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104

REGULATION OF TRANSCRIPTION OF THE PHENOL DEGRADATION *pheBA* OPERON IN *PSEUDOMONAS PUTIDA*

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CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I **Tover A**., Zernant J., Chugani S.A., Chakrabarty A.M. and 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: 173–83.

II **Tover A**., Ojangu E-L. and Kivisaar M. (2001) Growth medium composition-determined regulatory mechanisms are superimposed on CatR-mediated transcription from the *pheBA* and *catBCA* promoters in *Pseudomonas putida*. Microbiology. 147: 2149–56.

III Ojangu E-L., **Tover A**., Teras R. and Kivisaar M. (2000) Effects of combination of different –10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in *Pseudomonas putida*. J. Bacteriol. 182: 6707–13.

IV Putrinš M., **Tover A**., Tegova R., Saks Ü. and Kivisaar M. Physiological control mechanisms regulating transcription from the CatR-regulated *pheBA* promoter in *Pseudomonas putida* operate through reduction of the synthesis of the inducer-producing enzyme catechol 1,2-dioxygenase. Manuscript.

ABBREVATIONS

IBS	Internal Binding Sequence
ABS	Activation Binding Sequence
RBS	Recognition Binding Sequence
CCM	*cis,cis*-muconate
IHF	Integration Host Factor
HU	Histone like protein
UAS	Upstream Activating Sequence
bp	basepair(s)
Km	Kanamycine
CAA	Casaminoacids
C12O	Catechol 1,2-dioxygenase

INTRODUCTION

Bacteria can live in and adapt to various ecosystems. In their natural environment bacterial populations spend most of the time under conditions of nutrient limitation. Limitation of nutrient forces the bacteria to develop a number of catabolic genes for degradation of compounds which presence is rare in nature e.g. several hydrocarbons or aromatic compounds including phenols and to use them as a source of energy. Aromatic compounds have been around a world already several hundred millions of years since plants appeared onto the surface of the Earth. Phenolic compounds are found in the composition of several esthers or glycosides which are synthesised in plants.

Activation of transcription of the catabolic genes is usually controlled by specific regulator proteins. The transcription of catabolic genes for degradation of phenolic compounds is activated in response to the presence of corresponding aromatic compound in the bacterial growth medium. Generally, the proteins activating the transcription of the catabolic operons in *Pseudomonas putida* are divided into three classes: NtrC/XylR, XylS/AraC and LysR regulators. The transcriptional activation from the promoters of catabolic operons regulated by these proteins needs the presence of inducer molecule. The inducer molecule may be either the substrate of the regulated catabolic pathway (e.g. in the case of NtrC/XylR or XylS/AraC regulators) or intermediate of the degraded aromatic compound (LysR-type regulators). Binding of the inducer molecule to the activator protein causes conformational changes in the activator and thereby the regulator protein is able to activate the transcription from appropriate promoter. This specific regulation of gene expression by the regulator protein and inducer molecule is not the only mode to control the expression of catabolic genes.

Several global regulatory mechanisms have been developed by bacterial cells to optimize the gene expression of the catabolic operons. The global control of the expression in *P. putida* acts via repression of transcription of the catabolic operons in bacteria growing fast in nutritionally rich medium. This type of regulation has been studied in details for the XylR/NtrC or AraC/XylS regulons (Cases *et al.*, 1996; Sze *et al*., 1996; Yuste *et al.*, 1998) but not in the case of LysR-type regulons.

The plasmidial *pheBA* operon in cooperation with chromosomal catechol degradation *catBCA* operon is responsible for the degradation of phenol in *P. putida.* The transcription from the promoters of these operons is positively regulated by LysR-type regulator protein CatR (Rothmel *et al*., 1990 Kasak *et al*., 1993). The intermediate of phenol degradation, *cis,cis*-muconate, binds to the CatR protein and thereby allows the CatR-mediated activation of the transcription from both the *pheBA* and the *catBCA* promoters. The DNA sequences of the promoter regions of the *pheBA* and *catBCA* operons share

great similarity. However, the precise nucleotides necessary for binding of regulator protein and critical for activation of transcription from the *pheBA* and *catBCA* promoters are different.

First part of this thesis examines the similarities of the CatR-mediated regulatory mechanisms between the *catBCA* and *pheBA* promoters. Whereas, the second part of my thesis is dedicated to the investigation of physiological control mechanisms of the *pheBA* and *catBCA* promoters.

1. REVIEW OF LITERATURE

1.1. Bacterial RNA polymerase and initiation of transcription

The bacterial DNA-dependent RNA polymerase is responsible for the synthesis of all cellular RNA. The eubacterial RNA polymerase core enzyme consists of $\alpha_2\beta\beta'$ subunits, which are responsible for elongation and termination of the RNA synthesis (reviewed in Browning and Busby, 2004). Two domains of the α subunit are connected with ~20 amino acid-long linker. The N-terminal part of the α subunit (α-NTD) is responsible for assembly of β and β' subunits. The C-terminal part of α subunit (α-CTD) binds with DNA molecule and several activator proteins. The β and β' subunits together with two α-subunits form the transcriptionally active RNA polymerase which contains the active site for synthesis of RNA. The core enzyme of the RNA polymerase can bind DNA non-specifically and is not able to initiate the transcription. The exact binding of the RNA polymerase to the promoter region needs σ factor, which binds to the RNA polymerase core enzyme and leads to the initiation of transcription. The RNA polymerase contains also fifth subunit, ω, which does not participate in the synthesis of RNA but acts as a chaperon on the folding of the β' subunit (Browning and Busby, 2004).

1.1.1. Sigma factors in gram-negative bacteria

The σ factor bound to RNA polymerase core enzyme can recognize and specifically bind the promoter sequence to form the catalytically active RNA polymerase complex. The binding affinity of the sigma factor to the promoter sequence defines the strength of the promoter and thereby the efficiency of transcription from the appropriate gene. The bacterial σ factors are classified widely as housekeeping and alternative σ factors. Based on the structure, the sigma factors characterized in gram-negative bacteria are divided in two subclasses: σ^{70} and σ^{54}. Most genes in bacteria are transcribed from promoters which, are recognized by σ^{70} family factors (Merrick, 1993). The major sigma factor of σ^{70} class sigma factors in gram-negative bacteria is σ^{70} which recognize the promoter sequence of two 6 bp-long elements called –35 and –10 hexameric sequences (Harley *et al.* 1987; Dombroski *et al.* 1993). Between the –35 and –10 hexameric sequence remains 16–18 bp DNA region which is not conserved. The consensus sequence for the binding of *E. coli* σ^{70} is TTGACA-$N_{16\text{-}18}$-TATAAT (Wösten, 1998). The σ^{70} class includes also several stress-induced sigma factors, e.g. σ^{S}, which regulates the expression of the genes

necessary for surviving under conditions of starvation (Mulvey and Loewen, 1989; Loewen and Hengge-Aronis, 1994; Hengge-Aronis, 1996), or σ^{32}, which is responsible for the regulation of heat-shock genes (Yura *et al.* 1993; Nakahigashi *et al.* 1995). Activation of transcription from the σ^{70}-type promoter does not necessarily require an additional activator protein. However, in many cases (e.g. lack of σ^{70}-recognized –35 hexameric sequence) the activator is needed to enhance the binding of the RNA polymerase holoenzyme to the promoter and to form an open complex.

Another large class of σ factors are the σ^{54}-type sigma factors (Merrick, 1993). σ^{54}-type sigma factors have no homology with σ^{70} class sigma factors. σ^{54}-containing RNA polymerase recognizes the conserved GG and GC motifs which are locating at positions –12 to –24 from the transcription start point, respectively (Thony and Hennecke, 1989). In contrast to the σ^{70} -type sigma factors the σ^{54} is unable to form an open complex and always requires the activator protein bound usually about 100 bp upstream from the promoter sequence (Morris *et al.* 1994; Carmona *et al.* 1997). Additionally, DNA looping is required for the interaction of the RNA polymerase and regulator protein (reviewed in Xu and Hoover, 2001).

1.1.2. Sigma factors in *P. putida*

Most of the sigma factors characterized in pseudomonads are similar to their counterparts identified in enterobacteria. The σ factors found in *P. putida* are listed in Table 1. The genome of *P. putida* carries 24 different σ factor-encoding genes. 19 of these encode proteins, which belong to the class of extracytoplasmatic sigma factors (ECF) (Martinez-Bueno *et al.,* 2002). 13 of these factors share similarity with *E. coli* FecI sigma factor which is involved in iron acquisition (Martinez-Bueno *et al.,* 2002).

σ^{70} is responsible for the transcription initiation from the promoters of housekeeping genes. The first identified alternative σ factor in *P. putida* is the *rpoS*-encoded σ^{S} (Ramos-Gonzáles and Molin 1998) which is responsible for the activation of transcription of above 50 genes under C-limitation. The second alternative sigma factor is heat-shock-induced RpoH (σ^{32}) which is identified in *P. putida* by Manzanera *et al.* (2001). Gene regulation mechanisms controlled by σ^{32} are remained still unclear because *rpoH*-deficient *P. putida* is not viable. σ^{28} (also known as FliA and SigD) regulates the transcription of the genes responsible for flagellin biosynthesis (Kieboom *et al.*, 2001). The *rpoN*-encoded σ^{54} is involved in the regulation of operons responsible for C- and N-metabolism, and this sigma factor also regulates the expression of several catabolic pathways (Inouye, 1989).

Table 1. Sigma factors in *P. putida* cells.

σ factor	Protein name	Function
σ^{70} family		
Housekeeping σ factor	σ^{70}, RpoD	Major sigma factor, regulates the expression of most genes in exponential and stationary phase
Stationary-phase σ factor	σ^{S}, RpoS	Up-regulated during entry into the stationary phase of the bacterial growth. Responsible for the regulation of more than 50 genes during carbon starvation (Ramos-Gonzalez and Molin, 1998).
Heat shock σ factor	σ^{32}, RpoH	Regulates several heat shock genes but also transcription from the Pm promoter of the m-toluate degradation genes in TOL plasmid (Manzanera *et al.*, 2001, Marques *et al.*, 1999).
Flagella synthesis factor	^{28}, FliA, SigD	Flagellin biosynthesis (Kieboom *et al.*, 2001)
Extracytoplasmatic factors		19 membrane-bound sigma factors, 13 of them probably participate on the iron uptake and belong to the *fecI/fecR/*iron receptor cluster (Martinez-Bueno *et al.*, 2002)
σ^{54} family		
C and N utilization-regulating σ factor	σ^{54}, RpoN	Utilization of several carbon and nitrogen sources. Regulation of transcription from the *xyl* and *dmp* operons (Cases and de Lorenzo, 2001). Flagellin biosynthesis (Inouye, 1989)

1.2. Transcriptional regulation of phenolic compounds degradation operons in *P. putida* by specific regulators

1.2.1. Transcriptional regulators in bacteria

The transcriptional regulators control the level of gene expression by modulating recruitment of RNA polymerase to the promoter sequence. The activation of transcription from the σ^{70}-type promoter may not necessarily require the regulator protein but in the case of the σ^{54}-dependent promoters the activation of transcription is strictly dependent on the presence of activator protein (Browning and Busby, 2004). The most common transcriptional regulator proteins in gram-negative bacteria belong to the three main classes: NtrC/XylR,

XylS/AraC or LysR. The mechanisms of transcriptional activation of catabolic genes in *P. putida* mediated by these proteins are described below. The corresponding regulatory proteins and their target genes found in *P. putida* are listed in Table 2.

1.2.2. Domain organization of LysR-type transcriptional regulators and the mechanism of regulation of transcription by the LysR-type regulators

The LysR type transcriptional regulators have protein size between 27 to 33 kDa. In most cases the regulator-encoding gene and the operon, which expression is controlled by appropriate regulator locate closely in bacterial chromosome and their promoters are transcribed divergently (reviewed in Schell, 1993; Tropel and van der Meer, 2004). Usually, the transcription of the regulator gene is negatively auto-regulated by binding of the corresponding gene product to the promoter of its gene. The precise structure of the LysR type regulator is still not well defined. It is known that the N-terminal region of the regulator protein has helix-turn-helix motif for the binding of DNA molecule (Schell, 1993) (Fig. 1B). Central part of the protein is responsible for the binding of an inducer molecule. The C-terminal region is probably responsible for the changing of the conformation of the regulator protein (Fig. 1.). The amino acid substitutions in this region lead either to inducer-independent activation of transcription (NahR, NodD) or loss of DNA binding ability (AmpR) (reviewed in the Shell, 1993).

The mechanism of activation of transcription by the LysR type regulators can be described on the example of *catBCA* operon. *catBCA* operon encodes enzymes for further degradation of catechol to TCA-cycle intermediate (Aldrich and Chakrabarty, 1988). *catA* gene encodes the enzyme catechol 1,2-dioxygenase which degrades the catechol to *cis,cis*-muconate (CCM) (Parsek *et al.* 1992). CCM is converted to muconolactone and β-ketoadipate enol-lactone by *cis,cis*-muconate lactonizing enzyme and muconolactone isomeraze encoded by *catB* and *catC* genes, respectively (Houghton *et al.*, 1995). The initiation of the transcription from the *catBCA* promoter requires the regulator protein CatR and the catechol degradation intermediate CCM which interacts with the CatR protein and acts as an inducer. In the absence of CCM the CatR binds to the 26 bp region called RBS (recognition binding sequence), which locates at the positions –79 to –54 upstream from the transcription start-point of the *catB* gene (Fig. 1A. and Fig. 2A.) (Parsek *et al.*, 1994).

Table 2. Transcription factors involved in the regulation of biodegradation operons in *P. putida*.

Catabolic genes regulated by the LysR-family regulators in *P. putida*			
Regulator	Genes/Localization	Inducer	Function
CatR	*catBCA*/chromosome	*cis,cis*-muconate	Catechol degradation (Rothmel *et al.*, 1990)
	pheBA/plasmid pEST1001	*cis,cis*-muconate	Phenol degradation (Kasak *et al.*, 1993)
ClcR	*clcABD*/plasmid pAC27	2-chloromuconate	Chlorocatechol degradation (Coco *et al.*, 1993)
NahR	*nahAF*/plasmid NAH7	salicylate	Naphtalen degradation, salicylate degradation (Schell, 1985)
SalR	*sal* operon/chromosome	salicylate	Degradation of salicylate (Sato *et al*., 2001)
TfdR	*tfdCB*/plasmid pEST4011	2,4-dichloro-*cis,cis*-muconate	2,4-dichlorophenoxyacetic acid degradation (Vedler *et al.*, 2000)
TodR	*todFC1C2BADEGIH*	toluene	Toluene degradation (Wang *et al.*, 1995)
TcbR	*tcbAB, tcbCDEF/* plasmid p51	chlorobenzoate	Chlorobenzoate degradation (van der Meer *et al.*, 1991)
Catabolic genes regulated by the AraC family regulators in *P. putida*			
Regulator	Genes/Localization	Inducer	Function
BenR	*benABC*/chromosome	benzoate, methyl-bensoate	Degradation of benzoate, methylbensoate, 4-hydroxybenzoate and 4-hydroxybenzoate (Cowles *et al.*, 2000)
XylS	*xyl* operon/TOL plasmid pWW0	alkylbenzoates	Degradation of benzoate or alkylbenzoates (Inouye *et al.*, 1981)
AlkS	*alk* operon/OCT plasmid	*n*-alkanes	Degradation of n-alkanes (van Beilen *et al*., 2001)
Catabolic genes regulated by the NtrC family regulators in *P. putida*			
Regulator	Genes/Localization	Inducer	Function
DmpR	*dmp* operon/plasmid pVI150	dimethylphenols/methylphenol	Degradation of methylated or non-methylated phenols (Schingler *et al.*, 1993)
XylR	*xyl* operon/TOL plasmid pWW0	toluene, methylto-luenes etc.	Degradation of substituted and non-substituted toluene (Abril *et al.*,1989)
PhlR	*phlA-L*/plasmid pPGH1	phenol	Phenol degradation (Müller *et al.*, 1996)
PhhR	phh/chromosome	phenol/methylphenol	Phenol and methylphenol degradation (Ng *et al.*, 1995)

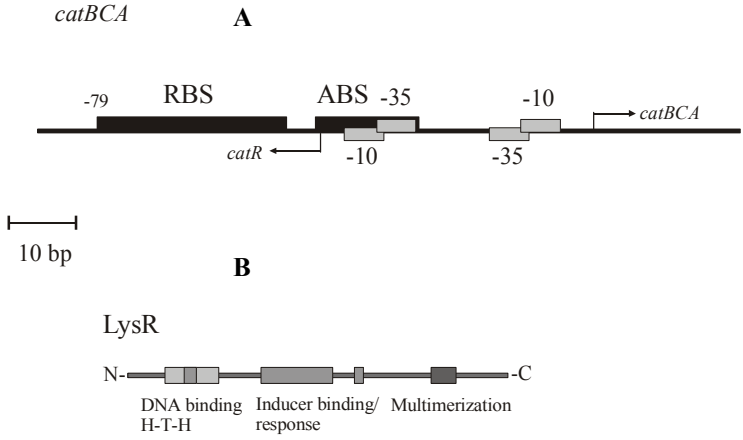

Fig. 1. Schematic representation of the *catBCA* promoter region (A) and domain organization of the LysR-family transcription regulators (B). (A) Black boxes represent the binding sequences RBS and ABS of the regulator protein CatR. Grey boxes indicate the hexameric sequences of the overlapped promoters of the *catBCA* operon and the *catR* gene. Transcription start points of the *catBCA* operon and *catR* gene are shown by arrows. (B) Different protein domains are represented by boxes and their functions are described below.

The RBS region of the LysR-family activator-regulated genes contains usually the T-N_{11}-A motif within the interrupted inverted repeat sequence. In the case of the *catBCA* promoter the DNA motif for the CatR binding is G-N_{11}-A (Parsek *et al.*, 1994). When CatR binds to the RBS region, it represses the transcription from the promoter of the *catR* gene (Fig. 1A.) (Parsek *et al.*, 1994). Therefore, the RBS region is also called as a repressor binding sequence. In the presence of inducer molecule (CCM) in addition to RBS region the CatR binds also to the region called ABS (activation binding sequence) located at –48 to –34 bp upstream from the transcription start point of the *catBCA* operon (Parsek *et al.* 1994). Binding of the CatR to the RBS and ABS regions results in the bending of the promoter region (Fig. 2B.) which is important for the activation of the transcription from the *catBCA* promoter (Parsek *et al.* 1995).

Several LysR-family transcriptional regulators (OxyR, TrpI and also CatR) require for the activation a contact with the C-terminal part of the RNA polymerase α-subunit (Chugani *et al.*, 1997; Storz *et al.*, 1990; Gussin *et al.*, 1992) (Fig. 2B).

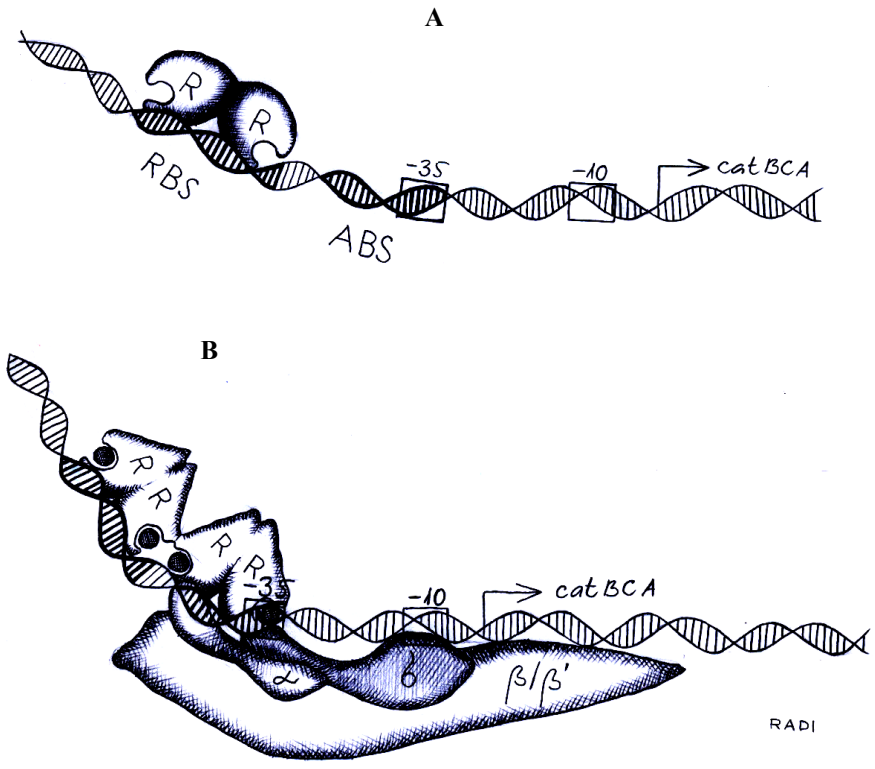

Fig. 2. Mechanism of the initiation of transcription by the LysR-family regulator proteins exampled by the *catBCA* promoter. (A) In the absence of the inducer molecul *cis,cis*-muconate (CCM) the regulator protein CatR binds only to the recognition binding site (RBS) and the RNA polymerase is unable to initiate the transcription. (B) In the presence of inducer molecule (CCM) the regulator protein CatR changes the conformation and binds to the activation binding site (ABS) and causes the activation of transcription. The interaction between RNA polymerase α subunit and the CatR protein is required for the activation of transcription from the *catBCA* promoter.

1.2.3. Domain organization of NtrC/XylR-type transcriptional regulators and the mechanism of activation of transcription from the Pu promoter of the upper *xyl* operon present in TOL plasmid pWW0

The NtrC/XylR family proteins are 500–600 amino acid residues-long. Proteins of this family have four functional domains (Fig. 3 B). The N-terminal A-domain is the most divergent part of the NtrC-family regulators. It acts as a signal receptor and interacts directly with the effector molecule such as xylene

or toluene (XylR protein) (Abril *et al.*, 1989) and phenol/cresols (DmpR protein) (Shingler *et al.*, 1993). Central domain, the highly conserved C-domain, is involved in ATP hydrolysis (Wedel and Kustu, 1995), interaction with the σ^{54} factor of the RNA polymerase holoenzyme (Berger *et al.*, 1994) and the oligomerization of the protein (Porter *et. al.* 1993). The most C-terminal domain of the NtrC-type protein contains helix-turn-helix motif for binding with DNA. Typically the A- and C-domains of the XylR-family proteins are connected with the flexible Q-linker or "hinge" region, which is a short hydrophobic region of the protein (Fernandez *et al.*, 1995). In the case of XylR the A-domain acts like an intramolecular repressor. Without the effector molecule the A-domain interacts with the central domain and thereby inhibits the constitutive activity of the XylR protein (Fernandez *et al.*1995, Perez-Martin and de Lorenzo 1995b).

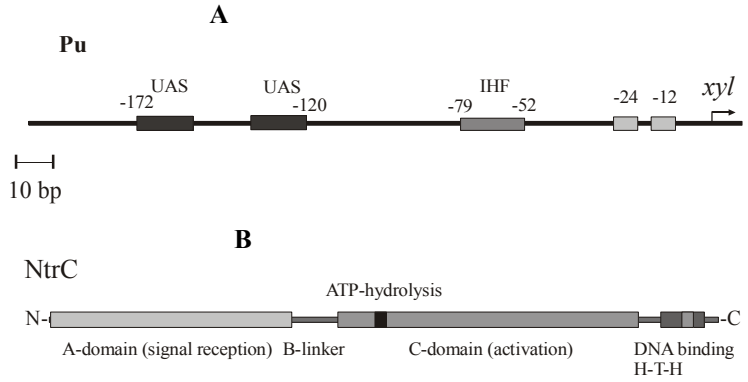

Figure 3. Schematic representation of the Pu promoter region of the TOL plasmid pWW0 (A) and domain organization of the NtrC-family transcription regulators (B). (A) Boxes represent the binding sequences of the XylR protein (UAS), binding sequence of IHF and –24/–12 recognition sequences of the σ^{54}. Transcription start point of the *xylCAB* genes is shown by arrow. (B) Domains indicated by boxes and their functions are described in the text.

To explain the mechanism of activation of transcription by NtrC/XylR-family regulators, I will discuss the well-investigated XylR-mediated initiation of transcription from the Pu promoter of the xylene degradation genes in TOL plasmid pWW0. The activation of transcription from the Pu promoter requires the XylR protein. XylR activates transcription in the presence of several aromatic compounds, e.g. toluene, xylene, benzyl alcohol and different substituted toluenes (Abril *et al.*1989). Particular aromatic effectors can activate XylR-mediated transcription at different levels (Garmendia *et al.*, 2001).

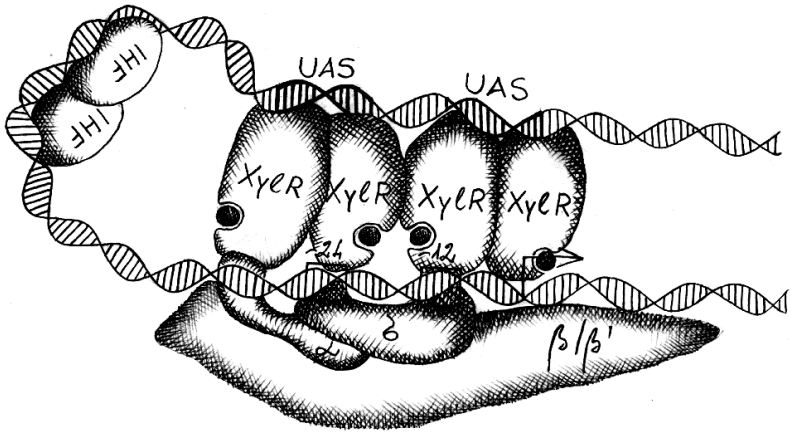

Fig. 4. Mechanisms of the activation of transcription by NtrC/XylR-family regulator proteins exampled by the Pu promoter. In the presence of effector-molecule (e.g. xylene) the XylR protein oligomerizes and interacts with upstream activating sequences (UAS). The interaction with UAS sequences requires the presence of IHF molecule which participates in looping of DNA. Activation of the transcription from the Pu promoter requires also the interaction between the XylR protein and the RNA polymerase α-subunit. Multimerization of the XylR proteins is not shown in this figure.

For the activation of transcription from the Pu promoter XylR protein binds to the two sites called UAS (upstream activating sequence) locating distant to the Pu promoter (Perez-Martin and de Lorenzo, 1996) (Fig. 3A). The activation of the transcription from the Pu promoter requires the formation of the DNA loop between the regulator binding region UAS and the Pu promoter sequence. The looping of the DNA is assisted by the DNA-bending proteins such as IHF (integration host factor) (de Lorenzo *et. al.* 1991) and HU (Perez-Martin and de Lorenzo 1995). Interactions between XylR bound to the UAS elements and RNA polymerase results in the initiation of transcription from the Pu promoter. This step needs the hydrolysis of ATP, stimulated by the binding of effector-molecule to the XylR protein. ATP hydrolysis is required also in the step of XylR multimerization that makes possible the interaction between the XylR and the σ^{54} subunit of RNA polymerase (Perez-Martin and de Lorenzo, 1996).

1.2.4. Domain organization of AraC/XylS-family transcriptional regulators and the mechanisms of activation of transcription from the Pm promoter of the "lower" *xyl* operon present on TOL plasmid pWW0

Catabolic genes controlled by this type of regulators in *P. putida* are listed in Table 2. The AraC/XylS proteins are approximately 300 amino acids-long. The C-terminal part of the AraC/XylS-family regulators contains two helix-turn-helix motifs for DNA binding (Fig. 6B). The C-terminus can probably interact also with the RNA polymerase (Bertoni *et al.*, 1998). The N-terminal part of the AraC-family proteins is not well conserved. It locates the domain responsible for effector binding, activation of transcription and dimerization. The AraC/XylS-family contains more than 100 different regulators that are involved in the regulation of transcription of the genes for carbon metabolism and pathogenesis (reviewed in Ramos *et al.*, 1997). The best-described AraC/XylS family regulator protein in *P. putida* is XylS which regulates the transcription from the Pm promoter of the "lower" *xyl* operon present in TOL plasmid pWW0 (Ramos *et al.*, 1997). This operon encodes proteins for degradation of benzoate and alkylbenzoate (Ramos *et al.*, 1997). The amount of the XylS in *P. putida* cells is controlled at the level of transcription by the XylR protein, the transcriptional regulator of the Pu promoter (Inouye *et al.*, 1987). The transcription of the *xylS* gene is controlled by two promoters, Ps1 and Ps2. The Ps1 promoter is distal from the *xylS* gene. The transcription from the Ps1 promoter is constitutive allowing low level of the expression of the XylS. The transcription from the Ps2 promoter is positively controlled by the XylR protein and requires the presence of effector molecule (Marques *et al.* 1998). The level of transcription from the Ps2 promoter is high, resulting in the high cellular amount of the XylS protein and activation of transcription from the Pm promoter. Marques *et al.* (1998) has shown that artificial increase of the XylS protein makes possible the effector-independent activation of the transcription from the Pm promoter.

Within the Pm promoter region, two XylS-binding sites were identified which are located at positions –35 to –78 nucleotides from the transcriptional start point (Fig. 6A) (Kaldalu *et al.*, 1996; Gonzales-Perez *et al.*, 1999). The binding region of the XylS contains two 15 bp-long direct repeats (Fig. 6B). For the activation of the transcription from the Pm promoter the XylS requires the contact to the RNA polymerase α-subunit (Ruiz *et al.*, 2001) (Fig. 5). The activation of transcription from the Pm promoter is stimulated by several substituted benzoates. It is suggested that the binding of an effector molecule to the XylS protein changes the conformation or forces the oligomerization of regulator protein and thereby activates the transcription from the Pm promoter (Ramos *et al.* 1997).

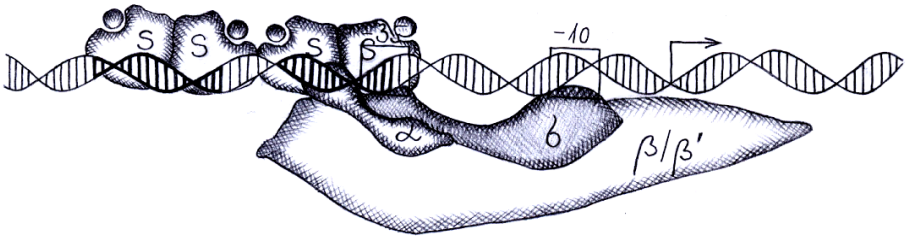

Fig. 5. Mechanism of the activation of transcription by AraC/XylS-family regulator proteins exampled by the Pm promoter. For the activation of transcription from the Pm promoter the transcriptional activator protein XylS in the presence of effector molecule (e.g. alkylbenzoate) binds to the two direct repeats and activates the transcription from the Pm promoter. The interaction between the XylS protein and RNA polymerase α-subunit is required for activation of transcription from the Pm promoter as well.

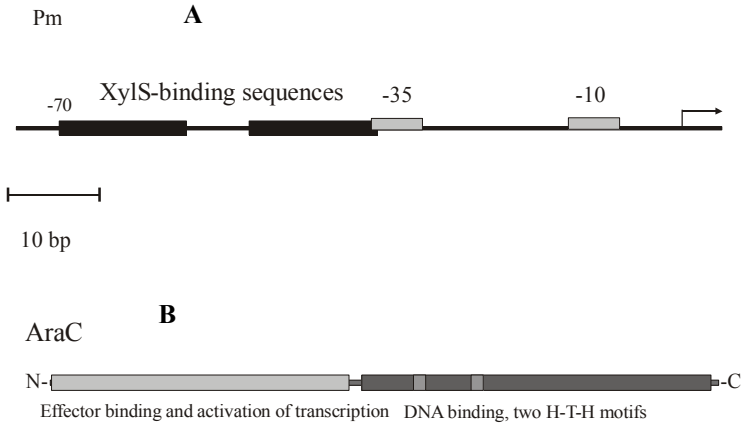

Figure 6. Schematic representation of the Pm promoter of the TOL plasmid pWW0 (A) Black boxes represent the binding sequences of the XylS protein at the Pm promoter and grey boxes indicate the –35 and –10 hexameric sequences of the σ^{70}-type promoter. Transcriptional start point of the *xyl* genes is shown by arrow. (B) The domain organization of XylS/AraC-type transcriptional regulator protein. Domains are indicated in the figure by boxes and their functions are described in the text.

1.3. Physiological control mechanisms of transcription of catabolic operons in *Pseudomonas*

1.3.1. Regulation of transcription of the catabolic operons by alternative σ factors

The transcription of the catabolic operons in *P. putida* is controlled by different sets of σ factors (Marques *et al*., 1994; Marques *et al*., 1995; Marques *et al*., 1999; Chugani *et.al.* 1997). Transcription initiation from the above-described Pu promoter is under the control of σ^{54} (de Lorenzo *et al.*, 1991). Other promoters are controlled by σ^{70}-class sigma factors. As already discussed in the chapter 1, the σ^{70}-class includes several alternative sigma factors, e.g. σ^{S} and σ^{32}. Use of alternative σ factors gives bacteria better opportunity to control more tightly the gene expression resulting in rapid adaptation of bacteria to stress, which in turn increases survival of bacteria in various environments (Venturi, 2003). Several promoter sequences of the hydrocarbon degradation genes do not display similarity to the typical σ^{70}-consensus sequence. In such case several other σ factors could be responsible for recognition of promoters by RNA polymerase. One example is the regulation of transcription from Pm promoter of the TOL plasmid pWW0. The transcription from the Pm promoter is regulated by the heat-shock σ factor in exponentially growing bacteria and by the σ^{S} in stationary-phase cells (Marques *et al.,* 1999). Another example of the regulation of transcription from the promoters of the genes responsible for degradation of hydrocarbons by the alternative sigma factors is the regulation of degradation of *n*-alkanes (Canosa *et al.*, 1998). Involvement of stationary-phase sigma factor σ^{S} in the regulation of the *alk* operon in *P. oleovoranse* is described by Canosa *et al.* (1999). Transcription from the P*alk* promoter is activated by the AraC/XylS-family transcriptional regulator AlkS in the presence of alkanes in the growth medium of bacteria. When bacterial culture is growing exponentially, the transcription from the P*alk* promoter is reduced but then increases drastically when the culture reaches to the stationary phase. This is because the transcription of the *alkS* is under the control of σ^{S} (Canosa *et al.* 1999).

1.3.2. Regulation of transcription of catabolic operons by exponential silencing

The regulation mechanism where the composition of growth medium of bacteria and the physiological state of bacteria could influence the transcription is described for many catabolic operons (Cases and de Lorenzo, 2000; Cases and de Lorenzo, 2001; Cases *et al.*, 1996; Cases and de Lorenzo, 2005; Dinamarca

et al., 2003; de Lorenzo *et al.*, 1993; Marques *et al.*, 1994; Santos *et al.*, 2000; Sze and Shingler, 1999; Sze *et al.*, 1996; Sze *et al.*, 2002; Yuste and Rojo, 2001; Yuste *et al.*, 1998; Rescalli *et al.*, 2004; Velazquez *et al.*, 2004; Rojo and Dinamarca, 2004). Basically, the phenomenon of exponential silencing is defined as down-regulation of transcription from catabolic operons during exponential growth of bacteria in rich medium containing amino acids. The phenomenon of exponential silencing has been observed in the case of the Pu promoter of TOL plasmid pWW0 (Cases *et al.*, 1996; Cases and de Lorenzo, 2000), Po promoter of the phenol degradation operon *dmp* (Sze *et al.*, 1996; Sze and Shingler, 1999) and also in the case of alkane degradation promoter *PalkB* (Yuste and Rojo, 2001). The specific regulation of transcription from the Po promoter is similar to that described for the Pu promoter (Sze *et al.*, 2002). Both these promoters require the presence of alternative sigma factor σ^{54} and with effector molecule-bound NtrC/XylR class transcriptional activator protein for the activation of transcription (for details, see table 2). The phenomenon of exponential silencing is not specific only to the promoters regulated by σ^{54}. For example, the PalkB promoter is recognized by σ^{70} and is also under the control of exponential silencing.

1.3.2.1. The role of σ^{54} in the exponential silencing of the transcription

Several studies on the exponential silencing of σ^{54} promoters have discovered the involvement of the amount of σ^{54} on physiological control of transcription (Cases *et al.*, 1999). Jurado *et al.* (2003) investigated the possibility that the down-regulation of the transcription from the Pu promoter in the rich medium could be mediated by the level of σ^{54} in the cells. They found that the amount of σ^{54}, which in *P. putida* cells is approximately 80 molecules per cell through the growth phases is approximately ten times lower if compared it to the amount of σ^{70} (750 molecules per cell). It is possible that the level of σ^{54}-containing RNA polymerase does not saturate all available σ^{54}-dependent promoters in *P. putida* cells. Any growth conditions which lead to the better occupancy of the Pu promoter by the σ^{54} –RNA polymerase may increase the initiation of transcription from the Pu promoter. This hypothesis is in accordance with data of Cases *et al.* (1999), where was shown that artificial increase of σ^{54} in bacterial cells relieves the exponential silencing of Pu promoter probably due to the better occupancy by the σ^{54}-RNA polymerase.

1.3.2.2. Involvement of proteases in the exponential silencing

Proteolysis is an important mechanism to control the gene regulation at post-translational level. Several regulatory proteins that are required only in short time under certain physiological conditions are sensitive to proteolytic degradation. For instance, it has been shown that the inner membrane-bound protease FtsH controls the level of heat-shock sigma factor σ^{32} in *E. coli* cells (Herman *et al.* 1995). The role of FtsH on the exponential silencing of the Pu promoter has been investigated in *E. coli* system (Carmona and de Lorenzo, 1999). Overproduction of FtsH relieves the exponential silencing of the Pu promoter. In *E. coli* strain deficient for *ftsH* the transcription from the Pu promoter is down-regulated but overproducing of σ^{54} restored the Pu promoter activity (Carmona and de Lorenzo., 1999). The mechanism of this phenomenon is still not clear. In the review by Cases and de Lorenzo (2001) it is hypothesized that the action of anti-sigma factors or connection between the activity of the σ^{54} and turnover of σ^{32} by FtsH protease and DnaK chaperone could be directly involved into the physiological control of the transcription from the Pu promoter. Whether FtsH could influence the activity of σ^{54} and σ^{32} in *P. putida* cells is still unclear.

1.3.2.3. Stringent response and involvement of ppGpp on the regulation of catabolic operons

Guanosine tetraphosphate (ppGpp) or guanosine pentaphosphate pppGpp (collectively called (p)ppGpp) is well-studied bacterial global signal molecules. Under stressful conditions (e.g. amino acid starvation) the synthesis and accumulation of (p)ppGpp in bacterial cells initiates the stringent response – a change in cellular metabolism (Chatterji and Ojha 2001). The (p)ppGpp binds to the β or β´ subunits of RNA polymerase thereby modulating the transcription from the specific promoters. (p)ppGpp can also operate indirectly by laying some regulatory cascade in bacterial cell. The (p)ppGpp stimulates the transcription from Po and Pu promoters both *in vivo* and *in vitro* (Laurie *et al.*, 2003; Carmona *et al.*, 2000). The effect of stimulation of transcription is higher in the case of Po promoter than in the case of Pu promoter (Carmona *et al.* 2000; Sze *et al.* 2002). Over-expression of (p)ppGpp synthetase gene *relA* in *P. putida* cells increased the transcription and relieved the exponential silencing from both, Po (Sze and Shingler, 1999) and Pu promoters(Carmona *et al.*, 2000), but in the case of Pu the effect of (p)ppGpp was lower.

1.3.2.4. Carbon source-mediated regulation of transcription of catabolic operons in P. putida

The inhibition of the gene expression by the certain carbon source in bacterial growth media is called catabolic repression. The glucose is the universal inducer of catabolite repression in *E. coli.* Pseudomonads lack classical cAMP/CRP mediated catabolic repression mechanisms described in *E. coli*. Vfr protein in Pseudomonads which share the homologue with *E. coli* catabolite repression protein CRP is responsible in regulation of quorum sensing but is not involved in catabolic repression (reviewed from Cases and de Lorenzo, 2001; Cases and de Lorenzo, 2005). In *Pseudomonas* unlike from *E. coli* the glucose is first transformed to the gluconic acid and only after that it is transported into bacterial cells. The transcription from the Pu promoter is down-regulated in the presence of glucose or gluconate in growth medium of *P. putida* (Cases *et al.*, 1999; Cases *et al.* 2001). It is shown that the inhibitory effect goes through the transport of the gluconate into the cells of *P. putida* or through the sensing of glucose or gluconate in growth medium of bacteria (Cases *et al.*, 1999). Cases *et al.* (1999) showed that the components of phosphoenolpyruvate-sugar phosphotransferase system (PTS) could be involved in the regulation of transcription from the Pu promoter in the presence of glucose. However, the inactivation of *ptsN*, encodes the IIA^{Ntr}, the protein which is a part of PTS relieved the glucose-mediated inhibition by sensing the presence of glucose but did not affect the exponential silencing in rich medium exponentially growing cells (Cases *et al.,* 1999). The precise mechanisms of PTS system mediated inhibition of the transcription from the Pu promoter in the presence of glucose is still unclear. Recent data by Velázquez *et al.* (2004) has showed that the repressive effect of glucose to the transcription from the Pu promoter is achieved through the catabolites of Entner-Doudoroff pathway 6-phosphogluconate and/or 2-dehydro-3-deoxyphosphogluconate.

1.3.2.5. The role of cytochrome o ubiquinol oxidase in the physiological control

The aerobic respiratory chain of *P. putida* contains a number of membrane-bound dehydrogenases which transfer the electron to the ubiquinone reducing it to the ubiquinol. Ubiquinol is oxidized by cytochrome *o* or cytochrome *d* complex (Dinamarca *et al.*, 2002). In exponential growth of bacteria the cytochrome *o* oxidase is responsible for the electron transport. Dinamarca *et al.* (2002) demonstrated that the exponential silencing of the Palk promoter of alkane degradation genes was relieved in the *P. putida* strain deficient for *cyoB* gene (encodes the cytochrome *o* ubiquinol oxidase) independently from the presence of repressing carbon source in the growth medium (Dinamarca *et al.*, 2002). This shows that the physiological control of catabolic operons could

monitor the physiological- or metabolic status of bacteria by using signal from the electron transport chain or from the redox state.

1.3.2.6. Physiological control of gene expression by the histone-like molecules

Recently, Rescalli *et al.* (2003) found the new protein in *P. putida*, TurA, which can bind to the Pu promoter and inhibits the transcription from it. It was shown that inactivation of TurA resulted in increased level of transcription from the Pu promoter. At the same time the TurA protein did not influence the exponential silencing or repression of transcription from the Pu promoter in the presence of glucose. TurA protein exhibits similarity to the H-NS protein of *E. coli* (Rescally *et al.*, 2003). The H-NS is a nucleoid-associated protein, which is involved in the control of many so called "environment-regulated" genes in *E. coli*, e.g. under conditions of low growth temperature, anaerobiosis, pH, etc. It was discussed that TurA protein might be required as a modulator of transcription from the Pu promoter especially at the low temperatures of growth (Rescally *et al.*, 2003).

1.3.2.7. The role of crc in the physiological regulation of catabolic genes

Crc protein (catabolite repression control) was first described as participant in catabolic repression mediated by succinate and lactate (reviewed in Collier *et al.*, 1996). In pseudomonads, the *crc* gene regulates the assimilation of several hydrocarbons, sugars and nitrogenated compounds (Collier *et al.*, 1996). The Crc protein mediates the down-regulation of alkane degradation pathway in *P. putida* (Yuste and Rojo, 2001). Recently, Morales *et al.* (2004) performed the proteome analysis of *P. putida* and they found that Crc could repress the expression of genes including the benzoate degradation *benABC* and the catechol degradation *catBCA* operons if bacteria were grown exponentially in rich medium (Morales *et al.*, 2004). The Crc protein is not the classical DNA-binding repressor because no detectable binding of Crc to DNA has been discovered. The mechanism of Crc mediated catabolic repression or the down-regulation of transcription in bacteria grown in rich growth medium remains still unclear.

2. RESULTS AND DISCUSSION

The regulation of the expression of biodegradative genes in *P. putida* has been investigated for many decades. Most studied mechanism of regulation of the biodegradative operon in *P. putida* is the initiation of transcription from the Pu promoter which controls the expression from the *xyl* operon, the "upper" pathway of xylene degradation of the TOL plasmid pWW0. *xyl* operon belongs to the σ^{54}-regulon together with methylphenol degradation *dmp* operon (Shingler *et al.*, 1993). The regulation of transcription from the promoters of these two operons is similar. Transcription from both promoters is activated by NtrC/XylR-family regulator protein in the presence of effector molecule which is usually the substrate of proteins encoded by appropriate operon. The *xyl* operon of "lower" pathway of TOL plasmid pWW0 and alkane degradation *alk* operon are also regulated by the presence of degradation substrate in growth medium of bacteria (Abril *et al.*, 1989; Inouye *et al.*, 1981; van Beilen *et. al.*, 2001). These operons belong to the σ^{70}-regulon and transcription from the promoters of these operons is activated by XylS/AraC-family regulator proteins. The regulation of transcription of catabolic operons in *P. putida* by LysR-family regulators is less studied. In this thesis I describe the regulation of transcription from plasmidial phenol degradation *pheBA* operon, which is positively controlled by LysR-family regulator and intermediate of the catabolic pathway (Kasak *et. al.*, 1993).

2.1. Study of binding specificity of the transcriptional regulator CatR to the promoter region of the *pheBA* operon and mechanisms of activation of transcription from the *pheBA* promoter (Ref. I)

To compare how similar or dissimilar the *pheBA* and *catBCA* promoter regions both in terms of homology and functionality, we conducted site-directed mutagenesis in the RBS, ABS and the putative IBS (Internal Binding Sequence) regions of the *pheBA* promoter.

The DNA sequences bound by CatR within the *pheBA* promoter region have been localised by DNase I footprinting (Parsek *et al*., 1995). In order to specify the critical nucleotides involved in DNA-protein interactions at the *pheBA* promoter, this DNA sequence was subjected to site-directed mutagenesis (Fig. 2 in Ref. I). The DNA fragments carrying the *pheBA* promoter with mutated RBS, ABS or "hinge" regions were cloned into the promoter-probe vector pKTLacZ (Hõrak and Kivisaar, 1998). The effect of the mutations on promoter activity was tested by measuring the β-galactosidase activity in cells of *P. putida*

PaW85 grown in LB in the presence or absence of benzoate. CCM, the inducer of the *pheBA* operon, is an intermediate of the benzoate degradation pathway and is produced during benzoate degradation. Gel shift assays were used to determine the effect of the mutations on the binding efficiency of CatR. The results of the study of the effect of the mutations on the *pheBA* promoter activity and CatR binding are shown in Table 1 (Ref. I) and are discussed below.

2.1.1. Mutations in the RBS region of the *pheBA* promoter affect the binding of CatR and activation of the transcription from the *pheBA* promoter (Ref. I)

Mutations in the interrupted inverted repeat of the RBS had drastic effects on CatR binding and activation of the *pheBA* promoter (Fig. 2 and Table 1 in Ref .I). At the same time, the base substitutions 67GA and 66CA between the repeat sequence did not have any effect. This indicates that the nucleotides in the interrupted inverted repeat may play an important role in the sequence-specific recognition of RBS by CatR. Similar results were seen in a previous site-directed mutagenesis study of the *catBCA* promoter (Parsek *et al*., 1994). Interestingly, the mutation 74CT which does not lie in the repeat had also a negative effect on the *in vivo* activity of the promoter and resulted in a lower CatR binding efficiency. Some mutations outside of the inverted repeat of the *catBCA* promoter also had negative effects on the *in vivo* activity of the promoter (Parsek *et al*., 1994).

The RBS sequence of the *catBCA* promoter contains the imperfect inverted repeat AGACC-N_5-GGTAT. It harbours a G-N_{11}-A motif instead of the consensus T-N_{11}-A of the LysR-family binding motifs. The *pheBA* promoter has a perfect inverted repeat ATACC-N_5-GGTAT and CatR binds the *pheBA* promoter with greater affinity than the *catBCA* promoter region (Parsek *et al*., 1995). Changing the G to the consensus T in the *catBCA* RBS resulted in slightly elevated levels of the CatR binding and the promoter activation. In contrast, the mutation 72TG which made the repeat sequence of the *pheBA* RBS identical to that of the *catBCA* promoter significantly abolished CatR binding and only 2% of the wild type promoter activity was observed under activating conditions (Table 1. in Ref. I). Moreover, the CatR-binding motif AGACC-N_5-GGTAT is conserved between the RBS and the low-affinity binding site IBS of the *catBCA* promoter (Chugani *et al*., 1998). This finding also indicates that the conserved interrupted inverted repeat sequence is not the sole important feature for the high-affinity binding of CatR.

2.1.2. Mutations in the ABS region of the *pheBA* promoter affect the binding of CatR and activation of the transcription from the *pheBA* promoter (Ref. I)

Under activating conditions, in the presence of the inducer CCM, CatR binds to the *pheBA* promoter and the *catBCA* promoter as tetramer. One dimer binds to the RBS and the second dimer binds cooperatively to the ABS (Parsek *et al*., 1994, Parsek *et al*., 1995). The CatR binding site ABS encompasses the promoter –35 element. Since the CatR recognition elements are located on the opposite surface of the DNA helix than the –35 element, both CatR and RNA polymerase may simultaneously interact with the same sequences from opposite sides of the DNA helix. Mutations in the ABS of the *pheBA* promoter fall in two groups on the basis of their effects: (1) mutations that affect both the promoter activity and CatR binding, and (2) mutations that affect negatively only the expression of the promoter (Fig. 2 and Table 1). For example, the mutations 36GC and 35GA which encompass the –35 hexameric sequence TTGGAT of the *pheBA* promoter drastically reduced the level of promoter expression both in the presence and the absence of the inducer but did not affect CatR binding. This indicates that these two nucleotides may be involved in interaction of RNA polymerase with the promoter sequence. The –35 hexameric sequence TTGGAT of the *pheBA* promoter deviates from the –35 consensus sequence TTGACA at three positions. Therefore, it was unexpected that changing the nucleotide from G to A at the position –35 relative to the *pheBA* transcriptional start (mutation 35GA) would inactivate the promoter, since such alteration is making the *pheBA* promoter sequence more similar to the σ^{70}-recognized promoter consensus. Comparison of the sequences of the –35 elements of the *pheBA* promoter and the *catBCA* promoter revealed that they are highly conserved (sequences TTGGAT and TTGGAC, respectively). The –35 hexameric sequence of the promoter of the chlorocatechol degradative genes *clcABD* is identical to that of the *catBCA* promoter and it was shown that CatR and ClcR activate transcription via a conserved mechanism (McFall *et al*., 1997). The elimination of expression from the *pheBA* promoter as a result of the 35GA mutation and conservation of G nucleotide instead of consensus nucleotide A in these three promoters indicate that this G nucleotide is important for RNA polymerase interactions with the CatR and ClcR-regulated promoters.

2.1.3. CatR as a negative regulator of the transcription from the *pheBA* promoter (Ref. I)

The CatR protein can also act as a repressor of the transcription from the *pheBA* or *catBCA* promoters. The third binding site, IBS (Internal Binding Sequence) of the CatR protein was identified in the case of the *catBCA* promoter (Chugani *et al*., 1998). The IBS regulates negatively the expression of the *catBCA* promoter. Occupation of the IBS by CatR was facilitated in the presence of the RBS and the ABS on the same DNA fragment and the maintenance of phasing between the promoter and the IBS was important for the IBS-mediated repression (Chugani *et al*., 1998). On the basis of these data it was proposed that CatR bound to the DNA at the *catBCA* promoter, through formation of a DNA loop, could interact with CatR bound to the IBS, and that this interaction could cause impaired transcriptional activation from the *catBCA* promoter (Chugani *et al*., 1998). On the basis of DNase I footprint data a weak CatR-binding site downstream of the transcriptional start site of the *pheBA* operon (+204 to +221) was found as well (Parsek *et al*., 1996). In order to examine the effect of the *pheBA* IBS (Fig.1C in Ref. I) on the expression of the *pheBA* operon, this potential CatR binding site was deleted. The resulting construct, pIBS18del contains an 18-bp deletion of the IBS sequence and is replaced by an 8-bp foreign sequence. The DNA fragment containing the *pheBA* promoter with the IBS sequence (designated as pIBS) and the deletion variant lacking this sequence were cloned upstream to the *lacZ* gene. The expression of the *lacZ* transcriptional fusions in the cells of *P. putida* PaW85 grown in the presence of benzoate revealed approximately 2-fold higher level of β-Galactosidase activity in the case of the IBS deletion construct pIBS18del when compared with the original construct pIBS (Fig. 3, Ref I). This indicates that the IBS of the *pheBA* operon could function as the *cis*-acting repressing element analogous to the IBS of the *catBCA* operon. However, the effect observed in this study was somewhat weaker (2-fold increase in comparison with 3 to 4-fold increase found in the case of the *catBCA* system). The IBS of the *catBCA* promoter closely matches the consensus sequence of the CatR-binding site RBS (Chugani *et al*., 1998). The IBS region of the *pheBA* operon contains the sequence ATACC at positions +207 to +211 which is identical to one half of the interrupted inverted repeat of the RBS sequence (Fig. 1 in Ref. I). The location of an A at position +220 (11 nucleotides from the T of the sequence ATACC) matches the LysR-binding consensus T-N_{11}-A motif. We generated two mutations in the IBS region of the *pheBA* operon: the 208TC mutation which substituted the T residue in the IBS to a C residue, and the 220A-GG mutation which replaced the A residue at position +220 with two G residues (Fig. 1C in Ref. I). The effect of these mutations was tested using the pKTLacZ reporter system (constructs pIBS208TC and pIBS220A-GG, respectively). Results of the β-Galactosidase assay in *P. putida* cells grown in the presence of inducer are

shown in Table 1 (Ref. I). Only a slight increase of the β-Galactosidase activity was observed when the 208TC mutation was compared with the wild type sequence. However, the 220A-GG mutation resulted in a 2-fold increase in the expression of the β-Galactosidase activity in comparison with the wild type. The 2-fold positive effect of the IBS deletion and mutation 220A-GG was observed also in cells that were grown without the inducer.

The presence of IBS region could give the repressing effect to the transcription from the *pheBA* promoter also without the inducer molecule. In this case the CatR protein is bound only to the RBS region and initiation of transcription from the *pheBA* promoter occurs at the basal level. The biological demand for this additional down-regulation mechanism could be that the IBS region-bound CatR protein gives more tight control for regulation of the expression of catabolic genes in conditions where the expression of these proteins in bacteria is not required.

2.1.4. Effect of changing of spacer length between the –35 and –10 hexameric region of the *pheBA* promoter (Ref. I)

The optimal distance between the –35 and –10 hexamers of the RNA polymerase σ^{70}-recognized promoters is usually 17 bp (Stefano and Gralla, 1982). The spacer sequence between the –35 and –10 hexamers of the *pheBA* promoter is unusually long, 19 bp. The 19-bp spacer adds an additional twist angle of at least 34° and the two hexamers may be out of phase with respect to each other. This raised the question whether optimisation of the distance between the –35 and –10 elements of the *pheBA* promoter could compensate for the requirement of CatR for transcriptional activation of this promoter. We made deletions in the spacer sequence of the *pheBA* promoter that reduced the distance between the hexamers from 19 bp to either 18, 17, or 16 bp and cloned the mutated promoters into the plasmid pKTLacZ (constructs pDEL18, pDEL17, and pDEL16, respectively, Fig. 2, Ref. I). Like the wild type promoter, the deletion mutants exhibited only a low basal level of β-Galactosidase activity both in the wild type and the CatR minus background when bacteria were grown without the inducer. When benzoate (a source of inducer CCM) was added to the growth medium, higher level of β-Galactosidase activity was observed in the case of the 18-bp spacer mutant. Reducing the distance between the –35 and –10 elements to 17 bp or 16 bp had a negative effect on transcriptional activation of the *pheBA* promoter. pDEL17 showed a 2-fold lower level of β-Galactosidase activity than the construct carrying the wild type promoter with the 19-bp spacer, and pDEL16 demonstrated only the low basal level of activity. Thus, optimising the distance between the –35 and –10 hexamers of the *pheBA* promoter is not sufficient for CatR-independent transcriptional activation of this promoter.

The promoter sequences of the *pheBA* and *catBCA* operons are TTGGAT-N_{19}-TAGCTT and TTGGAC-N_{19}-AATCCT respectively (Fig. 1B, Ref. I). These promoter sequences differ significantly from the typical σ^{70}-type promoter consensus sequence TTGACA-$N_{16\text{-}18}$-TATAAT. The finding that the transcription from the *pheBA* promoter is not activated in CatR-independent mode in the case when the spacer region between the –35 and –10 hexameric sequences is optimal for the initiation of transcription means that the CatR-mediated activation of transcription from *pheBA* promoter needs the exact orientation between the CatR protein and RNA polymerase for the activation of transcription.

For some promoters about 20 bp-long DNA region is located upstream of –35 hexameric sequence. UP-element gives the contact with C-terminal part of α-subunit of RNA polymerase resulting in increased initiation of transcription (Browning and Busby, 2004; Murakami *et al.*, 1996). The requirement of the carboxy-terminal domain of α subunit (α-CTD) of RNA polymerase was demonstrated for the activation of the *pheBA* and the *catBCA* promoters (Chugani *et al*., 1997). The α-CTD might interact directly not only with CatR but also with the DNA at the putative UP-element (Chugani *et al*., 1997). This hypothesis is supported by the dual effect of point mutation in the "hinge" region of the *pheBA* promoter where in position 52 G was replaced with A (Table 1. Ref. I). While this substitution does not affect the binding-efficiency of CatR to the promoter region of *pheBA* it makes the transcription from the *pheBA* promoter 2.5 times more efficient. This increase in the transcription from the *pheBA* promoter might be caused by the efficient binding of the RNA polymerase to the potential UP-element.

2.2. The global regulation of *pheBA* and *catBCA* operons

2.2.1. Growth phase-dependent activation of the transcription from the *pheBA* and *catBCA* operons (Ref. II)

The transcription of several catabolic genes in *P. putida* is up-regulated when bacterial culture enters to the stationary phase. Examples for such type of regulation are described in the case of σ^{54}-type Pu or Po promoters and also in the case of σ^{70}-type Pm and *alk* operon promoters (Cases *et al.*, 1996; Sze *et al*., 1996; Yuste *et al.*, 1998). The regulation of transcription and physiological control-mechanisms of these promoters were described above, in the review of literature.

We investigated the possibility whether the transcription from the *pheBA* and *catBCA* promoters depends on growth phase of bacteria. For that, the promoters of the *pheBA* and *catBCA* operons were cloned into plasmid pKTlacZ carrying

the *lacZ* reporter gene and β-Galactosidase activity was measured to examine transcription from these promoters at different growth phases. *P. putida* PaW85 cells containing the *lacZ* transcriptional fusion plasmids pZ-pheBA and pZ-catBCA were grown in rich media (LB) to the late stationary phase. Na-benzoate was always added into the growth medium to induce transcription from the *pheBA* and *catBCA* promoters. Results presented in Figure 2, Ref. II show that transcription from the *pheBA* and *catBCA* promoters remained at low level during the first 9 hours of growth of bacteria. The level of expression of β-Galactosidase activity was remarkably elevated in stationary-phase cells and it remained high in stationary-phase cultures during 24 hrs studied. This indicated that the *pheBA* and *catBCA* promoters are stationary-phase-inducible and they are down-regulated during exponential growth of bacteria.

2.2.2. Study on the involvement of stationary-phase sigma factor σ^S on the activation of transcription from the *pheBA* and *catBCA* promoters (Ref. II and III)

Stationary phase-specific sigma factor σ^S encoded by the *rpoS* gene 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 in Hengge-Aronis, 2000). The repression of transcription from the *pheBA* and the *catBCA* promoters in cells growing exponentially in rich medium gave us an idea to investigate the role of σ^S in the activation of the transcription from these promoters.

To investigate the possibility that the up-regulation of transcription from the *pheBA* and *catBCA* promoters in bacterial culture reaches at stationary phase could be due to alternative sigma factor σ^S, we constructed the *P. putida* strain lacking functional *rpoS* gene. *P. putida rpoS*-knockout mutant PKS54 was constructed as a derivative of PaW85 by interrupting *rpoS* gene with kanamycin resistance-encoding gene (*Km*^{r}) (Ref. III).

Plasmids pZ-pheBA and pZ-catBCA containing the *pheBA*- and *catBCA*-promoter-*lacZ* transcriptional fusions, respectively, were introduced into the *rpoS*-deficient strain PKS54 and into the wild-type strain PaW85 (Ref. II). Level of expression of β-Galactosidase activity measured in exponential-phase cells and stationary-phase cells of PKS54 was compared with that estimated in PaW85. The results presented in the Fig. 2, Ref. II show that in the case of the *pheBA* promoter, the β-Galactosidase activity remained approximately 3-fold lower in the σ^S-deficient strain than that in the wild type strain in stationary-phase cultures (Fig 2A, Ref. II). At the same time, lack of the expression of σ^S in *P. putida* did not affect transcription from the *catBCA* promoter (Fig. 2B, Ref. II). The sequence of the –10 region of the *catBCA* promoter (CAATCCT)

shows more similarity to the consensus CTATACT proposed for the promoters recognized by Eσ^S than that of the *pheBA* promoter (CTAGCTT). Based on *in vivo* experiments presented in this work we cannot state that the *pheBA* promoter is recognized by σ^S. However, the nucleotide sequence of the –10 region of the promoter is not the only one component that determines σ^S-dependent transcription. There is increasing evidence that additional regulators play crucial role in establishing sigma factor specificity at stress-inducible promoters (Hengge-Aronis, 1999). 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 C and energy under stressful conditions. However, this effect is insufficient to account for the inhibition of the *pheBA* promoter during exponential growth in rich medium. Moreover, transcription from the *catBCA* promoter was not influenced by the presence of σ^S in *P. putida* cells. It is obvious, therefore, that the reduced level of transcription from the *pheBA* and *catBCA* promoters in exponential phase must be achieved by some other mechanism than σ^S-mediated control.

2.2.3. Exponential silencing of the transcription initiation from the *pheBA* and *catBCA* promoters is mediated by the presence of amino acids in the growth medium of bacteria (Ref. II)

The role of growth medium composition in the regulation of gene expression has been shown in many cases. As already discussed above, the growth media composition (either rich or minimal media) determines the level of transcription from the Po promoter of the operon encoding dimethylphenol degradation enzymes (Sze & Shingler, 1999; Sze, *et al*., 1996), Pu promoter of the TOL plasmid pWW0 (Cases *et al*., 1996; de Lorenzo *et al*., 1993), and P*alk* promoter of the operon of alkane degradation pathway from the *Pseudomonas oleovorans* (Yuste, *et al*., 1998). We found that transcription from of the *pheBA* and *catBCA* promoters was rapidly activated when bacteria were grown in minimal medium (Fig. 4, Ref. II.). Measurement of β-Galactosidase activity in cells growing exponentially in minimal medium allowed us to detect the enzyme activity as early as 20 min after addition of Na-benzoate. When 0.5% Casamino Acids (CAA) solution was added to the minimal medium, the activation of transcription from the *pheBA* and *catBCA* promoters was delayed similarly to that observed in cells grown in LB medium (compare Fig. 2 and Fig. 4 in Ref. II). In the presence of CAA in the minimal medium, the level of transcription from the *pheBA* promoter was very low during the first 4 hrs of cultivation (Fig. 4A, Ref. II). The repression effect of CAA on transcription activation became apparent also for 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 the β-Galactosidase activity was detectable already after 60 minutes since addition of the benzoate (Fig. 4B, Ref. II).

We investigated the possible role of single amino acids separately on the exponential silencing of the transcription from the *pheBA* and *catBCA* promoters. Bacteria were grown in minimal medium containing glucose for 60 min in the presence of Na-benzoate. No amino acids or different number of amino acids (all 20, 15 or 5 amino acids) were added into the growth medium. The sets of amino acids were designed according to their biosynthetic pathways. First 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 rest of 15 amino acids. In the absence of amino acids, the level of β-Galactosidase activity was approximately 950 Miller units (MU) for the *pheBA* promoter and 400 Miller units for the *catBCA* promoter. In the case of the *pheBA* promoter, no β-Galactosidase activity could be detected when all amino acids were added. However, the presence of 5 and 15 amino acids allowed partial expression of this promoter: the β-Galactosidase activities were lowered about ten times (90 Miller units) and four times (200 Miller units), respectively. The repressive effect of amino acids on transcription from the *catBCA* promoter was lower (12 Miller units), than that observed for the *pheBA* promoter. Also the repressive effect of groups of amino acids was only about two times if compared it with β-galactosidase activity measured from bacterial cells grown in the presence of all amino acids. This indicates that the physiological control on the *pheBA* and *catBCA* promoters mediated by the presence of amino acids might be different from 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 exclude the possibility that one particular amino acid could mediate this effect.

2.2.4. The effect of the amount of the CatR protein on the exponential silencing of the transcription from the *pheBA* promoter (Ref. II and Ref. IV)

The effect of the amount of regulatory protein on the exponential silencing of transcription from catabolic operons in *P. putida* cells has been investigated in several cases. Artificial overexpression of regulatory protein relieves the exponential silencing in the case of Pu (Perez-Martin and de Lorenzo, 1996) and Po (Sze *et al.* 1996) promoters in *P. putida*. The same was described for the regulation of transcription of the alkane degradation operon when the overexpression of the regulatory protein AlkS relieved the exponential silencing (Yuste *et al.* 1998).

To investigate the role of the amount of the regulatory protein CatR on the exponential silencing of *pheBA* promoter we examined the possibility whether the transcription from the promoter of the *catR* gene could be down-regulated during the exponential growth of bacteria in rich medium and thereby cause the exponential silencing of the transcription from the *catBCA* and *pheBA* promoters. To study the expression of the *catR* gene during the growth cycle of bacteria, the promoter of the *catR* gene was cloned into the plasmid pKTlacZ to generate *catR-lacZ* transcriptional fusion. The level of the β-Galactosidase expression increased five times in stationary phase cells if compared to that observed in exponentially growing cells (Fig. 3, Ref. II). Therefore, although the *catR* promoter activity remained at very low level during the growth phases of bacteria, the intracellular amount of CatR may be somewhat increased in stationary phase cells. This result gave us an idea to investigate the regulation of transcription from the *pheBA* promoter under conditions when the level of CatR in bacterial cells is artificially increased.

To examine the possibility that over-expression of transcriptional regulator protein CatR could relieve the exponential silencing of the transcription from the *pheBA* promoter, the *catR* gene, placed under the control of the IPTG-inducible P*tac* promoter was introduced into the chromosome of the *P. putida* strain PaW85 (Ref. II). Since the *lacZ* reporter did not allow us to detect transcription from the *pheBA* promoter in cells growing exponentially in the presence of amino acids irrespectively of CatR was overexpressed or not (Ref. II). Therefore, the alternative test-system based on usage of more sensitive *luxAB* marker genes has been constructed (Ref. IV). The *pheBA* promoter-*luxAB* reporter system was introduced into the chromosome of *P. putida* and the transcription from the *pheBA* promoter was detected even in the case when bacteria were grown exponentially in rich medium.

The *P. putida* strain PaWlux-pheBA-catR carrying in the chromosome the *pheBA* operon, the *pheBA* promoter cloned upstream from the *luxAB* reporter and the *catR* gene placed under the control of P*tac* promoter, was grown in the presence or absence of 0.5 mM IPTG in phenol minimal medium supplemented or not with CAA, and the luciferase activities were measured in cells sampled at different time points. As shown in Table 2, Ref IV., the overexpression of CatR protein had positive effect on transcriptional activation from the *pheBA* promoter. In the presence of CAA in phenol-containing growth medium the cells with elevated CatR expression exhibited about 30 to 40-fold higher level of transcription from the *pheBA* promoter than the wild type cells. This effect was also significant in this case when bacteria were cultivated on phenol containing minimal medium lacking amino acids: the elevated amount of CatR resulted in about 25-fold higher level of transcription from the *pheBA* promoter in cells sampled one hour after addition of phenol. Moreover, the *pheBA* promoter expressed approximately two-fold higher level of transcription also in this case if activities in cells sampled on hour three after addition of phenol were measured. CatR is unable to activate transcription without binding the

effector molecule CCM (Rothmel et al., 1991). It is possible, therefore, that the cellular amount of CCM might be limiting factor of transcriptional activation from the *pheBA* promoter, but as a number of CatR molecules per cell increases, small amount of CCM molecules would be more easily captured by CatR. As a next step of our studies, we decided to test whether this hypothesis is correct, i.e., whether the level of transcription from the *pheBA* promoter would be limited by the amount of CCM in a cell.

2.2.5. The accumulation of inducer molecule *cis,cis*-muconate in *P. putida* cells relieves the exponential silencing of the *pheBA* promoter activation (Ref. IV)

To control the hypothesis that the amount of CCM could limit regulation of transcription from the *pheBA* promoter we constructed the *catB*-deficient strain. When the *catB* gene is inactivated in the chromosome of *P. putida*, the phenol and benzoate degradation pathway ends with the formation of CCM. The wild type strain PaWlux-pheBA and its *catB*::*tet* derivative PaWlux-pheBA-CatB::tet were grown either in the presence or absence of amino acids and bioluminescence was measured during the first three hours of growth of bacteria after the addition of phenol into the growth medium. As shown in Fig. 2C, Ref. IV, the transcription from the *pheBA* promoter was rapidly induced in *catB*-minus background both in the presence and absence of amino acids. During the first hour of cultivation of bacteria in phenol minimal medium lacking amino acids, the level of transcription increased more than 400-fold in the *catB*-defective strain compared to that in the wild type (Fig. 2B and C, Ref. IV). The difference was 3-fold after three hour growth. The effect of CCM accumulation in cells on transcription from the *pheBA* promoter was even more remarkable in the presence of CAA in the growth medium. In this case the *pheBA* promoter was also rapidly activated after the addition of phenol into the growth medium – up to three orders of magnitude higher level of transcription was observed in the *catB*-deficient mutant if compared to that measured in the wild type strain. These data led to the conclusion that the level of transcription from the *pheBA* promoter in *P. putida* grown under different nutritional conditions is controlled by cellular amount of the inducer molecule CCM.

2.2.6. The level of catechol-1,2 dioxygenase (C12O) is reduced in the presence of amino acids in the growth medium of bacteria (Ref. IV)

A lower amount of CCM in *P. putida* cells resulting in reduced level of the transcription from the *pheBA* promoter in bacteria growing in minimal medium supplemented with amino acids may be a consequence of decreased activities of enzymes involved in degradation phenol and benzoate to CCM. C12O is a common enzyme for the both benzoate and phenol degradation pathways, and the physiological control of transcription of the *pheBA* promoter became evident in the case of both substrates (see results presented in Fig. 2A and B, Ref. IV). Therefore, we decided to study whether the cellular amount and/or activity of C12O encoded by the *pheB* gene would be affected by growth conditions. For that we fused the *pheB* gene with the heterologous promoter P*tac* controlled artificially by the amount of IPTG added to the growth medium of bacteria. As a control, the *lacZ* transcriptional fusion was used. *P. putida* strains carrying either the P*tac-pheB* or P*tac-lacZ* expression cassettes were grown in glucose-containing M9 medium in the presence or absence of amino acids. Cells were sampled one hour after the addition of 1 mM IPTG and were examined for the expression of enzymes. The results presented in Fig. 3A, Ref. IV, demonstrate that the level of expression of the *pheB*-encoded C12O in the strain PaWPheB carrying the P*tac-pheB* expression cassette was strongly affected by the composition of bacterial growth medium. When grown exponentially in the medium supplemented with amino acids, bacteria showed about 10-fold lower level of C12O activity if compared to that measured in cells grown in minimal medium without amino acids. Only later, if the cells were sampled from overnight-grown stationary phase cultures, the levels of C12O were equal in the presence and absence of amino acids grown bacterial cells. Parallel experiments monitoring the β-Galactosidase activities in bacteria expressing the *lacZ* gene from the P*tac* promoter eliminated the possibility confirmed that the P*tac* promoter itself could be repressed in *P. putida* grown in amino acids-containing medium (Fig. 3B, Ref. IV). These data indicated that the presence of amino acids in the growth medium decreased the level of expression of the *pheB* gene by a mechanism acting after transcription initiation.

2.2.7. The growth media composition of bacteria affects the amount of synthesis of the *pheB* gene-encoded C12O (Ref. IV)

There might be a number of reasons for the reduced C12O activity in cells growing in amino acids-containing environment. If thinking about possible posttranslational control mechanisms, the enzymatic activity of C12O might be somehow inhibited in bacteria grown in nutritionally rich medium. Also the amount of C12O protein may be down-regulated under growth of bacteria in the presence of amino acids. To monitor the cellular amount of C12O in bacteria grown under different nutritional conditions, we performed Western blot analysis. Cell lysates sampled from cultures of PaWPheB grown in the presence or absence of amino acids were tested using *P. putida* anti-PheB polyclonal antibodies. Results of Western blot analysis presented in Fig. 4C, Ref. IV clearly demonstrated that the amount of C12O is drastically reduced in cells grown in amino acids-containing medium. These data excluded the possibility that presence of amino acids in the growth medium might inhibit the enzymatic activity of C12O. Rather, while the amount of the PheB protein in cells grown in rich medium was reduced proportionally to the level of C12O activity, the expression of C12O can most likely be controlled either by proteolysis of C12O or by the amount of its synthesis. The latter, in turn, may be affected either by the reduction of the half-life of the *pheB*-specific mRNA or by decreased efficiency of translation of this mRNA in bacteria grown in the presence of amino acids.

If the *pheB*-encoded C12O is degraded in nutritionally rich medium-grown cells, the cellular amount of C12O would rapidly decline after the transfer of bacteria from poor growth medium into amino acids-containing medium. To examine this possibility exponentially growing IPTG-induced cells of PaWPheB carrying the *pheB* gene under the control of P*tac* promoter were re-inoculated into fresh glucose containing minimal medium with or without supplementation by amino acids. Cells sampled one hour after the transfer expressed slightly increased C12O activity when grown in the absence of amino acids. Only slight decrease in C12O activity corresponding to the dilution of the synthesized protein due to cell division could be detected in bacteria grown in the presence of amino acids (Fig. 4, Ref. IV). The latter argued against the possibility that the decreased amount of C12O in bacteria grown in rich medium might be controlled at the level of proteolysis of C12O. Rather, the synthesis of the *pheB* gene-encoded C12O may be reduced in rich medium-grown bacteria.

2.2.8. Possible mechanisms of the physiological control of transcription from the *pheBA* promoter

The basic mechanism of the initiation of transcription from the promoters of the *pheBA* and *catBCA* operons is similar (Ref. I). Transcription from the *pheBA* and the *catBCA* promoters is activated by the LysR-type transcriptional regulator protein CatR in the presence of CCM, the intermediate of phenol and benzoate degradation (Kasak *et al.* 1993, Rothmel *et al.* 1990). The enzymes encoded by these operons are required in bacterial cells only when the aromatic substrate e.g. phenol or benzoate is present in the growth medium of *P. putida*. When growth medium of bacteria contains amino acids, the consumption of aromatic compounds is down-regulated even in the presence of effector molecule of the catabolic operon. This phenomenon is called as a physiological control or exponential silencing of catabolic operons. It is quite common that the transcription of certain catabolic operon in *Pseudomonas* is influenced by the physiological state of bacteria. The exponential silencing has been described in the case of Pu promoter (Cases and de Lorenzo, 2000), Po promoter (Sze and Shingler, 2001) and P*alk* promoter (Yuste and Rojo, 2001). The Pu and Po promoters belong to the σ^{54} regulon and the transcription from these promoters is controlled by the XylR/NtrC-family regulators. The exponential silencing is described not only for σ^{54}-dependent promoters. The transcription from the σ^{70}-dependent alkane degradation *alk* operon promoter which is regulated by the XylS/AraC-family regulator is also under physiological control (Yuste *et al.*, 1998).

In this thesis I have described for the first time that similarly to the Pu, Po and Palk promoters the transcription from the LysR family regulator-controlled promoters is also down-regulated in the presence of amino-acids in exponentially growing bacteria. Thus, the phenomenon of exponential silencing in *Pseudomonas* is even more general.

As the specific regulation of the transcription from the *pheBA* and *catBCA* promoters is similar, I have chosen here the *pheBA* promoter as an example to discuss the possible mechanism of physiological control of transcription from the CatR-regulated promoters.

The degradation of phenol in *P. putida* cells may be controlled at different levels:

(1) the uptake of phenol may be affected by growth medium composition of bacteria,
(2) the initiation of transcription from the promoter of the *pheBA* operon may be controlled by the amount of the regulator protein CatR
(3) the amount and activity of the enzyme degrading phenol to the *pheBA* operon inducer molecule CCM may be affected by growth medium.

Below, I will evaluate separately these three possibilities.

(1) Control via uptake of phenol. There is only little to know about the membrane transport of aromatic and especially phenolic compounds. There are some examples that aromatic compounds can enter into the bacterial cells by an active transport (Thayer and Wheelis, 1982; Cowles *et al.* 2000) but transport mechanisms characterized so far in *Pseudomonas* appeared to be inefficient (Bugg *et al.*, 2000). Aromatic compounds are supposed to enter into bacterial cell mostly by passive diffusion. Whether and how the growth phase of bacteria or growth medium composition could regulate the uptake of aromatic compounds in bacteria is still unknown. Data described in Ref. IV do not support the possibility that the uptake of phenol from the environment could influence the transcriptional regulation of the *pheBA* operon. Results presented in Ref. IV demonstrate that the amount of the inducer molecule CCM relieves the exponential silencing of the transcription from the *pheBA* promoter. In the case of the *catB*-deficient mutant, CCM accumulated into the bacterial cells and consequently the transcription from the *pheBA* promoter was rapidly induced both in the presence or absence of amino acids. If uptake of phenol would be influenced by the presence of amino acids in the growth medium of bacteria the transcription from the *pheBA* promoter in the *catB* deficient cells would be repressed as well as it occurred in the case of wild-type cells. Thus, there is no reason to believe that the growth phase of bacteria or the presence of amino acids in growth medium might influence the uptake of phenol.

(2) Initiation of transcription from the *pheBA* promoter is controlled by the amount of CatR protein. When the level of CatR was artificially increased in *P. putida* cells the transcription from the *pheBA* promoter was activated more rapidly than in this case when the cellular amount of CatR was not changed (Table 2. Ref. IV). Although the transcription from the *pheBA* promoter occurred at higher level under conditions of increased cellular amount of CatR, the overexpression of CatR did not completely relieve the repression of transcription from the *pheBA* promoter in the presence of amino acids in the growth medium of bacteria (Ref. IV). This indicates that the physiological control mechanisms, which occur in the case of the *pheBA* promoter are different from those discovered in the case of the Pu or Po promoters where the increase in amount of regulator protein almost entirely relieved exponential silencing (Perez-Martin and de Lorenzo, 1996; Sze *et al.* 1996). The faster activation of the transcription from *pheBA* promoter under the conditions of higher level of CatR protein in bacterial cells hints that the cellular amount of the inducer-molecule CCM might be limiting on the regulation of transcription from the *pheBA* promoter. Thus, the increased amount of the CatR protein might allow more efficiently capture limiting molecules of CCM and thereby activate the transcription from the *pheBA* operon at a higher level.

(3) Physiological control mechanisms acting at posttranscriptional/posttranslational level. If the cellular level of CCM is critical on the activation of transcription from the *pheBA* promoter, the question remains how the presence of amino acids could influence the amount of CCM in bacterial cells. Most obviously the amino acids may somehow affect either the cellular amount or activity of catechol 1,2-dioxygenase. Our results indicate that the presence of amino acids in growth medium of bacteria does not directly affect initiation of transcription from the *pheBA* promoter. Rather, the amount of C12O, necessary for the production of the inducer molecule, is negatively affected by the presence of amino acids at later steps influencing synthesis of this enzyme. The lower cellular amount of C12O results in reduced amount of the inducer which in turn causes reduced level of transcription initiation from the *pheBA* promoter.

CONCLUSIONS

The results discussed in this thesis can be summarized as follows:

1. The site-directed mutagenesis of the *pheBA* promoter region was performed to find the nucleotides necessary for the binding of CatR to the promoter region of the *pheBA* operon and critical on activation of transcription from this promoter. The main points on specific regulation of the transcription from the *pheBA* promoter are:
   1.1. We found that the effective binding of CatR protein to the promoter region and activation of the transcription from the *pheBA* promoter requires not only nucleotides of inverted repeat but also nucleotides outside this region.
   1.2. Mutations in activation binding site (ABS) reduced the transcription from the *pheBA* promoter. These substitutions in ABS which overlap with the –35 hexameric sequence reduced the transcription from the *pheBA* promoter but not the binding of CatR protein to this region. Point-mutations which are located outside of the –35 hexameric sequence reduced significantly the binding of CatR protein and thereby also the activation of transcription from the *pheBA* promoter.
2. The third CatR binding sequence (IBS) locates downstream from the transcriptional start point of the *pheB* gene. Interaction of the CatR protein to the IBS region slightly represses the transcription from the *pheBA* promoter.
3. The transcription from both the *pheBA* and *catBCA* promoters is under the control of physiological state of bacteria. The main points on the physiological control found by us are:
   3.1. The stationary-phase sigma factor σ^S has slight positive effect in the transcription from the *pheBA* promoter but not from the *catBCA* promoter.
   3.2. The transcription from the *pheBA* and *catBCA* promoters is silenced in exponentially growing bacteria in the presence of amino acids in the growth medium but in minimal medium-grown bacteria the transcription from the *pheBA* promoter is activated immediately.
   3.3. The reduced transcription from the *pheBA* promoter in bacteria grown in rich medium is caused by low cellular amount of inducer molecule CCM.
   3.4. The low cellular amount of CCM in *P. putida* is caused by the decreased amount of the *pheB*-encoded catechol 1,2-dioxygenase (the enzyme which converts catechol to CCM) in bacterial cells grown exponentially in the presence of amino acids.
   3.5. The decrease in the amount of C12O in rich medium exponentially growing cells might be caused by some so far unknown mechanism acting most likely at post-transcriptional level.

The results presented in my thesis show the conservation between the specific regulatory mechanisms of two catabolic operons, chromosomal *catBCA* and plasmid-born *pheBA* operons. Besides the similar specific regulatory mechanisms provided by the CatR protein both promoters are similarly repressed in the presence of amino acids in growth medium of bacteria. In this dissertation I have discussed the one possible mechanism of the phenomenon of exponential silencing of the transcription from the *pheBA* promoter. This is a first time to show that the physiological regulation of transcription of the catabolic operons in *Pseudomonas* might be achieved via down-regulation of the enzyme responsible for the synthesis of the inducer-molecule of the operon.

REFERENCES

Abril M.A., Michan C., Timmis K.N. and Ramos J.L. 1989. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol. 171: 6782–90.

Aldrich T.L., Chakrabarty A.M. 1988. Transcriptional regulation, nucleotide sequence, and localization of the promoter of the *catBC* operon in *Pseudomonas putida*. J. Bacteriol. 170: 1297–304.

Bertoni G., Fujita N., Ishihama A. and de Lorenzo V. 1998. Active recruitment of sigma54-RNA polymerase to the Pu promoter of *Pseudomonas putida*: role of IHF and alpha CTD. EMBO J. 17: 5120–8.

Browning, D.F. and Busby, S.J. 2004. The regulation of bacterial transcription initiation. Nat Rev Microbiol. 1 :57–65.

Bugg T, Foght JM, Pickard MA, Gray MR. 2000. Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Appl Environ Microbiol. 66: 5387–92.

Canosa I., Yuste L. and Rojo F. 1999. Role of the alternative sigma factor sigma S in expression of the AlkS regulator of the *Pseudomonas oleovorans* alkane degradation pathway. J. Bacteriol. 181: 1748–54.

Carmona M, Claverie-Martin F, Magasanik B. 1997. DNA bending and the initiation of transcription at sigma 54-dependent bacterial promoters. Proc Natl Acad Sci U S A. 94: 9568–72.

Carmona M, de Lorenzo V. 1999. Involvement of the FtsH (HflB) protease in the activity of sigma 54 promoters. Mol Microbiol. 31: 261–70.

Carmona M., Rodriguez M.J., Martinez-Costa O. and De Lorenzo V. 2000. *In vivo* and *in vitro* effects of (p)ppGpp on the sigma(54) promoter Pu of the TOL plasmid of *Pseudomonas putida*. J. Bacteriol. 182: 4711–8.

Cases I., Lopez J.A., Albar J.P. and de Lorenzo V. 2001. Evidence of multiple regulatory functions for the PtsN (IIA (Ntr)) protein of *Pseudomonas putida*. J. Bacteriol. 183: 1032–7.

Cases I. and de Lorenzo V. 2000. Genetic evidence of distinct physiological regulation mechanisms in the sigma (54) Pu promoter of *Pseudomonas putida*. J. Bacteriol. 182: 956–60.

Cases I, de Lorenzo V. 2001. The black cat/white cat principle of signal integration in bacterial promoters. EMBO J. 20 (1–2): 1–11.

Cases I., de Lorenzo V. and Perez-Martin J. 1996. Involvement of sigma 54 in exponential silencing of the *Pseudomonas putida* TOL plasmid Pu promoter. Mol Microbiol. 19: 7–17.

Cases I., Perez-Martin J. and de Lorenzo V. 1999. The IIANtr (PtsN) protein of Pseudomonas putida mediates the C source inhibition of the sigma 54-dependent Pu promoter of the TOL plasmid. J Biol Chem. 274: 15562–8.

Cases I. and de Lorenzo V. 2005. Promoters in the environment: transcriptional regulation in its natural context. Nat Rev Microbiol. 3: 105–18.

Chatterji D. and Ojha A.K. 2001. Revisiting the stringent response, ppGpp and starvation signaling. Curr Opin Microbiol. 4: 160–5.

Chugani S.A., Parsek M.R. and Chakrabarty A.M. 1998. Transcriptional repression mediated by LysR-type regulator CatR bound at multiple binding sites. J. Bacteriol. 180: 2367–72.

Chugani S.A., Parsek M.R., Hershberger C.D., Murakami K., Ishihama A. and Chakrabarty A.M. 1997. Activation of the *catBCA* promoter: probing the interaction of CatR and RNA polymerase through *in vitro* transcription. J. Bacteriol. 179: 2221–7.

Coco W.M., Rothmel R.K., Henikoff S. and Chakrabarty A.M. 1993. Nucleotide sequence and initial functional characterization of the *clcR* gene encoding a LysR family activator of the clcABD chlorocatechol operon in *Pseudomonas putida*. J. Bacteriol.175: 417–27.

Collier DN, Hager PW, Phibbs PV Jr. 1996. Catabolite repression control in the Pseudomonads. Res Microbiol 147: 551–61.

Cowles C.E., Nichols N.N. and Harwood C.S. 2000. BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida* .J. Bacteriol. 182: 6339–46.

de Lorenzo V., Herrero M., Metzke M. and Timmis K.N. 1991. An upstream XylR- and IHF-induced nucleoprotein complex regulates the sigma 54-dependent Pu promoter of TOL plasmid. EMBO J. 10: 1159–67.

de Lorenzo V., Cases I., Herrero M. and 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.

Dinamarca MA, Ruiz-Manzano A, Rojo F. 2002. Inactivation of cytochrome o ubiquinol oxidase relieves catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. J. Bacteriol. 184: 3785–93.

Dinamarca, M. A., I. Aranda-Olmedo, A. Puyet, and F. Rojo. 2003. Expression of the *Pseudomonas putida* OCT plasmid alkane degradation pathway is modulated by two different global control signals: evidence from continuous cultures. J. Bacteriol. **185:**4772–4778.

Dombroski A.J., Walter W.A. and Gross C.A. 1993. The role of the sigma subunit in promoter recognition by RNA polymerase. Cell Mol Biol Res.;39: 311–7.

Fernandez S., de Lorenzo V. and Perez-Martin J. 1995. Activation of the transcriptional regulator XylR of *Pseudomonas putida* by release of repression between functional domains. Mol Microbiol 16: 205–13.

Garmendia J., Devos D., Valencia A. and de Lorenzo V. 2001. A la carte transcriptional regulators: unlocking responses of the prokaryotic enhancer-binding protein XylR to non-natural effectors. Mol Microbiol. 42: 47–59.

Gonzalez-Perez M.M., Ramos J.L., Gallegos M.T. and Marques S. 1999. Critical nucleotides in the upstream region of the XylS-dependent TOL meta-cleavage pathway operon promoter as deduced from analysis of mutants. J Biol Chem. 274: 2286–90.

Gussin GN, Olson C, Igarashi K, Ishihama A. 1992. Activation defects caused by mutations in *Escherichia coli rpoA* are promoter specific.J. Bacteriol. 174: 5156–60.

Harley C.B. and Reynolds R.P. 1987. Analysis of *E. coli* promoter sequences. Nucleic Acids Res. 11: 2343–61.

Hengge-Aronis R. 1999. Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. Curr Opin Microbiol. 2: 148–52.

Hengge-Aronis R. 2000. A role for the sigma S subunit of RNA polymerase in the regulation of bacterial virulence. Adv Exp Med Biol. 485: 85–93.

Herman C., Thevenet D., D'Ari R. and Bouloc P. 1995. Degradation of sigma 32, the heat shock regulator in *Escherichia coli*, is governed by HflB. Proc Natl Acad Sci U S A. 92: 3516–20.

Horak R. and Kivisaar M. 1998. Expression of the transposase gene *tnpA* of Tn4652 is positively affected by integration host factor. J. Bacteriol. 180: 2822–9.

Houghton J.E, Brown T.M, Appel A.J, Hughes E.J and Ornston L.N. 1995. Discontinuities in the evolution of Pseudomonas putida *cat* genes.J. Bacteriol.177: 401–12.

Inouye S., Nakazawa A. and Nakazawa T. 1981. Molecular cloning of gene *xylS* of the TOL plasmid: evidence for positive regulation of the *xylDEGF* operon by *xylS*.J. Bacteriol. 148: 413–8.

Inouye S., Yamada M., Nakazawa A. and Nakazawa T. 1989. Cloning and sequence analysis of the *ntrA* (rpoN) gene of *Pseudomonas putida*. Gene. 85(1):145–52.

Inouye S., Nakazawa A. and Nakazawa T. 1987. Expression of the regulatory gene *xylS* on the TOL plasmid is positively controlled by the *xylR* gene product. Proc Natl Acad Sci U S A. 84: 5182–6.

Jeffrey W.H., Cuskey S.M., Chapman P.J., Resnick S. and Olsen R.H. 1992. Characterization of *Pseudomonas putida* mutants unable to catabolize benzoate: cloning and characterization of *Pseudomonas* genes involved in benzoate catabolism and isolation of a chromosomal DNA fragment able to substitute for *xylS* in activation of the TOL lower-pathway promoter. J. Bacteriol. 174: 4986–96.

Kahnert A., Mirleau P., Wait R. and Kertesz M.A. 2002 The LysR-type regulator SftR is involved in soil survival and sulphate ester metabolism in *Pseudomonas putida*. Environ Microbiol. 4: 225–37.

Kaldalu N., Mandel T. and Ustav M. 1996. TOL plasmid transcription factor XylS binds specifically to the Pm operator sequence Mol Microbiol. 20: 569–79.

Kasak L., Horak R., Nurk A., Talvik K. and Kivisaar M. 1993. Regulation of the catechol 1,2-dioxygenase- and phenol monooxygenase-encoding *pheBA* operon in *Pseudomonas putida* PaW85.J. Bacteriol. 175: 8038–42.

Kieboom J., Bruinenberg R., Keizer-Gunnink I. and de Bont J.A. 2001. Transposon mutations in the flagella biosynthetic pathway of the solvent-tolerant *Pseudomonas putida* S12 result in a decreased expression of solvent efflux genes. FEMS Microbiol Lett. 198: 117–22.

Kivisaar M., Horak R., Kasak L., Heinaru A. and 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.

Laurie A.D., Bernardo L.M., Sze C.C., Skarfstad E., Szalewska-Palasz A., Nystrom T. and Shingler V. 2003. The role of the alarmone (p)ppGpp in sigma N competition for core RNA polymerase. J Biol Chem. 278: 1494–503.

Loewen PC, Hengge-Aronis R. 1994. The role of the sigma factor sigma S (KatF) in bacterial global regulation. Annu Rev Microbiol. 48: 53–80.

Manzanera M., Aranda-Olmedo I., Ramos J.L. and Marques S. 2001. Molecular characterization of *Pseudomonas putida* KT2440 rpoH gene regulation. Microbiology. 147(Pt 5): 1323–30.

Marques, S., A. Holtel, K. N. Timmis, and J. Ramos. 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., Gallegos M. T. and Ramos J. L. (1995) Role of sigma S in transcription from the positively controlled Pm promoter of the TOL plasmid of Pseudomonas putida. Mol Microbiol. 18: 851–7.

Marques S., Manzanera M., Gonzalez-Perez M.M., Gallegos M.T. and Ramos J.L. 1999. The XylS-dependent Pm promoter is transcribed *in vivo* by RNA polymerase with sigma 32 or sigma 38 depending on the growth phase. Mol Microbiol. 31(4): 1105–13.

Martinez-Bueno M.A., Tobes R., Rey M. and Ramos J.L. 2002. Detection of multiple extracytoplasmic function (ECF) sigma factors in the genome of *Pseudomonas putida* KT2440 and their counterparts in *Pseudomonas aeruginosa* PA01. Environ Microbiol. 4(12): 842–55.

McFall S.M., Klem T.J., Fujita N., Ishihama A. and Chakrabarty A.M. 1997. DNase I footprinting, DNA bending and *in vitro* transcription analyses of ClcR and CatR interactions with the *clcABD* promoter: evidence of a conserved transcriptional activation mechanism. Mol Microbiol. 24: 965–76.

Merrick M.J. 1993. In a class of its own – the RNA polymerase sigma factor sigma 54 (sigma N). Mol Microbiol. 10: 903–9.

Morales G., Wiehlmann L., Gudowius P., van Delden C., Tummler B., Martinez J.L. and Rojo F. 2004. Structure of *Pseudomonas aeruginosa* populations analyzed by single nucleotide polymorphism and pulsed-field gel electrophoresis genotyping. J. Bacteriol. 186: 4228–37.

Morris L., Cannon W., Claverie-Martin F., Austin S. and Buck M. 1994. DNA distortion and nucleation of local DNA unwinding within sigma-54 (sigma N) holoenzyme closed promoter complexes. J Biol Chem. Apr 15;269(15): 11563–71.

Muller C., Petruschka L., Cuypers H., Burchhardt G. and Herrmann H. 1996. Carbon catabolite repression of phenol degradation in Pseudomonas putida is mediated by the inhibition of the activator protein PhlR. J. Bacteriol. 178: 2030–6.

Mulvey M.R. and Loewen P.C. 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel sigma transcription factor. Nucleic Acids Res. 11: 9979–91.

Murakami K., Fujita N., and Ishihama A. 1996. Transcription factor recognition surface on the RNA polymerase alpha subunit is involved in contact with the DNA enhancer element. EMBO J. 15: 4358–67.

Nakahigashi K., Yanagi H. and Yura T. 1995. Isolation and sequence analysis of *rpoH* genes encoding sigma 32 homologs from gram negative bacteria: conserved mRNA and protein segments for heat shock regulation. Nucleic Acids Res. 11: 4383–90.

Ng L.C., Poh C.L. and Shingler V. 1995. Aromatic effector activation of the NtrC-like transcriptional regulator PhhR limits the catabolic potential of the (methyl) phenol degradative pathway it controls. J. Bacteriol.177: 1485–90.

Parsek M.R., Shinabarger D.L., Rothmel R.K. and Chakrabarty A.M. 1992. Roles of CatR and *cis,cis*-muconate in activation of the *catBC* operon, which is involved in benzoate degradation in *Pseudomonas putida*. J. Bacteriol. 174: 7798–806.

Parsek M.R., Ye R.W., Pun P. and Chakrabarty A.M. 1994. Critical nucleotides in the interaction of a LysR-type regulator with its target promoter region. *catBC* promoter activation by CatR. J Biol Chem. 269: 11279–84.

Parsek M.R., Kivisaar M. and 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: 819–28.

Parsek M.R., McFall S.M. and Chakrabarty A.M. 1996. Evolution of Regulatory Systems of Biodegradative Pathways. *In*: Nakazawa T., Furukawa K., Haas D. and Silver S. (Eds.), Molecular Biology of Pseudomonads. ASM Press, Washington, D.C. pp. 135–152.

Parsek M.R., Ye R.W., Pun P. and Chakrabarty A.M. 1994. Critical nucleotides in the interaction of a LysR-type regulator with its target promoter region. *catBC* promoter activation by CatR. J Biol Chem. 269:11279–84.

Perez-Martin J. and de Lorenzo V. 1995. The amino-terminal domain of the prokaryotic enhancer-binding protein XylR is a specific intramolecular repressor. Proc Natl Acad Sci U S A. 92: 9392–6.

Perez-Martin J. and de Lorenzo V. 1996. ATP binding to the sigma 54-dependent activator XylR triggers a protein multimerization cycle catalyzed by UAS DNA.Cell. 86 331–9.

Porter S.C., North A.K., Wedel A.B. and Kustu S. 1993. Oligomerization of NTRC at the glnA enhancer is required for transcriptional activation.Genes Dev. 7: 2258–73.

Ramos J.L., Marques S. and 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. 51:341–73.

Ramos-Gonzalez M.I. and Molin, S. 1998. Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440.J. Bacteriol. 180(13):3421–31.

Rescalli E., Saini S., Bartocci C., Rychlewski L., De Lorenzo V. and Bertoni G. 2004. Novel physiological modulation of the Pu promoter of TOL plasmid: negative regulatory role of the TurA protein of *Pseudomonas putida* in the response to suboptimal growth temperatures. J Biol Chem. 279: 7777–84.

Rojo F. and Dinamarca A.M. 2004. Catabolite Repression and Physiological Control. *In*: Ramos J.L. (Eds.), *Pseudomonas*, Vol 2, Kluwer Academic/Plenum Publishers, New York 365–387.

Rothmel R.K., Aldrich T.L., Houghton J.E., Coco W.M., Ornston L.N. and Chakrabarty A.M. 1990. Nucleotide sequencing and characterization of *Pseudomonas putida* catR: a positive regulator of the *catBC* operon is a member of the LysR family. J. Bacteriol. 172: 922–31.

Rothmel R.K., Aldrich T.L., Houghton J.E., Coco W.M., Ornston L.N. and Chakrabarty A.M. 1990. Nucleotide sequencing and characterization of Pseudomonas putida catR: a positive regulator of the *catBC* operon is a member of the LysR family. J. Bacteriol.172: 922–31.

Rothmel R.K., Shinabarger D.L., Parsek M.R., Aldrich T.L. and 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: 4717–24.

Ruiz R., Ramos J.L. and Egan S.M. 2001. Interactions of the XylS regulators with the C-terminal domain of the RNA polymerase alpha subunit influence the expression level from the cognate Pm promoter. FEBS Lett. 491: 207–11.

Santos, P. M., J. M. Blatny, I. Di Bartolo, S. Valla, and E. Zennaro. 2000. Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens* ST. Appl. Environ. Microbiol. **66:**1305–1310.

Sato H., Kudo S., Ohnishi K., Mizuguchi M., Goto E. and Suzuki K. 2001. Nucleotide sequence analysis of 5'-flanking region of salicylate hydroxylase gene, and identification and purification of a LysR-type regulator, SalR. Eur J Biochem. 268: 2229–38.

Schell M.A. 1993. Molecular biology of the LysR family of transcriptional regulators. Annu Rev Microbiol. 47:597–626.

Shingler V., Bartilson M. and Moore T. 1993. Cloning and nucleotide sequence of the gene encoding the positive regulator (DmpR) of the phenol catabolic pathway encoded by pVI150 and identification of DmpR as a member of the NtrC family of transcriptional activators. J. Bacteriol.175: 1596–604.

Stefano J.E. and Gralla J.D. 1982. Spacer mutations in the *lac* ps promoter. Proc Natl Acad Sci U S A. 79: 1069–72.

Storz G, Tartaglia LA, Ames BN. 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science. 13: 189–94.

Sze C.C., Moore T. and Shingler V. 1996. Growth phase-dependent transcription of the sigma(54)-dependent Po promoter controlling the Pseudomonas-derived (methyl) phenol dmp operon of pVI150. J. Bacteriol. 178: 3727–35.

Sze CC, Bernardo LM, Shingler V. 2002. Integration of global regulation of two aromatic-responsive sigma (54)-dependent systems: a common phenotype by different mechanisms. J. Bacteriol. 184: 760–70.

Sze CC, Shingler V. 1999. The alarmone (p)ppGpp mediates physiological-responsive control at the sigma 54-dependent Po promoter. Mol Microbiol. 31: 1217–28.

Thayer J.R., and Wheelis M.L. 1982. Active transport of benzoate in *Pseudomonas putida*. J Gen Microbiol. 128 (Pt 8): 1749–53.

Thony B. and Hennecke H. 1989. The -24/-12 promoter comes of age. FEMS Microbiol Rev. 5: 341–57.

Tropel D. and van der Meer J.R. 2004. Bacterial transcriptional regulators for degradation pathways of aromatic compounds. Microbiol Mol Biol Rev. 68: 474–500.

van Beilen J.B., Panke S., Lucchini S., Franchini A.G., Rothlisberger M. and Witholt B. 2001. Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. Microbiology. 147: 1621–30.

van der Meer J.R., Eggen R.I., Zehnder A.J. and de Vos W.M. 1991. Sequence analysis of the *Pseudomonas* sp. strain P51 tcb gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. J. Bacteriol.173: 2425–34.

Vedler E., Koiv V. and Heinaru A. 2000. Analysis of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pEST4011 of *Achromobacter xylosoxidans* subsp. *denitrificans* strain EST4002.Gene. 255: 281–8.

Velázquez F., di Bartolo I. and de Lorenzo V. 2004. Genetic evidence that catabolites of the Entner-Doudoroff pathway signal C source repression of the sigma 54 Pu promoter of *Pseudomonas putida*. J. Bacteriol. 186: 8267–75.

Venturi V. 2003. Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? Mol Microbiol. 49: 1–9.

Wang Y., Rawlings M., Gibson D.T., Labbe D., Bergeron H., Brousseau R. and Lau P.C. 1995. Identification of a membrane protein and a truncated LysR-type regulator associated with the toluene degradation pathway in *Pseudomonas putida* F1. Mol Gen Genet. 246: 570–9.

Wedel A. and Kustu S. 1995. The bacterial enhancer-binding protein NTRC is a molecular machine: ATP hydrolysis is coupled to transcriptional activation.Genes Dev. 9: 2042–52.

Wösten M.M. 1998. Eubacterial sigma-factors. FEMS Microbiol Rev. 22: 127–50.

Xu H. and Hoover T.R. 2001. Transcriptional regulation at a distance in bacteria. Curr Opin Microbiol. 4: 138–44.

Yura T., Nagai H. and Mori H. 1993. Regulation of the heat-shock response in bacteria. Annu Rev Microbiol. 47: 321–50.

Yuste L. and Rojo F. 2001. Role of the *crc* gene in catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. J. Bacteriol. 183: 6197–206.

Yuste L., Canosa I. and Rojo F. 1998. Carbon-source-dependent expression of the P*alkB* promoter from the *Pseudomonas oleovorans* alkane degradation pathway. J. Bacteriol. 180: 5218–26.

SUMMARY IN ESTONIAN

Aromaatsete ühendite degradatsiooni ja selle eest vastutavate geenide regulatsiooni bakterirakkudes on uuritud pikka aega. Kataboolsete operonide transkriptsiooni spetsiifilist regulatsiooni aktivaatorvalgu ja induktormolekuli abil on põhjalikult uuritud. Viimasel ajal on hakatud aga üha rohkem tähelepanu pöörama kataboolsete operonide kontrollile bakteriraku füsioloogilise seisundi kaudu.

P. putida suudab lagundada katehhooli kromosoomis paiknevate *orto* raja geenide abil. Juhul kui bakterirakkudesse on viidud plasmiidne *pheBA* operon suudab *P. putida* lagundada ka fenooli. Fenool lagundatakse plasmiidse *pheBA* ja kromosomaalse *catBCA* operoni poolt kodeeritud ensüümide abil tsitraaditsükli vaheühenditeni. Transkriptsioon nende operonide promootoritelt on aktiveeritud transkriptsiooni regulaatorvalgu CatR abil, mis seondub fenooli lagundamise vaheühendi, *cis,cis*-mukonaadi juuresolekul *pheBA* ja *catBCA* operoni promootorregioonidega (Kasak jt. 1993).

Käesolevas doktoritöös on uuritud *pheBA* operoni spetsiifilist regulatsiooni CatR valgu ja *cis,cis*-mukonaadi abil. Samuti on selgitatud *pheBA* operoni globaalse regulatsiooni mehhanisme.

Tulemused, mis selles töös on esitatud võib kokku võtta järgmiselt:

1. *pheBA* promootorregiooni koht-suunatud mutagenees näitas, et:
   1.1 CatR valgu sidumiseks *pheBA* operoni promootorregiooni ei ole vajalikud mitte ainult nukleotiidid konserveerunud pöördkordusjärjestuses vaid ka väljaspoole seda regiooni jäävad nukleotiidid.
   1.2 Mutatsioonid aktivaatori seondumiskohas ABS, (*Activator Binding Sequence*) vähendasid transkriptsiooni *pheBA* promootorilt. Need mutatsioonid ABS regioonis, mis kattuvad –35 heksameerse järjestusega, vähendavad küll transkriptsiooni *pheBA* promootorilt, kuid ei mõjuta CatR valgu seondumist sellesse regiooni. Punktmutatsioonid väljapoole –35 heksameerset järjestust vähendasid oluliselt CatR valgu seondumist ning seetõttu ka transkriptsiooni aktivatsiooni *pheBA* promootorilt.
2. Leidsime, et *pheBA* operoni transkriptsiooni alguspunktist allpool paikneb kolmas CatR valgu seondumisjärjestus IBS (*Internal Binding Sequence*), kuhu seondudes käitub CatR kui nõrk transkriptsiooni repressor.
3. Transkriptsioon nii *pheBA* kui ka *catBCA* promootoritelt on kontrollitud bakterite füsioloogilise seisundi poolt. Nendelt promootoritelt lähtuva transkriptsiooni füsioloogiliste kontrollmehhanismide uurimisel selgus, et:
   3.1. Statsionaarse kasvufaasi sigma factor σ^S mõjutab positiivselt transkriptsiooni aktivatsiooni *pheBA* promootorilt, kuid mitte *catBCA* promootorilt.

3.2. Statsionaarse kasvufaasi sigma factor σ^S mõjutab positiivselt transkriptsiooni aktivatsiooni *pheBA* promootorilt, kuid mitte *catBCA* promootorilt.
3.3. Transkriptsioon *pheBA* ja *catBCA* promootoritelt on eksponentsiaalselt kasvavates bakterirakkudes pärsitud aminohapete juuresolekul, kuid minimaalsöötmes on transkriptsioon nendelt promootoritelt koheselt aktiveeritud.
3.4. *pheBA* promootorilt lähtuva transkriptsiooni vaigistamine aminohapete juuresolekul kasvavates rakkudes on põhjustatud *pheBA* operoni induktormolekuli *cis,cis*-mukonaadi madalast tasemest bakterirakkudes.
3.5. Geeni *pheB* poolt kodeeritud katehhooli CCM-iks lagundava ensüümi, katehhooli 1,2-dioksügenaasi tase on aminohapete juuresolekul kasvavates bakterirakkudes madal, mille tulemusena ei teki katehhoolist piisaval hulgal CCM-i, et aktiveerida transkriptsiooni *pheBA* promootorilt.
3.6. Meie poolt saadud tulemused viitavad sellele, et katehhooli 1,2-dioksügenaasi madal tase rikkas söötmes eksponentsiaalselt kasvavates bakterirakkudes võiks olla põhjustatud mehhanismide poolt, mis kontrollivad *pheB* geeni ekspressiooni transkriptsiooni initsiatsiooni järgsel tasemel.

Tulemused, mis on esitatud minu doktoritöös, käsitlevad kahe kataboolse operoni, *pheBA* ja *catBCA* operoni sarnaseid jooni transkriptsiooni initsiatsiooni spetsiifilisel regulatsioonil. Lisaks CatR valgu poolt vahendatud spetsiifilisele kontrollile alluvad need operonid sarnaselt ka füsioloogilisele kontrollile. Oma doktoritöös uurisin ma võimalikke mehhanisme, mis võiksid põhjustada *pheBA* promootorilt lähtuva transkriptsiooni initisiatsiooni pärssimist toitainete poolt rikkas keskkonnas kasvanud bakterites. Selles töös käsitletakse esmakordselt võimalust, et kataboolsete operonide transkriptsioon *Pseudomonas*'es võiks olla saavutatud selle ensüümi hulga negatiivse regulatsiooni kaudu, mis on vastutav vastava operoni induktsiooniks vajaliku ühendi sünteesi eest.

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Tover A., Ojangu E-L. and Kivisaar M. (2001) Growth medium composition-determined regulatory mechanisms are superimposed on CatR-mediated transcription from the *pheBA* and *catBCA* promoters in *Pseudomonas putida*. Microbiology. 147: 2149–56.

Ojangu E-L., **Tover A.**, Teras R. and Kivisaar M. (2000) Effects of combination of different –10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in *Pseudomonas putida*. J. Bacteriol. 182: 6707–13.

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Physiological control mechanisms regulating transcription from the CatR-regulated *pheBA* promoter in *Pseudomonas putida* operate through reduction of the synthesis of the inducer-producing enzyme catechol 1,2-dioxygenase

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ABSTRACT

During growth of *Pseudomonas* in nutritionally rich environment many catabolic operons are repressed by physiological control mechanisms. In the current report we have studied mechanisms that down-regulate transcription from the CatR-controlled promoter of phenol degradation genes *pheBA* in *P. putida* when bacteria are growing in the presence of amino acids. We found that the amount of *cis,cis*-muconate (CCM) which is an effector molecule for the CatR regulator and an intermediate of the catechol degradation pathway in *P. putida*, limits transcription from the *pheBA* promoter. Comparison of expression profiles from the *pheBA* promoter in *P. putida* wild type strain PaW85 and in its *catB* mutant derivative unable to degrade CCM demonstrated that cellular concentration of CCM is a limiting factor determining the level of transcription from the *pheBA* promoter. Although the amount of CCM is probably always below the levels that would allow maximal rate of transcription from the *pheBA* promoter, the limitation became most significant in rich medium-grown cells. Results of the current study provide a new experimental evidence that composition of the growth media influences transcription from the *pheBA* promoter by modulating the expression of the *pheB*-encoded catechol 1,2-dioxygenase which degrades catechol to CCM at the level of synthesis of this enzyme. To our knowledge, this is a first time to show that physiological control of catabolic operons in

Pseudomonas may act by regulating the synthesis of key enzymes that are necessary for the production of the inducer molecule for a catabolic operon.

INTRODUCTION

In *Pseudomonas* species, phenolic compounds are transformed by different enzymes to central intermediates such as protocatechuate and (substituted) catechols which are further degraded either by a *meta* ring cleavage pathway or an *ortho* ring cleavage pathway (14). Expression of these 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 (8, 28). These pathways are controlled at transcriptional level by regulatory proteins which bind specifically to promoter regions of catabolic operons and activate transcription in the presence of effector molecule(s), but physiological control mechanisms are superimposed on this specific control. Various reports have demonstrated that promoters of biodegradative operons in *Pseudomonas* are downregulated during exponential growth in rich nutrient media irrespective of the presence of a catabolic pathway substrate (see, e.g., references 7, 9–11, 21–23, 25, 33, 35–38, 40, 41). Disparate mechanisms of physiological control of catabolic operons in *Pseudomonas* described so far include: (i) control through the amount of a transcription regulator, (ii) by recruitment of alternative sigma factors by RNA polymerase, or (iii) via modulation of the efficiency of electron transport chain. Even promoters that contain the same control elements and show similar physiological regulation pattern may have evolved to employ different regulatory mechanisms. For example, the most thoroughly studied biodegradative pathways subjected to physiological control in *Pseudomonas* are toluene degradative upper pathway encoded by the TOL plasmid pWW0 (7, 9, 21) and dimethylphenol degradative pathway encoded by the plasmid pVI150 (35–37). However, despite similarities in specific regulation of corresponding operons (both are activated by a σ^{54}-dependent mechanism in the presence of regulators which can efficiently cross-regulate each other's target promoter), their interactions with the global regulatory network of the host differ (7, 37).

The *catBCA* operon encodes three enzymes required for catechol degradation via *ortho*-pathway, *cis,cis*-muconate lactonizing enzyme, muconolactone isomerase, and catechol 1,2-dioxygenase (C12O), respectively (17). The induction of this operon requires a LysR family transcriptional activator CatR, and an inducer molecule, *cis,cis*-muconate (CCM) that is intermediate of the *ortho*-pathway (29, 30). When the plasmid-borne *pheBA* operon encoding catechol 1,2-dioxygenase and phenol monooxygenase, respectively, is introduced into *P. putida,* bacteria acquire the ability to degrade phenol (19, 20). The *pheBA* promoter resembles *catBCA* promoter and is also activated by CatR (18, 26, 39). We have previously shown that transcriptional activation from the

pheBA and *catBCA* promoters is repressed in *P. putida* cells grown on minimal medium in the presence of amino acids (38). Here, we have studied mechanisms of physiological control of the transcription from the *pheBA* promoter. We show that the amount of the *pheBA* operon inducer CCM limits the transcription from the *pheBA* promoter. Results obtained in the current study allow us to propose a novel mechanism for the coupling of a specific regulation to cell physiology. This mechanism might operate through modulation of expression of key enzymes necessary for the production of an operon inducer at the level of synthesis of these enzymes. Specifically, the results of the current study provide a new experimental evidence indicating that a reduced cellular amount of CCM in rich medium-grown bacteria is a consequence of the reduced level of synthesis of the *pheB*-encoded C12O in these cells, controlled at posttranscriptional level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *E. coli* cells were grown at 37˚C in Luria broth (LB) and *P. putida* cells were grown at 30˚C in M9 minimal medium (1). Antibiotics were added at the following final concentrations: for *Escherichia coli,* ampicillin at 100 μg/ml; tetracycline, 10 μg/ml; for *P. putida*, carbenicillin at 1,000–3,000 μg/ml, tetracycline at 80 μg/ml; for both organisms, kanamycin at 50 μg/ml, streptomycin 50 μg/ml, and potassium tellurite 40 μg/ml. *E. coli* was transformed with plasmid DNA as described by Hanahan (13). *P. putida* was electrotransformed as described by Sharma and Schimke (34). *E. coli* strains TG1 (6) was used for the DNA cloning procedures and HB101 (4) as a host for helper plasmid pRK2013 (12) necessary for mobilisation of non-conjugative plasmids.

Cloning procedures. To construct *P. putida* strain, capable to degrade phenol, the *pheBA* operon was inserted into the chromosome of *P. putida* strain PaW85. About 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 (18) as the *Sac*I- and *Eco*RI-generated DNA fragment into pUC18Not (16) cleaved with same enzymes, to obtain plasmid pUCNot$P_i$pheBA. As a next step, the *Not*I fragment containing the inducible *pheBA* operon was subcloned from pUCNot$P_i$pheBA into plasmid pUTmini-Tn5 Km2 (10). The resulting plasmid, named pUT$P_i$pheBAKm, was used as a donor plasmid for the insertion of the *pheBA* operon into the chromosome of *P. putida* strain PaW85 to obtain strain PaWpheBA.

To study the regulation of transcription from the *pheBA* promoter, the *pheBA* promoter P_i was cloned upstream to the *luxAB* reporter system. The promoterless *luxAB* genes were cloned within the 2050-bp *Hin*dIII and *Pae*I restriction fragment from plasmid pGP704L (27) into the vector pUC18Not

cleaved with same restrictases to obtain plasmid pUCNotluxAB. The 158-bp *pheBA* promoter region was excised from the plasmid carrying the *pheBA* promoter in pBluescript SK (+) vector (39) using restrictases *Sma*I and *Rsa*I and inserted into the *Sma*I site of the plasmid pUCNotluxAB. Then, the *pheBA* promoter-*luxAB* transcription fusion P_i*luxAB* was inserted into the *Not*I site of the plasmid pUTmini-Tn*5* Sm (10) to generate plasmid pUT$P_i$luxABSm. Finally the plasmid pUT$P_i$luxABSm was used as a donor plasmid for the insertion of the P_i*luxAB* transcriptional fusion into the chromosome of *P. putida* strain PaWpheBA to obtain PaWlux-pheBA.

For the construction of *P. putida* strain PaWlux-pheBA-CatR carrying the *catR* gene under the control of P*tac* promoter and $LacI^q$ repressor, the *lacI*^{q}-P*tac-catR* expression cassette was inserted into the chromosome of *P. putida* strain PaWlux-pheBA. At first, the 913-bp DNA fragment containing the *catR* gene was PCR-amplified from *P. putida* PaW85 chromosome using the primers 5′-CCCACCATACCCTGGAGG-3′ and 5′-GGAGCGCGAAGCTTTTCGGCCTGTTGTCAATCAA-3′, complementary to the regions locating at positions between -4 to +14 bp and +875 to +909 relative of the *catR* gene transcription start site, respectively. The PCR product was cloned into the *Eco*RV site of pBluescript KS(+) vector, yielding plasmid pKScatR. Then, the *catR* sequence was excised from pKScatR with *Eco*RI and *Hind*III and inserted into the plasmid pBRlacItac (24) opened with the same restrictases. The resulting plasmid pBRtacCatR was cleaved with *Eco*RI and *Nhe*I to subclone the *lacI*^{q}-P*tac-catR* expression cassette into the plasmid pUC18Not to generate plasmid pUCNotPtacCatR. Finally, the expression cassette *lacI*^{q}-P*tac-catR* was inserted into the *Not*I site of the plasmid pUTmini-Tn5 Tel (31) to obtain plasmid pUTPtacCatR which was used as the donor plasmid for the insertion of the *lacI*^{q}-P*tac-catR* cassette into the chromosome of *P. putida* strain PaWlux-pheBA.

Inactivation of the *catB* gene in the chromosome of *P. putida* was performed by homologous recombination using the plasmid pGPcatB::tet carrying the *catB* sequence interrupted by 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 codon, respectively. The PCR product was cloned into the *Eco*RV site of the plasmid pBluescript KS(+) resulting in plasmid pKScatB. About 1.3-kb DNA fragment containing the tetracycline resistance gene was obtained from the plasmid pBR322 (3) and inserted into the *Ehe*I site locating 513 bp downstream from the *catB* translation start codon within the plasmid pKScatB to obtain plasmid pKScatB::tet. Finally, using the restrictases *Ecl*136II and *Acc*65I, the DNA fragment containing the interrupted *catB* gene was cloned from pBluescript KS(+) construct into the plasmid pGP704del (derivative of plasmid pGP704L

obtained by deletion of the 1-kb *Eco*RI fragment from the *luxA* gene). The resulting plasmid was named pGPcatB::tet.

For the construction of *P. putida* strain PaWPheB expressing the *pheB*-encoded catechol 1,2-dioxygenase under the control of P*tac* promoter, the *lacI*^{q}-P*tac-pheB* expression cassette was inserted into the chromosome of *P. putida* strain PaW85. For construction of this expression cassette, the 1011-bp DNA fragment from the plasmid pAT1442 containing the *pheB* gene was excised with restrictases *Eco*47II and *Hinc*II and inserted into the plasmid pBRlacItac cleaved with *Sma*I restrictase, yielding the plasmid pBRtacPheB. The *Ecl*136II-*Bam*HI DNA fragment containing the *lacI*^{q}-P*tac-pheB* expression cassette excised from the plasmid pBRtacPheB was cloned into the plasmid pUC18Not resulting in plasmid pUCNotPtacPheB. Finally, the expression cassette *lacI*^{q}-P*tac-pheB* was inserted into the *Not*I site of the plasmid pUTmini-Tn5 Tel and the generated plasmid, named pUTtacPheB, was used as donor plasmid for the insertion of the *pheB* expression cassette into the chromosome of *P. putida* strain PaW85.

To test the expression profile of the P*tac* promoter in *P. putida* cells grown under different nutritional conditions, the *lacZ* reporter gene was inserted downstream from the P*tac* promoter in the single-copy plasmid pPR9TT (32). The original *lacZ* gene lacking the translation initiation signals and residing in the plasmid pPR9TT was replaced with the functional *lacZ* gene originated from the plasmid pKRZ-1 (30). For this replacement, pPR9TT was cleaved with *Bam*HI to delete the defective *lacZ* gene resulting in plasmid pPR9TTdelBam. Thereafter, the *lacZ* gene, excised from pKRZ-1 with restrictases *Pst*I and *Xba*I, was inserted into the plasmid pPR9TTdelBam cleaved with restictases *Ecl*136II and *Xba*I to generate pPR9TTlacZ. In order to have the *lacZ* gene under the control of P*tac* promoter, the *Bam*HI fragment carrying the *lacI*^{q}-P*tac* sequence from pBRlacItac was cloned into pPR9TTlacZ opened with *Bam*HI, resulting in plasmid pPR9TTtac-lacZ.

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 % CAA. To ensure that bacterial culture is in exponential growth phase, the overnight culture was diluted 1:100 to the fresh medium supplemented with 0.02 % CAA. After 3 h the culture was diluted once more to obtain an OD_{580} of 0.05. To induce the transcription from P_i-*pheBA* and P_i-*luxAB* transcriptional fusions, 2.5 mM sodium benzoate or 2.5 mM phenol was added to the growth medium. To investigate the role of amino acids on the transcription from the *pheBA* promoter, 0.33% Casamino Acids (CAA) solution was added when indicated. To investigate the effect of overexpression of the CatR protein on transcription from the *pheBA* promoter, 1 mM IPTG was added to the growth medium of the strain PaWlux-pheBA-CatR when overnight-grown cells were diluted first and second time. To study the effect of the

presence of amino acids in bacterial growth medium to the enzymatic activity of the *pheB*-encoded catechol 1,2-dioxygenase (C12O), the *P. putida* strain PaWtacPheB was grown exponentially in M9 minimal medium supplemented with 0.02 % CAA (and with 1 mM IPTG when indicated) until OD_{580} of 0.3-0.4. Bacterial culture was divided in two, and 1 mM IPTG was added to induce the expression of the *pheB* gene. One of these cultures was supplemented with amino acids. C12O activity was measured in cells sampled after one and third hour of growth (exponential growth phase) and in 24 hours grown cells (late stationary phase). All cultures were grown in 100-ml flasks on the Infros HT RC-TK shaker at 180 rpm. The amount of growth medium in flasks was 30 ml.

Enzyme assays. Samples for luciferase assay were taken from exponentially grown cultures. Luciferase assay was performed as follows: 990 µl phosphate buffer (100 mM Na_2HPO_4/KH_2PO_4; pH 7.5), 10 µl decanal (5 mM decanal in ethanol) were mixed into the test tube. 10 µl of bacterial culture was added to the reaction mixture and light emission was measured after 5 minutes by a luminometer TD-20/20 "Turner designs". C12O assay was carried out as described by Hegeman (15). β-galactosidase measurement in cell suspensions was performed as described previously (38).

Preparation of cell lysates and immunoblotting of PheB. Cell lysates used for C12O assay and for separation of proteins by gel electrophoresis were prepared from 30-ml exponential phase cultures (first and third hour) and from 3-ml stationary-phase cultures of *P. putida* PaWtac-pheB. Cells were pelleted and sonicated in 300 µl volumes of 100 mM phosphate buffer, pH 7.5. The protein concentration in cleared lysates was estimated as described in Bradford (5). Equal amounts (10 µg) of total protein were used for a Western immunoblotting assay. Proteins were separated by sodium dodecyl sulfate polyacrylamide (10%) gel electrophoresis and transferred to nitrocellulose membrane (Hybond ECL, Amersham). For Western blotting, the membrane was probed with mouse anti-PheB polyclonal serum diluted 1:500, followed by alkaline phosphatase-conjugated goat anti-mouse immunoglobuline G diluted 1:5,000 (LabAS Ltd., Tartu, Estonia). The blots were developed using 5-bromo-4-chloro-indolylphosphate-nitroblue tetrazolium.

RESULTS AND DISCUSSION

Transcriptional profile from the *pheBA* promoter using the *luxAB* reporter. We have previously shown that presence of amino acids in the growth medium of a bacterial culture represses the transcription from the *pheBA* promoter in exponentially growing *P. putida* (38). CatR activates transcription from the *pheBA* promoter only in the presence of an inducer molecule, CCM (18). CCM is an intermediate of the benzoate degradation pathway (Fig. 1.). Therefore, to induce the transcription from the *pheBA* promoter, sodium ben-

zoate was added to the growth medium (38). Consequently, we were unable to separate the effects operating through the CatR-dependent transcriptional activation of the *pheBA* promoter from those that would affect expression of the first step of benzoate degradation, conversion of benzoate to catechol. To examine the possibility that reduction of transcription from the *pheBA* promoter could occur due to ineffective functioning of the first step of benzoate degradation pathway in bacteria grown in the presence of amino acids, we constructed a new strain PaWlux-pheBA that allows us to use an alternate substrate, phenol, as a source of the inducer (see Materials and Methods). This strain was able to metabolise phenol to CCM due to the presence of enzymes encoded by the *pheBA* operon inserted into bacterial chromosome under the control of its natural promoter Pi. To measure the activity of the *pheBA* promoter under conditions when the level of transcription from this promoter is very low (without inducer or in the presence of amino acids in the culture medium of exponentially growing bacteria), the Pi promoter was cloned upstream of the sensitive *luxAB* reporter system.

The strain PaWlux-pheBA harbouring the *pheBA* operon and the P_i-*luxAB* reporter system in the chromosome was grown exponentially in M9 glucose minimal medium with benzoate or phenol. Parallel experiments were carried out with cultures supplemented or not with amino acids (CAA solution). Bioluminescence was measured in cells sampled at hours 1, 2, and 3 after the addition of phenol or benzoate. As shown in Fig. 2, addition of amino acids into the growth medium of bacteria essentially decreased transcription from the *pheBA* promoter irrespective of which source of the inducer, phenol or benzoate, was used. Therefore, the decrease in transcription from the *pheBA* promoter observed in bacteria growing in benzoate-containing minimal CAA medium cannot be simply explained by a mechanism reducing degradation of benzoate to catechol. Rather, as the repression of transcription became apparent in the case of both substrates, expression of the enzymes responsible for the degradation of catechol to CCM might be subjected to physiological control.

Interestingly, the transcription profile of the *pheBA* promoter was remarkably different if cells grown in the presence of phenol or benzoate were comparatively analysed (Fig. 2). In the case when bacteria were grown on minimal medium lacking CAA, addition of benzoate into growth medium allowed rapid activation of transcription from the *pheBA* promoter (Fig. 2A). At the same time, after addition of phenol into growth medium not supplemented with amino acids, during the first hour of growth of bacteria the level of transcription from the *pheBA* promoter did not increase significantly above the level detected in non-induced bacteria. Only later, on hours 2 and 3, the level of transcription from the *pheBA* promoter rapidly increased (Fig. 2B). In the presence of CAA in the growth medium, the benzoate-grown cells expressed 6 to 60-fold lower levels of luciferase activities if compared to the cells grown in the medium lacking CAA (Fig. 2A). At the same time, if phenol was added to the amino acids-containing growth medium, only low basal level of transcription from the

pheBA promoter (similar to that obtained in non-induced cells) was detected during the three hours examined (Fig. 2B).

CatR overexpression elevates the rate of transcription from *pheBA* promoter. In the previous report (38) we examined whether the cellular amount of the activator protein CatR can limit transcriptional activation from the *pheBA* promoter by monitoring the expression of the *lacZ* reporter in *P. putida* cells grown in the presence of benzoate as a precursor of CCM. The *lacZ* reporter did not allow us to detect transcription from the *pheBA* promoter in cells grown exponentially in amino acids-containing medium irrespective of the CatR expression level. As already shown in Fig. 2, the *luxAB* reporter system designed for the current study is for more sensitive, enabling evaluation of transcription from the *pheBA* promoter under various growth conditions of bacteria. Thus, in order to test whether the artificial overexpression of CatR would influence transcription from the *pheBA* promoter if transcription is induced by the addition of phenol, the *catR* gene under the control of the IPTG-inducible P*tac* promoter was introduced into the chromosome of the strain PaWlux-pheBA. The resulting *P. putida* strain PaWlux-pheBA-catR was grown in the presence of 1 mM IPTG in phenol-containing medium supplemented or not with CAA, and the luciferase activities were measured in cells sampled from different time points.

As shown in Table 2, overexpression of CatR protein had positive effect on transcriptional activation from the *pheBA* promoter. In the presence of CAA in phenol-containing growth medium, the cells with elevated CatR expression exhibited about 30 to 40-fold higher level of transcription from the *pheBA* promoter than the wild type cells. This effect was also significant if bacteria were cultivated on phenol in minimal medium lacking amino acids: the elevated amount of CatR resulted in about 25-fold higher level of transcription from the *pheBA* promoter in cells sampled one hour after the addition of phenol. Moreover, the *pheBA* promoter showed approximately two-fold higher level of transcription also in this case if activities in cells sampled on hour three after the addition of phenol were measured. CatR is unable to activate transcription unless the effector molecule CCM is bound (30). It is possible, therefore, that cellular amount of CCM might limit the transcriptional activation from the *pheBA* promoter, but if the number of CatR molecules per cell increases, even low amount of CCM molecules can be more easily captured by CatR. In the next step of our studies, we decided to test whether this hypothesis is correct, i.e., whether the level of transcription from the *pheBA* promoter would be limited by the amount of CCM in a cell.

Higher amount of the inducer molecule CCM relieves exponential silencing of the *pheBA* promoter. CCM-lactonizing enzyme encoded by the *catB* gene converts CCM to muconolactone (Fig. 1). When the *catB* gene is inactivated in the chromosome of *P. putida*, the phenol and benzoate degradation pathway ends in the formation of CCM. To investigate whether the higher amount of CCM would enhance the level of transcription from *pheBA*

promoter, we constructed the *catB*-deficient mutant PaWlux-pheBA-CatB::tet by interrupting the chromosomal *catB* gene in PaWlux-pheBA strain with the Tet-resistance encoding gene.

To investigate the effect of increased cellular amount of CCM on the level of transcription from the *pheBA* promoter, the wild type strain PaWlux-pheBA and its *catB*::*tet* derivative PaWlux-pheBA-CatB::tet were grown either in the presence or absence of amino acids and bioluminescence was measured during the first three hours of growth of bacteria after the addition of phenol into the growth medium. As shown in Fig. 2C, in the case of the *catB*-deficient mutant, the transcription from the *pheBA* promoter was rapidly induced both in the presence or absence of amino acids. During the first hour of cultivation of bacteria in phenol minimal medium lacking amino acids, the level of transcription increased by more than 400-fold in the *catB*-defective strain if compared to that in the wild type (Fig. 2B and C). The *catB* mutant grown for three hours exhibited three times higher level of transcription than the wild type. In the presence of CAA in the growth medium, the effect of CCM accumulation in cells was even more remarkable. In this case the *pheBA* promoter was also rapidly activated after the addition of phenol to the growth medium and up to three orders of magnitude higher levels of transcription were observed in the *catB*-deficient mutant if compared to those measured in the wild type. These data led to the conclusion that the level of transcription from the *pheBA* promoter in *P. putida* grown under different nutritional conditions is controlled by cellular amount of the inducer molecule CCM.

The presence of amino acids in growth medium of bacteria affects negatively the expression of the *pheB*-encoded C12O. A lower amount of CCM in *P. putida* cells resulting in reduced level of the transcription from the *pheBA* promoter may be a consequence of decreased activities of enzymes involved in phenol and benzoate degradation to CCM when bacteria are grown in minimal medium supplemented with amino acids. C12O is a common enzyme for both benzoate and phenol degradation pathways, and the physiological control of transcription became evident in the case of both substrates (see results presented in Fig. 2A and B). Therefore, we decided to study whether the cellular amount and/or activity of C12O encoded by the *pheB* gene would be affected by growth conditions of bacteria. For that, we fused the *pheB* gene with the heterologous promoter P*tac* controlled artificially by the amount of IPTG added to the growth medium of bacteria. As a control, the *lacZ* transcriptional fusion was used. *P. putida* strains carrying either the P*tac-pheB* or P*tac-lacZ* expression cassettes were grown in glucose-containing medium in the presence or absence of amino acids and cells sampled one hour after the addition of 1 mM IPTG were examined for the expression of enzymes. Results presented in Fig. 3A demonstrate that the level of expression of the *pheB*-encoded C12O in the strain PaWPheB carrying the P*tac-pheB* expression cassette was strongly affected by the composition of bacterial growth medium. When grown exponentially in the medium supplemented with amino acids,

bacteria showed about 10-fold lower level of C12O activity if compared to that measured in cells grown in minimal medium without amino acids. Only later, in cells sampled from overnight-grown stationary phase cultures, the levels of C12O were equal between the presence and absence of amino acids growing cells. Parallel experiments monitoring the β-galactosidase activities in bacteria expressing the *lacZ* gene from the P*tac* promoter confirmed that the P*tac* promoter itself was not repressed in *P. putida* grown in amino acids-containing medium (Fig. 3B). These data indicated that presence of amino acids in the growth medium decreased the level of expression of the *pheB* gene at post-transcriptional level.

There might be two reasons for the reduced C12O activity in cells growing in amino acids-containing environment. First, enzymatic activity of C12O might somehow be inhibited in bacteria grown in nutritionally rich medium. Secondly, the amount of C12O protein may be down-regulated under growth of bacteria in the presence of amino acids. To monitor the cellular amount of C12O in bacteria grown under different nutritional conditions, we performed Western blot analysis of cell lysates sampled from cultures of PaWPheB grown in the presence or absence of amino acids by using *P. putida* anti-PheB polyclonal antibodies. Results of Western blot analysis presented in Fig. 3C clearly demonstrated that the amount of C12O is drastically reduced in cells grown in amino acids-containing medium. These data excluded the possibility that presence of amino acids in the growth medium might inhibit the enzymatic activity of C12O. Rather, while amount of the PheB protein in cells grown in rich medium was reduced proportionally to the level of C12O activity, we concluded that the expression of C12O can most likely be controlled either by proteolysis of C12O or by the amount of its synthesis. The latter, in turn, may be affected either by the reduction of the half-life of the *pheB*-specific mRNA or by decreased efficiency of translation of this mRNA in bacteria grown in the presence of amino acids.

If the *pheB*-encoded C12O is degraded in nutritionally rich medium-grown cells, the cellular amount of C12O would rapidly decline after the transfer of bacteria from poor growth medium into amino acids-containing medium. To examine that possibility, exponentially growing IPTG-induced cells of PaWPheB carrying the *pheB* gene under the control of P*tac* promoter were inoculated into fresh glucose minimal medium. In the parallel experiment, the medium was supplemented with amino acids. Cells sampled one hour after the transfer expressed slightly increased C12O activity when grown in the absence of amino acids. Also, only slight decrease in C12O activity corresponding to the dilution of the synthesised protein due to cell division, could be detected if bacteria grown in the presence of amino acids were analysed (Fig. 4). The latter argued against the possibility that the decreased amount of C12O in rich medium-grown bacteria can be controlled at the level of proteolysis of C12O. Rather, the synthesis of the *pheB* gene-encoded C12O may be reduced in rich medium-grown bacteria.

Possible mechanisms of regulation of transcription from the *pheBA* promoter in bacteria grown under different nutritional conditions. The molecular bases of physiological control mechanisms that are superimposed on transcriptional activation of catabolic operons by specific regulators in the presence of effector molecules vary largely, depending on a particular operon studied. In some cases, this control operates through the level of expression of a transcription activator protein (see, e.g., references 23, 36, 41). 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 (41). Results presented in the current report demonstrate that activation of the transcription from the *pheBA* promoter is also influenced by the amount of the operon regulator, CatR. Artificial overexpression of the *catR* gene in *P. putida* strain PaWlux-pheBA-CatR led to the increased level of transcription from the *pheBA* promoter (Table 2). The positive effect of the increased amount of CatR protein became apparent also in minimal medium-grown cells. However, other experimental data obtained in the current study (Fig. 2C) argue against the possibility that the amount of CatR itself is limiting on the transcriptional activation from the *pheBA* promoter. We found that transcription from the *pheBA* promoter was rapidly induced under both growth conditions of bacteria (in minimal medium and in medium supplemented with amino acids) when cellular amount of the CatR effector molecule CCM was artificially increased due to the lack of further metabolism of CCM in the *catB*-deficient mutant strain. The observed increase was at least ten times higher than observed by simple CatR overexpression. We have shown that high constitutive expression of the *pheBA* genes also led to rapid activation of the transcription from the *pheBA* promoter in minimal medium-grown bacteria, and overproduction of respective proteins almost completely relieved repressive effect of amino acids on transcription already during the first hour of cultivation of bacteria after the addition of phenol into the growth medium (data not shown). Based on the results of these experiments we concluded that the cellular amount of CCM limits the level of transcription from the *pheBA* promoter. This could also explain why artificial overexpression of CatR resulted in faster activation of the transcription from the *pheBA* promoter in bacteria grown on phenol-containing minimal medium. We suppose that initially, after addition of phenol, only few molecules of CCM might be available for the CatR-mediated transcriptional activation. Thus, the increased cellular amount of CatR would increase a chance that CCM molecules available at that moment could be bound by CatR. Moreover, our results indicate that CCM, more or less, always limits the transcription from the CatR-regulated promoters: even if bacteria were grown in nutritionally poor environment, transcription from the *pheBA* promoter was 2 to 3-fold higher with excess of CCM if compared to that with “normal” cellular amount of the inducer (Fig. 2B and C).

Although the cellular amount of CCM is probably always below the level that would allow the maximal rate of transcription from the *pheBA* promoter, the limitation became most significant in rich medium-grown cells. Results presented in this report indicate that the effect of growth media composition on transcription from the *pheBA* promoter operates through the amount of synthesis of the enzymes necessary for the degradation of phenol to CCM. Precisely, we have demonstrated that when bacteria were grown exponentially on amino acids-containing environment (even if the level of transcription of the *pheB* gene was kept constant) the cellular amount of the *pheB*-encoded C12O was at least 10-fold lower than that measured in cells grown in minimal medium. The artificial overexpression of the *pheB* gene reduced the repressive effect caused by physiological control mechanisms by at least 10-fold but not entirely (data not shown). At the same time, as already mentioned above, the overexpression of both *pheA* and *pheB* genes completely relieved this effect. This indicates that the reduced amount of the *pheB*-encoded C12O is not the only bottleneck of transcriptional activation of the *pheBA* promoter in bacteria grown in amino acids-containing medium. It is very likely, although not proved in the current study, that mechanisms similar to that controlling the abundance of the *pheB*-encoded C12O may regulate expression of the *pheA*-encoded phenol monooxygenase as well.

To summarize the results presented in the current report, and draw parallels with mechanisms concerning growth medium composition-determined regulation of transcriptional activation of other catabolic operons in *Pseudomonas* (see, e.g., examples reviewed in reference 8), a novel mechanism became evident here. So far, the growth medium composition-determined regulatory mechanisms have been shown to operate mostly by influencing the activity of components of RNA polymerase and/or transcription activators. Data obtained by us indicate that the synthesis of catabolic enzymes necessary for the production of the inducer molecule for the catabolic operon can also be also a subject to physiological control. *P. putida* strain used in our study, like other pseudomonads, carries chromosomally encoded *ortho* pathway genes including *catA* which is a close homolog to the *pheB* gene. We have previously shown (38) that transcriptional activation from the CatR-regulated *catBCA* promoter is also under physiological control. Further studies are under way to examine whether the other catabolic operons, e.g., the chromosomally encoded benzoate and catechol degradation operons, would also be regulated by physiological control mechanisms similar to that elucidated by us on transcriptional regulation of the *pheBA* promoter.

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REFERENCES

1. **Adams, M. H.** 1959. Bacteriophages. p. 445–447. Interscience Publishers Inc. New York, N.Y.
2. **Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda.** 1977. Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154:**203–204.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
4. **Boyer H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in Escherichia coli. J. Mol.Biol. **41:**459–472.
5. **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.
6. **Carter, P.**, **H. Bedouelle, and G. Winter.** 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. Nucleic Acids Res. **13:**4431–4443.
7. **Cases, I., and V. de Lorenzo.** 2000. Genetic evidence of distinct physiological regulation mechanisms in the σ^{54} *Pu* promoter of *Pseudomonas putida*. J. Bacteriol. **182:**956–960.
8. **Cases I., and V. de Lorenzo.** 2001. The black cat/white cat principle of signal integration in bacterial promoters. EMBO J. **20:**1–11.
9. **Cases, I., V. de Lorenzo, and J. Perez-Martin.** 1996. Involvement of σ^{54} in exponential silencing of the *Pseudomonas putida* TOL plasmid *Pu* promoter. Mol. Microbiol. **19:**7–17.
10. **de Lorenzo, V., I. Cases, M. Herrero, and K. N. Timmis.** 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.
11. **Dinamarca, M. A., I. Aranda-Olmedo, A. Puyet, and F. Rojo.** 2003. Expression of the *Pseudomonas putida* OCT plasmid alkane degradation pathway is modulated by two different global control signals: evidence from continuous cultures. J Bacteriol. **185:**4772–4778.
12. **Figurski D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A. **76:**1648–1652.
13. **Hanahan, D.** 1983. Studies on the transformation of *E. coli* with plasmids. J. Mol. Biol. **166:**577–580.

14. **Harayama, S., and K. N. Timmis.** 1989. Catabolism of aromatic hydrocarbons by *Pseudomonas*, p. 151–174. *In* D. A. Hopwood and K. E. Chater (ed.), Genetics of bacterial diversity. Academic Press Ltd., London.
15. **Hegeman, G. D.** 1966. Synthesis of the enzymes of the mandelate pathway by Pseudomonas putida. I. Synthesis of enzymes by the wild type. J. Bacteriol. **91:**1140–1154.
16. **Herrero, M, V. de Lorenzo, and K. N. Timmis.** 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.
17. **Houghton, J. E., T. M. Brown, A. J. Appel, E. J. Hughes, and L. N. Ornston.** 1995. Discontinuities in the evolution of *Pseudomonas putida cat* genes. J. Bacteriol. **177:**401–412.
18. **Kasak, L., R. Hõrak, A. Nurk, K. Talvik, and M. Kivisaar.** 1993. Regulation of the catechol 1,2-dioxygenase- and phenol monooxygenase-encoding *pheBA* operon in *Pseudomonas putida* PaW85. J. Bacteriol. **175:**8038–8042.
19. **Kivisaar, M., R. Hõrak, L. Kasak, A. Heinaru, and J. Habicht**. 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.
20. **Kivisaar, M., L. Kasak, and A. Nurk.** 1991. Sequence of the plasmid-encoded catechol 1,2-dioxygenase-expressing gene, *pheB*, of phenol-degrading *Pseudomonas* sp. strain EST1001. Gene **98:**15–20.
21. **Marques, S., A. Holtel, K. N. Timmis, and J. Ramos.** 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.
22. **Marques, S., M. Manzanera, M.-M. Gonzalez-Perez, M.-T. Gallegos, and J. L. Ramos.** 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.
23. **Müller, C., L. Petruschka, H. Cuypers, G. Burchhardt, and H. Herrmann.** 1996. Carbon catabolite repression of phenol degradation in Pseudomonas putida is mediated by the inhibition of the activator protein PhlR. J Bacteriol. **178:**2030–2036.
24. **Ojangu, E., A. Tover, and M. Kivisaar.** 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.
25. **Petruschka, L., G. Burchhardt, C. Muller, C. Weihe, and H. Herrmann.** 2001. The cyo operon of *Pseudomonas putida* is involved in carbon catabolite repression of phenol degradation. Mol. Genet. Genomics. **266:**199–206.
26. **Parsek, M. R., M. Kivisaar, and A. M. Chakrabarty.** 1995. Differental 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.
27. **Pavel H., M. Forsman, and V. Shingler.** 1994. An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of

Pseudomonas sp. strain CF600 on para-substituted methylphenols. J Bacteriol. **176:**7550–7557.

28. **Ramos, J. L., S. Marques, and K. N. Timmis.** 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.
29. **Rothmel, R. K., T. L. Aldrich, J. E. Houghton, W. M. Coco, L. N. Ornston, and A. M. Chakrabarty.** 1990. Nucleotide sequencing of *Pseudomonas putida catR*: positive regulator of the *catBC* operon is a member of LysR family. J. Bacteriol. **172:**922–931.
30. **Rothmel, R. K., D. L. Shinabarger, M. R. Parsek, T. L. Aldrich, and A. M. Chakrabarty.** 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.
31. **Sanchez-Romero J. M., R. Diaz-Orejas, and V. De Lorenzo.** 1998. Resistance to tellurite as a selection marker for genetic manipulations of Pseudomonas strains. Appl. Environ. Microbiol. **64:**4040–4046.
32. **Santos, P. M., I. Di Bartolo, J. M. Blatny, E. Zennaro, and S. Valla**. 2001. New broad-host-range promoter probe vectors based on the plasmid RK2 replicon. FEMS Microbiol. Lett. **195:**91–96.
33. **Santos, P. M., J. M. Blatny, I. Di Bartolo, S. Valla, and E. Zennaro.** 2000. Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens* ST. Appl. Environ. Microbiol. **66:**1305–1310.
34. **Sharma, R. C., and R. T. Schimke.** 1996. Preparation of electro-competent *E. coli* using salt-free growth medium. BioTechniques **20:**42–44.
35. **Sze, C. C., and V. Shingler.** 1999. The alarmone (p)ppGpp mediates physiological-responsive control at the σ^{54}-dependent Po promoter. Mol. Microbiol. **31:**1217–1228.
36. **Sze, C. C., T. Moore, and V. Shingler.** 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.
37. **Sze, C.C., L.M.D. Bernardo, and V. Shingler.** 2002. Integration of global regulation of two aromatic-responsive σ^{54}-dependent systems: a common phenotype by different mechanisms. J. Bacteriol. **184:**760–770.
38. **Tover, A., E.-L. Ojangu, and M. Kivisaar.** 2001. Growth medium composition-determined regulatory mechanisms are superimposed on CatR-mediated transcription from the *pheBA* and *catBCA* promoters in *Pseudomonas putida*. Microbiology **147:**2149–2156.
39. **Tover, A., J. Zernant, S. A. Chugani, A. M. Chakrabarty, and M. Kivisaar.** 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.
40. **Yuste, L., and F. Rojo.** 2001. Role of the crc gene in catabolic repression of the Pseudomonas putida GPo1 alkane degradation pathway. J Bacteriol. **183:**6197–6206.
41. **Yuste, L., I. Canosa, and F. Rojo.** 1998. Carbon-source-dependent expression of the *PalkB* promoter from the *Pseudomonas oleovorans* alkane degradation pathway. J. Bacteriol. **180:**5218–5226.

TABLES AND FIGURES

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or construction	Source or reference
Strains		
E. coli		
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F´ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	6
HB101	*supE*44 *hsd*20(r_B^- m_B^-) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	4
CC118λpir	*galE galK phoA*20 *thi*-1 *rpsE rpoB argE*(Am) *recA*1, λ*pir* phage lysogen	16
P. putida		
PaW85	Wild type	2
PaWpheBA	P_i*pheBA* fusion in the chromosome of PaW85, Km^r	This work
PaWlux-pheBA	P_i*luxAB* fusion in the chromosome of PaWpheBA, Km^r Sm^r	This work
PaWlux-pheBA-CatB::tet	PaWlux-pheBA with an inactivated *catB*::*tet*, Km^r Sm^r Tet^r	This work
PaWlux-pheBA-CatR	*catR* gene under control of P*tac* promoter in chromosome of PaWlux-pheBA, Km^r Sm^r Tel^r	This work
PaWPheB	*pheB* gene under control of P*tac* promoter in chromosome of PaW85, Tel^r	This work
Plasmids		
pBluescript KS(+)	cloning vector (Ap^r)	Stratagene
pGP704L	Ap^r; *luxAB*	27
pUC18Not	Cloning vector (Ap^r)	16
pRK2013	Km^r Mob^+ Tra^+, donor transfer functions	12
pKRZ-1	Cloning vector (Ap^r Km^r)	30
pPR9TT	Single-copy broad-host-range cloning vector (Ap^r Cm^r)	32
pBR322	Cloning vector (Ap^r Tet^r)	3
pBRlacItac	*Ptac* promoter and *lacI*^{q} repressor in pBR322	24
pUTmini- Tn5 Sm	Delivery plasmid for mini Tn5 Sm	10
pUTmini-Tn5 Km2	Delivery plasmid for mini Tn5 Km2	10
pUTmini-Tn5 Tel	Delivery plasmid for mini Tn5 Tel	31
pUCNotluxAB	2050 bp promoterless *luxAB* genes containing HindIII and PaeI restriction fragment from plasmid pGP704L cloned into pUC18Not	This work
pUCNot$P_i$luxAB	*pheBA* promoter region in SmaI-RsaI fragment from pKSP_i inserted at the SmaI site of pUCNotluxAB	This work
pUT$P_i$luxABSm	*pheBA* promoter-*luxAB* fusion from pUCNot$P_i$luxAB into pUTmini-Tn5 Sm	This work
pAT1142	*pheBA* genes under control of their native inducible promoter into pAYC32	18
pUCNot$P_i$pheBA	6,3 kbp SacI-EcoRI fragment containing inducible *pheBA* genes from plasmid pAT1142 into the pUC18Not	This work
pUT$P_i$pheBAKm	Inducible *pheBA* genes from pUCNot$P_i$pheBA into pUTmini-Tn5Km2	This work
pKScatB	1122 bp *P. putida* PaW85 *catB* gene cloned into pBluescript KS(+) EcoRV site	This work
pKScatB::tet	2 kbp tetracycline resistance (Tet^r) determinant from plasmid pBR233 inserted at the EheI site of pKScatB, located 513 bp downstream of the *catB* translation start codon	This work

pGP704del	Derivative of pGP704 by EcoRV deletion of 1kbp from *luxA* gene	This work
pGPcatB::tet	EcI136II-Acc65I fragment from pKScatB::tet containing *catB*::*tet* allele cloned into pGP704del	This work
pKScatR	913 bp *P. putida* PaW85 *catR* gene cloned into pBluescript KS(+) EcoRV site	This work
pBRtacCatR	EcoRI-HindIII fragment containing *catR* gene from pKScatR inserted into pBRlacItac	This work
pUCNotPtacCatR	EcoRI-NheI fragment containing *lacI*^{q}-*Ptac-catR* from pBRtacCatR into pUC18Not	This work
pUTtacCatRTel	*lacI*^{q}-P*tac-catR* from pUC18Not into pUTmini-Tn5Tel	This work
pBRtacPheB	HincII-Eco47II fragment containing *pheB* gene from pAT1142 inserted into pBRlacItac	This work
pUCNotPtacPheB	EcI136II-BamHI fragment containing *lacI*^{q}-P*tac-pheB* from pBRtacPheB into pUC18Not	This work
pUTtacPheBTel	*lacI*^{q}-P*tac-pheB* from pUC18NotPtacPheB into pUTmini-Tn5Tel	This work
pPR9TTdelBam	Derivative of pPR9TT by BamHI deletion of *lacZ* gene	This work
pPR9TTlacZ	Promoter probe vector containing *lacZ* gene from pKRZ-1 cloned into p9TTdelBam	This work
pPR9TTtac-lacZ	P*tac* promoter and *lacI*^{q} repressor in BamHI fragment from pBRlacItac cloned into p9TTlacZ	This work

Table 2. Effect of CatR overexpression on transcription from the *pheBA* promoter in *P. putida* exponentially growing cells[a].

	Wild type		CatR overexpression			
Hours	–CAA	+ CAA	–CAA	CatR/wt	+ CAA	CatR/wt
1	13 ± 5.0	4.0 ± 0.3	307 ± 39	24	109 ± 1.0	27
2	655 ± 141	5.0 ± 1.0	2111 ± 32	3.2	182 ± 2.3	36
3	2157 ± 474	8.0 ± 2.2	4729 ± 826	2.2	341 ± 1.9	43

[a] Transcription from the *pheBA* promoter was assayed by measuring the luciferase activity (relative luciferase units/optical density unit at 580 nm) in glucose and phenol-containing minimal medium in the presence or absence of amino acids (CAA solution) in the growth medium.

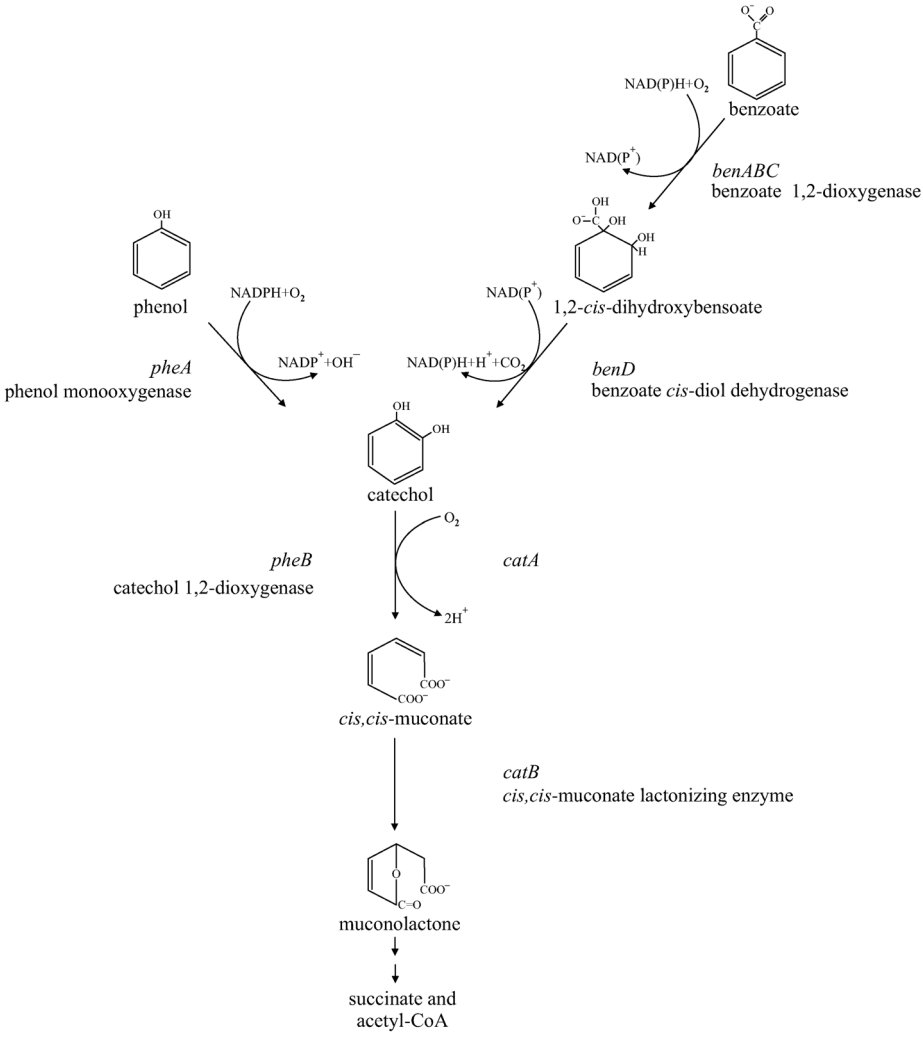

Fig. 1. The part of β-ketoadipate pathway by which phenol and benzoate are converted to succinate and acetyl-CoA.

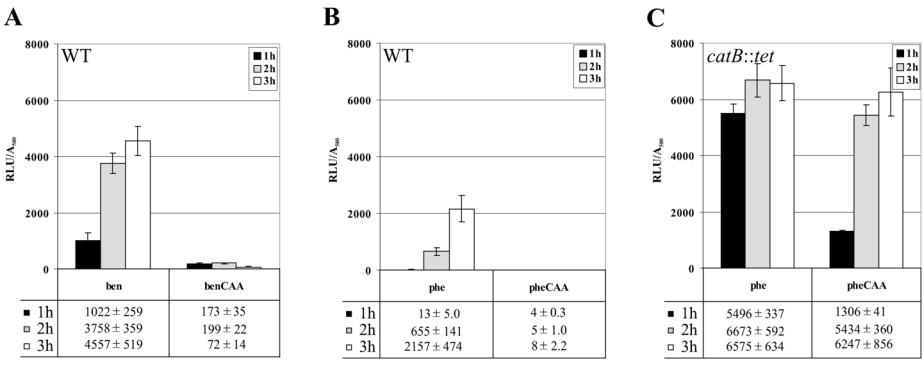

Fig. 2. (A) and **(B)** Effect of growth medium composition on the transcription from the *pheBA* promoter. Bacteria were grown in M9 minimal medium containing glucose. Sodium benzoate (ben) or phenol (phe) was added to growth medium to induce the transcription from the *pheBA* promoter. **(C)** Effect of *catB* knockout mutation on the level of transcription from the *pheBA* promoter in *P. putida*. Bioluminescence (relative luciferase units/optical density unit at 580 nm) was measured in exponentially growing *P. putida* wild type strain PaWlux-pheBA and a *catB*-defective mutant PaWlux-pheBA-CatB::tet grown either in the presence or absence of amino acids (CAA) in the growth medium. Data (means ± standard deviations) of five independent experiments are presented.

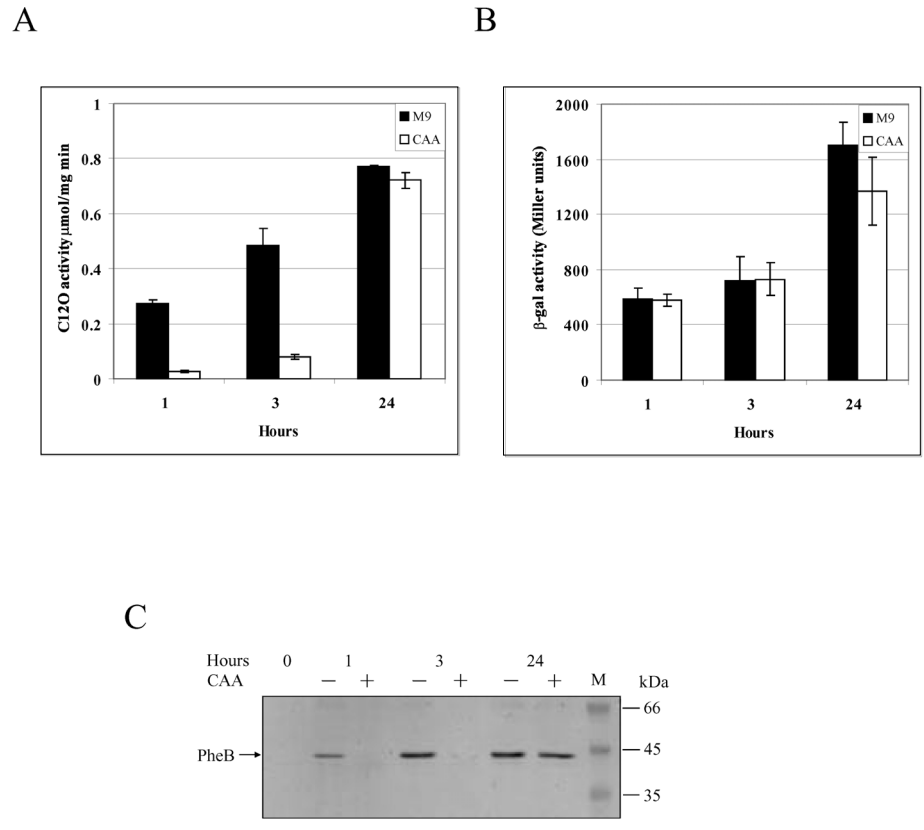

Fig. 3. Effect of growth medium composition on the level of expression of the *pheB*-encoded C12O. **(A)** C12O activity was measured at different time points after the addition of 1 mM IPTG to the growth medium of *P. putida* strain PaWPheB carrying the *pheB* gene under the control of P*tac* promoter in the chromosome. **(B)** β-galactosidase (β-gal) activity measured in *P. putida* PaW85 carrying a single-copy plasmid p9TTtac-lacZ to examine whether the growth media composition would influence level of transcription from the P*tac* promoter. **(C)** Western immunoblot analysis of cellular amounts of C12O in cells sampled from same cultures of PaWPheB that were analysed in **(A).** About 10 µg of clared cell lysate was loaded per lane. Data (means ± standard deviations) of at least three independent experiments are presented in **(A)** and **(B)**.

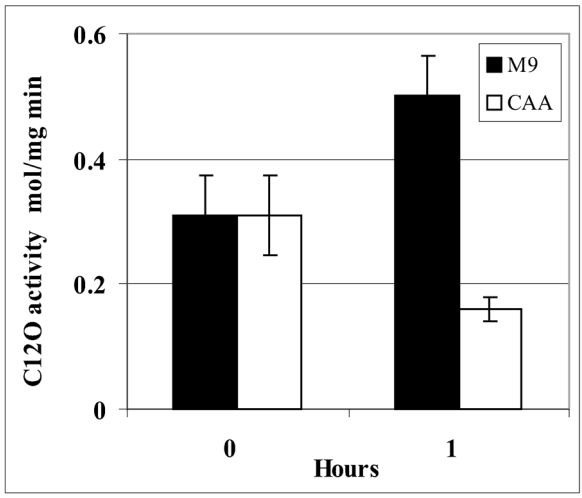

Fig. 4. Study of the effects of growth media composition of bacteria on the stability of *pheB*-encoded C12O in *P. putida*. Cells of PaWPheB expressing the *pheB* gene under the control of IPTG-induced P*tac* promoter were analysed at the zero point (cells grown exponentially in glucose minimal medium supplemented with 1 mM IPTG) and one hour after the dilution of initial culture into two fresh media supplemented or not with amino acids (CAA). Data (means ± standard deviations) of three independent experiments are presented.

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List of publications

1. **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 :173–83.
2. **Ojangu E.L., Tover A., Teras R. and Kivisaar M.** (2000) Effects of combination of different –10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in *Pseudomonas putida*. J. Bacteriol. 182: 6707–13.
3. **Tover A., Ojangu E.L. and Kivisaar M.** (2001) Growth medium composition-determined regulatory mechanisms are superimposed on CatR-mediated transcription from the *pheBA* and *catBCA* promoters in *Pseudomonas putida*.Microbiology. 147: 2149–56.
4. **Saumaa S., Tover A., Kasak L. and Kivisaar M.** (2002) Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and the stationary-phase sigma factor RpoS. J. Bacteriol. 184: 6957–65.
5. **Tegova R., Tover A., Tarassova K., Tark M. and Kivisaar M.** (2004) Involvement of error-prone DNA polymerase IV in stationary-phase mutagenesis in *Pseudomonas putida*. J. Bacteriol. 186 :2735–44.
6. **Tark M., Tover A., Tarassova K., Tegova R., Kivi G., Hõrak R. and Kivisaar M.** TOL plasmid pWW0-encoded DNA polymerase V homologue confers evolutionary fitness on *Pseudomonas putida* under conditions of environmental stress J. Bacteriol. JB00365-05 version 2 (in press).
7. **Putrinš M., Tover A., Tegova R., Saks Ü. and Kivisaar M.** Physiological control mechanisms regulating transcription from the CatR-regulated *pheBA* promoter in *Pseudomonas putida* operate through reduction of the synthesis of the inducer-producing enzyme catechol 1,2-dioxygenase (manuscript).

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Minu huvi teadustöös on seotud kahe teemaga: 1) *Pseudomonas putida* rakkudes toimuvad mutatsiooniprotsessid ja geneetiline adaptatsioon stressitingimustes ning 2) fenooli degradatsiooniks vajaliku *pheBA* operoni regulatsioon *P. putida* rakkudes.

Publikatsioonide nimekiri

1. **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 :173–83.
2. **Ojangu E.L., Tover A., Teras R. and Kivisaar M.** (2000) Effects of combination of different –10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in *Pseudomonas putida*. J. Bacteriol. 182 :6707–13.
3. **Tover A., Ojangu E.L. and Kivisaar M.** (2001) Growth medium composition-determined regulatory mechanisms are superimposed on CatR-mediated transcription from the *pheBA* and *catBCA* promoters in *Pseudomonas putida*. Microbiology. 147 :2149–56.
4. **Saumaa S., Tover A., Kasak L. and Kivisaar M.** (2002) Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and the stationary-phase sigma factor RpoS. J. Bacteriol. 184 :6957–65.
5. **Tegova R., Tover A., Tarassova K., Tark M. and Kivisaar M.** (2004) Involvement of error-prone DNA polymerase IV in stationary-phase mutagenesis in *Pseudomonas putida*. J. Bacteriol. 186 :2735–44.
6. **Tark M., Tover A., Tarassova K., Tegova R., Kivi G., Hõrak R. and Kivisaar M.** TOL plasmid pWW0-encoded DNA polymerase V homologue confers evolutionary fitness on *Pseudomonas putida* under conditions of environmental stress J. Bacteriol. JB00365-05 version 2 (trükis).
7. **Putrinš M., Tover A., Tegova R., Saks Ü. and Kivisaar M.** Physiological control mechanisms regulating transcription from the CatR-regulated *pheBA* promoter in *Pseudomonas putida* operate through reduction of the synthesis of the inducer-producing enzyme catechol 1,2-dioxygenase (manuscript).