



Transcriptional kinetics of the cross-talk between the *ortho*-cleavage and TOL pathways of toluene biodegradation in *Pseudomonas putida* mt-2



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ABSTRACT

The TOL plasmid promoters are activated by toluene leading to gene expression responsible for the degradation of the environmental signal. Benzoate is formed as an intermediate, activating the BenR protein of the chromosomal *ortho*-cleavage pathway that up-regulates the chromosomal *PbenA* promoter and the TOL *Pm* promoter resulting in cross-talk between the two networks. Herein, the transcriptional kinetics of the *PbenR* and *PbenA* promoters in conjunction with TOL promoters was monitored by real-time PCR during toluene biodegradation of different concentrations in batch cultures. The cross-talk between the two pathways was indicated by the simultaneous maximal expression of the *Pm* and *PbenR* promoters, as well as the transcriptional activation from *PbenA* occurring prior to *PbenR*, which indicates the potential up-regulation of *PbenA* by the TOL XylS protein. The repressory effect of toluene on *Pr* was evident for concentrations higher than 0.3 mM suggesting a threshold value for restoring the promoter's activity, while all the other promoters followed a specific expression pattern, regardless of the initial inducer concentration. Induction of the system with higher toluene concentrations revealed an oscillatory behaviour of *Pm*, the expression of which remained at high levels until the late exponential phase, demonstrating a novel function of this network.

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1. Introduction

The successful activation of a specific metabolic pathway in the presence of an aromatic compound relies mainly on two significant factors: i) the catabolic enzymes catalysing the degradation of the compound, and ii) the promoters of genes and operons subject to regulation by specific transcription factors (de Lorenzo and Perez-Martin, 1996). Transcriptional regulation is a key step in the biodegradation process of an aromatic compound acting as a controller regulating the appropriate metabolic cascades in response to the availability of specific substrate(s) (Díaz and Prieto, 2000).

Pseudomonas putida is a metabolically versatile soil bacterium capable of thriving in diverse habitats (Timmis, 2002) as well as

an industrially significant strain producing a series of fine and bulk chemicals, which has resulted in a growing interest in understanding its specific metabolic pathways (Ballerstedt et al., 2007). Among the several *P. putida* strains, mt-2 contains the TOL plasmid (pWW0), which specifies metabolic pathways for toluene and *m*-xylene degradation (Timmis, 2002). The latter compounds belong to the BTEX (benzene, toluene, ethylbenzene and the three isomers of xylene) group of pollutants; their biodegradation leads to Krebs cycle intermediates, which are essential for biomass growth (Jindrova et al., 2002). The TOL is considered as a paradigm of global and specific gene regulation due to the interactions that occur between DNA-bending proteins, a set of sigma factors and the regulators encoded in the system (Aranda-Olmedo et al., 2006). The transcriptional regulatory network of TOL has been described in detail by Ramos et al. (1997) and constitutes of four transcriptional units (*xylR*, *xylS*, *upper* and *meta* operon) controlled by four promoters (*Pr*, *Ps*, *Pu* and *Pm*), respectively. Entry of an aromatic compound in the TOL provokes a cascade of regulatory events, as presented in Fig. 1 and described briefly below.

Abbreviations: *P. putida*, *Pseudomonas putida*; PCR, Polymerase chain reaction; BTEX, Benzene; i, inactive; a, active; GC, Gas chromatograph; OD, Optical density; XMO, Xylene monooxygenase; 3MBA, 3-methyl-benzyl alcohol; IHF, Integration host factor; LB, Luria Broth (rich medium); CDK1, Cyclin B dependent kinase 1.

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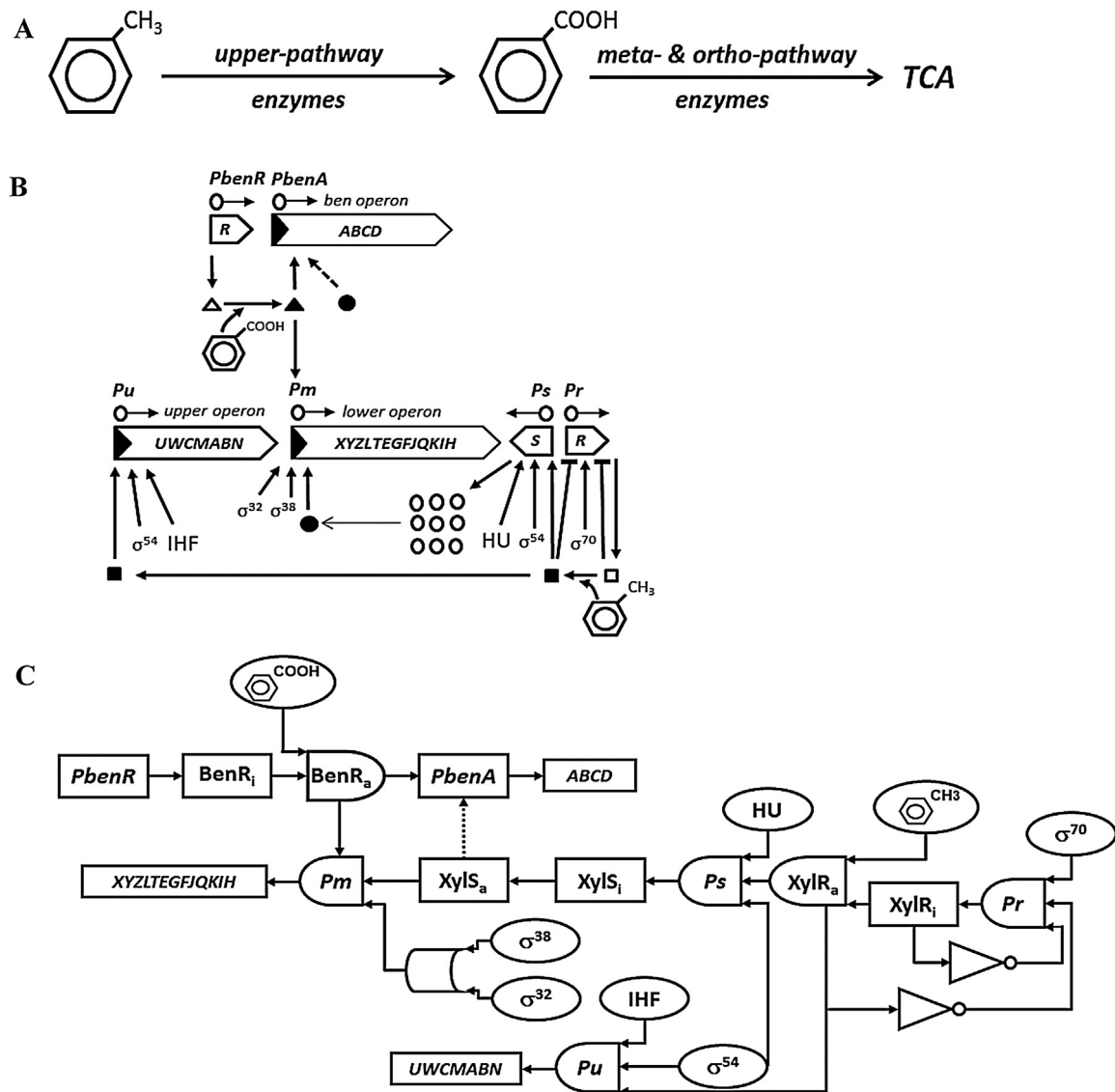


Fig. 1. Cross talk of the chromosomal and TOL genetic networks during toluene induction. The overimposed regulation of the promoters is additionally presented. (A) The enzymes encoded in the upper operon sequentially transform toluene into benzoate. The latter is then transformed into acetate and pyruvate through the action of the enzymes synthesised by the meta operon. The meta pathway products are channelled into the Krebs cycle yielding the precursor molecules required to support biomass growth. (B) The biochemical and (C) logic representations of the two pathways. □: inactive form of XylR (XylR_i); ■: active form of XylR (XylR_a); ○: inactive form of XylS (XylS_i); ●: active form of XylS (XylS_a); △: inactive form of BenR (BenR_i); ▲: active form of BenR (BenR_a); ○: input; □: output; ▭: AND; ▨: OR; ⊗: NOT.

The *Pr* controls the expression of the *xylR* gene, which encodes for the XylR protein. In the absence of an environmental signal the XylR protein is produced in an inactive form (XylR_i), while the entry of an aromatic compound results in oligomerisation of 3 inactive XylR dimers forming an active molecule of XylR (XylR_a). XylR_a activates *Pu* and *Ps* promoters. Upon *Pu* activation the genes of the upper operon are expressed resulting in the production of the corresponding enzymes catalysing the oxidative catabolism of toluene to benzoate. The activation of *Ps*, as well as the presence of benzoate, results in overexpression of the *xylS* gene, which is constitutively expressed, leading to the dimerisation of the inactive XylS protein to the active protein form. The presence of benzoate is known to boost mRNA expression from the meta operon, which encodes for the corresponding enzymes catalysing the catabolism of benzoate to Krebs cycle intermediates, acetate and pyruvate (Ramos et al., 1997). Furthermore, benzoate activates the BenR protein of the ortho-cleavage pathway, which is encoded by the *benR* gene (Cowles et al., 2000; Cuskey and Sprengle, 1988).

The activation of BenR triggers the *ben* operon expression (Cowles et al., 2000) of the ortho-cleavage pathway followed by a cascade of metabolic events in the chromosome, which further catabolise benzoate to Krebs cycle intermediates, such as succinyl-coA and acetyl-CoA (Chugani et al., 1997). Furthermore, BenR protein acts as an up-regulator of TOL *Pm* (Cowles et al., 2000). The *benR* gene and *ben* operon are controlled by *PbenR* and *PbenA* promoters. Although, *PbenR* and *PbenA* are essential in the catabolic process of toluene their transcriptional activity until substrate depletion has not been systematically monitored and *PbenR* expression has not been demonstrated yet. The transformation of toluene to Krebs cycle intermediates through the enzymes produced by the genetic elements of the system is presented in Fig. 1A. The information of Fig. 1B was used to construct a logic representation of the interactions between the genetic elements (Fig. 1C), using a direct analogy to electrical circuits (Weiss et al., 2003).

The effect of gene regulation and transcriptional response on toluene catabolism through activation of the TOL and chromosomal *ben* operon genes has been previously studied in batch cultures

(Dominguez-Cuevas et al., 2006; Gerischer, 2002). The TOL transcriptional kinetics has been evaluated in batch cultures using *m*-xylene (Koutinas et al., 2011; Koutinas et al., 2010) and (3-) benzyl-alcohol (Marques et al., 1994) as the inducer of the pathway. But the simultaneous assessment of transcriptional kinetics of TOL and *ortho*-cleavage key promoters upon induction with toluene, which is one of the most common TOL substrates (Timmis, 2002), has not been evaluated yet. Monitoring of transcriptional kinetics in *P. putida* provides valuable information about the activity of catabolic genes enhancing our knowledge of the interplay between the two pathways during toluene degradation and the biodegradation process itself.

Therefore, the objective of this study was to investigate the expression levels of the promoters involved in toluene catabolism (*Pr*, *Ps*, *Pu*, *Pm*, *PbenR* and *PbenA*) by *P. putida* mt-2 over time through the cross-talk of the chromosomal and TOL metabolic pathways. The transcriptional kinetic profiles of the promoters involved are presented in measurements obtained in 30 min time intervals, in conjunction with the dynamic profiles of toluene consumption and biomass growth. Various toluene concentrations were tested in batch cultures by performing three independent experiments at each concentration to clarify the effect of the environmental signal on the transcriptional regulation as well as to reveal the behaviour of the promoters in a wide range of conditions. Therefore, the effect of toluene concentration on promoters' activity was assessed, which unravelled specific expression patterns and provided an opportunity to explore the regulatory mechanisms involved.

2. Materials and methods

2.1. Microbial cultures

Subcultures of *P. putida* mt-2 (c) were pre-grown for 23 h at 30 °C in M9 minimal medium (Sambrook et al., 1989) supplemented with 10 mM of succinate. Three independent cultures were prepared by diluting the overnight culture in minimal medium to an initial optical density (OD) of 0.1 (0.41 culture volume) at 600 nm (UV-2101PC, Shimadzu UK Ltd, UK). The minimal medium was supplemented with toluene at a different concentration level in each experiment. Cultures were performed using conical Erlenmeyer flasks with 2.35 l total volume, which were continuously stirred at 1000 rpm via a Heidolph MR3001 K (Heidolph, UK) magnetic stirrer. The flasks were filled with medium to one-sixth of their volume, to ensure that sufficient oxygen is available, and closed gas-tight with Teflon coated lids to avoid volatile organic compound losses. Temperature was maintained constant at 30 °C. All chemicals used were obtained from Sigma-Aldrich Company Ltd and were of ANALAR grade.

2.2. Substrate and biomass analyses

Gas Chromatograph (GC) analysis was employed for determination of toluene concentration in the gaseous and aqueous samples using an Agilent 6850 Series II Gas Chromatograph with a FID detector and a 'J&W Scientific' (Agilent Technologies UK Limited, UK) column with HP-1 stationary phase (30 m × 0.32 mm × 0.25 mm). The samples for the measurement of toluene concentration were collected using a glass syringe injected to the Teflon coated lids and released immediately in GC vials (Agilent Technologies UK Limited, UK) to avoid any toluene loss. Following that, gaseous samples of 25 µl were injected into the GC and the temperature program run at 70 °C for 3 min and then increased to 80 °C at a rate of 5 °C min⁻¹. Toluene concentration in the culture was determined experimentally interpolating from an established toluene calibration curve and partition coefficient, as previously described (Koutinas et al.,

Table 1
Primers used in quantitative real time-PCR.

Pair of primers	Description	Source
<i>xyIR</i>	5'-AACTGTTTGGTGTGCGATAAAGG-3' 3'-ATCACCTCATCAAGAAAGATGG-5'	Koutinas et al. (2011) Koutinas et al. (2011)
<i>xyIS</i>	5'-GGATTAGAGACCTGTATCATCTG-3' 3'-GATTGAGCAGCAATAGTTCG-5'	Koutinas et al. (2011) Koutinas et al. (2011)
<i>xyIU</i>	5'-GCAGTTATCCGGCTTCATCTC-3' 3'-CATATAGTCGGTTGAGGTAGC-5'	Koutinas et al. (2011) Koutinas et al. (2011)
<i>xyIX</i>	5'-TGAAGAAGATGAGAACCAGG-3' 3'-AGATAAATCCAGTTGCCCTC-5'	Koutinas et al. (2011) Koutinas et al. (2011)
<i>benR</i>	5'-TCATTACCGGCTGGATGAGC-3' 3'-CTGGCGACAATCTGGCTGTA-5'	This study This study
<i>benA</i>	5'-CTCGAGGACGACCGTGAAA3' 5'CAGTTTGGCCGTGTTGTA-3'	This study This study
<i>rpoN</i>	5'-TAACGAAACCCTGATGAAGG-3' 3'-AATGTCATGCAGTACCAACG-5'	Koutinas et al. (2011) Koutinas et al. (2011)

2010). The coefficient of variation for 4 samples was 4.6% at a concentration level of 0.2 mM toluene. The samples applied for the measurement of biomass concentration were taken using a plastic syringe injected to the Teflon coated lids and released to Bijou sample containers (Sigma-Aldrich Company Limited). Biomass concentration was determined by absorbance at 600 nm on a UV-1800 scanning spectrophotometer (Shimadzu, UK) interpolating from an established dry cell weight calibration curve. Dry cell weight calibration curve was established by preparing a microbial culture overnight. Multiple samples were applied and diluted at different OD obtaining 5 ml of total volume. The samples were dried for eight days at 105 °C and were placed in a furnace for 45 min at 600 °C to remove the organic content. The coefficient of variation for 3 samples was 3.4% at a concentration level of 1233 mg_{biomass} L⁻¹.

2.3. Preparation and isolation of total RNA, cDNA synthesis, quantitative real-time PCR and gradient PCR

Culture samples of 3–4.5 ml (depending on cell density) were placed in cryogenic vials (Sigma-Aldrich Company Ltd., UK) and cell pellet was harvested by centrifugation on 15000 rpm for 10 min at 4 °C. The supernatant was discarded and the vials were immersed in liquid nitrogen for 1 min and stored at –80 °C until use. Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR) was performed to determine the expression of *xyIR* (*Pr* promoter), *xyIS* (*Ps* promoter), *xyIU* (*Pu* promoter), *xyIX* (*Pm* promoter) and *rpoN* (housekeeping) genes during the course of the experiments. The Q-PCR method as well as the calculation of the relative mRNA expression based on threshold cycle (C_T) values was conducted as previously described (Koutinas et al., 2011; Koutinas et al., 2010). Q-PCR analysis of promoters' kinetics was conducted in triplicate measurements for each time point. Gradient PCR was performed to select the best primer pair for *PbenR* and *PbenA* promoters (primers listed on Table 1) and the best cDNA annealing temperature for amplification. The method for isolation of total RNA and cDNA synthesis has been previously described by Koutinas et al. (2010). The PCR reaction was carried out in an Eppendorf thermocycler (Fisher Scientific, UK). The denaturation temperature was set at 95 °C for 3 min, followed by of 95 °C (20 s). In the next step different annealing temperatures were employed in each column of the thermocycler. The annealing temperatures ranged from 50 to 65 °C for 30 s, and 72 °C for 30 s. The amplification last for 50 cycles. The best annealing temperature for *PbenR* and *PbenA* promoters was 60 and 66 °C, respectively.

2.4. Gel electrophoresis

Gel electrophoresis was conducted for the PCR products to identify which pair of primers (Table 1) for the *benR* (*PbenR* promoter)

and *benA* (*PbenA* promoter) genes was capable of elongating the DNA synthesised. The sequence of *benR* constitutes approximately of 1200 Kbp. Thus, a mixture of 1% agarose (BD) in $1 \times$ TE buffer (Ambion, UK) supplemented with EtBr (15 μ l/250 ml) was prepared.

2.5. Statistical analysis

One way ANOVA (SigmaStat version 3.5, Systat Software UK Ltd, UK) was conducted for elucidation of the relative mRNA expression profiles of all promoters. Since the mRNA was measured in triplicates for each of the three cultures at each concentration level, P-values were calculated through comparison of the mean relative mRNA expression between two given time points of the three independent cultures performed. The level of significance was accepted at P-values lower than 0.05.

3. Results

3.1. Evolution of toluene biodegradation and biomass growth kinetics upon induction with different toluene concentrations

Cells were pre-grown overnight in succinate to ensure that TOL and *ortho*-cleavage pathways were not expressed prior to toluene induction. The consumption of toluene, which was added in concentrations that ranged between 0.4–1.2 mM, was investigated in batch cultures of mt-2 (Fig. 2). The batch cultures were repeated three times at each concentration level. The results demonstrated that for lower aromatic compound concentrations the mt-2 metabolised the substrate faster. Specifically, in 0.4 mM (Fig. 2A), which was the lowest inducer concentration tested, 90% of toluene was degraded within 290 min, while degradation of the same amount of inducer was achieved within 330 min when 1.2 mM was fed (Fig. 2D). Additionally, for higher inducer concentrations the duration of the lag-phase, which was observed in all experiments performed, was substantially increased. For instance, the lag phase was 90 min and 180 min for 0.4 mM (Fig. 2A) and 1.2 mM (Fig. 2D) toluene concentrations, respectively. It is expected that as soon as the aromatic compound interacts with the phospholipid bilayer of the cell membrane, a stress response is produced since the pollutant is known to disturb the cytoplasmic membrane structure (Dominguez-Cuevas et al., 2006; Sikkema et al., 1995). Xylene monooxygenase (XMO) is a membrane-bound enzyme found in *P. putida* mt-2 (Tizzard and Lloyd-Jones, 2007) responsible for the initiation of pollutant's catabolism, which following the disturbance in the membrane structure its synthesis stops for a certain time period (Buhler et al., 2006). The lag-phase could also be caused due to the inhibition of protein synthesis occurring when the culture is exposed to toluene. A comparison between the consumption of toluene and 3-methyl-benzoate by *P. putida* mt-2 cells revealed the effect of toluene concentration on protein synthesis (Vercellone-Smith and Herson, 1997). The complete inhibition of protein synthesis for the first 20 min of cultivation for cells exposed to sub-lethal toluene concentrations was evident. Thereafter, protein synthesis resumed and completely recovered after 1.5–3 h of cultivation.

3.2. Pr promoter

Herein, the two σ^{70} -dependent tandem *Pr* promoters (Marques et al., 1998) have been integrated into a single promoter. The active and inactive forms of the XylR protein repress *Pr* (Marques et al., 1998). This effect was also observed in this study where the activity of *Pr* decreased significantly ($P < 0.05$) following induction of the culture with toluene (Fig. 3). A low level of *Pr* expression was maintained for the rest of the experiment until the concentration of

toluene was reduced to approximately 0.3 mM. However, when the concentration of toluene was reduced below 0.3 mM, the repression of *Pr* was alleviated and its activity was increased reaching a similar level of expression as prior to the induction with the environmental signal ($P < 0.05$). Furthermore, the difference between the mean relative mRNA expression at 0 min and the last time point was not statistically significant demonstrating that a similar level of expression existed at both time points ($P > 0.05$). The recovery of *Pr* activity could be due to the end of auto-repression of XylR occurring at lower pollutant concentrations, while a toluene concentration of 0.3 mM could serve as a threshold level initiating this response.

Vercellone-Smith and Herson (1997) revealed that protein synthesis by 3-methyl benzoate, which activates directly *Ps* and indirectly *Pm* through the activation of XylS, is high since the start of the culture. However, when toluene was used as the inducer, *Pr* and *Pu* promoters were activated prior to *Ps* and *Pm*. Furthermore, transcription from *Ps* and *Pm* occurred at a later stage and protein synthesis reached comparable levels to the experiments performed with 3-methyl benzoate 4–6 h after induction. This delay was attributed to the XylR protein activity, the master regulator of the system, which is required for initiation of toluene degradation acting as both the direct and indirect activator of *Pu*, *Ps* and *Pm*, as well as an auto-repressor (*Pr*) (Silva-Rocha et al., 2011). Therefore XylR plays a key role in the TOL regulatory network.

3.3. Ps promoter

Transcription of *xylS* is triggered by two tandem promoters *Ps1* and *Ps2* (Marques et al., 1998). The σ^{70} -dependent *Ps2* promoter is expressed at a basal level indicating that the *xylS* gene is constitutively expressed even in the absence of a chemical signal (Mermod et al., 1987). Following toluene entry into the cell, XylR_a up-regulates the σ^{54} -dependent *Ps1* promoter triggering *xylS* over expression and XylS protein over production due to the presence of benzoate (Gonzalez-Perez et al., 2004). In the present study the introduction of toluene into the culture initiated an increase in the activity of *Ps*, due to *Ps1* transcription, reaching a peak of maximal mRNA expression at 60 min regardless of the concentration of toluene used ($P < 0.05$) (Fig. 4, Table 2). Following that relative maximal expression observed within an hour of cultivation, the activity of *Ps* decreased significantly and it was gradually reduced to low transcription levels. The increase in the mRNA expression level of *Ps* suggests that its activator, XylR_a protein, is activated rapidly following induction with toluene (Inouye et al., 1987) and binds strongly *Ps1* sequence of the promoter. Furthermore, as toluene concentration increased, the maximal relative mRNA expression and the amplitude of relative mRNA expression increased.

3.4. Pu promoter

As shown on Fig. 5, *Pu* is not expressed prior to the introduction of toluene into the culture. Similarly to the response of *Ps* following the addition of toluene, the activity of *Pu* increased and reached a maximal expression at 60 min ($P < 0.05$). Furthermore, the maximal activity of *Pu* was observed at 60 min in every toluene concentration tested (Table 2) and increased in higher toluene concentrations. Subsequently, the activity of *Pu* gradually reduced highlighting the fact that since both *Pu* and *Ps* promoters are activated by XylR_a (Abril et al., 1991), their activation through a common transcription factor results in similar transcription kinetic profiles. The mRNA expression amplitude was increased in conjunction with increased inductive toluene concentrations. Due to the activation of *Pu*, the TOL upper operon is expressed followed by the production of the enzymes catalysing the conversion of toluene into benzoate. The latter compound acts as an effector of TOL *Pm* (Kessler et al., 1994)

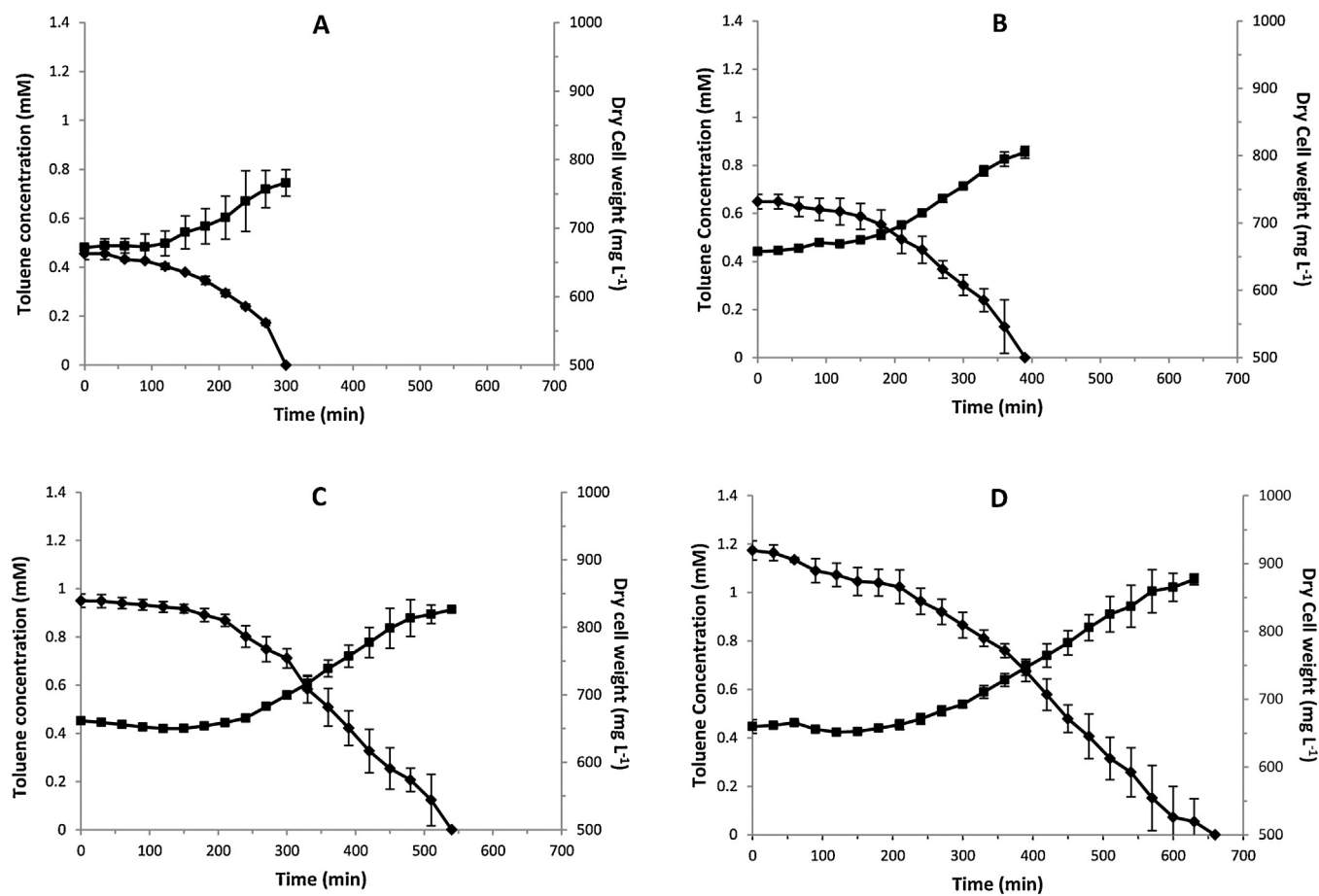


Fig. 2. Concentration of toluene and dry cell weight in the experiments. (A) 0.4, (B) 0.7, (C) 1.0, and (D) 1.2 mM of toluene concentration.

Table 2

The time points at which *Pu*, *Ps*, *Pm*, *PbenR* and *PbenA* promoters present their maximal relative mRNA expression.

Toluene concentration (mM)	Maximal relative mRNA expression				
	<i>Pu</i> (min)	<i>Ps</i> (min)	<i>Pm</i> (min)	<i>PbenR</i> (min)	<i>PbenA</i> (min)
0.4	60	60	60–90	90	180
0.7	60	60	60–90	90	180
1.0	60	60	60–90	90	180
1.2	60	60	60–90	90	180

and of the chromosomal *benR* gene (Cowles et al., 2000; Cuskey and Sprenkle, 1988) activating efficiently both pathways (Silva-Rocha and de Lorenzo, 2012a).

3.5. *Pm* promoter

The active form of XylS, due to benzoate presence, mediates *Pm* activation by RNA polymerase with σ^{32} or σ^{38} depending on the growth phase (Marques et al., 1999). During the exponential phase σ^{32} is involved in the transcription (Marques et al., 1999), while as biomass growth enters the stationary phase σ^{38} is engaged (Marqués et al., 1995). During the first 10 min of induction the increase in the activity of *Pm* was relatively low compared to that of *Pu* and *Ps* ($P > 0.05$) (Fig. 6). The delay in the increase of mRNA expression from *Pm* was previously observed by Velazquez et al. (2005) for mt-2 cultures exposed to *m*-xylene, and Silva-Rocha et al. (2011) and Marques et al. (1994) for mt-2 cultures fed with 3-methyl-benzyl alcohol (3MBA) and benzyl-alcohol, respectively. Following the initial delay, the activity of *Pm* gradually increased reaching a peak at 60 min in all experiments performed ($P < 0.05$)

(Table 2), remaining at high levels until the late exponential growth phase. Prior to the stationary phase the activity of *Pm* was sharply reduced to its basal level. Interestingly the response of *Pm* to the two higher concentrations of toluene tested (Figs. 6C, D) was oscillating in a narrow range close to the maximum value. The activity of *Pm* oscillated with a regular frequency of approximately 150 min when exposed to 1 mM of toluene, while the frequency varied when the concentration of the inducer was increased to 1.2 mM. The oscillatory expression of *Pm* was clearly observed in the higher toluene concentrations tested (1.0, 1.2 mM) (Fig. 6C, D) and occurred following activation of the *ortho*-cleavage pathway encoded in the chromosome. Both the systematic experimental work (triplicate experiments at each concentration level) and the statistical analysis ($P < 0.05$ between the oscillatory points) ensured that this behaviour is not an artifact.

Transcription from *Pm* was delayed compared to *Pu* and *Ps*, since benzoate should be produced and XylS activated to drive increasing activation of *Pm*. The XylS protein production triggered through *Ps1* activation is sufficient for *Pm* stimulation upon toluene induction (Dominguez-Cuevas et al., 2008). Despite the minor delay of 10 min,

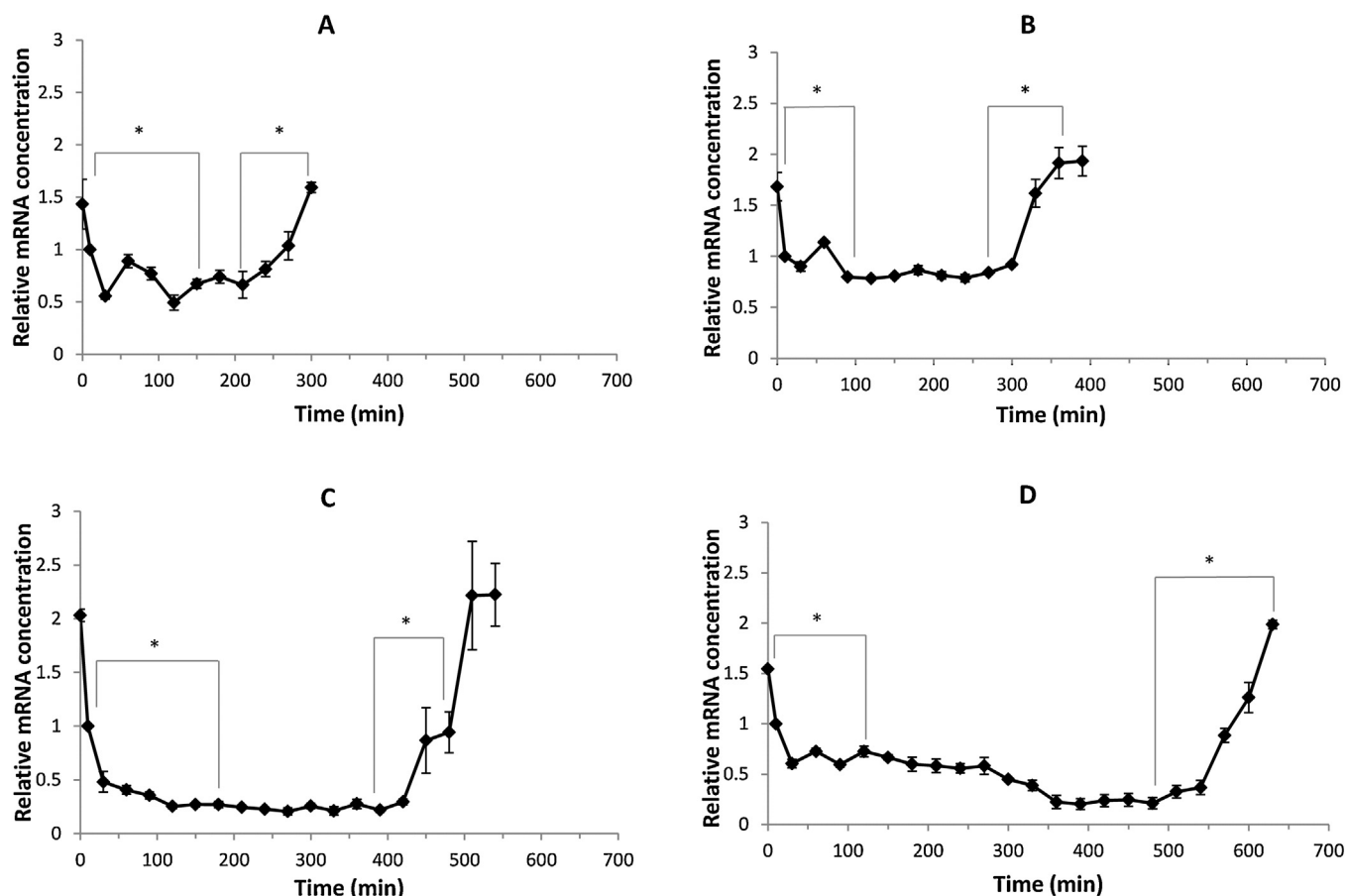


Fig. 3. *Pr* promoter relative mRNA expression in the experiments. (A) 0.4, (B) 0.7, (C) 1.0, and (D) 1.2 mM of toluene concentration, where *: statistical significant difference of relative mRNA expression between different time points.

the highest expression of *Pm* was observed at 60 min similarly to *Ps* and *Pu* remaining at the same level up to 90 min. Transcription from *Pm* was maintained at high levels after 90 min ($P > 0.05$) (Fig. 6), a time point where *PbenR* reached its maximal expression (see below). Furthermore, the level of transcription from *Pm* was substantially higher compared to *Pu* and *Ps* in agreement with fluorescence measurements by Silva-Rocha and de Lorenzo (2012b). The remarkably high maximal *Pm* expression levels compared to *Pu* and *Ps* could be attributed to the activation of XylS protein due to benzoate formation which leads to over-production of this protein stimulating *Pm* at high level of expression. *Pm* expression level remained high possibly due to the requirement for further transformation of benzoate into Krebs cycle metabolites through the production of the 9 catabolic enzymes (Ramos et al., 1997) encoded in the lower-operon. In addition, *Pm* activity was markedly affected by the concentration of toluene employed, increasing its relative mRNA expression at higher inducer concentrations.

3.6. *PbenR* promoter

PbenR drives transcription of the *benR* gene, which is activated due to the presence of benzoate and encodes for the production of the BenR protein (Cuskey and Sprengle, 1988). Prior to the introduction of toluene, expression from *PbenR* was at a basal level. The promoter was not immediately activated following the addition of toluene remaining at low expression levels for a period that ranged between 10 and 30 min after induction with the substrate (Fig. 7). The statistical significance of the data obtained at 0, 10 and 30 min was not significant ($P > 0.05$). The presence of benzoate activates BenR protein (Cowles et al., 2000). According to our results the first

10–30 min the constant *PbenR* expression leads to a constitutive level of *benR* expression and, thus, BenR constant production. BenR is expected to be activated following activation of *Pu*, which drives the transcription of the upper operon encoding for the enzymes that convert toluene to benzoate.

However, following that period expression of *PbenR* increased reaching its peak level within 90 min of pollutant addition, which was evident for all experiments performed ($P < 0.05$) (Table 2). After 90 min, mRNA expression decreased and gradually reached the initial basal level ($P > 0.05$) remaining almost at a stable level ($P > 0.05$) until the complete depletion of the substrate. This expression pattern of *PbenR* indicated that there is a transcription factor up-regulating its activity with great efficiency until 90 min and then the decrease of the mRNA expression could be attributed to the decrease of the activity of the transcription factor.

The results obtained for *Ps*, *Pu* and *PbenR* indicated that the three promoters had similar behaviour and that there was approximately a 30 min delay between transcription initiation and the maximum expression levels observed in the TOL and chromosomal pathway promoters. Thus, although the two promoters subject to XylR regulation were activated immediately following the addition of the inducer, expression from *PbenR* was delayed by 30 min due to the requirement for benzoate formation, which is possible through transcription from *Pu* that controls the function of the upper pathway and catalyses the conversion of toluene to benzoate. This time delay indicated that following the activation of a given promoter in the specific pathway, the time required for the synthesis of its respective enzyme(s) could be up to a period of 30 min (Golding et al., 2005).

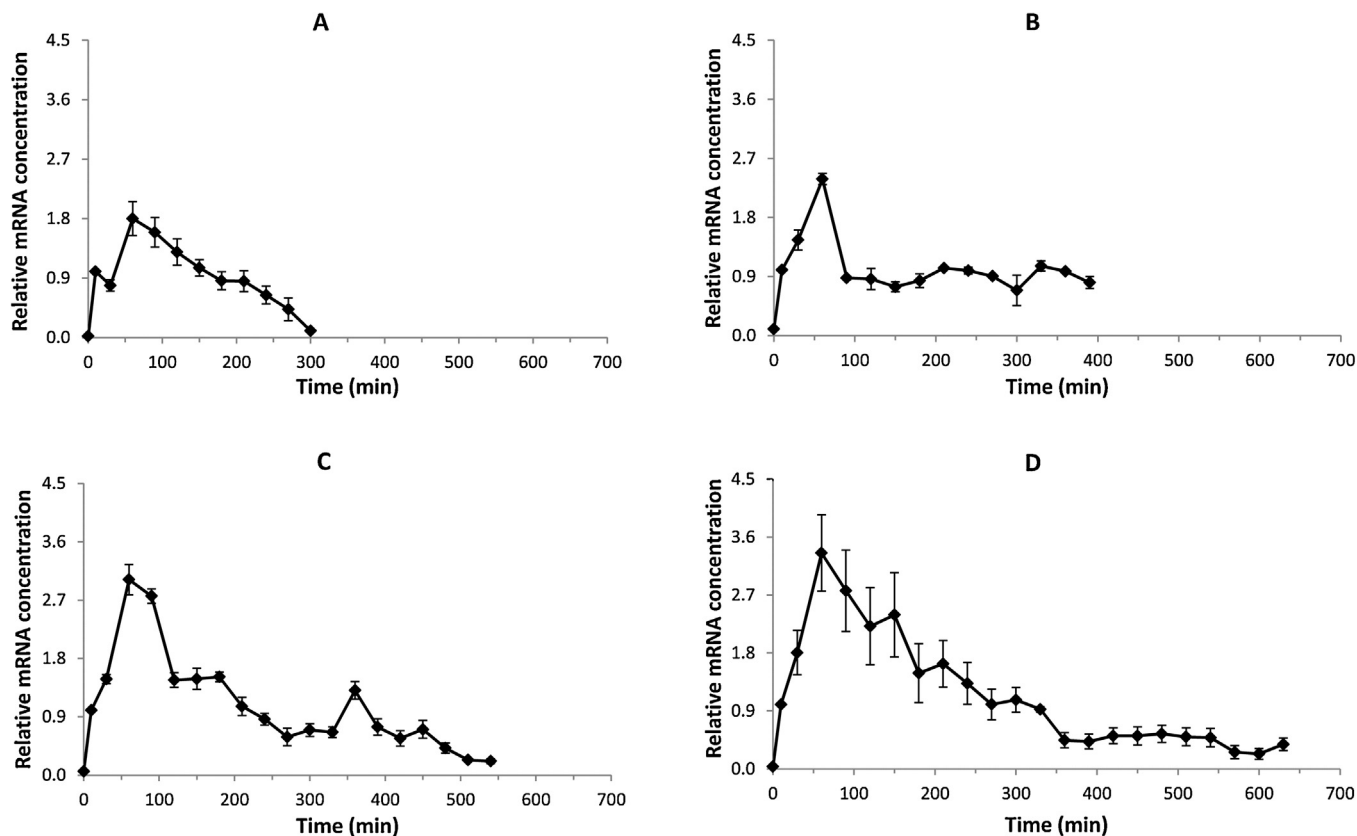


Fig. 4. *Ps* promoter relative mRNA expression in the experiments. (A) 0.4, (B) 0.7, (C) 1.0, and (D) 1.2 mM of toluene concentration.

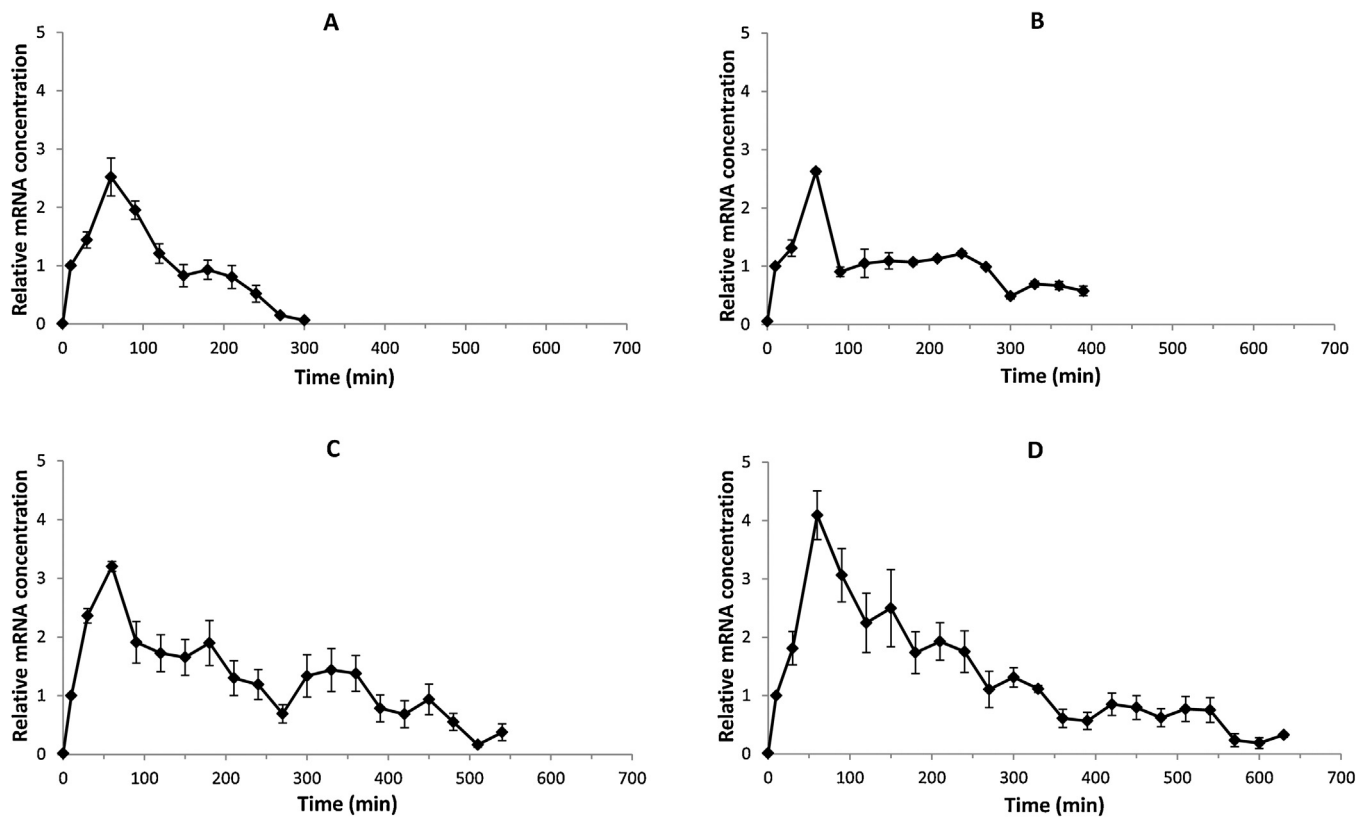


Fig. 5. *Pu* promoter relative mRNA expression in the experiments. (A) 0.4, (B) 0.7, (C) 1.0, and (D) 1.2 mM of toluene concentration.

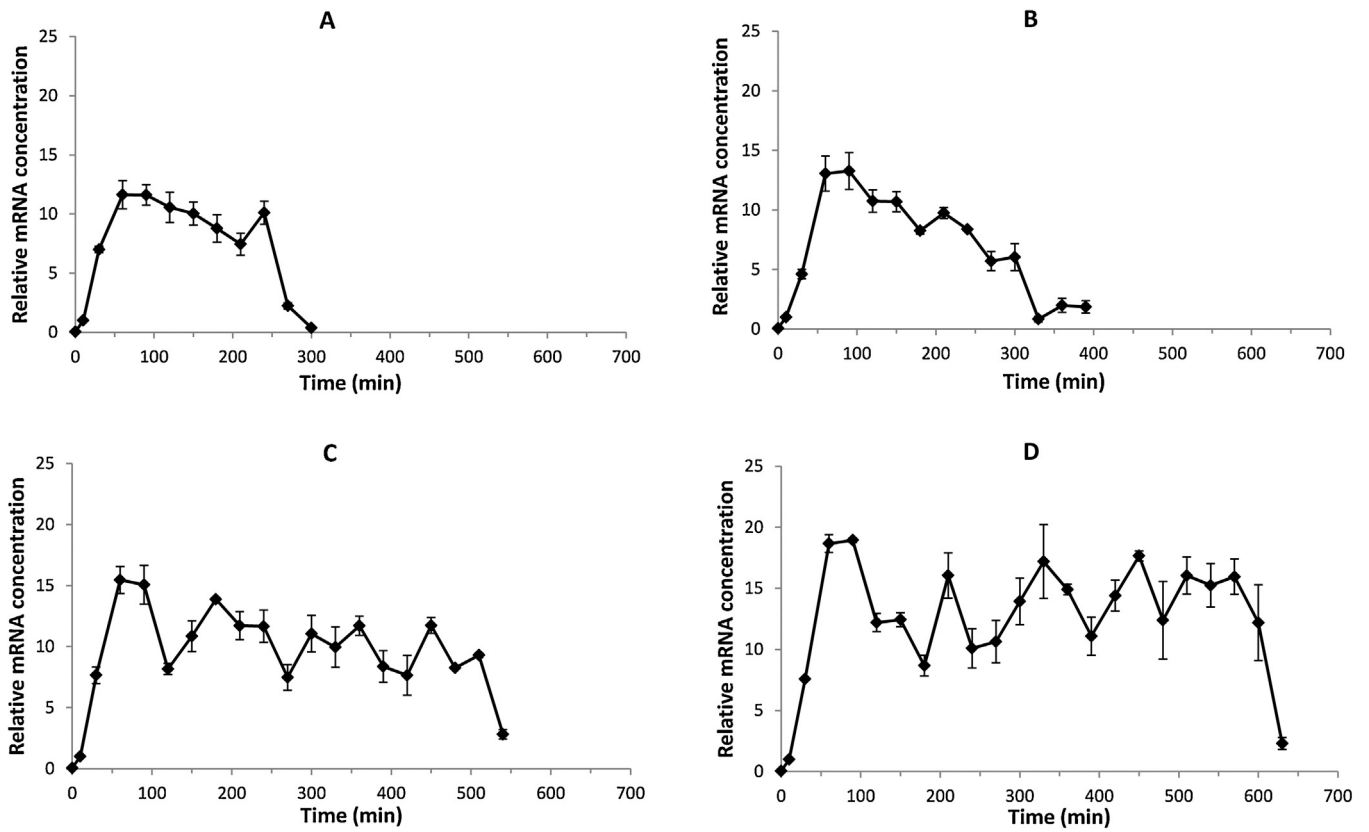


Fig. 6. *Pm* promoter relative mRNA expression in the experiments. (A) 0.4, (B) 0.7, (C) 1.0, and (D) 1.2 mM of toluene concentration.

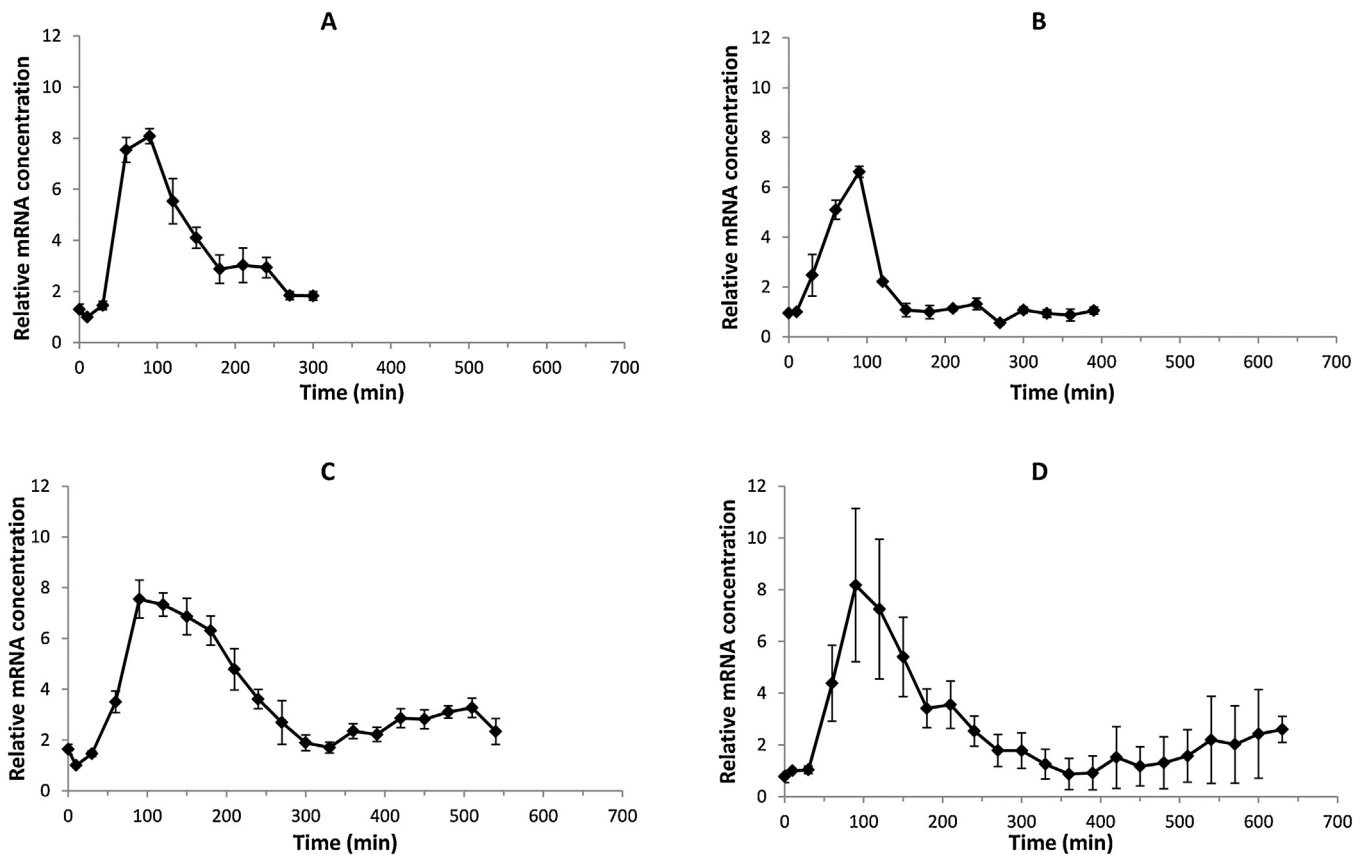


Fig. 7. *PbenR* promoter relative mRNA expression in the experiments. (A) 0.4, (B) 0.7, (C) 1.0, and (D) 1.2 mM of toluene concentration.

Furthermore, contrary to *Ps* and *Pu* relative mRNA expression profiles, the maximum relative mRNA expression level of *PbenR* was similar for each experiment and it was not influenced by the concentration of toluene used. However, the amplitude of expression was higher than *Ps* and *Pu* indicating a difference of expression between chromosomally and TOL encoded promoters.

3.7. *PbenA* promoter

PbenA is activated by BenR driving the transcription of the *benABCD* operon in the *ortho*-cleavage pathway (Chugani et al., 1997). Prior to toluene induction, expression from *PbenA* was not present. Similarly to the response of *Pm*, during the first 10 min of induction the increase in the activity of *PbenA* was relatively low ($P > 0.05$) compared to the activity of *Ps* and *Pu* TOL promoters (Fig. 8). Following that time point transcription from *PbenA* started and its expression level reached a maximal level at 180 min ($P < 0.05$). BenR is currently the only transcription factor known to up-regulate *PbenA*. Nevertheless, *PbenA* activity did not remain at a basal level for the first 30 min ($P < 0.05$) of toluene introduction, where transcription from *PbenR* was expressed at a constitutive level and hence BenR protein was produced at a constant concentration. The response of *PbenA* indicates its potential up-regulation by TOL XylS_a, thus highlighting the interdependent cross-activation of the two networks.

Following 180 min of cultivation the relative mRNA expression of *PbenA* gradually decreased to the basal level prior to the stationary phase. Similarly to *Pm* the level of transcription from *PbenA* was substantially higher compared to *Pu* and *Ps* and it was significantly affected ($P < 0.05$) by the concentration of toluene introduced, increasing its relative mRNA expression at higher pollutant concentrations. Comparing the dependency of *PbenR* and *PbenA* on the concentration of toluene in the *ortho*-cleavage pathway, the response of *PbenA* is similar to that of TOL promoters. On the contrary *PbenR* is activated reaching a maximal expression level independently of the toluene concentration employed. Furthermore *PbenA* expression levels are substantially higher than *Pu* and *Ps* and lower than *Pm*. The lower activity of chromosomal *PbenA* compared to TOL *Pm* was observed by Silva-Rocha and de Lorenzo (2014) upon benzoate induction.

The operons *benABCD* and *xylXYZL* encoded in the *ortho*- and *meta*-cleavage pathways respectively, are simultaneously activated by benzoate (Perez-Pantoja et al., 2015; Silva-Rocha and de Lorenzo, 2014) stimulating the transformation of benzoate to catechol which is further catabolised into Krebs cycle intermediates (Burlage et al., 1989; Harwood and Parales, 2000). Transcription from *benABCD* results in the formation of the BenD enzyme, which catabolises the transformation of catechol into *cis-cis*-muconate enabling the activation of subsequent *ortho*-pathway genes (Jeffrey et al., 1992).

4. Discussion

The complex genetic circuits of TOL and *ortho*-cleavage pathways interplay with the overimposed regulation being depicted in Fig. 1B and C. The integration host factor (IHF) has been also indicated as a regulator of *Pr* and *Ps* activity upon induction with a chemical signal. In cultures using LB medium the binding sites of IHF were not relevant to the transcriptional control of both promoters (Holtel et al., 1992). However, a clear repressory effect on *Ps* became evident under the presence of an inducer with M9 minimal and low-LB medium, respectively (Holtel et al., 1995; Marques et al., 1998). In contrast, this effect was weakly observed by (de Lorenzo et al., 1991; Gomada et al., 1994) rendering *Ps* activation independent of IHF regulation. Therefore, additional *in vitro* and

in vivo studies are required to elicit the explicit regulatory effect of IHF on *Ps*. Herein, toluene consumption (Fig. 2) was presented in conjunction with continuous quantitative information, obtained by real-time PCR, of the promoters pertinent to this genetic circuit (Figs. 3–8).

The first promoter activated upon toluene entry into the cells was *Pr*, which was down-regulated due to XylR auto-repression. The activated form of XylR activates both σ^{54} -dependent *Ps* and *Pu* promoters (Figs. 4, 5). The transcriptional kinetics of these promoters has been presented by Marques et al. (1994) in cultures pre-grown overnight in glucose and cultivated in M9 minimal medium supplemented with 8 mM of 3-methylbenzyl alcohol with an initial OD₆₆₀ of 0.3. Maximal mRNA expression was achieved following 10 min of induction, while similar results were obtained upon benzyl-alcohol induction. Herein, mt-2 was pre-grown in succinate and diluted to an OD₆₀₀ of 0.1 in M9 supplemented with toluene. Since 3-methylbenzyl alcohol and benzyl-alcohol were the first intermediates of *m*-xylene and toluene oxidative catabolism in TOL, respectively, their presence was expected to lead to faster induction of *Ps* and *Pu* compared to the addition of *m*-xylene/toluene. Furthermore, observing the transcriptional kinetics of *Ps* and *Pu* under 3-methylbenzyl alcohol and benzyl-alcohol induction, similarly to the results presented herein, the maximal expression level was followed by a sharp decrease in the promoters' activity.

The expression pattern of *Ps* and *Pu* indicated a significant reduction in the efficiency of XylR_a to up-regulate the promoters following their maximal activity level. The specific expression pattern has been previously reported by Bar-Joseph et al. (2012) where the increase of an up-regulated promoter activity reached a plateau followed by a consequent activity reduction. The fluctuation of promoters' level may be attributed to the role they play in a metabolic pathway acting as controllers. As soon as the environmental signal enters the cells the relevant promoter's expression is boosted triggering gene expression and, thus, enzyme production which catalyses effector's degradation to Krebs cycle intermediates. Following metabolic pathway activation, a promoter (as well as the relevant transcription factor) may have fulfilled its purpose, followed by gradual activity decrease. Another common characteristic between the present study and Marques et al. (1994) is the higher maximal level of relative mRNA concentration of *Pu* compared to *Ps*, suggesting that *Pu* is expressed at a higher level regardless of the growth conditions.

Following that, *Pm* expression is triggered. *Pm* underwent an oscillatory expression when induced at high toluene concentrations (Fig. 6C,D), which was a novel observation. The predominant mechanism causing oscillatory behaviour in genetic circuits is the presence of a composite negative feedback loop regulating both the transcription and protein levels (Alon, 2006). Oscillations often arise when the composite negative feedback loop exists in systems also containing a positive feedback loop that operates at a slower timescale. A well-studied oscillatory expression caused by a composite-negative feedback loop exists in the Notch pathway through the signalling effector Hes1 (Hirata et al., 2002; Kiparissides et al., 2011), where *de novo* synthesis and degradation of this protein is required for *hes1* mRNA oscillation to occur. Interlinked positive-negative feedback loops may act as effectors of oscillatory behaviour in the cell cycle, such as the *Xenopus* embryonic cell cycle, where the reporting system is the cyclin B dependent kinase 1 (CDK1) (Ferrell et al., 2009; Ferrell et al., 2011) or the Ca²⁺ spikes in the GS-NS0 mammalian cell cycle (García Münzer et al., 2013). Furthermore, the oscillations can be either overrun or damned, while the more un-damned an oscillation the stronger the regulation (Alon, 2006). Therefore, it is possible that in concentrations of toluene higher than 1 mM a regulator could be activated down-regulating the *meta*-operon and resulting in the

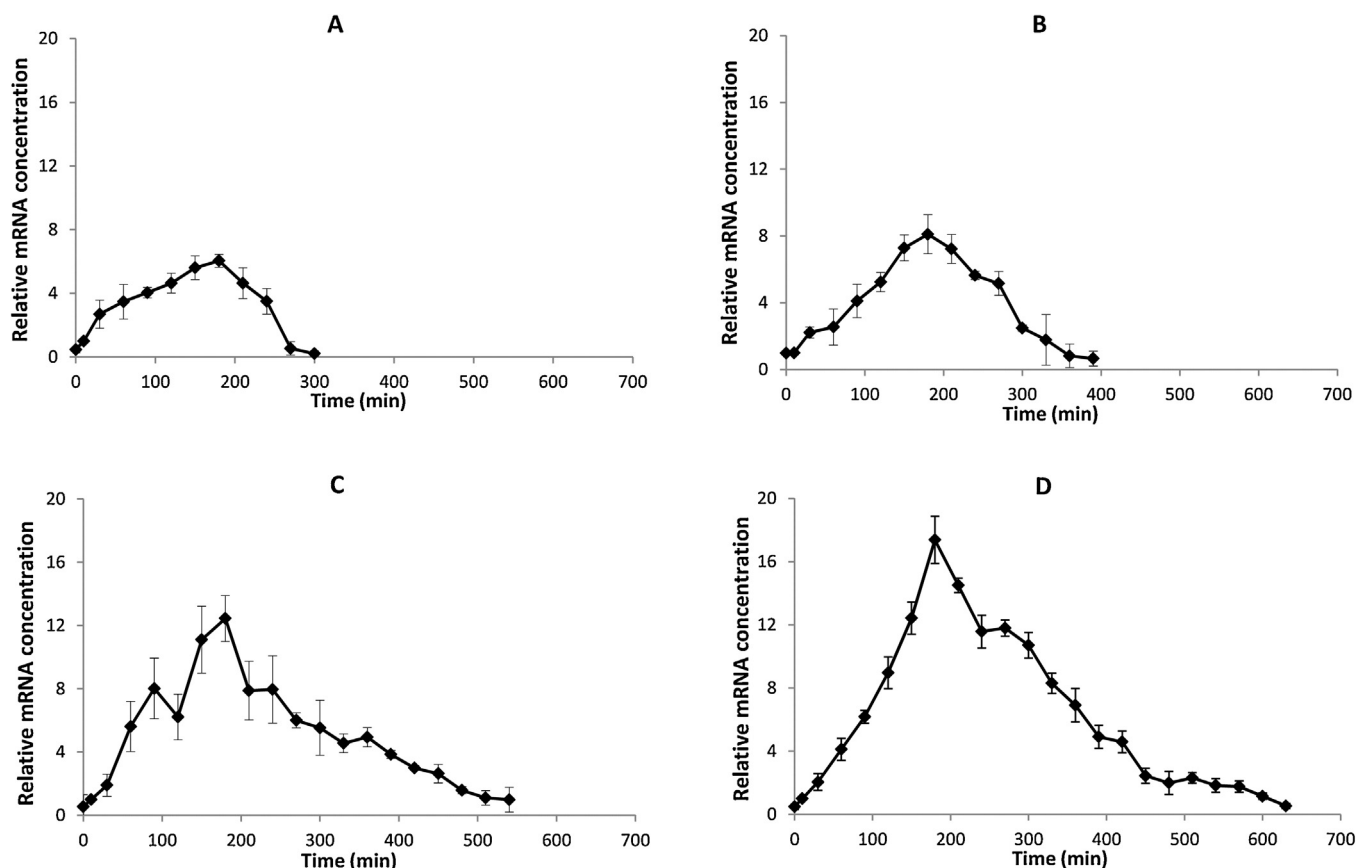


Fig. 8. *PbenA* promoter relative mRNA expression in the experiments. (A) 0.4, (B) 0.7, (C) 1.0, and (D) 1.2 mM of toluene concentration.

formation of a composite feedback loop together with the two up-regulators of *Pm*. To our knowledge, this is the first time that *Pm* has been demonstrated to exhibit oscillatory behaviour and thus future research should focus on characterisation of the biological mechanism(s) causing this effect through experimental and modelling approaches.

Pu controls upper operon expression encoding for the catalytic enzymes of toluene to benzoate transformation. Benzoate induces BenR protein activity (Cowles et al., 2000). The *benR* gene has been reported to be activated by the presence of benzoate (Cuskey and Sprengle, 1988) which was consistent with the *PbenR* expression pattern observed (Fig. 7) triggered by benzoate and up-regulated by a yet unknown transcription factor. According to the present study and that of Marques et al. (1994), regardless of the pathway inducer and pre-culture conditions employed, *Ps* and *Pu*, which are up-regulated by the same transcription factor ($XylR_a$), reached a maximal level of expression at the same time point. This fact suggests that promoters activated by the same transcription factor may achieve maximal activity simultaneously. Therefore, *Pm* and *PbenR* that reached their maximal level of expression at 90 min in all concentration levels of toluene tested could be up-regulated by the same transcription factor. Since BenR is the chromosomally encoded transcription factor of *Pm* (Cowles et al., 2000), the results of the present study suggest that BenR could serve as the transcription factor of *PbenR* indicating the interplay of the two pathways as well as BenR auto-regulation.

PbenR activation resulted in BenR protein production, which is the known transcription factor of *PbenA*. Alas, *PbenA* activation occurred prior to *PbenR* increasing activity suggesting up-regulation by TOL XylS protein (Fig. 8). The cross-talk of *PbenA* and XylS in *P. putida* PRS2000 strain has been suggested before (Cowles et al., 2000; Jeffrey et al., 1992). Additionally, the up-

regulation of *PbenA* by XylS has been previously reported for *P. putida* mt-2 with the application of a plasmid harbouring a *Pben-lacZ* fusion (Dominguez-Cuevas et al., 2006). However, the particular interaction has not been entirely validated yet. Herein, we suggest that *PbenA* would be activated even in the absence of BenR driving to further activation of *ortho*-cleavage pathway and its utilisation for benzoate degradation. Therefore, the presence of toluene could activate both catabolic pathways in *P. putida* mt-2.

It was also observed that *PbenA* reached higher levels of expression compared to *Ps* and *Pu*. However *PbenA* mRNA expression level is lower than *Pm* in every toluene concentration tested similarly to Silva-Rocha and de Lorenzo (2014) results of single-cell dynamics upon testing both promoters activity up to 4 h of *P. putida* mt-2 cultivation using benzoate as the inducer, which is formed by *Pu* controlled upper pathway activity upon toluene entry. Furthermore Dominguez-Cuevas et al. (2006) showed the fold change of mRNA expression of chromosomal *ben*- and TOL *meta*- operon genes after 15 min of toluene induction observing lower folding change of *benA* than *xylX* gene controlled by *PbenA* and *Pm* promoter, respectively. Possible reasons for the remarkably higher mRNA expression levels of the chromosomal *PbenA* compared to TOL *Ps* and *Pu* could be the low-copy of TOL plasmid in the bacterial culture or, taking into consideration activation of *PbenA* by XylS protein, high maximal expression levels of *PbenA* may be triggered by the activated and over-produced XylS due to benzoate formation.

During *m*-xylene catabolism in TOL the transcriptional response from *PbenA*, which controls *ben* operon, due to the final product (3-methyl-benzoate) of the upper pathway was tested by Perez-Pantoja et al. (2015). It was shown that 3-methyl-benzoate cannot serve as a substrate of the chromosomal pathway, since *PbenA* is only marginally activated resulting in the formation of a product causing cell death (Schmidt et al., 1985). Thus, toluene, which is

currently considered as the predominant substrate for degradation by *P. putida* (Nicolaou et al., 2010), is currently the only inducer of the complete TOL regulatory mechanism triggering expression of the chromosomal pathway.

Activation of the *ortho*-cleavage pathway due to toluene leads to the production of muconic acid (Xie et al., 2014), which is a value-added product with a wide range of applications highlighting the industrial importance of toluene catabolism through TOL and chromosomal pathways. Furthermore, acquiring the capacity to understand the properties of specific and global regulation in this genetic network paves the way towards *de novo* (Diaz et al., 2013) and *in silico* (Koutinas et al., 2011) engineering of aromatic degradation pathways by employing a combination of computational and synthetic biology approaches. The activation of the *ortho*-cleavage pathway upon toluene entry in *P. putida* mt-2 has been studied by Silva-Rocha and de Lorenzo (2013) to engineer cell-to-cell metabolic wiring of *P. putida* mt-2 and KT2440 strains. While modelling of the transcriptional regulation in TOL and *ortho*-cleavage pathways could lead to *in silico* prediction of toluene consumption and biomass growth patterns similarly to Koutinas et al. (2011) upon *m*-xylene entry to TOL pathway. Transcriptional regulation could be the controller of macroscopic phenomena since the promoters of *upper*, *meta* and *ben* catabolic operons *Pu*, *Pm* and *PbenA*, respectively were sensitive to the concentration of the inducer used.

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