by exposure to a single aromatic inducer. As shown below, these predictions were examined in detail by following emission of bioluminescence by a *Pu-luxCDABE* reporter system as well as monitoring XylR and σ^{54} levels *in vivo* with specific antibodies for each protein.

Simultaneous increase of σ^{54} and XylR levels in response to *Pu* inducer *m*-xylene. In order to test our model we engineered a mini-Tn*5* transposon determining transcription of the *rpoN* gene under the

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control of *Pu* (Fig. 3a). The transposon Tn*5* [Pu•RpoN] (Fig. 3a, module 3) was then delivered to the chromosome of the previously described *P. putida* Pu•RBX strain[6] bearing in its chromosome transcriptional fusions *Pu→luxCDABE* (Fig. 3a, module #1) and *Pu→xylR* (Fig. 3a, module 2). The resulting strain (*P. putida* Pu•RpoN•Pu•RBX) thus bears an extra copy of *rpoN* transcribed from *Pu* (and thus sensitive to XylR-mediated induction with *m*-xylene) besides the native *rpoN* gene present in the extant genomic location. In order to test whether this new regulatory architecture raised intracellular σ^{54} and XylR concentrations we grew both strains in LB and we exposed them or not to saturating vapours of *m*-xylene. After induction, protein extracts from each strain were prepared at different time points and levels of the σ^{54} factor and XylR were examined in Western blot assays (Fig. 3b) with recombinant antibodies[22] either against σ^{54} or XylR[23]. The results of Fig. 3b showed an increase of the σ^{54} molecules 3 hours after the induction of strain *P. putida* Pu•RpoN•Pu•RBX with *m*-xylene in respect to the one lacking the *Pu→rpoN* module (Fig. 3b, upper panel). Concerning XylR contents, both strains showed an increase after *m*-xylene (Fig. 3b lower panel) induction in accordance with the results predicted using the model (Fig. 2b) regarding the presence in both strains of the module *Pu→xylR*. Nevertheless, the augmentation of XylR in *P. putida* Pu•RpoN•Pu•RBX was higher than the one observed in *P. putida* Pu•RBX strain due to the effect of the overexpression intracellular σ^{54}. These data confirmed that it was possible to obtain increased levels of both XylR and σ^{54} by implementing the regulatory architecture of Fig. 2d in which expression of both proteins is magnified in response to a single input. But how does this translate in actual performance of *Pu* promoter activity?

Effect of co-occurrent rise of XylR and σ^{54} in transcriptional in *Pu* readout. *P. putida* strains Pu•RBX and Pu•RpoN•Pu•RBX were tested in parallel for *Pu* activity using light emission stemming from their *Pu→luxCDABE* insert as a proxy of transcription initiation. The data shown in Fig. 4a revealed that *Pu* output in the *P. putida* strain with *m*-xylene inducible expression of *rpoN* increased after 5 h by about twofold when compared to the reference strain *P. putida* Pu•RBX. These experimental figures were consistent with the predictions of Fig. 2, in which (within a certain parameter set), increasing σ^{54} on top of the already exacerbated XylR was expected to augment *Pu* output by a factor ~2. To examine whether this improved responsiveness was dependent on having a prime effector of the XylR protein (ie. *m*-xylene) or could be maintained also with a suboptimal inducer we recorded light emission of *P. putida* strains Pu•RBX and Pu•RpoN•Pu•RBX along time but using 3-methylbenzyl alcohol (3MBA) instead of *m*-xylene as the aromatic inducer. The results are shown in Fig. 4. For a

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1 more rigorous comparison of the two conditions fold-induction in respect to non-inducing conditions
2 (rather than specific luminescence) was plotted *vs.* time. While *P. putida* Pu•RBX displayed an
3 inducibility of 60-80 fold, the strain carrying the *Pu*→*rpoN* module (*P. putida* PuRpoN•Pu•RBX) reached
4 ~ 120-fold at the peak of its activity. Note, however that light emission caused by 3MBA did not start
5 taking off until 6 h after inducer addition (in contrast with the much earlier response to *m*-xylene, Fig.
6 4a). Since the regulatory architecture of Fig. 2c sets *Pu* activation by XylR to happen earlier than σ^{54}
7 overproduction, it is possible that a less efficient inducer delay accumulation of both factors until they
8 reach a critical level. But once this happens (by 5-6 h after induction in Fig. 4b), the same architecture
9 causes a much faster induction rate.
10
11 **Outlook.** The work above shows that one can amplify dramatically the net transcriptional activity of the
12 *Pu* promoter of the TOL plasmid in response to *m*-xylene by rationally rewiring the connectivity of its key
13 components: the *Pu* promoter proper, XylR and σ^{54}. This is in contrast to habitual approaches with the
14 same purpose, which typically rely on either generation of mutants in the promoter DNA or in the amino
15 acid sequence of the cognate transcriptional factors. In our case we have re-connected the constituents
16 by means of two intertwined positive FFLs that deliver high amounts of the two limiting proteins in a self-
17 activation fashion. Simultaneous escalation of both the signal-specific (XylR) factor and one or more
18 global regulatory components (the σ^{54} in our case), are likely to take this promoter to its maximum
19 possible transcriptional activity *in vivo.* In reality, as the circuit lacks any restraining feedback loop, once
20 the forward cascade of Fig. 2c get started upon *m*-xylene induction the engineered regulatory node
21 cannot but amplify itself over time. But eventually, the hyper-activity of this σ^{54}-dependent system is
22 likely to reach its ceiling by competing for the host's gene expression machinery. This may occur by [i]
23 displacing other sigma factors out of the RNAP pool and/or [ii] draining the metabolic currency that fuels
24 the synthetic implant. Current efforts try to tackle this problem (named *retroactivity*) with additional
25 genetic isolation devices[24,25] so that the functioning of the genetic constructs has a minimal influence in
26 the physiology and viability of the host.
27
28 **EXPERIMENTAL PROCEDURES**
29
30 **Strains, culture conditions, and general procedures.** *P. putida* strains used in study are derivatives
31 of the reference strain KT2440 inserted with various combinations of the genetic cassettes indicated in

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1 each case. *E. coli* CC118λ*pir* was used as the host for propagating plasmids based on a R6K origin of
2 replication[26]. Bacteria were grown in Luria-Bertani (LB) medium. When required, the media was
3 amended with specified concentrations of 3-methylbenzylalcohol (3MBA) or *m*-xylene vapours.
4 Antibiotics were used at the following concentrations: piperacilin (Pip) 40 μg/ml, chloramphenicol (Cm)
5 30 μg/ml, gentamycin (Gm) 10 μg/ml, Streptomycin (Sm) 50 μg/ml, and potassium tellurite (Tel) at 80
6 μg/ml. For PCR reactions, 50-100 ng of the DNA template indicated in each case was mixed in a 50 μl
7 mixture with 0.2 μM of each of the primers specified and 2.5 units of Pfu DNA polymerase (Stratagene).
8 Samples were then subject to 30 cycles of 1 min at 95°C, 30 sec at 58°C and 1 min at 72°C. Clones
9 were first checked by colony PCR [27] using 1.25 units Taq DNA polymerase (Roche) and later confirmed
10 by DNA sequencing. Other gene cloning techniques and Molecular Biology procedures were carried out
11 according to standard methods[27].
12
13 **Bioluminescence assays**. *P. putida* strains were first pre-grown in test tubes overnight in LB media at
14 30ºC. Then they were diluted to an OD_{600} of 0.05 in 100 ml flasks and cultured to an OD_{600}= 1.0. At that
15 point they were exposed, where indicated, to diverse amounts of *m*-xylene vapours or 1 mM 3MBA.
16 Then, at the indicated time points, 200 μl aliquots of the cultures were placed in 96 well plates (NUNC)
17 and light emission and OD_{600} were measured in a Victor II 1420 Multilabel Counter (Perkin Elmer). The
18 specific bioluminescence values were calculated by dividing the obtained values of total light emission
19 (in arbitrary units) by the ones that reflect the optical density of the culture (OD_{600}). The specific
20 bioluminescence values shown represent the average of at least three biological replicates.
21
22 **Protein techniques.** Whole-cell protein extracts were prepared by pelleting the cells (10,000 × *g*, 5 min)
23 from 1 ml of LB cultures and re-suspending them in 50 μl Tris HCl 10 mM pH 7.5 and then 50 μl of 2×
24 SDS-sample buffer (Tris–HCl 120 mM pH 6.8, SDS 2%, w/v, glycerol 10%, v/v, bromophenol blue
25 0.01%, w/v, 2-mercapto-ethanol 2%, v/v). After resuspension, samples were boiled for 10 min,
26 sonicated briefly (~5 s) and centrifuged (14,000 × *g*, 10 min). Samples with thereby prepared extracts
27 equivalent to ~10^8 cells/lane were loaded in SDS-PAGE gels (Miniprotean system, Bio-Rad). Following
28 electrophoresis, gels were transferred to polyvinylidene difluoride membranes using a semi-dry
29 electrophoresis transfer apparatus (Bio-Rad). Membranes were next blocked for 2 h at room
30 temperature with MBT buffer (0.1% Tween and 5% skimmed milk in phosphate-buffered saline, PBS).
31 For inmmunodetection of XylR, phage-based antibodies (Phab[23]) were used following the method

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1 described before[6]. For immunodetection of σ^{54}, we used the recombinant antibody scFv C2[22] according
2 to a previously described protocol[4].
3
4 **Mathematical methods.** The simple models (*toy models*) presented in this work were made by setting
5 a number of ordinary differential equations describing the TOL control network. Simulations and other
6 calculations were done with MATLAB®. (See Supplementary Information for further details).
7
8 **Genetic constructs.** The transposon bearing a *Pu-luxCDABE* reporter system (which is present in all
9 the strains used in this study) used to engineer *P. putida* PuLUX has been described before[6]. Also the
10 mini-Tn7 derivative bearing a cassette expressing *xylR* wild type version under the control of its native
11 *Pu* promoter and the *P. putida* strain engineered with it *P. putida* Pu•RBX (i.e., subject to a XylR self-
12 amplifying loop[6]) is described in a previous work. pTn5 [Pu•RpoN] construct for *Pu* dependent
13 overexpression of the σ^{54} sigma factor was engineered using pUT mini-Tn5 Sm/Sp [26] as the assembly
14 vector as follows: a 238 bp fragment containing the *Pu* promoter was amplified with the primers Pu8F
15 (*Xba*I) (gcTCTAGACCCGGGAAAGCGCGATGA) and Pu9R (*BamH*I)
16 (cgcGGATCCTGAAGGGTCACCACTATTTTT) using pMAD plasmid as template[12]. This fragment was
17 then cloned *Xba*I/*Bam*HI into pUC18Not[26] rendering pPu2. Then a 1551 bp fragment containing the
18 *rpoN* gene was obtained by PCR using RpoN 7F (gcGGATCCTTACACTTAGTTAAATTGCTAAC) and
19 RpoN 5R (GgGGTACCCTACATCAGTCGCTTGCGTT) primers and pTn5 [Psal•RpoN] as template [4]
20 and inserted into pPu2 as a *Bam*HI/*Kpn*I fragment generating pPuRpoN. Finally to construct pTn5
21 [Pu•RpoN] a *Not*I fragment containing the *Pu-rpoN* fusion was excised from pPuRpoN and cloned into
22 pUTmini-Tn5 Sm/Sp. This pTn5 [Pu•RpoN] was then mobilized into the *P. putida* Pu•RBX, generating *P.*
23 *putida* Pu•RpoN•Pu•RBX.
24
25 **Plasmid transfer and mini-transposon delivery into *P. putida*.** Plasmids and transposons were
26 conjugally passed from the donor *E. coli* strain indicated in each case into the different *P. putida*
27 recipients with a filter mating technique[26]. To this end, a mixture of donor, recipient and helper strain *E.*
28 *coli* HB101 (pRK600) was laid on 0.45 μm filters in a 1:1:3 ratio and incubated for 8 h at 30°C on the
29 surface of LB-agar plates. Mini-Tn7 derivatives were co-mobilized along with the transposase-encoding
30 genes *tnsABCD* into the recipient strains by including *E. coli*CC118 λ*pir* (pTNS1[28]) in the mating
31 mixture. After incubation, cells were resuspended in 10 mM $MgSO_4$ in either case, and appropriate

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1 dilutions plated on M9/succinate amended with suitable antibiotics for counter-selection of the donor and
2 helper strains and growth of the *P. putida* clones that had acquired the desired plasmids or insertions.
3 *Bona fide* transposition was verified in every case by checking the sensitivity of individual exconjugants
4 to the delivery vector marker, piperacillin.
5
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9
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1 **Table 1.** Strains and plasmids

2

Strain or plasmid(s)	Relevant characteristics	Reference
Strains		
E. coli CC118 λ*pir*	CC118 lysogenized with λ*pir* phage	[29]
E. coli DH5α	routine cloning host strain	[27]
P. putida Pu•LUX	*P. putida* strain carrying in the chromosome a *Pu-luxCDABE* fusion	[6]
P. putida Pu•RBX	*P. putida* strain carrying in the chromosome a *Pu-luxCDABE* fusion and *xylR* under the control of *Pu* (positive feedback loop)	[6]
P. putida Pu•RpoN•Pu•RBX	*P. putida* Pu•RBX carrying *rpoN* under the control of *Pu* promoter	This study
Plasmids		
RK600	Cm^R; ColE1*oriV* RK2*mob*+ *tra*+	[30]
pUT/mini-Tn5 Sm/Sp	Mini-Tn5 Sm/Sp delivery plasmid	[31]
pUC18Not	pUC18 with NotI sites flanking the polylinker	[29]
pPu2	pUC18Not containing the *Pu* promoter	This study
pPuMRpoN	pUC18Not containing a fusion *Pu-rpoN*	This study
pTn5 [Pu•RpoN]	mini-Tn5 delivery vector carrying the *Pu* promoter controlling *rpoN* expression.	This study
pPu•RBX	pUC18NotI carrying a *Pu-xylR*fusion	[6]
pTn7-PuRBX	mini-Tn7 delivery vector carrying a *Pu-xylR*fusion	[6]

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1 **Figure legends**

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3 **Figure 1.** Relational scheme of the key components of the natural and synthetic regulatory architectures

4 of the XylR/*Pu* regulatory node.

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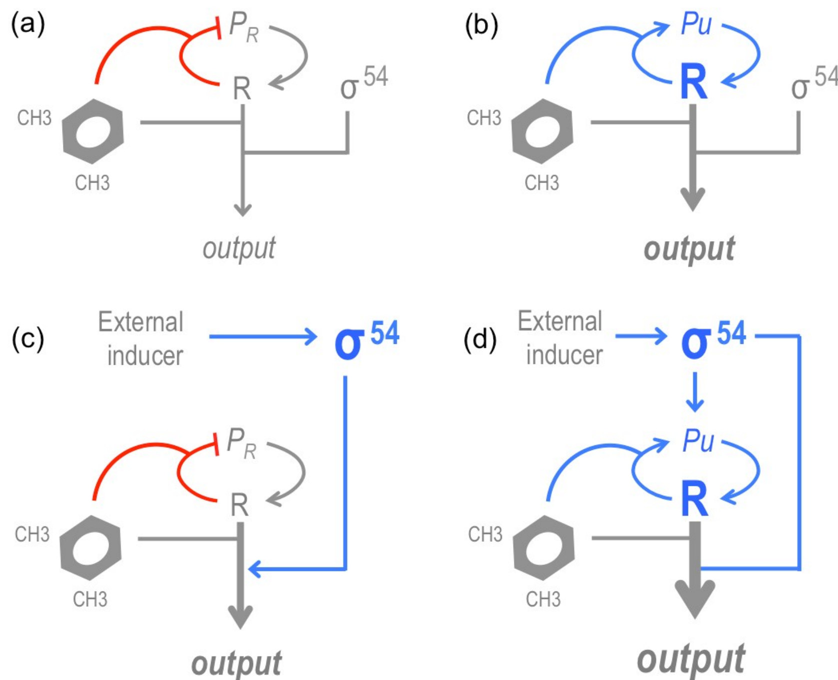

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8 (a) Natural regulatory architecture of XylR/*Pu* node: in the presence of *m*-xylene XylR (R) activates *Pu*

9 (output) and inhibits its own expression via P_R. In this natural configuration, σ^{54} is a necessary factor for

10 expression of *Pu* but its input comes separately from the rest of the components. (b) Synthetic amplifier

11 of *Pu* performance based on changes of XylR amount: in the presence of *m*-xylene, XylR (R) both turns

12 *Pu* on (output) and self-activates its expression through the *Pu* promoter also. In this configuration, the

13 natural amount of σ^{54} also contributes to the final output. (c) Synthetic amplifier of *Pu* performance

14 (output) based on changes of σ^{54} amount: in the same native regulatory architecture described before, it

15 is possible to modify the output of the system by increasing the amount of σ^{54} with an external inducer.

16 (d) Finally, rearrangement of the XylR/*Pu* node combining both synthetic amplifiers.

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1 **Figure 2.** Modeling the reshaped XylR/*Pu* regulatory node with alternative configurations of σ^{54}
2 expression where two intertwined positive feedback loops influence the output of the system.
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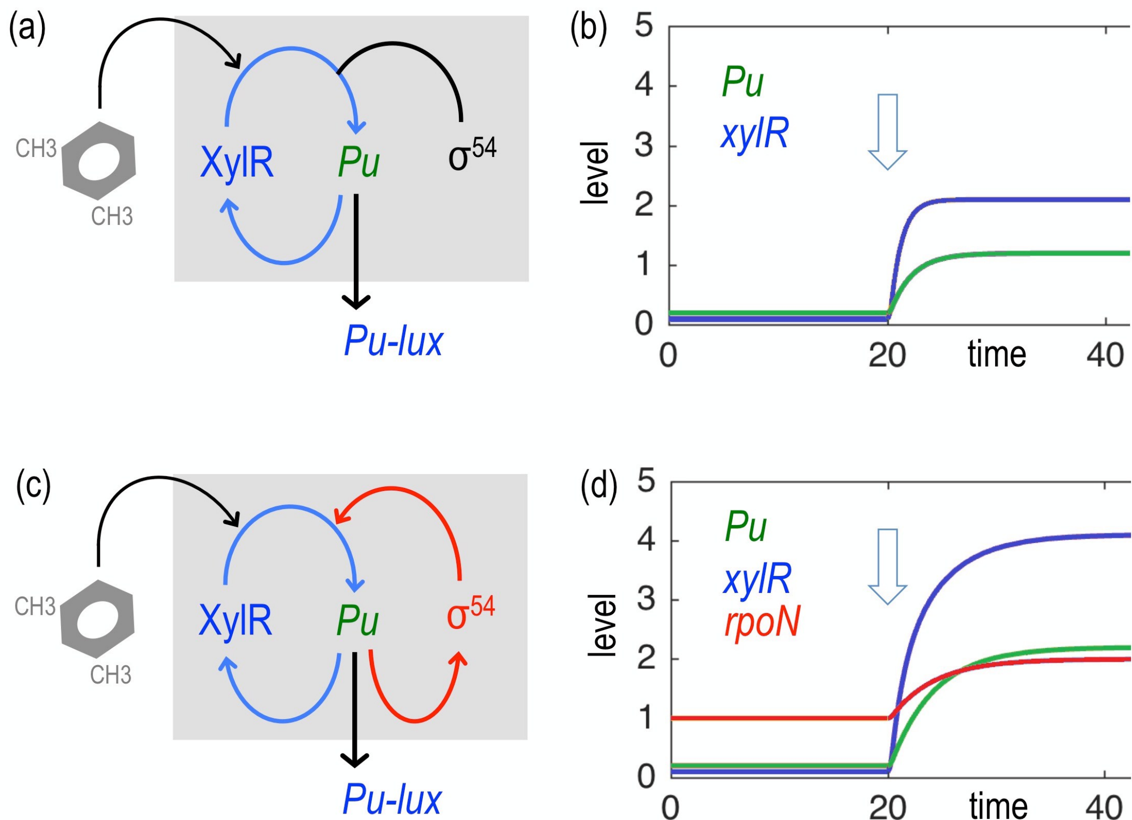

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5
6 (a) Relational map of reference: in the presence of *m*-xylene, XylR self-activates its expression but the
7 levels of σ^{54} are kept constitutive (and thus not represented as a variable in the system). (b) Dynamic
8 model. Arrows signals the moment of induction by *m*-xylene. (c) Alternative regulatory configuration in
9 which *m*-xylene causes *Pu* to activate *xylR* and σ^{54} expression. Augmentation of σ^{54} intensifies its own
10 expression and therefore the amount of XylR and *Pu* activity in a positive merge of two FFLs. (d)
11 Dynamic model.
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1 **Figure 3:** Augmentation of XylR and σ^{54} in the XylR/*Pu* regulatory system.

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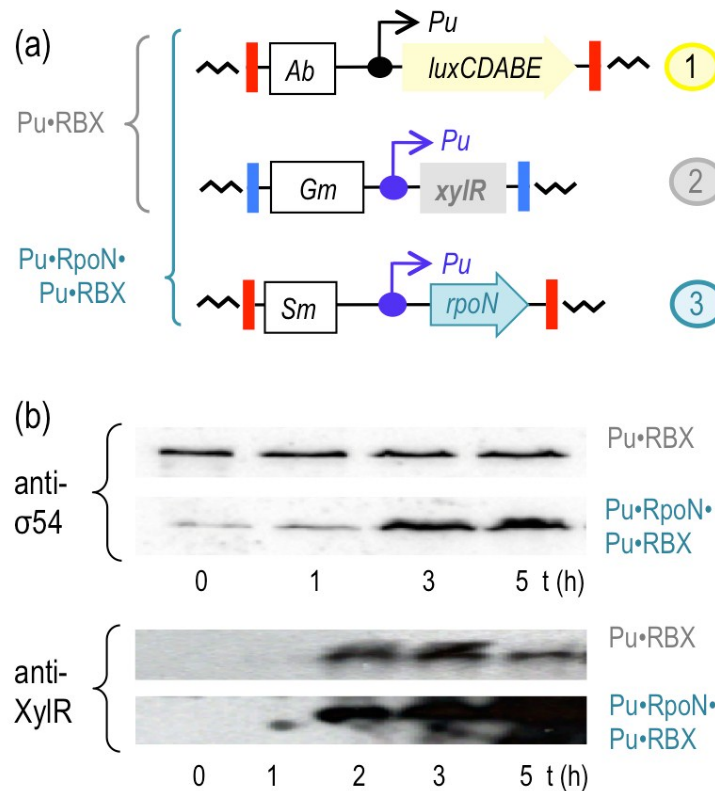

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5 (a) Genetic constructs. The figure shows a sketch (not to scale) of the genetic modules born by the *P.*
6 *putida* strains used in the experiment: The *Pu-luxCDABE* reporter (module #1) has a promoterless
7 luminescence-determining operon controlled by the *Pu* promoter. Module #2 determines *xylR*
8 transcription engineered in an auto-activation loop in which the gene is transcribed through the *Pu*
9 promoter. Module #3 is an specialized device in which expression of the the *rpoN* gene (encoding σ^{54})
10 has been placed under the control of *Pu*. The *P. putida* strains used in this experiment are *P. putida*
11 Pu•RBX and *P. putida* Pu•RpoN•Pu•RBX. Both bear in the chromosome modules #1 and #2 and *P.*
12 *putida* Pu•RpoN•Pu•RBX also carries module #3. (b) Western blot of *P. putida* Pu•RBX and *P. putida*
13 Pu•RpoN•Pu•RBX extracts prepared from cells collected at different time points after exposing cultures
14 to saturating vapours of *m*-xylene and probed with an anti-σ^{54} antibody (upper panel) and anti-XylR
15 (lower panel).

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1 **Figure 4**. Dynamics of *Pu* response to XylR inducers in *P. putida* Pu•RBX and *P. putida*
2 Pu•RpoN•Pu•RBX.
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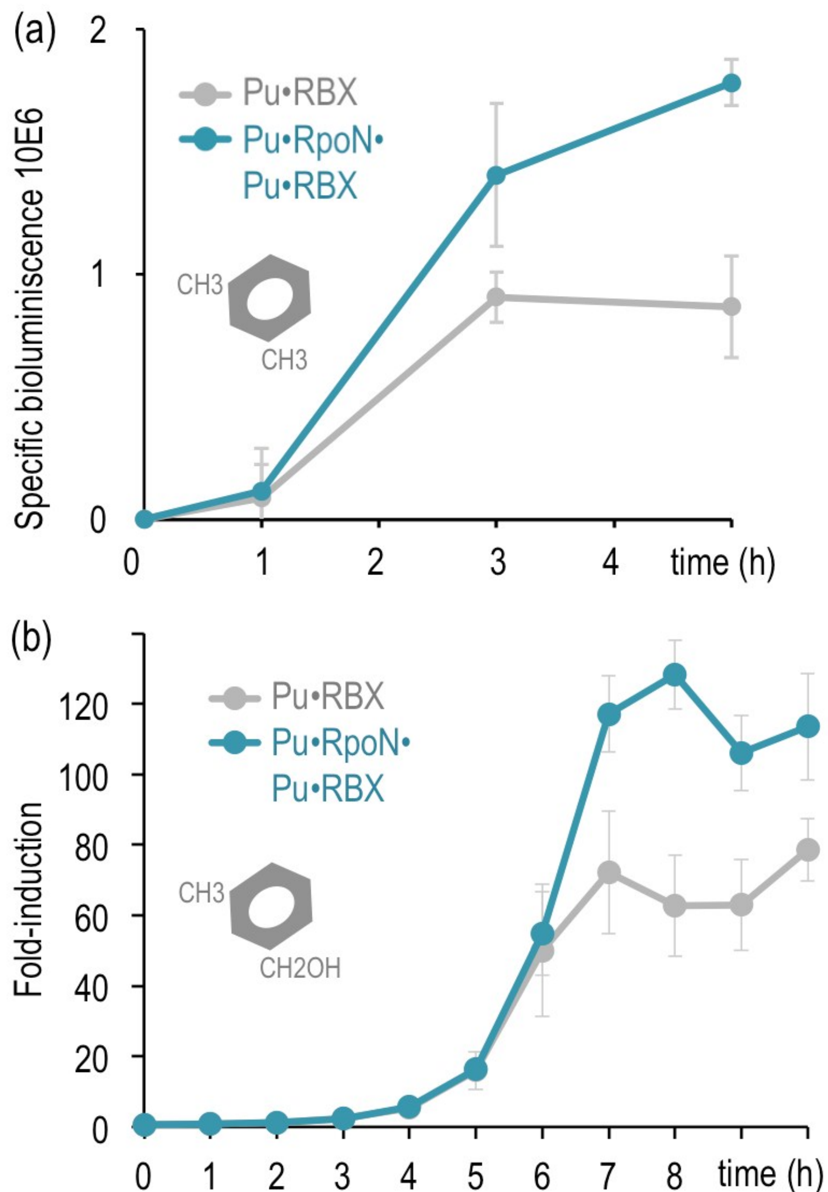

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6 (a) *Pu-luxCDABE* activity upon induction of cells with the optimal effector *m*-xylene. The strains
7 indicated were grown in LB medium and exposed to saturating vapours of *m*-xylene at t =0 and then for
8 the period of time indicated. (b) Fold-induction with 3-methylbenzylacohol (suboptimal effector) in
9 respect to non-induced conditions (baseline at t = 0 was 0.66). The same bacteria were grown in the
10 presence of the 1 mM 3MBA for the times indicated and luminescent emissions recorded as described
11 in Experimental Procedures.

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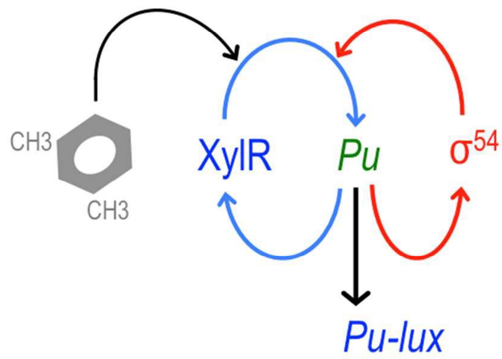

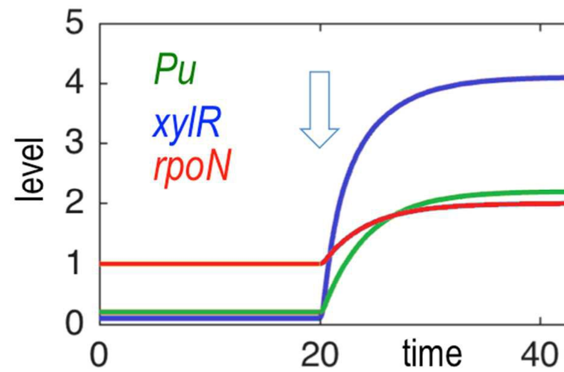

Rational rewiring of the components of the sigma-54 dependent promoter *Pu* makes transcriptional output to reach its physiological limit

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