Growth medium composition-determined regulatory mechanisms are superimposed on CatR-mediated transcription from the *pheBA* and *catBCA* promoters in *Pseudomonas putida*

Andres Tover, Eve-Ly Ojangu and Maia Kivisaar

Author for correspondence: Maia Kivisaar. Tel: +372 7 375015. Fax: +372 7 420286.
e-mail: maiak@ebc.ee

Department of Genetics,
Institute of Molecular and
Cell Biology, Estonian
Biocentre and Tartu
University, Riia 23,
51010 Tartu, Estonia

Expression of the phenol degradation pathway in *Pseudomonas putida* strain PaW85 requires coordinated transcription of the plasmid-borne *pheBA* operon encoding catechol 1,2-dioxygenase and phenol monooxygenase, respectively, and the chromosomally encoded catechol degradation *catBCA* operon. Transcriptional activation from the *pheBA* and *catBCA* promoters is regulated by CatR and the catechol degradation pathway intermediate *cis,cis*-muconate. Here it is shown that physiological control mechanisms are superimposed on this regulatory system. Transcriptional activation from the *pheBA* and *catBCA* promoters is growth-phase-regulated in *P. putida* cells grown on rich medium (LB medium). CatR-mediated transcription from these promoters is silenced on rich medium until the transition from exponential to stationary phase. A slight positive effect (threefold) of stationary-phase-specific sigma factor σ^S on transcription from the *pheBA* promoter was observed. Expression of the *catBCA* promoter was not influenced by the activity of this sigma factor. In contrast to rich growth medium, transcription from the *pheBA* and *catBCA* promoters in minimal medium containing a mixture of glucose and sodium benzoate was rapidly induced in exponential culture. It was shown that the presence of amino acids in the culture medium causes exponential silencing of the *pheBA* and *catBCA* promoters. The possibility that a hypothetical repressor protein could be involved in physiological control of transcription from the *pheBA* and *catBCA* promoters is discussed.

Keywords: *pheBA* and *catBCA* operons, exponential silencing of transcription

INTRODUCTION

Expression of catabolic pathways in *Pseudomonas* is inhibited by a number of growth conditions that adjust the activity of specific promoters to a given metabolic and physiological status (Cases & de Lorenzo, 1998). The best studied examples of growth-condition-dependent expression of aromatic compound degradation genes in soil pseudomonads are catabolic operons of TOL plasmid pWW0 (Cases & de Lorenzo, 2000; Marques *et al.*, 1994, 1999; Ramos *et al.*, 1997 and references cited therein) and dimethylphenol degradation genes, *dmp*, from megaplasmid pVI150 (Sze & Shingler, 1999). The strong repressive effect of LB

medium or amino acids has been observed for the promoters of these operons (Cases *et al.*, 1996; de Lorenzo *et al.*, 1993; Sze & Shingler, 1999; Sze *et al.*, 1996). The physiological down-regulation of the Pu promoter of TOL plasmids is a complex process controlled by two or more independent mechanisms (Cases & de Lorenzo, 2000). For example, PtsN-mediated carbon source inhibition (Cases *et al.*, 1999) and the control of the activity of σ^{54} (Cases *et al.*, 1996) are involved in regulation of transcription from Pu. Promoter Po of the *dmp* operon drives the expression of the dimethylphenol degradation pathway very similarly to that of Pu for the upper pathway of TOL plasmids (Sze *et al.*, 1996). The stimulating effect of (p)ppGpp on transcriptional activation was recently demonstrated for Po (Carmona *et al.*, 2000). At the same time, this signal molecule, which triggers the stringent response to

Abbreviations: CCM, *cis,cis*-muconate; β -Gal, β -galactosidase.

Fig. 1. (a) The β -ketoadipate pathway by which phenol and benzoate are converted to catechol and to the common metabolic intermediates succinate and acetyl-CoA. (b) Schematic representation of the promoter region of the *pheBA* and *catBCA* operons. The bracketed sequences represent nucleotides protected by CatR from cleavage by DNaseI (Chugani *et al.*, 1997; Parsek *et al.*, 1995). The protected regions are designated as RBS (recognition binding sites) and ABS (activation binding sites). Bold/italic type designates the T and A residues of the conserved T-N₁₁-A sequence of the LysR family regulatory proteins binding motif of the RBS. The *catBCA* promoter has a G instead of the conserved T. The nucleotides of the interrupted inverted repeat within the RBS are underlined. The nucleotides of the -35 and -10 hexamers of the promoters are in bold type and boxed. The transcriptional start sites are denoted by asterisks.

amino acid starvation, had only a moderate effect on the activity of the very similar promoter Pu (Carmona *et al.*, 2000).

In *Pseudomonas* species, phenolic compounds are transformed by different enzymes to central intermediates, such as protocatechuate and (substituted) catechols (Harayama & Timmis, 1989). Typically, unsubstituted compounds, such as benzoate, are metabolized by an *ortho*-cleavage pathway (Fig. 1). The genes for benzoate

metabolism, including *ortho*-pathway genes, are chromosomally encoded in *P. putida* (Harwood & Parales, 1996). The *catBCA* operon encodes three enzymes of the *ortho*-pathway required for benzoate catabolism, namely muconate lactonizing enzyme I, muconolactone isomerase and catechol 1,2-dioxygenase, respectively (Houghton *et al.*, 1995). The induction of this operon, which is σ^{54} -independent, requires a LysR family transcriptional activator, CatR, and an inducer molecule, *cis,cis*-muconate (CCM), an intermediate of the *ortho*-pathway (Rothmel *et al.*, 1990, 1991). The *pheB* and *pheA* genes originating from plasmid DNA of *Pseudomonas* sp. EST1001 encode catechol 1,2-dioxygenase and phenol monooxygenase, respectively (Kivisaar *et al.*, 1990). When the *pheBA* operon is introduced into *P. putida*, 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). Comparative studies of the interaction of CatR with 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).

In this study we have investigated (i) how the physiological status of the cells controls expression of the *pheBA* and *catBCA* promoters in *P. putida* and (ii) the similarity of these control mechanisms on transcription from these promoters. We show that transcription from the *pheBA* and *catBCA* promoters is strongly influenced by the composition of the growth medium (rich versus minimal medium). The presence of amino acids in the growth medium is one component that causes exponential silencing of both the *pheBA* and the *catBCA* promoters.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* TG1 (Carter *et al.*, 1985) was used for DNA cloning procedures. *E. coli* was incubated at 37 °C and *P. putida* at 30 °C. *E. coli* was transformed with plasmid DNA as described by Hanahan (1983). *P. putida* was electrotransformed according to the protocol described by Sharma & Schimke (1996). Antibiotics were added at the following final concentrations: ampicillin at 100 μ g ml⁻¹ for *E. coli*; carbenicillin at 1500 μ g ml⁻¹ and kanamycin at 50 μ g ml⁻¹ for *P. putida*.

Growth conditions and β -galactosidase (β -Gal) assay. To investigate the role of rich medium on transcription from the *pheBA* and *catBCA* promoters, *P. putida* cells were grown in LB medium (Miller, 1992). Cells were grown overnight in LB medium and diluted into fresh medium to obtain an OD₅₈₀ of 0.02. After 2 h cultivation sodium benzoate was added at a final concentration of 2.5 mM to induce the transcription of the *pheBA-lacZ* and *catBCA-lacZ* transcriptional fusions. The inducer molecule, CCM, required for CatR-dependent transcriptional activation is an intermediate of the benzoate degradation pathway. Because *P. putida* uses sodium benzoate as a carbon source, this compound was added again after 5

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or construction	Source of reference
<i>E. coli</i>		
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F' (traD36 proAB⁺ lacI^a lacZΔM15)</i>	Carter <i>et al.</i> (1985)
S17-1 λ pir	Tp ^r Sm ^r <i>recA thi pro</i> (r ⁻ m ⁺) RP4::2 Tc::Mu::Km Tn7 λ pir	Miller & Mekalanos (1988)
C118 λ pir	$\Delta(ara-leu)$ <i>araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE</i> (Am) <i>recA1</i> λ pir phage lysogen	Herrero <i>et al.</i> (1990)
<i>P. putida</i>		
PaW85	Tn4652	Bayley <i>et al.</i> (1977)
PKS54	Tn4652 <i>rpoS</i> ::Km ^r	Ojangu <i>et al.</i> (2000)
PaW85CatR ⁺	PaW85 <i>catR</i> gene under control of <i>P</i> _{tac} promoter and <i>lacI</i> ^a repressor, Km ^r	This study
Plasmids		
pBluescript SK(+)	Cloning vector (Ap ^r)	Stratagene
pKTLacZ	Cloning vector (Ap ^r)	Hörak & Kivisaar (1998)
pZ-pheBA	<i>pheBA</i> promoter cloned into pKTLacZ	This study
pZ-catBCA	<i>catBCA</i> promoter cloned into pKTLacZ	This study
pUTmini-Tn5 Km2	Delivery plasmid for mini Tn5 Km2	de Lorenzo <i>et al.</i> (1993)
pUC18Not	pUC18 with <i>NotI</i> restriction sites in multicloning region (Ap ^r)	Herrero <i>et al.</i> (1990)
pBRLacIac	<i>P</i> _{tac} promoter and <i>lacI</i> ^a repressor in 2.2 kb <i>NruI</i> - <i>EcoRI</i> fragment from plasmid pMMB208 cloned into <i>EcoRV</i> / <i>EcoRI</i> -cleaved pBR322	Ojangu <i>et al.</i> (2000)
pKZΔHf	pKR1300 derivative containing <i>catR</i> gene	Rothmel <i>et al.</i> (1990)
pBRLacIac-catR	<i>catR</i> in 1 kb <i>EcoRI</i> - <i>HindIII</i> fragment cloned into pBRLacIac to obtain <i>lacI</i> ^a - <i>P</i> _{tac} - <i>catR</i>	This study
pUCPac-catR	<i>lacI</i> ^a - <i>P</i> _{tac} - <i>catR</i> expression cassette cloned into pUC18Not	This study
pUT-lacIac-catR	<i>lacI</i> ^a - <i>P</i> _{tac} - <i>catR</i> expression cassette cloned from pUCPac-catR into pUT-miniTn5 Km2	This study
pVI536	<i>relA</i> gene of <i>E. coli</i> overexpressed from P _{BAD}	Sze & Schingler (1999)

and 12 h cultivation (at a final concentration of 5 mM) to maintain the source of the inducer in the growth medium. Samples for the β -Gal assay were taken from cultures grown for 5, 12 and 24 h.

To investigate the regulation of transcription from the *pheBA* and *catBCA* promoters in minimal medium, *P. putida* cells were grown overnight in MOPS medium (Neidhardt *et al.*, 1974) either with 0.5% CAA or amino acids at a final concentration of 200 ng each amino acid ml⁻¹. The overnight bacterial culture was diluted with fresh medium (1:20, v/v) grown to exponential phase and then diluted again in minimal medium containing 0.2% glucose. The OD₅₈₀ of the diluted culture was 0.1. Sodium benzoate was added at a final concentration of 10 mM at the beginning of the experiment and 1 ml samples were taken at different time points during growth to assay the β -Gal activity in cell suspensions.

To investigate the possible effect of overexpression of the CatR protein on the transcription from the *pheBA* and *catBCA* promoters, the culture was grown overnight in LB medium and thereafter diluted into fresh LB medium as indicated above. To induce the expression of CatR, IPTG (at a final concentration of 0.5 mM) was added to the growth medium. When the culture reached the exponential growth phase, it was diluted again into fresh LB medium containing 0.5 mM IPTG. At this step, 5 mM sodium benzoate was added for the induction of transcription from the *pheBA* and *catBCA* promoters.

A β -Gal assay with the cell lysates was carried out as specified by Miller (1992). In all cases at least three independent measurements were made. Protein concentration in crude lysates was measured by the method of Bradford (1976). β -Gal measurement in cell suspensions was performed by modification of the standard protocol of Miller (1992). The amount of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ and 50 mM β -mercaptoethanol) in a test tube was 1.6 ml. ONPG (2-nitrophenyl- β -D-galactopyranoside; 400 μ l of a 50 mg ml⁻¹ solution), 100 μ l 0.002% SDS and 100 μ l chloroform were added into the reaction mixture. Finally, bacteria were added into the test tube and mixed well. The β -Gal reaction was stopped by the addition of 1 ml 1 M Na₂CO₃. Data of at least three independent experiments are presented in all figures.

Cloning procedures. Construction of the *pheBA* promoter-*lacZ* transcriptional fusion (plasmid pZ-pheBA) was described previously (Tover *et al.*, 2000). For the cloning of the *catBCA* promoter into the promoter-probe plasmid pKTLacZ (Hörak & Kivisaar, 1998), the 207 bp *catBCA* promoter region from the *P. putida* chromosome was amplified by using oligonucleotides cat35 (5'-GGGCTGC-CAGCCGCGGGCCC-3') and AFC62 [5'-AGCGCGGCGG-CTCGACGACGCTG(*PstI*)CAGAGC-3'], complementary to the upstream and downstream regions of the *catBCA* promoter, respectively. The PCR amplification product was cleaved with *PstI* and cloned into pBluescript SK(+) cleaved with *PstI* and *EcoRV*. The sequence of the *catBCA* promoter was verified by DNA sequencing. Subsequently, the *catBCA*

promoter was inserted using *Bam*HI- and *Xho*I-generated ends into the promoter-probe vector pKTlacZ to obtain plasmid pZ-catBCA. For the construction of *P. putida* CatR overexpression strain PaWCatR⁺, the *catR* gene was cloned from plasmid pKRAHF (Rothmel *et al.*, 1991) by using *Hind*III- and *Eco*RI-generated ends to the vector pBRlac tac (Ojangu *et al.*, 2000) cleaved with same enzymes (pBRlac tac-catR in Table 1). The CatR expression cassette *lacI*^q-Ptac-catR was inserted into pUC18Not (Herrero *et al.*, 1990) using *Eco*RI- and *Bam*HI-generated ends. Thereafter, the *lacI*^q-Ptac-catR sequence was inserted into the *Not*I site of pUTmini-Tn5 Km2 (de Lorenzo *et al.*, 1990) and pUTlac tac-catR was selected in *E. coli* C118 λ pir (Herrero *et al.*, 1990). The *lacI*^q-Ptac-catR expression cassette was inserted into the chromosome of *P. putida* strain PaW85 (Bayley *et al.*, 1977) by the method of random insertion using *E. coli* S17-1 λ pir (Miller & Mekalanos, 1988) as donor strain. *P. putida* PaW85CatR⁺ was selected at 30 °C on glucose/kanamycin plates. The expression of CatR in PaW85CatR⁺ was verified by Western blot analysis using polyclonal antibodies against *P. putida* CatR protein.

RESULTS AND DISCUSSION

Growth-phase-dependent activation of transcription of the *pheBA* and *catBCA* operons

The promoters of the *pheBA* and *catBCA* operons were cloned into plasmid pKTlacZ carrying the *lacZ* reporter gene and β -Gal activity was measured to examine transcription from these promoters. *P. putida* PaW85 cells containing *lacZ* transcriptional fusion plasmids pZ-*pheBA* and pZ-*catBCA* were grown in rich medium (LB) to the late stationary phase. β -Gal activity was measured in cells sampled from different time points during culture (Fig. 2). Sodium benzoate was always added into the growth medium to induce transcription from the *pheBA* and *catBCA* promoters. Results presented in Fig. 2 show that transcription from the *pheBA* and *catBCA* promoters remained at a low level during the first 9 h of growth. The level of expression of β -Gal activity was remarkably elevated in stationary-phase cells and it remained high in stationary-phase cultures during the 24 h period of the study. This indicated that the *pheBA* and *catBCA* promoters are stationary-phase-inducible and down-regulated during exponential growth.

Transcription from the *pheBA* and *catBCA* promoters in a σ^S -deficient background

Use of an *in vitro* transcription assay has demonstrated that the only requirements for transcription from the *pheBA* and *catBCA* promoters are purified CatR, CCM and RNA polymerase- σ^{70} holoenzyme ($E\sigma^{70}$) (Chugani *et al.*, 1997). Stationary-phase-specific sigma factor σ^S , encoded by *rpoS*, activates transcription of several genes in stationary phase or otherwise stressed bacteria (Hengge-Aronis, 1999). Analysis of σ^{70} - and σ^S -promoter specificity has revealed that $E\sigma^{70}$ and $E\sigma^S$ recognize many promoter sequences equally well *in vitro*, but differentially control genes *in vivo* (reviewed by Hengge-Aronis, 2000). The exponential silencing of transcription from

Fig. 2. Effect of the growth phase of bacteria and σ^S on transcription from the *pheBA* promoter (a) and from the *catBCA* promoter (b) in *P. putida* strain PaW85 (wild-type; $\blacklozenge$, $\blacktriangle$) and in its *rpoS*-defective derivative PK554 ($\blacktriangle$, $\triangle$). β -Gal activity (filled symbols) was measured either in cells of *P. putida* strain PaW85 (wild-type) or the *rpoS*-deficient strain PK554 harbouring either of the plasmids pZ-*pheBA* or pZ-*catBCA*. Bacteria were grown in LB medium to stationary phase in the presence of sodium benzoate. The growth curves of *P. putida* cells are indicated by open symbols. The standard deviation is shown in the figure.

the *pheBA* and *catBCA* promoters gave us the idea to investigate the role of σ^S on transcription from these promoters.

We have previously constructed a *P. putida* σ^S -deficient strain, PK554, that is a derivative of the wild-type strain PaW85 (Ojangu *et al.*, 2000). Plasmids pZ-*pheBA* and pZ-*catBCA*, containing the *pheBA*-*lacZ* and *catBCA*-*lacZ* transcriptional fusions, respectively, were introduced into the *rpoS*-deficient strain, PK554, and into the wild-type strain, PaW85. The level of expression of β -Gal activity measured in exponential- and stationary-phase cells of PK554 was compared with that estimated in PaW85. The results presented in Fig. 2 show that in the case of the *pheBA* promoter, β -Gal activity remained approximately threefold lower in the σ^S -deficient strain than in the wild-type strain in stationary-phase cultures (Fig. 2a). At the same time, lack of expression of σ^S in *P. putida* cells did not affect transcription from the *catBCA* promoter (Fig. 2b). The sequence of the -10 region of the *catBCA* promoter (CAATCCT) shows more similarity to the consensus CTATACT proposed for the promoters recognized by $E\sigma^S$ than that of the *pheBA* promoter (CTAGCTT). Based on *in vivo* experiments presented in this paper we

Fig. 3. Effect of the growth phase of bacteria and σ^S on transcription from the *catR* promoter. β -Gal activity (filled symbols) was measured either in the cell lysates of *P. putida* strain PaW85 (wild-type; $\bullet$, $\circ$) or in *rpoS*-deficient strain PKS54 ($\blacktriangle$, $\triangle$) harbouring the *catR-lacZ* fusion in plasmid pKTlacZ. Bacteria were grown in LB medium to stationary phase in the presence of sodium benzoate. The growth curves of *P. putida* cells are indicated by open symbols. The standard deviation is shown in the figure.

cannot state that the *pheBA* promoter is recognized by σ^S . However, the nucleotide sequence of the -10 region of the promoter is only one component that determines σ^S -dependent transcription. There is increasing evidence that additional regulators play a crucial role in establishing sigma factor specificity at stress-inducible promoters (Hengge-Aronis, 1999). Moreover, coming back to the sequence determinants, we have recently shown on fusion promoters generated in a starving population of *P. putida* that not only the -10 hexameric sequence, but also sequence downstream from the -10 hexamer is important for σ^S -dependent transcription (Ojangu *et al.*, 2000).

The positive effect of σ^S observed in the case of the *pheBA* promoter can give *P. putida* cells a little advantage to use phenol as a single source of carbon and energy under stressful conditions. However, this effect is insufficient to account for the inhibition of the *pheBA* promoter during exponential growth. Moreover, transcription from the *catBCA* promoter was not influenced by the presence of σ^S in *P. putida* cells. It is obvious, therefore, that stationary-phase-specific transcription from the *pheBA* and *catBCA* promoters must be regulated by some other mechanism than σ^S -mediated control.

Modulation of *pheBA* and *catBCA* promoter activity by growth phase does not operate through the amount of regulator protein CatR

Transcriptional activation from the *pheBA* and *catBCA* promoters requires the presence of CatR and an inducer molecule CCM (Kasak *et al.*, 1993; Parsek *et al.*, 1995). Therefore, we examined whether the amount of the regulator protein CatR would be limiting in transcriptional activation from the *pheBA* and *catBCA* promoters in exponentially growing cells. To study the

expression of the *catR* gene during the growth cycle, the promoter of the *catR* gene was cloned into plasmid pKTlacZ to generate a *catR-lacZ* transcriptional fusion. The activity of this fusion was compared in the wild-type *P. putida* strain PaW85 and in its *rpoS*-deficient mutant PKS54. In both strains, the level of β -Gal expression increased in stationary-phase cells when compared to that observed in exponentially growing cells (Fig. 3). Therefore, although *catR* promoter activity remained at a very low level during all growth phases of the bacteria, the intracellular amount of CatR (undetectable by Western blot analysis) may be somewhat increased in stationary-phase cells.

To study whether exponential silencing of the *pheBA* and *catBCA* promoters could be related to the amount of CatR in cells, we constructed a *P. putida* strain enabling artificial modulation of the expression of the *catR* gene. For that purpose, the *catR* gene was cloned under the control of inducible *Ptac* promoter and the *lacI^q* repressor. The *catR* expression cassette was introduced into the chromosome of *P. putida* PaW85. Overexpression of CatR in the presence of 0.5 mM IPTG was verified by using Western blot analysis with polyclonal antibodies against CatR (data not shown). We found that overexpression of CatR does not affect transcription from the *pheBA* and *catBCA* promoters in stationary-phase cells. No transcription from these promoters could be detected in exponentially growing cells (sampled from 2-h-old cultures), irrespective of the addition of IPTG into the growth medium. Therefore we concluded that the exponential silencing of the *pheBA* and *catBCA* promoters is not determined by the cellular amount of CatR.

Exponential silencing of the *pheBA* and *catBCA* promoters is dependent on the nature of the culture medium

The role of growth medium in the regulation of gene expression has been shown in many cases. For example, growth medium composition (either rich or minimal) determines the level of transcription from the *Po* promoter of the operon (*dmp*) encoding dimethylphenol degradation (Sze & Shingler, 1999; Sze *et al.*, 1996), from the *Pu* promoter of the TOL plasmid pWW0 (Cases *et al.*, 1996; de Lorenzo *et al.*, 1993) and from the *PalkB* promoter from the *Pseudomonas oleovorans* alkane degradation pathway (Yuste *et al.*, 1998). We found that transcription from the *pheBA* and *catBCA* promoters was rapidly activated when bacteria were grown in MOPS minimal medium (Fig. 4). Measurement of β -Gal activity in cells sampled at different time points of an exponentially growing culture of *P. putida* PaW85 allowed us to detect the enzyme activity as early as 20 min after the addition of sodium benzoate.

When 0.5% CAA was added to minimal medium, the activation of transcription from the *pheBA* and *catBCA* promoters was delayed, similar to that observed in cells grown in LB medium (compare Figs 2 and 4). In the

Fig. 4. Effect of growth medium composition on transcription from the *pheBA* promoter (a) and from the *catBCA* promoter (b). Bacteria were grown in MOPS minimal medium containing glucose. Sodium benzoate was added as a source of inducer. β -Gal activity (filled symbols) was measured in exponentially growing cells of *P. putida* strain PaW85 grown either in the presence ($\blacklozenge$, $\blacktriangle$) or absence ($\triangleleft$, $\triangle$) of amino acids (0.5% CAA) in MOPS minimal medium. The growth curves of *P. putida* cells are indicated by open symbols. The standard deviation is shown in the figure.

presence of CAA in MOPS minimal medium, the level of transcription from the *pheBA* promoter was very low during the first 4 h of cultivation (Fig. 4a). The repression effect of CAA on transcription activation was also apparent on the *catBCA* promoter, but this promoter appeared to be less sensitive to the presence of CAA in the growth medium than the *pheBA* promoter. In the case of the *catBCA* promoter, expression of β -Gal activity was detectable 60 min after the addition of sodium benzoate (Fig. 4b).

Transcription from the σ^{54} -dependent Po promoter of the dimethylphenol (*dmp*)-degradation pathway is silenced in rich medium until amino acids become limiting (Sze & Schingler, 1999). The requirement of (p)ppGpp for activation of transcription from the Po promoter was demonstrated using overexpression of RelA and *E. coli* (p)ppGpp-deficient mutants (Sze & Schingler, 1999). We were not able to demonstrate the positive role of (p)ppGpp on transcriptional activation from the *pheBA* and *catBCA* promoters. The absence of an effect of (p)ppGpp on transcription from these promoters was confirmed by experiments where the

(p)ppGpp level was artificially increased either by adding 1 mM serine hydroxamate into the growth medium or by using RelA overexpression plasmid pVI536 (Sze & Schingler, 1999) in *P. putida* cells carrying the *pheBA-lacZ* transcriptional fusion in the chromosome (data not shown).

In the (p)ppGpp-dependent Po regulatory system, distinct groups of amino acids were not able to mediate the delay in transcription (Sze & Schingler, 1999). We found that specific groups of amino acids could cause partial silencing of transcription from the *pheBA* and *catBCA* promoters. Bacteria were grown in MOPS minimal medium containing glucose for 60 min in the presence of sodium benzoate. No amino acids or a different number of amino acids (all 20, 15 or 5) were added into the growth medium. The sets of amino acids were designed according to their biosynthetic pathways. The third group consisted of 5 amino acids (Asp, Asn, Glu, Gln and Ser) that are precursors for several other amino acids, and the second group contained the other 15 amino acids. In the absence of amino acids, the level of β -Gal activity was 950 ± 44 Miller units in the case of the *pheBA* promoter and 397 ± 50 Miller units in the case of the *catBCA* promoter. In the case of the *pheBA* promoter, no expression of β -Gal activity could be detected when all amino acids were added, but the presence of 5 and 15 amino acids allowed partial expression of this promoter: the β -Gal activities were 91 ± 3 and 196 ± 20 Miller units, respectively. The repressive effect of amino acids on transcription from the *catBCA* promoter was lower than that observed with the *pheBA* promoter. The β -Gal activity was 12 ± 2.5 Miller units when all amino acids were added into the growth medium and it was approximately half of that observed without amino acids: 178 ± 18 and 199 ± 50 Miller units in the presence of 5 and 15 amino acids, respectively. This also indicates that the physiological control on the *pheBA* and *catBCA* promoters mediated by the presence of amino acids might be different from the mechanism related to stringent response. The occurrence of the partial silencing effect by different groups of amino acids on the transcription from the *pheBA* and *catBCA* promoters excludes the possibility that one particular amino acid could mediate this effect.

At this stage the mechanism by which the presence of amino acids causes repression of these two promoters is unclear. Data presented in Fig. 4 and results obtained in experiments with different sets of amino acids show that the exponential silencing of the *pheBA* promoter is stronger than that of the *catBCA* promoter. The nucleotide sequences of the *pheBA* and *catBCA* promoters are similar, but not identical. Differences become most apparent on sequences located downstream from the CatR-binding sites and -35 hexamers of the promoters (Fig. 1). Analysis of the locations of regulatory sites of σ^{70} -dependent promoters has revealed that repressors usually bind downstream from -30 (Gralla *et al.*, 1996). It is possible that a hypothetical repressor protein could bind to the *pheBA* and *catBCA* promoters with different efficiency due to sequence

differences of the target DNA. However, despite the minor differences in the expression level of the *pheBA* and *catBCA* promoters under certain growth conditions, the general mechanisms for physiological control of these promoters seem to be well conserved to coordinate the expression of the hybrid plasmid-chromosome-encoded pathway for phenol degradation in *P. putida*.

ACKNOWLEDGEMENTS

We thank V. Shingler for kindly providing plasmid pVI536. We also thank T. Alamäe and R. Hörak for critically reading the manuscript. This work was supported by grants 2323 and 4481 from the Estonian Science Foundation.

REFERENCES

- Bayley, S. A., Duggleby, C. J., Worsey, M. J., Williams, P. A., Hardy, K. G. & Broda, P. (1977). Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. *Mol Gen Genet* **154**, 203–204.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Carmona, M., Rodriguez, M. J., Martinez-Costa, O. & de Lorenzo, V. (2000). *In vivo* and *in vitro* effects of (p)ppGpp on the σ^{54} promoter Pu of the TOL plasmid of *Pseudomonas putida*. *J Bacteriol* **182**, 4711–4718.
- Carter, P., Bedouelle, H. & Winter, G. (1985). Improved oligonucleotide site-directed mutagenesis using M13 vectors. *Nucleic Acids Res* **13**, 4431–4443.
- Cases, I. & de Lorenzo, V. (1998). Expression systems and physiological control of promoter activity in bacteria. *Curr Opin Microbiol* **1**, 303–310.
- Cases, I. & de Lorenzo, V. (2000). Genetic evidence of distinct physiological regulation mechanisms in the σ^{54} Pu promoter of *Pseudomonas putida*. *J Bacteriol* **182**, 956–960.
- Cases, I., de Lorenzo, V. & Perez-Martin, J. (1996). Involvement of σ^{54} in exponential silencing of the *Pseudomonas putida* TOL plasmid Pu promoter. *Mol Microbiol* **19**, 7–17.
- Cases, I., Perez-Martin, J. & de Lorenzo, V. (1999). The HIA^{Ntr} (Ptn) protein of *Pseudomonas putida* mediates the C source inhibition of the σ^{54} -dependent Pu promoter of the TOL plasmid. *J Biol Chem* **274**, 15562–15568.
- Chugani, S. A., Parsek, M. R., Hershberger, C. D., Murakami, K., Ishihama, A. & Chakrabarty, A. M. (1997). Activation of the *catBCA* promoter: probing the interaction of CatR and RNA polymerase through *in vitro* transcription. *J Bacteriol* **179**, 2221–2227.
- Gralla, J. D. & Collado-Vides, J. (1996). Organization and function of transcription regulatory elements. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, 2nd edn, pp. 1232–1245. Edited by F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger. Washington, DC: American Society for Microbiology.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Harayama, S. & Timmis, K. N. (1989). Catabolism of aromatic hydrocarbons by *Pseudomonas*. In *Genetics of Bacterial Diversity*, pp. 151–174. Edited by D. A. Hopwood & K. E. Chater. London: Academic Press.
- Harwood, C. S. & Parales, R. E. (1996). The β -ketoadipate pathway and the biology of self-identity. *Annu Rev Microbiol* **50**, 553–590.
- Hengge-Aronis, R. (1999). Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. *Curr Opin Microbiol* **2**, 148–152.
- Hengge-Aronis, R. (2000). The general stress response in *Escherichia coli*. In *Bacterial Stress Responses*, pp. 161–178. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.
- Herrero, M., de Lorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* **172**, 6557–6567.
- Hörak, R. & Kivisaar, M. (1998). Expression of the transposase gene *tnpA* of Tn4652 is positively affected by integration host factor. *J Bacteriol* **180**, 2822–2829.
- Houghton, J. E., Brown, T. M., Appel, A. J., Hughes, E. J. & Ornston, L. N. (1995). Discontinuities in the evolution of *Pseudomonas putida* cat genes. *J Bacteriol* **177**, 401–412.
- Kasak, L., Hörak, R., Nurk, A., Talvik, K. & Kivisaar, M. (1993). Regulation of the catechol 1,2-dioxygenase- and phenol monooxygenase-encoding *pheBA* operon in *Pseudomonas putida* PaW85. *J Bacteriol* **175**, 8038–8042.
- Kivisaar, M., Hörak, R., Kasak, L., Heinaru, A. & Habicht, J. (1990). Selection of independent plasmids determining phenol degradation in *Pseudomonas putida* and the cloning and expression of genes encoding phenol monooxygenase and catechol 1,2-dioxygenase. *Plasmid* **24**, 25–36.
- Kivisaar, M., Kasak, L. & Nurk, A. (1991). Sequence of the plasmid-encoded catechol 1,2-dioxygenase-expressing gene, *pheB*, of phenol-degrading *Pseudomonas* sp. strain EST1001. *Gene* **98**, 15–20.
- de Lorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K. N. (1990). Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* **172**, 6568–6572.
- de Lorenzo, V., Cases, I., Herrero, M. & Timmis, K. N. (1993). Early and late responses of TOL promoters to pathway inducers: identification of postexponential promoters in *Pseudomonas putida* with *lacZ-tet* bicistronic reporters. *J Bacteriol* **175**, 6902–6907.
- Marques, S., Holtel, A., Timmis, K. N. & Ramos, J. (1994). Transcriptional induction kinetics from the promoters of the catabolic pathways of TOL plasmid pWW0 of *Pseudomonas putida* for metabolism of aromatics. *J Bacteriol* **176**, 2517–2524.
- Marques, S., Manzanera, M., Gonzalez-Perez, M.-M., Gallegos, M.-T. & Ramos, J. L. (1999). The XylS-dependent Pm promoter is transcribed *in vivo* by RNA polymerase with σ^{32} or σ^{38} depending on the growth phase. *Mol Microbiol* **31**, 1105–1113.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Miller, V. L. & Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**, 2575–2583.
- Neidhardt, F. C., Bloch, P. L. & Smith, D. F. (1974). Culture medium for enterobacteria. *J Bacteriol* **119**, 736–747.
- Ojangu, E., Tover, A. & Kivisaar, M. (2000). Effects of combination of different –10 hexamers and downstream sequences on stationary phase-specific sigma factor σ^S -dependent transcription in *Pseudomonas putida*. *J Bacteriol* **182**, 6707–6713.
- Parsek, M. R., Kivisaar, M. & Chakrabarty, A. M. (1995).

Differential DNA bending introduced by the *Pseudomonas putida* LysR-type regulator, CatR, at the plasmid-borne *pheBA* and chromosomal *catBC* promoters. *Mol Microbiol* **15**, 819–828.

Ramos, J. L., Marques, S. & Timmis, K. N. (1997). Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators. *Annu Rev Microbiol* **51**, 341–373.

Rothmel, R. K., Aldrich, T. L., Houghton, J. E., Coco, W. M., Ornston, L. N. & Chakrabarty, A. M. (1990). Nucleotide sequencing of *Pseudomonas putida catR*: positive regulator of the *catBC* operon is a member of LysR family. *J Bacteriol* **172**, 922–931.

Rothmel, R. K., Shinabarger, D. L., Parsek, M. R., Aldrich, T. L. & Chakrabarty, A. M. (1991). Functional analysis of the *Pseudomonas putida* regulatory protein CatR: transcriptional studies and determination of the CatR DNA-binding site by hydroxyl-radical footprinting. *J Bacteriol* **173**, 4717–4724.

Sharma, R. C. & Schimke, R. T. (1996). Preparation of electro-competent *E. coli* using salt-free growth medium. *BioTechniques* **20**, 42–44.

Sze, C. C. & Shingler, V. (1999). The alarmone (p)ppGpp mediates physiological-responsive control at the σ^{54} -dependent Po promoter. *Mol Microbiol* **31**, 1217–1228.

Sze, C. C., Moore, T. & Shingler, V. (1996). Growth phase-dependent transcription of the σ^{54} -dependent Po promoter controlling the *Pseudomonas*-derived (methyl)phenol *dmp* operon of pVI150. *J Bacteriol* **178**, 3727–3735.

Tover, A., Zernant, J., Chugani, S. A., Chakrabarty, A. M. & Kivisaar, M. (2000). Critical nucleotides in the interaction of CatR with the *pheBA* promoter: conservation of the CatR-mediated regulation mechanisms between the *pheBA* and *catBCA* operons. *Microbiology* **146**, 173–183.

Yuste, L., Canosa, I. & Rojo, F. (1998). Carbon-source-dependent expression of the *PalkB* promoter from the *Pseudomonas oleovorans* alkane degradation pathway. *J Bacteriol* **180**, 5218–5226.

Received 11 December 2000; revised 19 March 2001; accepted 12 April 2001.