Study of factors which negatively affect expression of the phenol degradation operon *pheBA* in *Pseudomonas putida*

Marta Putrinš, Andres Tover, Radi Tegova, Ülle Saks and Maia Kivisaar

Department of Genetics, Institute of Molecular and Cell Biology, Tartu University and Estonian Biocentre, 51010 Tartu, Estonia

Correspondence
Maia Kivisaar
maiak@ebc.ee

Transcription of the plasmid-borne phenol catabolic operon *pheBA* in *Pseudomonas putida* is activated by the LysR-family regulator CatR in the presence of the effector molecule *cis*, *cis*-muconate (CCM), which is an intermediate of the phenol degradation pathway. In addition to the positive control of the operon, several factors negatively affect transcription initiation from the *pheBA* promoter. First, the activation of the *pheBA* operon depends on the extracellular concentration of phenol. The *pheBA* promoter is rapidly activated in the presence of micromolar concentrations of phenol in minimal growth medium, but the initiation of transcription from this promoter is severely delayed after sudden exposure of bacteria to 2.5 mM phenol. Second, the transcriptional activation from this promoter is impeded when the growth medium of bacteria contains amino acids. The negative effects of amino acids can be suppressed either by overproducing CatR or by increasing the intracellular amount of CCM. However, the intracellular amount of CCM is a major limiting factor for the transcriptional activation of the *pheBA* operon, as accumulation of CCM in a *P. putida* *catB*-defective strain, unable to metabolize CCM (but expressing CatR at a natural level), almost completely relieves the negative effects of amino acids. The intracellular amount of CCM is negatively affected by the catabolite repression control protein via downregulating at the post-transcriptional level the expression of the *pheBA*-encoded catechol 1,2-dioxygenase and the phenol monooxygenase, the enzymes needed for CCM production.

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INTRODUCTION

Aromatic compounds in the environment may be plant-derived or released from industry. Microbial transformation of aromatics is important in nature as well as in technological applications of micro-organisms, such as bioremediation. Nevertheless, in pseudomonads, transcription of genes encoding catabolic pathways degrading these chemicals is inhibited during rapid growth in nutritionally rich medium, irrespective of the presence of specific effector molecules (for reviews, see e.g. Cases & de Lorenzo, 2001, 2005; Marqués *et al.*, 2006; Rojo & Dinamarca, 2004; Shingler, 2003). Several global factors such as the Crc protein (Hester *et al.*, 2000; Morales *et al.*, 2004; Yuste & Rojo, 2001), CyoB (Dinamarca *et al.*, 2002; Petruschka *et al.*, 2001) and the FtsH protease (Carmona & de Lorenzo, 1999; Sze *et al.*, 2002) have been related to catabolite repression in *Pseudomonas putida*. As many aromatic compounds are toxic to living organisms, this also complicates their degradation. The toxicity of certain

aromatic compounds such as toluene, xylenes and phenol occurs above a certain threshold, since they easily dissolve in the cell membrane, disorganizing its structure and impairing vital functions (Domínguez-Cuevas *et al.*, 2006; Ramos *et al.*, 2002; Sikkema *et al.*, 1995). It has been demonstrated that sudden exposure of bacteria to sublethal inhibitory concentrations of phenol leads to reduction of growth rate of microbial culture until the bacteria are adapted to this concentration of phenol (Santos *et al.*, 2004). During the adaptation to phenol, the fatty acid composition of the bacterial membranes changes (Heipieper *et al.*, 2003; Sikkema *et al.*, 1995). Also, the results of quantitative proteomics have revealed that the response of *P. putida* KT2440 to phenol-induced stress results in upregulation of many proteins, e.g. those involved in the oxidative stress response and transport of small molecules (Santos *et al.*, 2004).

In *P. putida*, aromatic compounds are transformed by different enzymes to central intermediates such as proto-catechuate and (substituted) catechols, which are further degraded either by a *meta* ring cleavage pathway or by an *ortho* ring cleavage pathway (Harayama & Timmis, 1989). The *catBCA* operon encodes three enzymes required for

Abbreviations: C12O, catechol 1,2-dioxygenase; CAA, casamino acids; CCM, *cis*,*cis*-muconate; Crc, catabolite repression control; PMO, phenol monooxygenase; RLU, relative light unit.

catechol degradation via the *ortho* pathway: *cis,cis*-muconate (CCM) lactonizing enzyme, muconolactone isomerase and catechol 1,2-dioxygenase (C12O), respectively (Houghton *et al.*, 1995). The induction of this operon requires the LysR-family transcriptional activator CatR and an inducer molecule, CCM, which is an intermediate of the *ortho* pathway (Rothmel *et al.*, 1991, 1990).

The *pheB* and *pheA* genes, originating from the plasmid DNA of *Pseudomonas* sp. EST1001, encode C12O and phenol monooxygenase (PMO), respectively (Kivisaar *et al.*, 1990). When the *pheBA* operon is introduced into *P. putida* laboratory strain PaW85, the bacteria acquire the ability to degrade phenol (Kivisaar *et al.*, 1991, 1990). The *pheBA* promoter resembles the *catBCA* promoter and is also activated by CatR (Kasak *et al.*, 1993; Parsek *et al.*, 1995; Tover *et al.*, 2000). Comparative studies of the interaction of CatR at the promoters of the *pheBA* and *catBCA* operons have revealed that the CatR-mediated activation mechanism is well conserved, despite the different origins of these operons (Parsek *et al.*, 1995; Tover *et al.*, 2000). Transcriptional regulation of the *pheBA* operon in *P. putida* is illustrated in Fig. 1.

After release of the laboratory *P. putida* strain carrying the *pheBA* genes on a plasmid into a phenol-contaminated mining area in Estonia, horizontal transfer of the *pheBA* operon and its expression in different soil bacteria was observed (Peters *et al.*, 1997). The *pheBA* operon was redetected in different bacterial species in watershed continuously polluted by phenols, but not in non-contaminated areas. In all isolates degrading phenol via the *ortho* pathway and harbouring the *pheBA* genes integrated either into other plasmids or in the chromosome, the original *pheBA* promoter was present as before and the *pheBA* genes were induced in the presence of phenol and benzoate (Peters *et al.*, 1997). Thus, soil bacteria carrying the *ortho* pathway genes regulated by CatR may easily expand their substrate range via horizontal transfer of the *pheBA* genes without the need for subsequent extensive genetic rearrangements. Hence, the *pheBA* operon provides a good model for examining the regulation of phenol catabolic genes in soil bacteria.

Here, we have focused on mechanisms which negatively affect transcription from the *pheBA* promoter. We found that sudden exposure of *P. putida* to 2.5 mM phenol delayed the operon activation without affecting transcription in general, whereas micromolar concentrations allowed rapid induction of the phenol degradation pathway. Additionally, our previous studies have revealed that transcriptional activation from the *pheBA* and *catBCA* promoters is repressed in *P. putida* cells growing exponentially on minimal medium in the presence of amino acids (Tover *et al.*, 2001). The results of the current study indicate that the Crc protein downregulates at the post-transcriptional level the expression of the *pheBA* genes, resulting in reduced amounts of phenol degradation enzymes PheA and PheB needed for the production of the *pheBA* operon inducer molecule, CCM.

Fig. 1. Schematic representation of transcriptional regulation of the *pheBA* operon in *P. putida*. Phenol and benzoate are converted to *cis,cis*-muconate (CCM), an intermediate of the catechol degradation *ortho* pathway which is needed for CatR-mediated transcriptional activation from the *pheBA* promoter. PheA, phenol monooxygenase; PheB, catechol 1,2-dioxygenase; BenABC, benzoate dioxygenase; BenD, benzoate *cis*-diol dehydrogenase; CatA, catechol 1,2-dioxygenase; CatB, *cis,cis*-muconate lactonizing enzyme; CatR, LysR-family transcriptional activator.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* cells were grown at 37 °C in Luria broth and *P. putida* cells were grown at 30 °C in M9 minimal medium (Adams, 1959). Antibiotics were added at the following final concentrations: for *E. coli*, ampicillin at 100 µg ml⁻¹, tetracycline at 10 µg ml⁻¹; for *P. putida*, carbenicillin at 1000–3000 µg ml⁻¹, tetracycline at 80 µg ml⁻¹; for both organisms, kanamycin at 50 µg ml⁻¹, streptomycin at 100 µg ml⁻¹ and potassium tellurite at 40 µg ml⁻¹. *E. coli* was transformed with plasmid DNA as described by Hanahan (1983). *P. putida* was electrotransformed as described by Sharma & Schimke (1996). *E. coli* strains TG1 (Carter *et al.*, 1985) and CC118 λ pir (Herrero *et al.*, 1990) were used for the DNA cloning procedures, and strain HB101 (Boyer & Roulland-Dussoix, 1969) was used as a host for helper plasmid pRK2013 (Figurski & Helinski, 1979), necessary for mobilization of non-conjugative plasmids.

Cloning procedures and construction of bacterial strains. We have used Tn5-based mini-transposons and their delivery systems,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or construction	Source or reference
<i>E. coli</i>		
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F (traD36 proAB⁺ lacI^q lacZAM15)</i>	Carter <i>et al.</i> (1985)
HB101	<i>supE44 hsd20(r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	Boyer & Roulland-Dussoix (1969)
CC118λpir	<i>galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1, λpir</i> phage lysogen	Herrero <i>et al.</i> (1990)
<i>P. putida</i>		
PaW85	Wild-type	Bayley <i>et al.</i> (1977)
PaWlux	P _i - <i>luxAB</i> in the chromosome of PaW85, Sm ^r	This work
PaWpheBA-lux	P _i - <i>luxAB</i> and P _i - <i>pheBA</i> fusions in the chromosome of PaW85, Km ^r Sm ^r	This work
PaWpheBA-lux-catB::tet	PaWpheBA-lux with an inactivated <i>catB</i> gene, Km ^r Sm ^r Tet ^r	This work
PaWpheBA-lux-catR	<i>catR</i> gene under control of P _{tac} promoter in chromosome of PaWpheBA-lux, Km ^r Sm ^r Tel ^r	This work
PaWlux-pheBA-crc::tet	PaWpheBA-lux with an inactivated <i>crc</i> gene, Km ^r Sm ^r Tet ^r	This work
PaWtaclux	PaW85 with <i>lacI^q-P_{tac}-luxAB</i> expression cassette in the chromosome, Sm ^r	This work
PaWtaclux-pheB	PaW85 with <i>lacI^q-P_{tac}-luxAB</i> and <i>lacI^q-P_{tac}-pheB</i> expression cassettes in the chromosome, Sm ^r Tel ^r	This work
PaWtaclux-pheB-crc::tet	PaWtaclux-pheB with an inactivated <i>crc</i> gene, Tel ^r Sm ^r Tet ^r	This work
Plasmids		
pBluescript KS(+)	Cloning vector (Ap ^r)	Stratagene
pGP704L	Ap ^r ; <i>luxAB</i>	Pavel <i>et al.</i> (1994)
pGP704del	Derivative of pGP704 by deletion of 1 kb <i>EcoRV</i> fragment from <i>luxA</i> gene	This work
pUC18Not	Cloning vector (Ap ^r)	Herrero <i>et al.</i> (1990)
pRK2013	Km ^r Mob ⁺ Tra ⁺ , donor transfer functions	Figurski & Helinski (1979)
pCRC10	<i>P. putida</i> <i>crc</i> gene interrupted with Tet ^r gene in pKNG101	Yuste & Rojo (2001)
pBR322	Cloning vector (Ap ^r Tet ^r)	Bolivar <i>et al.</i> (1977)
pBRLacItac	P _{tac} promoter and <i>lacI^q</i> repressor in pBR322	Ojangu <i>et al.</i> (2000)
mTn5SSgusA40	Delivery plasmid for mini Tn5 Sm	Wilson <i>et al.</i> (1995)
pUTmini-Tn5Km2	Delivery plasmid for mini Tn5 Km2	de Lorenzo <i>et al.</i> (1990)
pUTmini-Tn5Tel	Delivery plasmid for mini Tn5 Tel	Sanchez-Romero <i>et al.</i> (1998)
pUCNotluxAB	2050 bp promoterless <i>luxAB</i> genes containing <i>HindIII</i> and <i>PaeI</i> restriction fragment from plasmid pGP704L cloned into pUC18Not	This work
pUCNotP _i luxAB	<i>pheBA</i> promoter region within <i>SmaI</i> - <i>RsaI</i> fragment inserted into the <i>SmaI</i> site of pUCNotluxAB	This work
pUTP _i luxABSm	<i>pheBA</i> promoter- <i>luxAB</i> fusion from pUCNotP _i luxAB cloned into mTn5SSgusA40	This work
pAT1142	<i>pheBA</i> genes under control of their natural promoter P _i in pAYC32	Kasak <i>et al.</i> (1993)
pUCNotP _i pheBA	6.3 kb <i>SacI</i> - <i>EcoRI</i> fragment containing inducible <i>pheBA</i> genes cloned from plasmid pAT1142 into pUC18Not	This work
pUTP _i pheBAKm	Inducible <i>pheBA</i> genes from pUCNotP _i pheBA into pUTmini-Tn5Km2	This work
pUCNottacluxAB	<i>Bam</i> HI fragment containing <i>lacI^q-P_{tac}</i> sequence cloned into pUCNotluxAB	This work
pUTtacluxABSm	<i>lacI^q-P_{tac}-luxAB</i> cassette from pUCNottacluxAB inserted into <i>NotI</i> site of mTn5SSgusA40	This work
pKScatR	913 bp <i>P. putida</i> <i>catR</i> gene cloned into pBluescript KS(+) <i>EcoRV</i> site	This work
pBRtacCatR	<i>EcoRI</i> - <i>HindIII</i> fragment containing <i>catR</i> gene from pKScatR inserted into pBRLacItac	This work
pUCNottacCatR	<i>EcoRI</i> - <i>NheI</i> fragment containing <i>lacI^q-P_{tac}-catR</i> cassette from pBRtacCatR cloned into pUC18Not	This work
pUTtacCatRTel	<i>lacI^q-P_{tac}-catR</i> cassette from pUCNotPtacCatR cloned into pUTmini-Tn5Tel	This work
pKScatB	1122 bp <i>P. putida</i> <i>catB</i> gene cloned into pBluescript KS(+) <i>EcoRV</i> site	This work
pKScatB : : tet	Tet ^r gene from plasmid pBR233 inserted into pKScatB into the <i>EheI</i> site locating 513 bp downstream of the <i>catB</i> translation start codon	This work
pGPcatB:tet	<i>Ecl136II</i> - <i>Acc65I</i> fragment from pKScatB:tet containing <i>catB</i> : : <i>tet</i> allele cloned into pGP704del	This work
pBRLacItacPheB	<i>HincII</i> - <i>Eco47II</i> fragment containing <i>pheB</i> gene from pAT1142 inserted into pBRLacItac	This work
pUCNotPtacPheB	<i>Ecl136II</i> - <i>Bam</i> HI fragment containing <i>lacI^q-P_{tac}-pheB</i> inserted from pBRLacItacPheB into pUC18Not	This work
pUTtacPheBTel	<i>lacI^q-P_{tac}-pheB</i> inserted from pUC18Not into pUTmini-Tn5Tel	This work

commonly used in genetic engineering of Gram-negative bacteria (de Lorenzo & Timmis, 1994; de Lorenzo *et al.*, 1990), to integrate desired transcription fusion constructs stably into the genome of *P. putida*. In order to be sure that the acquisition of the selected phenotype by *P. putida* is due to the transposition event and not to the integration of the whole delivery plasmid into a recipient chromosome, the transconjugants were tested for resistance to carbenicillin. Only clones which were sensitive to this antibiotic were used for further examination whether we had obtained the desired strains (see below for details of testing). Additionally, all strains were confirmed by PCR analysis for the presence and correct organization of the inserted cassettes. We determined chromosomal locations of the integrated transcription fusion cassettes by sequencing the DNA flanking the insertion sites. None of the targeted genes (PP0806, PP1103, PP1308, PP1775, PP2872, PP3483, PP3956, PP4063 and PP5300) are related to the metabolism of aromatic compounds or encode general transcription factors. We have obtained reproducible results with strains carrying the same transcriptional fusions in different chromosomal locations. This also indicates that there are no additional regulatory effects caused by the insertional inactivation of certain chromosomal genes.

To study the regulation of transcription from the *pheBA* promoter, the *pheBA* promoter P_i was cloned upstream from the *luxAB* reporter system. The promoterless *luxAB* genes (these genes originated from *Vibrio harveyi*) were cloned within the 2050 bp *HindIII* and *PaeI* restriction fragment from plasmid pGP704L (Pavel *et al.*, 1994) into vector pUC18Not (Herrero *et al.*, 1990), cleaved with the same restriction enzymes, to obtain plasmid pUCNotluxAB. The 158 bp *pheBA* promoter region was excised from the plasmid carrying the *pheBA* promoter in pBluescript SK(+) vector (Tover *et al.*, 2000) using restriction enzymes *SmaI* and *RsaI*, and inserted into the *SmaI* site of plasmid pUCNotluxAB. The *pheBA* promoter-*luxAB* transcription fusion P_i -*luxAB* was then inserted into the *NotI* site of plasmid mTn5SSgusA40 (Wilson *et al.*, 1995) to generate plasmid pUTP₁luxABSm. Finally, the plasmid pUTP₁luxABSm, which was not able to replicate in hosts other than *E. coli* CC118 λ pir, was conjugatively transferred into *P. putida* PaW85 (Bayley *et al.*, 1977) by using a helper plasmid, pRK2013, and transconjugants carrying random insertions of mini-transposon containing the P_i -*luxAB* fusion in the chromosome of PaW85 were isolated. We tested several independent clones in order to identify transconjugants whose luciferase expression was not affected by sequences flanking the inserted P_i -*luxAB* cassette [i.e. they expressed luciferase activity at a low basal level not exceeding 10 RLU (relative light units) per OD₅₈₀ unit]. One of these clones was chosen for future research and was named PaWlux.

To construct a *P. putida* strain capable of degrading phenol, the *pheBA* operon was inserted into the chromosome of *P. putida* strain PaWlux. An approximately 6.3 kb DNA fragment, containing the *pheBA* genes under the control of the native inducible *pheBA* promoter P_i , was cloned from plasmid pAT1142 (Kasak *et al.*, 1993) as a *SacI*- and *EcoRI*-generated DNA fragment into pUC18Not cleaved with same enzymes, to obtain plasmid pUCNotP₁pheBA. As a next step, the *NotI* fragment containing the inducible *pheBA* operon was subcloned from pUCNotP₁pheBA into plasmid pUTmini-Tn5 Km2 (de Lorenzo *et al.*, 1990). The resulting plasmid, named pUTP₁pheBAKm, was used as a donor plasmid for the insertion of the *pheBA* operon into the chromosome of *P. putida* strain PaWlux to obtain strain PaWpheBA-lux. The procedure for isolation of transconjugants was the same as described above. Several independent clones were tested and the expression of the *pheBA* genes and the *luxAB* reporter in these clones was dependent on the presence of the *pheBA* operon inducer (greater than 100-fold induction appeared when the growth medium of bacteria contained phenol or benzoate, whereas the basal level of luciferase activity was the same as in PaWlux).

In order to construct a control strain expressing the *luxAB* genes under the control of the P_{tac} promoter, the *BamHI*-generated DNA fragment containing the *lacI*^R- P_{tac} regulatory region was subcloned from pBRLacItac (Ojangu *et al.*, 2000) into plasmid pUCNotluxAB to obtain plasmid pUCNottacluxAB. Then, the *lacI*^R- P_{tac} -*luxAB* expression cassette was inserted into the *NotI* site of plasmid mTn5SSgusA40. Finally, the plasmid pUTtacluxABSm generated was used as a donor plasmid for the insertion of the *lacI*^R- P_{tac} -*luxAB* expression cassette into the chromosome of *P. putida* strain PaW85 to obtain strain PaWtaclux. Similar procedures as described above were used to obtain mini-transposon insertion into the chromosome of *P. putida*, and several independent clones were tested to be sure that transcription from the P_{tac} promoter was inducible by IPTG in the constructed strain.

For the construction of *P. putida* strain PaWpheBA-lux-catR, carrying the *catR* gene under control of the P_{tac} promoter and the *LacI*^R repressor, the *lacI*^R- P_{tac} -*catR* expression cassette was inserted into the chromosome of *P. putida* strain PaWpheBA-lux. In contrast with the *CatR*-overexpression cassette constructed in our previous study (Tover *et al.*, 2001), the new construct lacked the *CatR* auto-regulation site [i.e. an original *catR* gene is divergently transcribed from the *catBCA* promoter, which contains *CatR* binding sites overlapping the *catR* gene promoter region, thereby reducing transcription from its own gene (Rothmel *et al.*, 1991)]. First, the 913 bp DNA fragment containing the *catR* gene, but lacking the *CatR* binding site, was PCR-amplified from *P. putida* PaW85 chromosome using the primers 5'-CCCACCATACCCTGGAGG-3' and 5'-GG-AGCGCGAAGCTTTTCGGCCTGTTGTCAATCAA-3', complementary to the regions located at positions between -4 and +14 bp and +875 and +909 relative to the *catR* gene transcription start site, respectively. The PCR product was cloned into the *EcoRV* site of pBluescript KS(+) vector, yielding plasmid pKScatR. Then, the *catR* sequence was excised from pKScatR with *EcoRI* and *HindIII*, and inserted into the plasmid pBRLacItac, opened with the same enzymes. The resulting plasmid pBRtacCatR was cleaved with *EcoRI* and *NheI* to subclone the *lacI*^R- P_{tac} -*catR* expression cassette into plasmid pUC18Not to generate plasmid pUCNottaCatR. Finally, the expression cassette *lacI*^R- P_{tac} -*catR* was inserted into the *NotI* site of plasmid pUTmini-Tn5Tel (Sanchez-Romero *et al.*, 1998) to obtain plasmid pUTtacCatRTel, which was used as donor plasmid for the insertion of the *lacI*^R- P_{tac} -*catR* cassette into the chromosome of *P. putida* strain PaWpheBA-lux. We tested several independent *P. putida* PaWpheBA-lux transconjugants carrying random insertions of the *lacI*^R- P_{tac} -*catR* expression cassette in the chromosome, and clones giving a luciferase expression pattern similar to that measured in the wild-type strain PaWpheBA-lux if IPTG was not added (this was tested in both the presence and absence of phenol) were chosen for further examination. The results of PCR analysis confirmed the presence of the *lacI*^R- P_{tac} -*catR* expression cassette in the chromosome of transconjugants.

Inactivation of the *catB* gene in the chromosome of *P. putida* was performed by homologous recombination using plasmid pGPcatB::tet carrying the *catB* sequence interrupted by the Tet^r gene. To construct the plasmid pGPcatB::tet, at first the 1122 bp DNA fragment containing the *catB* gene was PCR-amplified from the chromosome of *P. putida* PaW85 with the primers 5'-ATGACAAGCGTGCTGATTGA-3' and 5'-TCAGCGACGGGCGAAG-3', complementary to the regions containing the *catB* start and stop codons, respectively. The PCR product was cloned into the *EcoRV* site of plasmid pBluescript KS(+), resulting in plasmid pKScatB. An approximately 1.3 kb DNA fragment, containing the tetracycline resistance gene, was obtained from plasmid pBR322 (Bolivar *et al.*, 1977) and inserted into the *EheI* site located 513 bp downstream from the *catB* translation start codon within plasmid pKScatB to obtain plasmid pKScatB::tet. Finally, using the restriction enzymes *Ecl136II* and *Acc65I*, the DNA fragment containing the interrupted

catB gene was cloned from pKScatB : : tet into plasmid pGP704del (a derivative of plasmid pGP704L obtained by deletion of the 1 kb *EcoRI* fragment from the *luxA* gene) to obtain plasmid pGPcatB : : tet. This plasmid, not able to replicate in hosts other than *E. coli* CC118 λ pir, was conjugatively transferred into *P. putida* PaWpheBA-lux by using the helper plasmid pRK2013. The integration of the whole delivery plasmid into a target site was excluded by testing transconjugants for resistance to carbenicillin (only those unable to grow in the presence of 1500 μ g carbenicillin ml⁻¹ were considered to be true recombinants, generated as a result of double recombination events). The *catB*-knockout strain PaWpheBA-lux-*catB* : : tet was verified by PCR analysis for the absence of the original *catB* gene and the presence of the *catB* : : tet allele. Additionally, we could confirm that this knockout strain was unable to grow on benzoate or phenol as a carbon source.

Inactivation of the *crc* gene in the chromosome of *P. putida* was performed by homologous recombination using plasmid pCRC10 carrying the *P. putida* *crc* gene interruption *crc* : : tet on plasmid pKNG101 (Yuste & Rojo, 2001). The plasmid pCRC10 was transferred to PaWpheBA-lux and sucrose-resistant colonies were selected according to the procedure described by Yuste & Rojo (2001) to isolate clones carrying the *crc* : : tet allele instead of the original *crc* gene. An isolated *crc*-defective strain was named PaWpheBA-lux-*crc* : : tet.

For the construction of *P. putida* strain PaWtaclux-pheB, where PheB is expressed in the presence of IPTG, the *lacI*^R-*P*_{lac}-*pheB* expression cassette was inserted into the chromosome of *P. putida* strain PaWtaclux. A 1011 bp DNA fragment from plasmid pAT1442, containing the *pheB* gene, was excised with restriction enzymes *Eco47II* and *HincII*, and inserted into plasmid pBRLactac cleaved with *SmaI*, yielding plasmid pBRLactacpheB. The *Ecl136II*-*BamHI* fragment, containing the *lacI*^R-*P*_{lac}-*pheB* expression cassette from plasmid pBRLactacpheB, was cloned into the plasmid pUC18Not, yielding plasmid pUCNotPtacPheB. Finally, the expression cassette *lacI*^R-*P*_{lac}-*pheB* was inserted into the *NotI* site of plasmid pUTmini-Tn5Tel, and the plasmid pUTPtacPheB generated was used as donor plasmid for the insertion of the *lacI*^R-*P*_{lac}-*pheB* cassette into the chromosome of *P. putida* strain PaWtaclux. Inactivation of the *crc* gene in the chromosome of *P. putida* PaWtaclux-pheB was performed by homologous recombination by using the same strategy as already described for the construction of strain PaWpheBA-lux-*crc* : : tet. The resulting strain was named PaWtaclux-pheB-*crc* : : tet.

Growth and culture conditions. To study the regulation of transcription from the *pheBA* promoter, all *P. putida* strains used in this study were grown in M9 minimal salts medium supplemented with 10 mM glucose. For enzyme assays, the bacterial cultures were grown overnight in M9 medium supplemented with 0.2 % casamino acids (CAA). To ensure that the bacterial culture was in exponential growth phase, the overnight culture was diluted 1 : 100 in fresh medium supplemented with 0.02 % CAA. After 3 h the exponential culture was diluted once more to obtain an OD₅₈₀ of 0.05. To induce the transcription from *P*_I-*pheBA* and *P*_I-*luxAB* transcriptional fusions, 2.5 mM sodium benzoate or phenol at different final concentrations (0.0025–2.5 mM) was added to the growth medium. In parallel, control cultures lacking these substrates were sampled for enzyme assays. To investigate the role of amino acids on the transcription from the *pheBA* promoter, 0.2 % CAA solution was added when indicated. To investigate the effect of overexpression of the CatR protein on transcriptional activation from the *pheBA* promoter, 1 mM IPTG was added to the growth medium of strain PaWpheBA-lux-catR when overnight-grown cells were diluted the first and second time. To study the effect of Crc protein on PheB levels, strains carrying the *lacI*^R-*P*_{lac}-*pheB* expression cassette were grown exponentially in M9 minimal medium supplemented with

0.02 % CAA until an OD₅₈₀ of 0.3–0.4 was reached. The bacterial culture was divided in two and 1 mM IPTG was added to induce the expression of PheB. One of these cultures was supplemented with amino acids (0.2 % CAA).

Luciferase assay. Samples for luciferase assay were taken from exponentially grown cultures. Luciferase assay was performed as follows. Phosphate buffer (990 μ l; 100 mM Na₂HPO₄/KH₂PO₄, pH 7.5) and 10 μ l decanal (5 mM decanal in ethanol) were mixed in a test tube. Bacterial culture (10 μ l) was then added, and light emission was measured after 5 min incubation with a luminometer (TD-20/20; Turner BioSystems). The luciferase activities were measured in at least three separate experiments, where each experiment included two parallel cultures.

Immunoblotting of PheA and PheB. Cell suspensions used for separation of proteins by gel electrophoresis were prepared from the same cultures that were used for luciferase assays. Crude lysates, used for quantification of PheA and PheB proteins, were prepared from an amount of cells which expressed approximately 10 000 RLU luciferase activity per 10 s. Proteins were separated by sodium SDS-polyacrylamide (10 %) gel electrophoresis and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences). For Western blotting, the membrane was probed simultaneously with mouse anti-PheA and anti-PheB polyclonal sera (the polyclonal antibodies against PheA and PheB proteins were prepared by LabAS) diluted 1 : 2000 and 1 : 1000 respectively, followed by alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1 : 5000 (LabAS). The blots were developed using 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium. The polyclonal antibodies against PheA and PheB were obtained by using PheA and PheB proteins which were overproduced in *E. coli* with pUC19 derivatives pPU1930 for PheA (Nurk *et al.*, 1991) and pBRLactacPheB for PheB. These proteins were detectable as major visible bands in SDS-polyacrylamide gel. The PheA and PheB bands were cut from the gel. The gel slices were dialysed against 1 $\times$ PBS, homogenized in the same buffer and the liquid was used for injection of mice. The polyclonal antibodies obtained were specific to PheA and PheB as we did not detect antibody reaction if cell lysates prepared from *P. putida* lacking the *pheBA* operon were analysed. We have controlled that PheA-specific antibodies do not react with polypeptide detected with PheB-specific antibodies and vice versa. To compare intracellular amounts of PheA and PheB in bacteria grown in the presence or absence of amino acids, gradual dilutions of crude lysates of PaWpheBA-lux-*catR* grown in the absence of amino acids were tested by Western blotting and compared with the amount of these enzymes detected in cells of the same strain grown in the absence of amino acids.

RESULTS AND DISCUSSION

The presence of phenol or benzoate in growth medium of *P. putida* results in different levels of transcription induction from the *pheBA* promoter

The phenol degradation genes *pheBA*, originated from the multiplasmid *Pseudomonas* sp. strain EST1001, enable *P. putida* strain PaW85 to degrade phenol and use it as a growth substrate via coordinated expression of the *pheBA* genes and the chromosomal catechol degradation *ortho* pathway genes *catBCA* (Kasak *et al.*, 1993; Parsek *et al.*, 1995). Transcription from the *pheBA* promoter in *P. putida* carrying the functional *pheBA* operon is activated in the presence of either phenol or benzoate in the growth

medium. Both phenol and benzoate are converted to catechol, which is subsequently oxidized by C12O to CCM, and the latter acts as an effector molecule on CatR-mediated transcriptional activation from the *catBCA* and *pheBA* promoters (Kasak *et al.*, 1993; Parsek *et al.*, 1995) (Fig. 1). Our previous studies have shown that addition of benzoate into minimal growth medium of *P. putida* leads to rapid induction of the *pheBA* operon, but transcription from the *pheBA* promoter was severely impaired in exponentially growing bacteria when amino acids were present in the growth medium (Tover *et al.*, 2001).

In order to compare the activation of transcription regulation from the *pheBA* promoter in bacteria growing in the presence of either phenol or benzoate, we constructed *P. putida* strain PaWpheBA-lux (for construction details, see Methods). This strain harbours in the chromosome the *pheBA* genes under the control of their natural promoter P_i (enabling the bacteria to degrade phenol to CCM) and a P_i -*luxAB* reporter system (the *pheBA* promoter cloned upstream of the *luxAB* reporter).

Strain PaWpheBA-lux was grown exponentially in M9 minimal medium containing glucose in the presence of benzoate or phenol. Parallel experiments were carried out with cultures supplemented with amino acids (CAA solution). Bioluminescence was measured in cells sampled 1, 2 and 3 h after addition of 2.5 mM phenol or benzoate. As shown in Fig. 2, transcription from the *pheBA* promoter was significantly lower in the presence of amino acids in the growth medium irrespective of which source of inducer, phenol or benzoate, was used. At the same time,

Fig. 2. Effect of growth medium composition on transcription initiation from the *pheBA* promoter P_i in *P. putida* strain PaWpheBA-lux containing the *pheBA* operon and the P_i -*luxAB* reporter system (*pheBA* promoter P_i upstream of the *luxAB* genes) in the chromosome. Bacteria were grown exponentially in M9 glucose minimal medium in the presence of 2.5 mM benzoate or phenol, or in the absence of these chemicals. Parallel experiments were carried out with cultures supplemented or not with amino acids (0.2% CAA solution), and bioluminescence was measured in cells sampled 1, 2 and 3 h after addition of phenol or sodium benzoate. Means $\pm$ SD of at least six independent measurements are presented.

the transcription profile of the *pheBA* promoter was remarkably different in cells grown in the presence of either phenol or benzoate (Fig. 2). Interestingly, the negative effect of amino acids was much stronger when phenol was added as an inducer. Only a low basal level of transcription from the *pheBA* promoter (comparable with that obtained in non-induced cells) was detected during the 3 h examined, if phenol was added into the amino acid-containing growth medium. At the same time, bacteria grown in CAA-containing medium with benzoate expressed much higher luciferase activities. The differences appeared also in minimal medium lacking CAA. Addition of benzoate into the growth medium allowed rapid activation of transcription from the *pheBA* promoter whereas phenol did not. During the first hour of growth of bacteria in the presence of phenol, the level of transcription from the *pheBA* promoter did not increase significantly above that detected in non-induced culture. Only later, at 2 and 3 h, was the level of transcription from the *pheBA* promoter remarkably increased. This raised the question whether addition of 2.5 mM phenol into the growth medium could somehow impede transcription initiation from the *pheBA* promoter.

Efficiency of transcription initiation from the *pheBA* promoter depends on the phenol concentration in the growth medium of *P. putida*

To test the possibility that addition of 2.5 mM phenol into growth medium is non-optimal for the induction of transcription from the *pheBA* promoter, we cultivated strain PaWpheBA-lux in glucose minimal medium in the presence of different concentrations of phenol. Samples were taken from exponential cultures after 1 h of growth with phenol. Control samples were analysed from cultures growing without phenol. We observed about 30-fold higher level of transcription from the *pheBA* promoter if the concentration of phenol was reduced 10- to 100-fold from the usual 2.5 mM that was added into the M9-glucose minimal growth medium (Fig. 3a). Thus, our results indicated that activation of the *pheBA* operon occurs rapidly in the presence of micromolar concentrations of phenol, but is severely delayed if bacteria are exposed to higher concentrations of this compound. At the same time, in the presence of amino acids in the growth medium, only basal level of transcription from the *pheBA* promoter occurred at any concentrations of phenol used (Fig. 3a).

To exclude the possibility that the presence of millimolar concentrations of phenol in minimal growth medium could inhibit the luciferase reaction or transcription in general, we performed control experiments by measuring luciferase activity in bacteria carrying the IPTG-inducible P_{tac} -*luxAB* transcriptional fusion and grown for 1 h in minimal medium after addition of 0.5 mM IPTG in the presence or absence of 2.5 mM phenol. Phenol and IPTG were added into the growth medium at the same time point. Comparison of luciferase activity measured in

Fig. 3. (a) Effect of phenol concentration on transcriptional activation from the *pheBA* promoter in *P. putida* strain PaWpheBA-lux. Bacteria were grown exponentially in M9 glucose minimal medium supplemented (open symbols) or not (filled symbols) with amino acids in the presence of different concentrations of phenol. Bioluminescence was measured in *P. putida* cells sampled 1 h after addition of phenol. (b) Effect of phenol on transcription initiation from the P_{tac} promoter. SD is shown in the figure. Means $\pm$ SD of at least six independent measurements are presented.

bacteria growing with or without IPTG (Fig. 3b) revealed that transcription from this promoter is strongly dependent on IPTG (we have observed 100-fold induction). The presence of phenol in the growth medium did not affect IPTG-dependent transcriptional activation from the P_{tac} promoter (Fig. 3b). These data confirmed that the exposure of bacteria to a high concentration of phenol influences specifically transcription from the *pheBA* promoter, but not the expression of the *luxAB* reporter or transcription from the P_{tac} promoter, which is unrelated to phenol catabolism.

The reason why the *pheBA* promoter is rapidly activated in the presence of 0.025–0.25 mM phenol in the growth medium, but the initiation of transcription is severely delayed when bacteria are exposed to 2.5 mM phenol, is not clear. It is possible that after sudden exposure of bacteria to a high concentration of phenol, the intracellular concentrations of this chemical may rise too high, leading to inactivation of the CatR activator protein or phenol degradation enzymes. However, the results of our studies do not support this explanation, because we have not seen

inactivation of the phenol degradation enzymes even in the presence of 5 mM phenol in the reaction buffer (data not shown). Inactivation of CatR by phenol is also less plausible, because transcription from the *pheBA* promoter was rapidly induced at any concentration of phenol in a CatB-deficient strain expressing CatR at a natural level (see below). Alternatively, one may also speculate that phenol could be rapidly extruded from cells before the bacteria are adapted to a toxic concentration of this compound, thereby making intracellular amounts of phenol limiting for the production of CCM, which is needed for the operon activation. Several studies have demonstrated that efflux pumps are induced to extrude deleterious compounds like toluene and other aromatic compounds (Ramos *et al.*, 2002; Santos *et al.*, 2004). However, although *P. putida* contains many efflux systems (Domínguez-Cuevas *et al.*, 2006; Ramos *et al.*, 2002), no efflux pumps for phenol export have been studied so far.

Negative effect of amino acids in the growth medium on transcription from the *pheBA* promoter is suppressed either by overproducing CatR or by increasing the cellular amount of CCM

Study of the effects of growth medium composition on the expression of the *pheBA* operon revealed that, despite the availability of the source of the *pheBA* operon inducer CCM (benzoate or phenol), the activation of transcription from the *pheBA* promoter is impeded if bacteria are grown exponentially in the presence of amino acids. The molecular basis of physiological control mechanisms that are superimposed on transcriptional activation of catabolic operons by specific regulators in the presence of effector molecules varies largely, depending on the particular operon studied. In some cases, this control operates through the level of expression of a transcription activator protein (see e.g. Müller *et al.*, 1996; Sze *et al.*, 1996; Yuste *et al.*, 1998). For example, expression of the n-alkanes catabolic pathway is repressed when cells grow exponentially in rich medium even if exposed to the inducer, but overproduction of the regulatory protein AlkS relieves the repression of transcription (Yuste *et al.*, 1998).

In order to test whether CatR would limit transcription from the *pheBA* promoter in bacteria grown in rich medium, the *catR* gene under the control of the IPTG-inducible P_{tac} promoter was introduced into the chromosome of strain PaWpheBA-lux. The resulting *P. putida* strain PaWpheBA-lux-catR was grown exponentially in the presence or absence of amino acids, and 1 mM IPTG was added to overexpress the *catR* gene. Luciferase activities were measured in cells sampled after 1 h of growth in the presence of different concentrations of phenol. The control samples analysed from the cultures grown in the absence of phenol revealed that the overexpression of CatR itself (in the absence of an external source of CCM) did not induce *pheBA* promoter expression. Although we observed somewhat higher levels of luciferase activity compared with

those measured in the wild-type strain (compare Fig. 3a and Fig. 4a), this change was not significant compared with the activity measured in the presence of phenol. When phenol was added into the amino acid-containing growth medium, the overexpression of CatR protein resulted in more than 100-fold higher level of the *pheBA* promoter activity (compare Fig. 3a and Fig. 4a). Notably, up to 20-fold positive effect of the CatR overexpression could be detected in minimal-medium-grown cells as well. This indicated that the intracellular amount of CatR is limiting the transcriptional activation from the *pheBA* promoter not only in the presence of amino acids, but also in bacteria grown in minimal medium.

The CCM-lactonizing enzyme encoded by the *catB* gene converts CCM to muconolactone (Fig. 1). When the *catB* gene is inactivated in *P. putida*, the phenol or benzoate degradation pathways end up with the formation of CCM. To investigate whether the quantity of CCM in cells grown in the presence of amino acids may limit the rate of transcriptional activation from the *pheBA* promoter, we constructed the *catB*-deficient mutant PaWpheBA-lux-catB : : tet by interrupting the chromosomal *catB* gene in strain PaWpheBA-lux with the tetracycline-resistance-encoding gene. Interestingly, when bacteria were grown in the absence of phenol, the transcription from the *pheBA* promoter occurred at about a 10-fold higher level in the *catB*-defective mutant than in the wild-type strain (compare Fig. 3a and Fig. 4b). One possible explanation for this phenomenon is that a small amount of CCM is formed endogenously, most likely as a result of metabolism of aromatic amino acids in *P. putida*. However, here we wish to note that the *pheBA* promoter was still about 100-fold inducible in PaWpheBA-lux-catB : : tet (Fig. 4b).

As shown in Fig. 4(b), transcription from the *pheBA* promoter was rapidly induced in the *catB*-deficient mutant strain in the presence of phenol, even when amino acids were added into the growth medium. The level of transcription observed in this strain in the presence of amino acids exceeded up to ten times the *pheBA* promoter activity measured in the strain overexpressing the CatR protein. Additionally, our results indicate that CCM almost always limits the transcription from the CatR-regulated promoters: even if bacteria were grown in a nutritionally poor environment, transcription from the *pheBA* promoter was more than fivefold higher with excess of CCM compared with that measured in the wild-type strain (compare Fig. 3a and Fig. 4b). Taken together, the results of the experiments with the CatB-defective strain indicate that the intracellular amounts of CCM primarily limit the level of transcription from the *pheBA* promoter. In the light of these data, the positive effect of CatR overproduction on the *pheBA* promoter activation might be interpreted as follows: if there is more CatR protein, then a limiting number of CCM molecules available at an early stage of induction of the phenol degradation pathway are more likely bound by CatR to activate transcription.

We also tried to increase the intracellular amounts of CCM by overproduction of the enzymes PheA and PheB, required for the degradation of phenol to CCM. Indeed, artificial overexpression of these enzymes on the medium-copy plasmid pAT1142 carrying the *pheBA* operon enabled rapid activation of the *pheBA* promoter in both the presence and absence of amino acids in the growth medium. The overexpression of these enzymes was verified by Western blot analysis (data not shown). Overproduction of PheA and PheB almost completely relieved

Fig. 4. Suppression of negative effects of amino acids on transcriptional activation from the *pheBA* promoter in *P. putida*. Effects of overexpression of *catR* gene (a), inactivation of the *catB* gene encoding CCM lactonizing enzyme (b), overexpression of the *pheBA* operon on plasmid pAT1142 (c) and inactivation of the *crc* gene encoding catabolite repression protein Crc (d) were investigated. Bacteria were grown exponentially in M9 glucose minimal medium supplemented (open symbols) or not (filled symbols) with amino acids in the presence of different concentrations of phenol. To overexpress the *catR* gene in strain PaWpheBA-lux-catR, bacteria were grown in the presence of 1 mM IPTG. Bioluminescence was measured in *P. putida* cells sampled 1 h after addition of phenol. Means $\pm$ SD of at least six independent measurements are presented.

the repressive effect of the presence of amino acids (Fig. 4c). However, the plasmid pAT1142-harboring cells always exhibited a lower level of expression of the *pheBA* promoter compared with the level that was detected when bacteria carried the *pheBA* operon as a single copy in the bacterial chromosome. Plasmid pAT1142 is an RSF1010 derivative which has more than 10 copies per cell. As already discussed above, the cellular amount of CatR is always below the level which allows maximal level of transcription from its target promoters. Therefore, we suppose that the presence of multiple binding sites for CatR in cells carrying the plasmid pAT1142 could titrate out this activator protein, resulting in a reduced level of expression of the chromosomal P_i -*luxAB* reporter system used to measure the *pheBA* promoter activity. Indeed, this reporter system expressed much higher luciferase activity when the *catR* gene was also present in the *pheBA* operon-carrying medium-copy plasmid (data not shown). However, the results of the overproduction of PheA and PheB also implied that the intracellular amount of CCM, but not CatR, is the most limiting factor determining the rate of transcription from the *pheBA* promoter, when bacteria are grown in the presence of amino acids. At the same time, we cannot exclude the possibility that amino acids reduce *catR* expression as well.

The importance of intracellular CCM concentration for the regulation of expression of catabolic operons is also seen in *Acinetobacter baylyi* ADP1 (Collier *et al.*, 1998; Cosper *et al.*, 2000; Ezezika *et al.*, 2006). At the same time, the accumulation of high levels of CCM is toxic to the cells (Gaines *et al.*, 1996). A complex regulatory circuit involving two LysR-family transcriptional regulators BenM and CatM has evolved to allow optimal expression of benzoate and catechol degradation genes, while at the same time keeping the concentration of potentially harmful CCM sufficiently low (Ezezika *et al.*, 2006).

Repression of transcription from the *pheBA* promoter in the presence of amino acids is caused by a mechanism affecting expression of the *pheBA* operon at the post-transcriptional level

There are several possible mechanisms which may reduce intracellular levels of CCM in the presence of amino acids: the intracellular amount of precursor molecule phenol may be too low, CCM is degraded fast, the enzymes for CCM production are inhibited or the levels of enzymes needed for the production of CCM are reduced. Here, we decided to compare the intracellular amounts of PheA and PheB under different nutritional conditions (i.e. in the presence or absence of amino acids). In order to separate the possible effects of growth medium composition on the cellular amounts of the PheA and PheB proteins from those which directly influence transcription initiation of the corresponding genes, we quantified these enzymes by Western blot analysis with anti-PheB and anti-PheA polyclonal antibodies by examining cell lysates prepared

from such amounts of bacteria which gave the same value of transcription from the *pheBA* promoter. More precisely, the level of transcription of the *pheBA* genes was monitored by luciferase activity as described above, and the samples of bacteria taken for quantification of PheA and PheB proteins expressed approximately 10 000 RLU/OD₅₈₀ luciferase activities per 10 s. The *catR*-overexpressing strain PaWpheBA-*luxAB*-*catR* was used in these assays to obtain high levels of transcription from the *pheBA* promoter in cells growing in the presence of amino acids. As shown in Fig. 5(a), despite the fact that bacteria derived from different growth conditions expressed comparable luciferase activities (which could reflect equal transcription of the *pheBA* genes from their promoter), the cellular amounts of the PheA and PheB proteins were remarkably affected by the growth medium composition. About four- and eight-fold lower amounts of the PheA and PheB proteins, respectively, were detected if bacteria were grown in the presence of amino acids (Fig. 5a, compare lanes 1 and 2). Thus, our results indicated that the cellular amounts of PheA and PheB enzymes are negatively controlled by amino acids at the post-transcriptional level of expression of the corresponding genes.

Fig. 5. (a) Quantification of cellular amounts of PheA and PheB proteins by Western blot analysis in crude lysates of *P. putida* strains PaWpheBA-*lux*-*catR* overexpressing CatR (P_{tac} -*catR*, lanes 1 and 2) and PaWpheBA-*lux*-*crc* : : *tet* lacking the Crc protein (*crc* : : *tet*, lanes 3 and 4). Bacteria were grown for 1 h in glucose minimal medium supplemented with 0.05 mM phenol in the presence or absence of amino acids (CAA, indicated by + or -). (b) Quantification of cellular amounts of PheB proteins by Western blot analysis in crude lysates of *P. putida* strain PaWtaclux-*pheB* and strain PaWtaclux-*pheB*-*crc* : : *tet* lacking the Crc protein. Bacteria were grown for 1 h in glucose minimal medium supplemented with 1 mM IPTG in the presence or absence of amino acids. M, molecular mass markers. Lanes 1–4 in (a) and (b) represent samples of bacteria taken for Western blot analysis expressing approximately 10 000 RLU luciferase activity per 10 s. About 20 µg crude lysates of PaWlux are analysed on lanes 5 and 6 of (a).

The predicted molecular mass of the PheA protein is 67.4 kDa. The Western blot analysis presented in Fig. 5(a) revealed the presence of three protein bands which reacted with PheA-specific antibodies, two of which migrated slower than the predicted *pheA* gene product. We did not detect these bands when cell lysate prepared from *P. putida* lacking the *pheBA* operon was analysed (Fig. 5a, lanes 5 and 6). These data exclude the possibility that some bands are derived from reaction of these antibodies with other proteins. The presence of three protein bands reacting with PheA-specific antibodies also appeared in the case of Western blot analysis of cell lysates prepared from *E. coli* cells expressing the *pheA* gene alone, but not when *pheA* was absent (data not shown). At present, the nature of the multiple PheA-specific bands remains unclear.

Crc protein is involved in regulation of expression of PMO and C12O

The Crc protein is involved in repression of several catabolic pathways responsible for degradation of aromatic compounds or other carbon sources in *Pseudomonas* when other preferred carbon sources are present in the culture medium (Rojo & Dinamarca, 2004). It has been demonstrated that the expression of *P. putida* chromosomal genes for the homogentisate, catechol and protocatechuate pathways is inhibited by Crc when cells grow in a complete medium (Morales *et al.*, 2004). The precise function and mechanism of Crc are still unknown, but it has been suggested that Crc modulates the expression of target genes posttranscriptionally (Hester *et al.*, 2000; Rojo & Dinamarca, 2004). The most recently published studies suggest that Crc acts through generating a metabolic signal (Aranda-Olmedo *et al.*, 2005; Morales *et al.*, 2004).

Comparison of the results of the Western blot analysis of the PheA and PheB enzymes in *P. putida* wild-type strain and its Crc-defective derivative grown in the presence or absence of amino acids revealed that, in contrast to the differences in cellular amounts of the phenol degradation enzymes in the wild-type strain (Fig. 5a, lanes 1 and 2), the abundance of these proteins was not affected by the growth medium composition in the Crc mutant strain (Fig. 5a, lanes 3 and 4). These data indicate that Crc may negatively affect the expression of the *pheBA* operon at the post-transcriptional level in the presence of amino acids. To verify this hypothesis, we performed an additional control experiment by monitoring intracellular amounts of PheB in wild-type and Crc-defective bacteria expressing the *pheB* gene under the control of the IPTG-inducible P_{tac} promoter, which is not dependent on levels of CCM. The results of the Western blot analysis presented in Fig. 5(b) revealed lower amounts of PheB in Crc-proficient cells grown in amino acid-containing medium compared with the minimal-medium-grown bacteria. At the same time, no negative effect of the amino acids on the levels of PheB could be detected in bacteria lacking the Crc protein, which is in accordance with the results presented in Fig. 5(a).

Based on the results of the Western blot analysis indicating that Crc controls negatively the intracellular amounts of the PheA and PheB proteins in bacteria growing in the presence of amino acids, we expected to see that transcription from the *pheBA* promoter was elevated in the Crc-deficient background. Indeed, data presented in Fig. 4(d) revealed remarkable differences between the level of transcription from the *pheBA* promoter in the wild-type and Crc-deficient *P. putida* strains grown in the presence of the amino acids solution (compare Fig. 3a and Fig. 4d). The most remarkable effects of Crc appeared in the presence of 0.05–0.5 mM phenol in the amino acid-containing growth medium. Whereas the transcription from the *pheBA* promoter occurred at a very low level under such growth conditions in the wild-type strain, an up to 150 times higher level of transcription was detected in the Crc-defective strain PaWpheBA-lux-crc : : tet. As lower intracellular amounts of phenol degradation enzymes in Crc-proficient background lead to decreased production of the pathway inducer CCM, this might be a mechanism by which this protein indirectly interferes with the transcription of the *pheBA* genes.

The lack of induction of the phenol degradation pathway in the presence of amino acids cannot be explained only by the action of the Crc protein, as the expression level of the promoter of the *pheBA* genes was still about four times lower in rich growth medium, compared with that when bacteria were grown in minimal medium (Fig. 4d). This indicates that some additional control mechanisms operating through transcriptional activation of the *pheBA* promoter may also exist. As a transfer of bacteria from minimal to rich growth medium recruits transcription machinery to promoters whose expression is greatly needed with an excess of nutrients (e.g. stable RNA promoters), it is possible that some other promoters, like the *pheBA* promoter, are less efficiently transcribed due to competition for free RNA polymerase.

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