The pWW0 plasmid imposes a stochastic expression regime to the chromosomal ortho pathway for benzoate metabolism in *Pseudomonas putida*

Rafael Silva-Rocha & Victor de Lorenzo

Systems Biology Program, Centro Nacional de Biotecnología CSIC, Cantoblanco-Madrid, Spain

**Abstract**

Environmental plasmids often expand the metabolic repertoire of bacteria that carry them, but they also interfere with the biochemical and genetic network of the host. The pWW0 plasmid born by *Pseudomonas putida* mt-2 encodes the TOL pathway for degradation of toluene/m-xylene through production of intermediate compounds benzoate/3-methylbenzoate. These can be also recognized as substrates by the chromosomally encoded *ben* and *cat* gene products, thereby creating a manifest regulatory and biochemical conflict. In this context, we have investigated how the introduction of the pWW0 plasmid into *P. putida* affects behaviour of the promoter of the *ben* pathway (Pb) in single cells. Using a series of standardized transcriptional fusions to green fluorescent protein, we found that acquisition of the TOL pathway switches the activation course of the Pb promoter from unimodal/graded to bimodal/stochastic when cells were exposed to benzoate. This behaviour was propagated downstream into the Pt promoter of the *cat* gene cluster, which responds to the benzoate-degradation intermediate cis,cis-muconate. The TOL plasmid thus imposes expression of the chromosomal Pb with a stochastic behaviour likely to result in biochemical heterogeneity of the otherwise genetically clonal population when exposed to benzoate as a growth substrate.

**Introduction**

The pWW0 plasmid of *Pseudomonas putida* mt-2 is one of the best characterized catabolic plasmids in terms of both metabolic and regulatory features encoded in the replicon (Ramos *et al.*, 1997; Greated *et al.*, 2002). The main feature of this system is the presence of two large polycistronic operons (*upper* and *meta* gene clusters) that form the TOL pathway for toluene/m-xylene and benzoate/3-methylbenzoate degradation (Ramos *et al.*, 1997). To this end, toluene/m-xylene is first transformed to the intermediates benzoate/3-methylbenzoate through the action of the *upper* metabolic route. Next, these compounds are further metabolized to TCA intermediates through the action of the *meta* pathway (Ramos *et al.*, 1997). The corresponding catabolic operons are controlled each by transcriptional factors (TFs) XylR and XylS, which activate the cognate promoters *Pu* (*upper* promoter) and *Pm* (*meta* promoter) in response to tolue-
ne/m-xylene and benzoate/3-methylbenzoate, respectively (Ramos *et al.*, 1997; Silva-Rocha *et al.*, 2011). This apparently simple regulatory system is, however, subject to an intricate regulatory architecture that involves a large number of global regulators (Silva-Rocha *et al.*, 2011). The *raison d’être* of this complex network seems to allow induction of the *upper* and *meta* pathways only under a very limited subset of environmental conditions (Silva-Rocha *et al.*, 2011; Silva-Rocha & de Lorenzo, 2013).

In addition to the TOL pathway encoded in the pWW0 plasmid, the chromosome of *P. putida* is equipped with a large set of catabolic genes that allow this bacterium to use a number of aromatic compounds as sole carbon source for growth (Jimenez *et al.*, 2002). In particular, this bacterium harbours a specialized pathway for the degradation of benzoate known as the ortho pathway, which is composed by the *ben*, *cat* and *pca* genes (Harwood & Parales, 1996; Jimenez *et al.*, 2002). While the degradation of 3-methylbenzoate (3MBz)
Regulatory interplay of the TOL plasmid with its host

Fig. 1. The meta and ortho pathways for benzoate degradation in Pseudomonas putida mt-2. (a) The top part of the figure highlights the structure of the ben and meta operons along with the cognate Pb and Pm promoters. These are regulated by the TFs BenR and XylS, respectively in response to benzoate (Bz), with the particularity that BenR can also trigger Pm activity (Cowles et al., 2000). At the metabolic level, the plasmid-encoded meta enzymes are capable of fully metabolizing benzoate to TCA cycle intermediates, while the chromosomally borne Ben enzymes perform the first part of the ortho pathway. Following the action of the Ben enzymes on benzoate and ensuing production of catechol, the pathway continues through the action of the Cat and Pca systems. (b) Reporter strategy to analyse activity of catabolic promoters. The architecture of the promoters under scrutiny is shown along with their main functional sequences (Parsek et al., 1992; Kessler et al., 1993; Cowles et al., 2000). Om-d and Om-p: distal and proximal binding sites, respectively for XylS in the Pm promoter of TOL plasmid. Ob-d and Ob-p: distal and proximal binding sites for BenR in the chromosomal Pb promoter. RBS and ABS: distal and proximal binding sites for CatR in the chromosomal Pc promoter. The three target promoters are placed in a dual (GFP-lacZ) reporter system placed into the host chromosome in single copy (Silva-Rocha & de Lorenzo, 2012c).

occurs exclusively through the meta pathway (Williams & Murray, 1974; Worsey & Williams, 1975), this state of affairs enables P. putida mt-2 to degrade benzoate using both the meta and the ortho pathways, each of them generating a different metabolic intermediate after the ring cleavage step. That the same bacterium has two competing routes for benzoate [lower TOL pathway, ben pathway (Pb)] resulting in different products (Jimenez et al., 1987; Cowles et al., 2000). The extended similarity between these two factors (about 60% in amino acid sequence) allows an unusual type of cross-regulation, where BenR is able to trigger both Pb and Pm activation in response to benzoate while XylS can only recognize Pm (Fig. 1a; Cowles et al., 2000; Perez-Pantoja et al., 2014). Despite such regulatory solution, growth of P. putida mt-2 in benzoate as sole carbon sources results in spontaneous loss of the TOL plasmid (Jimenez et al., 2013) indicating that the co-existence of the two benzo-degradation systems causes some inherent physiologic stress to the cell (Williams & Murray, 1974).

Recently, we have inspected the mode of activation of the ortho and meta pathways at the single-cell level in P. putida, and we found that these systems present different behaviours (Silva-Rocha & de Lorenzo, 2012b,c). In the case of ortho, the first two operons of the system (ben and cat) are expressed from the Pb and Pc promoters in P. putida strains lacking the TOL plasmid, displaying an unimodal/graded regime where all cells became activated concurrently (Silva-Rocha & de Lorenzo, 2012b). This indicated that, in the presence of benzoate, the entire population becomes simultaneously and homogeneously equipped for metabolism of this compound. In contrast, the main catabolic promoters (Pu and Pm) of the TOL system behave in bimodal/stochastic fashion in response to the pathway substrates (Silva-Rocha & de Lorenzo, 2012c). In this case, while most of the population expresses genes for catabolism of the aromatic compounds, an important portion of the cells seems to ignore the presence of the substrates and remains inactive. This intriguing process seems to be controlled by some still undetermined physiologic signal, as the phenomena were only observed in cells growing exponentially (Silva-Rocha & de Lorenzo, 2012c). Given the interplay between the Pm/XylS and Pben/BenR regulatory devices, the question at stake was whether acquisition of pWW0 by a
plasmid-less *P. putida* strain alters also the single-cell expression regime of the recipient cells for catabolism of benzoate and vice versa. Using a suite of transcriptional fusions to a green fluorescent protein (GFP) reporter in different genetic backgrounds, we show below that, contrary to the expected, the *Pm* promoter behaves in an unimodal way when activated by BenR only, while *Pb* and *Pc* promoters become bimodal when cells harbour the pWW0 plasmid. The results below not only expose one more turn of the regulatory intimacy between pWW0 and its host, but also raise one more challenge to the engineering of stable man-made whole-cell catalysts aimed at biodegradation of environmental pollutants.

**Materials and methods**

**Bacterial strains and growth conditions**

All induction experiments were performed in M9 minimal medium (Sambrook et al., 1989) supplemented with 25 mM succinate as the sole carbon source plus 2 mM MgSO₄, with occasional addition of trace elements (Abril et al., 1989). When required, streptomycin (Sm; 50 µg mL⁻¹) and kanamycin (Km 50 µg mL⁻¹) were added to the growth media. For induction experiments, benzoate was purchased from Sigma-Aldrich. All strains used in this study are listed in Table 1. To access the *meta* and *ortho* expression dynamics at single-cell level, *P. putida* reporter strains MEG3-*Pb* and MEG3-*Pc* (Silva-Rocha & de Lorenzo, 2012b), as well as *P. putida* MEG3-*Pm* and Mmt2-*Pm* (Silva-Rocha & de Lorenzo, 2012c) were used. For analysis of the *ortho* promoters (*Pb* and *Pc*) in strains harbouring the full TOL network, the pWW0 plasmid was introduced into *P. putida* MEG3-*Pb* and MEG3-*Pc* reporter strains by conjugation as previously described (Silva-Rocha & de Lorenzo, 2012b). The presence of the plasmid in the recipient strain was confirmed by the appearance of yellow colour upon spraying colonies with 1% catechol.

**GFP expression analyses in individual bacteria**

For the assay of GFP production at the single-cell level, reporter strains were inoculated into M9 medium supplemented with 25 mM succinate and grown overnight at 30 °C with vigorous shaking. After pregrowth, cell cultures were washed twice in 1X PBS buffer, diluted 1 : 20 in fresh M9-succinate medium as before and incubated for 4 additional hours to reach mid-exponential phase. Following this pre-incubation, the cultures were divided, added with 1 mM benzoate and allowed to grow. 500 µL samples was then collected at various time points (typically 1 h intervals) and centrifuged for 1 min. Then, cells were resuspended in 500 µL of PBS and stored on ice until analysis. For the experiments with varying levels of benzoate, after pre-incubation for 4 h, cultures were divided into five flasks and exposed to different concentrations of the inducer (0, 1, 10, 100 and 1000 µM). Following 4 h of exposure to the inducer, samples were collected and processed as before prior to flow cytometry analysis. Distribution of GFP in the cell population was analysed in a GALLIOS cytometer (Perkin Elmer). 15 000 cells were inspected for each sample. Data processing was performed using CYFLOGIC software (http://www.cyflogic.com/). For each condition, the average values and standard derivations for cell fluorescence were calculated using MICROSOFT® EXCEL®.

<table>
<thead>
<tr>
<th>Table 1. Pseudomonas putida strains used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td><em>P. putida</em> mt-2</td>
</tr>
<tr>
<td><em>P. putida</em> KT2440</td>
</tr>
<tr>
<td><em>P. putida</em> MEG3</td>
</tr>
<tr>
<td><em>P. putida</em> MEG3-<em>Pb</em></td>
</tr>
<tr>
<td><em>P. putida</em> MEG3-<em>Pc</em></td>
</tr>
<tr>
<td><em>P. putida</em> MEG3-<em>Pm</em></td>
</tr>
<tr>
<td><em>P. putida</em> Mmt2-<em>Pm</em></td>
</tr>
<tr>
<td><em>P. putida</em> Mmt2-<em>Pb</em></td>
</tr>
<tr>
<td><em>P. putida</em> Mmt2-<em>Pc</em></td>
</tr>
</tbody>
</table>
Results and discussion

Activation of Pm promoter by BenR generates a unimodal/graded regime

To examine the single-cell behaviour of the meta and ortho promoters in P. putida, we used a set of GFP reporter fusions placed in the host chromosome generated using a newly described system (Fig. 1b; Silva-Rocha & de Lorenzo, 2012b). Furthermore, two sets of P. putida strains were used, one that lacks the pWW0 plasmid (MEG3-derived strains) and another group were the plasmid is present (Mnt2-based). In this way, MEG3-derived strains harbour only the chromosomally encoded BenR regulator (the endogenous TF for Pb), while Mnt2-based strains are endowed with both BenR and the plasmid-encoded XylS (the cognate activator of Pm) along with XylR for the upper operon.

Using this strain set, we first investigated the single-cell activation dynamics of Pm and Pb in response to benzoate when activated by BenR only. For this, P. putida MEG3-Pm and MEG3-Pb strains were pregrown overnight in M9-succinate medium, diluted in fresh medium and regrown for 4 hours to reach mid-exponential phase. At this point, 1 mM of benzoate was added to the cultures and samples recovered at 1 hour time intervals analysed using flow cytometry. As shown in Fig. 2a, when Pm was assayed in a strain devoid of the TOL plasmid (and therefore, turned on exclusively by BenR), this promoter presented an unimodal/graded induction profile similar to the previously reported for the ortho promoters Pb (Fig. 2b) and Pc (Silva-Rocha & de Lorenzo, 2012b). Furthermore, comparison of Pm vs. Pb revealed a similar induction profile at the population level, as calculated by taking into account the average fluorescence level of all cells sampled, with a slight higher fold change for Pm (14.9 times) than Pb (10.5 times, Fig. 2c). This result indicated that in the absence of XylS, BenR brings about equivalent activation efficiencies for both promoters. These data indicated also that the bimodal/stochastic behaviour previously reported for Pm in its native context (Silva-Rocha & de Lorenzo, 2012c) is not an intrinsic property of the promoter sequence, but it depends on the upstream regulatory interactions (e.g. the nature and dynamics of the TF involved) that controls transcription initiation.

Pb switches to bimodality in strains harbouring the TOL plasmid

Once we observed that Pm presents a different expression profile when activated exclusively by BenR, we investigated how the ortho pathways are expressed when the complete TOL network is present in the host cells. For this, we first introduced pWW0 into the P. putida MEG3-Pb strain (Silva-Rocha & de Lorenzo, 2012b) by conjugation, thereby generating strain P. putida Mnt2-Pb. As a control, we used P. putida Mnt2-Pm strain that was previously reported to present a bimodal expression dynamics in response to benzoate (Silva-Rocha & de Lorenzo, 2012c). The single-cell expression dynamics of the Pm and Pb promoters was then tested under the same...
experimental conditions described in the previous section. As shown in Fig. 3, while Pm displayed the expected bimodal behaviour (Fig. 3a), Pb activity changed its behaviour in respect to the TOL-less strain above in that it also acquired a bimodal profile (Fig. 3b). This result was unexpected as it cannot be explained trivially on the basis of our current knowledge of Pb activation. While XylS (the TOL-encoded Pm activator) is known to trigger Pb activation in P. putida PRS2000 (Cowles et al., 2000), we recently observed that this was not the case in P. putida KT2440 (the parental strain of P. putida MEG3 and Mmt2 used here) due to the lack of a suitable distal binding site for XylS in Pb (Silva-Rocha & de Lorenzo, 2012a). According to this, the population-wide promoter fold change of Pb upon induction by benzoate changes very little between a strain lacking or harbouring the TOL plasmid (from 10.5 to 7.5, respectively). In contrast, the Pm fold change increases almost twice (from 14.9 to 26.1) due to the additional action of XylS at this promoter (Fig. 3c). It is thus unlikely that the bimodal behaviour of Pb in the presence of the TOL plasmid is the result of a direct interaction with XylS.

To gain more insight on the stochasticity of Pm and Pb in strains harbouring the TOL network, we analysed the single-cell promoter activation dynamics in response to different concentrations of benzoate. In systems presenting bimodal activation profiles, the concentration of the inducer modulates the number of cells that become active over time, so the overall fluorescence level of the population reflects the relative number of cells in the inactive (OFF) or active (ON) states (Siegel & Hu, 1997; Cai et al., 2006). To investigate this process in the Pm and Pb target promoters, overnight cultures of P. putida Mmt2-Pm and Mmt2-Pb strains were diluted into fresh media and grown for 4 hours. At this point, different concentrations of benzoate (1, 10, 100 and 1000 µM) were added and the incubation continued for 4 additional hours, after which samples were taken and analysed by flow cytometry. As shown in Fig. 4a–b, the number of cells in the population that transit to the ON state was strictly dependent on the concentration of induced present in the media. Under the experimental conditions used, significant level of induction was obtained with ≥ 10 µM benzoate, and the highest difference between Pm and Pb was observed at higher inducer dosage (Fig. 4c). Taken together, these results strengthens the notion that the presence of the TOL plasmid is able to impose on the Pb promoter a switch from unimodal to bimodal activation, the ON/OFF rate being dependent on inducer concentration.

### Bimodality of Pb activation is propagated to the downstream cat system

As mentioned in the Introduction above, the degradation of benzoate through the ortho route is performed by a set of subpathways known as Ben, Cat and Pca (Fig. 1a). The Ben enzymes are responsible for the first steps of ring modification, which generate the metabolic intermediate catechol (Ornston, 1966). In turn, the Cat enzymes further metabolize catechol to β-ketoacidipate, which then is processed by the Pca enzymes to generate the TCA intermediates pyruvate and acetaldehyde (Ornston, 1966). The cat operon is activated by its own regulator (CatR) in response to cis,cis-muconate, the first product of catechol metabolism by the Cat enzymes (Parsek et al., 1992).

---

**Fig. 3.** Expression of Pm and Pb in the presence of the TOL plasmid. Overnight grown cells of the strains indicated were processed as explained in the legend to Fig. 2. (a) Expression profile of Pseudomonas putida Mmt2-Pm exposed to benzoate. (b) Expression profile of P. putida Mmt2-Pb exposed to benzoate. (c) Fold change of P. putida Mmt2-Pm and Mmt2-Pb strains induced with benzoate over time.
This metabolic scenario allowed us to investigate whether the bimodal activation of \( P_b \) results into an equally bimodal production of the Ben enzymes, which produce the catechol necessary to trigger \( P_c \) activity in cells exposed to benzoate. The rationale for this is the following. If \( P_b \) bimodality translates into differences in Ben enzymes in individual cells, then the \( P_c \) promoter should also display bimodal activation due to the stochastic production of catechol from the Ben enzymes. To see whether the stochastic activation of \( P_b \) in a strain harbouring the TOL plasmid is thus propagated downstream in the pathway, we compared the expression pattern of the \( P_c \) in \( P.\ putida \) MEG3-derived and Mmt2-derived strains in response to different concentrations of benzoate as described above. As shown in Fig. 5, while \( P.\ putida \) MEG3-\( P_c \) strain presented an unimodal expression regime regardless of the amount of benzoate used (Fig. 5a), the TOL-carrying \( P.\ putida \) Mmt2-\( P_c \) strain displayed bimodal activation dependent of the level of inducer present in the medium (Fig. 5b). This result strongly suggested that the stochastic activation of \( P_b \) is indeed propagated into the Cat sub-pathway (and potentially downstream), supporting the notion that TOL network imposes strong stochastic effects and metabolic diversification in single cells of \( P.\ putida \) during benzoate degradation.

**Conclusion**

The natural interplay between chromosome-encoded and plasmid-encoded biochemical pathways provides an instance of how bacteria solve the impact caused by the implantation of foreign genes into a pre-existing metabolic and regulatory network (Nojiri, 2013). In the case of the pWW0 plasmid, a number of host global regulators are
recruited to control production of the TOL catabolic genes, allowing pathway expression only under a specific set of conditions (Aranda-Olmedo et al., 2005; Vitale et al., 2008; Silva-Rocha & de Lorenzo, 2013). In other cases (e.g. pCAR1; Nojiri, 2013), the acquired genetic device is capable of influencing the host regulatory network through expression of plasmid-encoded global regulators that target the chromosome of the host bacterium (Miyakoshi et al., 2007; Yun et al., 2010). In this sense, the wiring of the newly acquired system seems to be a bilateral process where both the new and the existing networks have to be reshaped for bringing about a proper function of the system. The results presented here demonstrated that the acquisition of a catabolic plasmid not only does influence the network connectivity of the host, but also is capable of affect basic properties of the regulatory components, such as the single-cell dynamics of the target promoters (Supporting Information, Fig. S1). In the case of the TOL system, this regulatory imposition seems to generate a populational diversification that could solve some of the metabolic conflicts generated by the co-existence of both meta and ortho pathways in the same host (Williams & Murray, 1974; Jimenez et al., 2013). While the phenomenon is well accredited by the results shown above, the molecular mechanism through which Pb transits from unimodality to bimodality upon co-existence with the TOL plasmid is currently under investigation.

**Acknowledgements**

This study was supported by the BIO Program of the Spanish Ministry of Science and Innovation, the ST-FLOW and ARYSIS Contracts of the EU, the ERANET-1B Program and the PROMT Project of the CAM. R.S.-R. holds a FAPESP postdoctoral Fellowship (FAPESP 2013/04125-2). Authors declare no conflict of interests.

**References**


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Adoption of bimodal expression regime upon pWW0 plasmid acquisition.