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Responses of *Pseudomonas putida* to phenol-induced metabolic and stress signals

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II **Kivistik PA, Putrinš M, Püvi K, Ilves H, Kivisaar M, Hõrak R**: The ColRS two-component system regulates membrane functions and protects *Pseudomonas putida* against phenol. J Bacteriol 2006, 188:8109–8117.

III **Putrinš M, Ilves H, Kivisaar M, Hõrak R**: ColRS two-component system prevents lysis of subpopulation of glucose-grown *Pseudomonas putida*. Environ Microbiol 2008, 10:2886–2893

IV **Putrinš M, Ilves H, Lilje L, Kivisaar M, Hõrak R**: Factors affecting phenol tolerance of *colR*-deficient *P. putida*. Manuscript

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My contribution to journal articles of current dissertation is following:

Ref. I – I planned and performed *in vivo* experiments, participated in construction of plasmids and strains, contributed to writing of the manuscript.

Ref. II – I designed and performed phenol-tolerance experiments, measured *in vivo* promoter activities and participated in writing of the manuscript.

Ref. III – I designed and performed the experiments and wrote the paper.

Ref. IV – I designed and performed the *in vivo* experiments and wrote the paper.

ABBREVATIONS

ABS	activation binding sequence of CatR protein
CCM	*cis,cis*-muconate
c-di-GMP	cyclic di-guanosine monophosphate
CL	cardiolipin
Crc	catabolite repression control protein, an RNA binding protein
ECF	extracytoplasmic functioning family of RNA polymerase sigma subunits
IBS	internal binding sequence of CatR protein
IHF	integration host factor, a global DNA binding protein
IPTG	isopropyl β-D–1-thiogalactopyranoside
LPS	lipopolysaccharide
MCP	methyl-accepting chemotaxis protein
MIC	minimal inhibitory concentration
PCF	pentachlorophenol
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
ppGpp	guanosine tetraphosphate
RBS	recognition binding sequence of CatR protein
RND	resistance-nodulation-cell division family of efflux pumps
TCA	tricarboxylic acid cycle
TCS	two-component signal transduction system

INTRODUCTION

Unlike higher organisms, bacterial cells are in direct contact with carbon and energy sources as well as with variety of noxious compounds. Aromatic hydrocarbons are deleterious to microbes, as they easily dissolve in bacterial cell membrane, inhibiting its function (Sikkema *et al.*, 1995). The consequence of accumulation of an organic solvent in the cell envelope is permeabilization of the membrane, leading to leakage of cellular metabolites and ions (Heipieper *et al.*, 1991; Isken & de Bont, 1998). Besides naturally occurring, mainly plant-derived aromatics, there are many man-made compounds present in the environment. Aromatic compounds consisting of one or more benzene rings are very stable. Therefore, they tend to accumulate in the environment, being a significant and concerning component of the environmental pollution (Diaz, 2004). At the same time, these toxic substances can serve as potential carbon sources for bacteria. However, although microorganisms have acquired the ability to use pollutants as carbon and energy sources which makes them important contributors to natural bioremediation process, the biodegradation efficiency is evolutionarily optimised for bacterial fitness and not for human needs. Therefore, knowledge of mechanisms that influence the tolerance to the aromatic compounds and expression of biodegradation operons is highly valuable for using bacteria at bioremediation of these compounds or as biosensors for *in situ* monitoring of polluted sites. Besides that, this knowledge contributes to the general understanding of functioning of the sophisticated regulatory network enabling bacteria to survive in constantly changing environment.

Pseudomonas putida is a soil-inhabiting bacterium that is able to degrade a wide variety of aromatic compounds (Jimenez *et al.*, 2002). Coordinated expression of plasmid-originated *pheBA* genes and the chromosomal catechol degradation *ortho*-pathway genes *catBCA* enables *P. putida* to degrade phenol and use it as a growth substrate (Kasak *et al.*, 1993; Parsek *et al.*, 1995). Previous studies have revealed that transcriptional activation from the *pheBA* and *catBCA* promoters is repressed in *P. putida* cells growing exponentially in minimal medium in the presence of amino acids despite of the presence of pathway inducer (Tover *et al.*, 2001). Therefore, my first studies were focused on the mechanisms which negatively affect transcription of the phenol degradation operon in *P. putida*.

Different tolerance mechanisms that enable bacteria to live in the presence of a high concentration of aromatic compounds have been thoroughly studied in *P. putida* (Ramos *et al.*, 2002). At the same time, much less is known about sensing and induction of aromatic compounds-mediated stress response. Many adaptive responses of bacteria to environmental changes are controlled by two-component signal transduction systems (Stock *et al.*, 2000). The two-component system ColRS has been previously shown to regulate transposition of Tn*4652* in starving *P. putida* cells since the frequency of appearance of transposition-linked mutants was severely reduced in bacteria lacking the

ColRS system (Hõrak *et al.*, 2004). As the transposition assay was carried out on phenol containing medium, a potential link between mutational processes in the presence of phenol and the ColRS system had been proposed earlier (Hõrak *et al.*, 2004; Ilves, 2006). To be precise, the transposition assay carried out at different phenol concentrations demonstrated that the frequency of transposition in *colR*-deficient strain was partially restored by lowering the phenol concentration in the selective medium (Ilves, 2006). This suggested that the ColRS two-component system might be somehow implicated in phenol tolerance of *P. putida.* Therefore, the second part of my thesis concentrates on the elucidation of the functions of the ColRS system and particularly on its role in phenol tolerance of *P. putida*.

1 REVIEW OF LITERATURE

1.1 Introduction to *Pseudomonas*

Current understanding of the functioning and surviving of bacteria in a complex and constantly changing environment is still far from complete. For historical reasons the most thoroughly investigated bacterium has remained *Escherichia coli*. Nevertheless, it is clear that bacteria living in different environments possess different capacities. To day several organisms from genera *Bacillus, Caulobacter, Pseudomonas* have become model objects, each of them being known for some specific research interest.

Bacterial species of the genus *Pseudomonas* belong to the gamma subclass of the Proteobacteria and are known for their metabolic versatility. These bacteria are involved in many important activities like degradation of xenobiotic compounds, growth promotion of plants and causing diseases in plants and animals. Pseudomonads are widely distributed in different ecological niches and therefore several of them have become model organisms to study adaptation of microbes to environmental stress.

One popular representative of the genus is *P. putida*. Bacteria from this species are present in water and soil and are able to colonize plant roots and seeds (Espinosa-Urgel *et al.*, 2002; Kuiper *et al.*, 2001; Vilchez *et al.*, 2000). Though *P. putida* is mostly non-pathogenic, there are several reports showing that some *P. putida* strains are able to colonize also human (Docquier *et al.*, 2003; Ladhani & Bhutta, 1998; Lombardi *et al.*, 2002; Martino *et al.*, 1996). The different *P. putida* strains have been mostly studied because of their ability to degrade a wide range of noxious organic compounds including chaotropic and aromatic pollutants (Mrozik *et al.*, 2004; Timmis *et al.*, 1994). Partial reason for the versatility of *P. putida* is the presence of multiple plasmids and transposons which carry the genes for hydrocarbon metabolism and antibiotic or heavy metal resistance, and which can be readily exchanged between *Pseudomonas* species (Timmis, 2002). The first fully sequenced strain of *P. putida* was KT2440 (Nelson *et al.*, 2002), which is plasmid-free derivative of strain mt–2 (Williams & Murray, 1974). *P. putida* KT2440 is remarkable also because it is the first Gram-negative soil bacterium to be certified as a safety strain by the Recombinant DNA Advisory Committee (Federal Register, 1982). This strain has been used in studies concerning global and specific transcription regulation, biodegradation, tolerance mechanisms and rizosphere colonization (Espinosa-Urgel *et al.*, 2002; O'Sullivan & O'Gara, 1992).

Comparison of five *Pseudomonas* species *P. entomophila* L48, *P. aeruginosa* PAO1, *P. putida* KT2440, *P. fluorescens* Pf–5 and *P. syringae* pv. tomato DC3000 has identified 2065 genes that are conserved among species and constitute core genome (Vodovar *et al.*, 2006). It means that for *P. putida* KT2440, with genome size of 6182 kb, the number of non-core genes is larger than that of core genes. Moreover, there are 1539 genes that are specific only to

P. putida and that mostly code for proteins with unknown functions. Importantly, there are more than 100 species-specific genes that code for regulatory and signal transduction functions as well as over 100 genes for metabolism and transport proteins (Vodovar *et al.*, 2006). These non-core functions are probably contributing to the fitness and versatility of *P. putida* in its natural habitat. The ability of *P. putida* to adapt to various and changing environmental conditions depends on its capacity to monitor the surrounding and integrate this information to regulatory network responsible for appropriate tuning of the cellular metabolism (Timmis, 2002).

The ability to degrade aromatic hydrocarbons is the most thoroughly studied aspect of *P. putida*. Because of the specific properties of aromatic hydrocarbons the studies of their degradation are tightly coupled with the studies of the mechanisms governing the tolerance to these compounds (Ramos *et al.*, 2002).

1.2 Sensing the environment

For an unicellular organism like bacterium, the ability to sense the environment and to respond to the changes is indispensable for survival. Sensing systems can be simple, consisting of one molecule that receives the signal and performs the response, or they can be complex, consisting of several proteins/RNAs that transduce the signal to their cognate response regulators.

The most simple, so called "one-component", signal sensing and transducing systems are more prevalent in bacteria (Ulrich *et al.*, 2005). The best-known example of simple signal sensing and responding mechanism is LacI protein, a regulator of the *lac* operon, which induces the expression of genes that are necessary for lactose assimilation in the presence of a substrate. Importantly, in this case the signal (substrate) must reach the cytoplasm before it is recognized. Thus, one-component signal transduction systems can receive signals that are synthesized in the cytoplasm or have reached cytoplasm while the extracellular or membrane-affecting signals are mostly sensed by more complex systems.

The same cell membrane that is involved in nutrient uptake and protection of the cell from harmful compounds also senses the environment. It means that in many cases the bacterial cell membrane is not only a stress target but also the first sensor activating a stress response. It has been shown that alterations in the composition of bacterial membrane can affect the transformation of environmental signals into the transcriptional activation of stress genes (Baysse & O'Gara, 2007; Los & Murata, 2004). In membrane the signal can emerge as a result of changes in membrane properties or specific signal molecules can be recognised by membrane-embedded signal transduction proteins.

It is important to keep in mind that the term "signal transduction" is mainly used for the two-component systems (TCSs) that are composed of a sensor histidine kinase and a cognate response regulator. However, the actual picture

of signal transduction systems is much more complicated. There are several signal transduction proteins – histidine kinases, methyl-accepting chemotaxis receptors, Ser/Thr/Tyr protein kinases, adenylate and diguanylate cyclases and c-di-GMP phosphodiesterases – that sense the environment (Galperin, 2005). Emerging data over the last years have also shown the importance of RNA, both non-coding and mRNA, in perception of environmental signals. Many small regulatory RNAs function via antisense mechanism that interferes with translation of target mRNA or interact directly with proteins influencing their performance (Altuvia & Wagner, 2000; Wassarman *et al.*, 1999). There are also many examples of so called "riboswitches", where the 5′ end of mRNA itself can be responsive to environmental stimulus regulating its own half-life or accessibility for ribosomes (Edwards *et al.*, 2007). Multiple extracytoplasmic function (ECF) sigma factors should also be considered as members of signal transduction systems since their activity is in many cases controlled by the membrane-embedded regulatory proteins (Helmann, 2002).

It is clear that in the more complex environment bacteria live, the more complex signalling network they have to encode. Parasitic bacteria that inhabit relatively stable host environments typically encode few signalling proteins while soil bacteria have hundreds of them (Galperin *et al.*, 2001; Galperin, 2004; Konstantinidis & Tiedje, 2004).

Compared to *E. coli*, *P. putida* is much more orientated to sensing of environment. This is illustrated by the fact that there are almost 450 genes coding for transcriptional regulators in *P. putida* while *E. coli* has only 266 (Martinez-Bueno *et al.*, 2002). Another characteristic showing the ability of particular bacterium to sense and to adapt to changing environment is the number of different sigma subunits of RNA polymerase. As each sigma factor is responsible for determining the specific set of promoters to which RNA polymerase binds for transcription to start, then the expression of multiple genes is controlled simultaneously by the level of each sigma factor (Ishihama, 2000). While *E. coli* has only 7 different sigma factors, the number of sigma factors is 24 in *P. putida* (Martinez-Bueno *et al.*, 2002). If we look at the most common devices specialized to the sensing and transducing environmental signals, the two-component systems, then *E. coli* employs 30 different histidine protein kinases and 34 response regulators that modulate various processes such as chemotaxis (CheA/CheY); cell-envelope permeability (EnvZ/OmpR); phosphorus uptake (PhoR/PhoB); oxidative metabolism (ArcA/ArcB), *etc* (Yamamoto *et al.*, 2005). At the same time, there are 68 histidine kinases (Alm *et al.*, 2006) and 75 response regulators (Galperin, 2005) in *P. putida* strain KT2440. It has been proposed that the fraction of signal transducers (TCS, methyl-accepting chemotaxis receptors, Ser/Thr/Tyr protein kinases, adenylate and diguanylate cyclases and c-di-GMP phosphodiesterases) among total proteins can be used as a measure of the organism's ability to adapt to diverse conditions and it has been named the "bacterial IQ", where IQ = 100 corresponds to 9 signal transducers in a 1000 kb genome and to 105 transducers

4

in a 5000 kb genome (Galperin, 2005). According to this sort of calculation, despite the differences in total signal transducers count, the IQ of *P. putida* KT2440 and *E. coli* K12 is quite similar being 116 and 104, respectively (Galperin, 2005).

1.2.1 Two-component systems

Two-component signal transduction systems (TCS) that enable bacteria to elicit an adaptive response to environment are widespread in these organisms. A TCS typically consists of a membrane-located sensor with histidine kinase activity and a cytoplasmic response-protein with receiver domain. Environmental signal sensed by membrane protein is transduced to the response regulator by phosphorylation. Although the basic chemistry of TCSs is quite well understood the nature of the environmental cues sensed by the TCSs has been experimentally verified in very few cases.

We can divide the signal sensing mechanism also in case of TCSs in discrete molecule sensing and indirect sensing of changes in physicochemical properties of cell membrane. Sensing of discrete molecules is a simple and better studied mechanism. Well-studied examples of direct molecule sensing are NarX sensor that binds nitrate/nitrite or CitA sensor for citrate (Janausch *et al.*, 2002; Stewart & Bledsoe, 2003). In a bit more complex situation the discrete molecule is sensed in the periplasm by another protein that after binding the signal molecule transduces the signal to the membrane-embedded sensor protein. One such example is from *Agrobacterium tumefaciens* where periplasmic ChvE protein binds glucose and then interacts with VirA sensor kinase (Shimoda *et al.*, 1993). Most complicated and less studied are the mechanisms where signal is triggered by some physicochemical changes in the membrane. To date the exact type of stimulus for most of TCSs is not known. Nevertheless, there is some possibility to predict what kind of stimulus can be sensed. Namely, the sensor proteins that do not possess large periplasmic domain (this domain contains less than 25 amino acids) are more likely to perceive the signal from the membrane (changes in membrane properties) (Mascher *et al.*, 2006).

Some of the TCSs are implicated in regulation of membrane properties. One of the well-studied examples is PhoPQ two-component system in *Salmonella typhimurium*, which is activated in response to low divalent cation concentrations and as a response regulates over 40 different genetic loci some of which are involved in LPS modification (Guo *et al.*, 1997). It has been shown that lipid-A-remodelling is necessary to produce an outer membrane that serves as effective permeability barrier in an environment that is poor in divalent cations (Murata *et al.*, 2007). On the other hand, the unmodified lipid-A makes the membrane a better barrier in condition of high concentration of cations, which shows the importance of PhoPQ mediated ability to regulate lipid A modifications (Murata *et al.*, 2007).

1.2.1.1 ColRS two-component system

ColRS two-component system is encoded by genes which orthologs are present in genomes of all so far sequenced *Pseudomonas* species (www.pseudomonas.com). Thus, these genes belong to the so-called *Pseudomonas* core genome. Taking into account that ColRS system is present in different pathogenic as well as commensal species one can hypothesize that it must regulate vital functions and sense signals that are common in different habitats. Considering the high conservation of *colRS* genes one can assume that the role of ColRS system is similar in different pseudomonads and therefore I will discuss here the facts that are known from different *Pseudomonas* species all together.

Three basic attempts have been made to elucidate the role of ColRS two-component system. First, several phenotypes of *colR*- or *colS*-deficient strains have been described in *P. fluorescens* and *P. putida* (de Weert *et al.*, 2006; Dekkers *et al.*, 1998; Hu & Zhao, 2007; Hõrak *et al.*, 2004). Secondly, the favoured expression conditions of the *colRS* genes have been reported in *P. putida* and *P. aeruginosa* (Duan *et al.*, 2003; Ramos-Gonzalez *et al.*, 2005). And finally, finding of ColR target genes in *P. putida* and *P. fluorescens* has widened the knowledge of ColRS regulated functions (de Weert *et al.*, 2006; Kivistik *et al.*, Manuscript).

It was first reported in *P. fluorescens* that *colS*-deficient strain is not able to compete with wild-type in root colonization experiments but has no colonization defects in monoculture (Dekkers *et al.*, 1998). It was also shown that *colS*-deficient *P. fluorescens* is more resistant to several antibiotics (rifampicin, spectinomycin, tetracycline) and paraquat but more sensitive to polymyxin B (de Weert *et al.*, 2006). For *P. putida* it has been reported that the *colR*-deficient strain is sensitive to elevated Mn ion concentrations (Hu & Zhao, 2007). In addition, the ColRS system was shown to be implicated in mutagenesis as transposition of Tn*4652* was repressed in starving *colS*- and *colR*-knockouts of *P. putida* (Hõrak *et al.*, 2004).

Two interesting facts are known about transcriptional regulation of the *colRS* genes. First, there is a report about *P. aeruginosa* clinical isolate in which the transcription from the *colRS* promoter was induced when co-cultivated with strains from oropharyngeal flora (Duan *et al.*, 2003). The second study with *P. putida* showed that transcription of the *colS* gene was enhanced during maize root colonization (Ramos-Gonzalez *et al.*, 2005). At the same time, despite the fact that ColRS system is important in Mn-resistance, its promoter is not induced by metal ions (Hu & Zhao, 2007).

Studies of ColRS-regulated genes in *P. fluorescens* have revealed that genes *orf222* and *wapQ* (they encode for hypothetical membrane functions associated proteins, methyltransferase and lipopolysaccharide kinase, respectively) locating just downstream from *colRS* are controlled by ColR (de Weert *et al.*, 2006). Importantly, the same genes are positively controlled by ColR protein also in *P. putida* (Kivistik *et al.*, Manuscript). In fact, the chromosomal location

of the operon next to the *colRS* genes is conserved in pseudomonads (www.pseudomonas.com) suggesting that these genes may be under the control of ColRS system also in other *Pseudomonas* species (Fig. 1). In addition, recent genome-wide search for ColR-regulated promoters in *P. putida* has revealed several new ColR target genes and operons, among them genes coding for the lipid A 3-O-deacylase PagL and diacylglycerol kinase DgkA (Kivistik *et al.*, Manuscript).

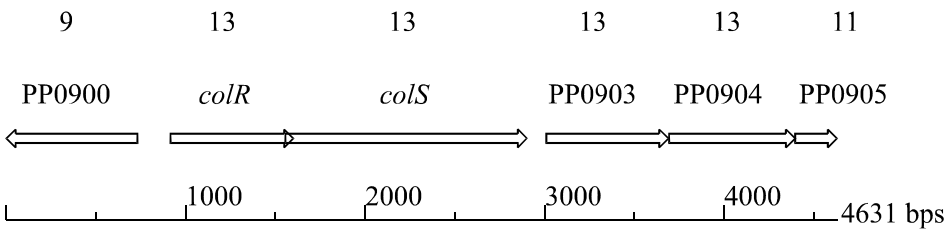

Figure 1. Chromosomal organization of *colRS* operon and adjacent genes in *P. putida* KT2440 genome. Numbers above genes indicate orthologs in the same chromosomal context in 13 different fully sequenced *Pseudomonas* species (www.pseudomonas.com).

The signal(s) sensed by ColS is not known. Nevertheless, some assumptions can be made considering the favoured expression conditions of ColRS-controlled genes. Namely, it has been shown that orthologous genes to PP0903 and PP0904 in *P. aeruginosa* are induced in the presence of antibiotic ceftazidime (Blazquez *et al.*, 2006). The induction of homologs of PP0903 and PP0904 was 5–7 and 4–8 fold, respectively, at low antibiotic concentrations where the growth rate of the cells was not affected (Blazquez *et al.*, 2006). The specific target of ceftazidime is FtsI (penicillin-binding protein 3) which is cytoplasmic membrane-locating protein that regulates cell division (Nakamura *et al.*, 1983). This indicates that some kind of disturbance of cell division could induce the expression of ColR-regulated genes. Altogether the facts up till now point to the regulation of cell membrane and its permeability by ColRS system.

1.3 Aromatic compounds in the environment

Aromatic compounds can be plant-derived or released to the environment as a result of human action, which is especially true for chlorinated hydrocarbons. Aromatic hydrocarbons consist of one or more planar sets of six carbon atoms that are connected by delocalised electrons. This structure is called benzene ring after the simplest aromatic compound benzene. As benzene ring is thermo-dynamically stable then aromatics can accumulate in the environment. In fact,

the benzene ring is one of the most widely distributed units of chemical structures in the nature (Jimenez *et al.*, 2004).

Some of the aromatic compounds are also called organic solvents. One of the best-known solvents is toluene which has characteristic features of an organic solvent – it evaporates easily and can be removed leaving dissolved substance behind. Another widely used term for some aromatic compounds is "xenobiotics", which by strict definition means that these compounds are man-made and unknown to the nature (Top & Springael, 2003). Xenobiotics are for example several polychlorinated phenols which despite their short existence in the environment are already recognised and degraded by some bacteria, thus being interesting research objects also from evolutionary aspect.

1.3.1 Aromatics as toxic compounds

Large amount of environmental aromatic compounds must be avoided because of their toxicity to cellular systems. The toxicity of phenol and other hydrophobic organic solvents depends on their concentration in the environment and on the tolerance level of bacteria. Chaotropic solutes like phenols that weaken electrostatic interactions in biological macromolecules can influence water availability without having a major impact on cell turgor (Hallsworth *et al.*, 2003). Mostly the toxicity of aromatic compounds like toluene, xylenes and phenol depends on their ability to dissolve easily in the cell membrane, disorganizing its structure and impairing vital functions (Dominguez-Cuevas *et al.*, 2006; Ramos *et al.*, 2002; Sikkema *et al.*, 1995). Disruption of membrane integrity affects different membrane functions like being a barrier, energy transducer and matrix for enzymes, and more or less it also affects cell division and DNA replication. The term "homeoviscous/homeophasic adaptation" is used to describe different mechanisms how bacteria actively keep the fluidity of their membrane at suitable level irrespective of the environmental conditions (Hartig *et al.*, 2005). The outer membrane of Gram-negative bacteria is poorly permeable to hydrophobic chemicals and therefore Gram-negative bacteria are slightly more tolerant to aromatics than Gram-positive bacteria (Denyer & Maillard, 2002).

Widely used parameter to describe toxicity of an organic solvent is log P_{ow} which is a logarithm of the partition coefficient of the compound in a standard n-octanol/water system. When log P_{ow} value of a compound is between 1.5 and 4 it is considered toxic because such a compound will partition preferentially in the cell membrane (Aono *et al.*, 2001; Inoue *et al.*, 1991; Segura *et al.*, 2004b). For example, the log P_{ow} value for phenol is 1.5, for benzene 2, for p-xylene 3.1 and for toluene 2.48 (Wery & de Bont, 2004).

1.3.1.1 Tolerance to the aromatic compounds

Tolerance to the organic solvents has several advantages to the bacteria. First, it enables colonization of specific environmental niches. Secondly, some degree of tolerance is a presumption for ability to use such compounds as carbon and energy sources. Metabolically versatile bacteria like Pseudomonads that can metabolise natural and xenobiotic pollutants play a major role in eliminating such pollutants from the environment. Most limiting to the biodegradation capacity can be the sensitivity to the aromatic compounds and therefore it has been an extensive research object. Additionally, unravelling the tolerance mechanisms for aromatic hydrocarbons is important because these are highly analogous to the mechanisms that enable bacteria to tolerate several antibiotics. The tolerance mechanisms of *P. putida* can be divided into three basic classes: those making cell surface more repellent to solvent molecules, restructuring the cell membrane to diminish damaging effects of the solvent and active efflux of the solvent from the cell (Wery & de Bont, 2004) (Fig. 2). Additionally, accumulation of several solutes like betaine, proline and trehalose inside the cell is proposed to participate in tolerance of aromatic compounds (Weber & de Bont, 1996). It has been shown that in solvent-tolerant *Pseudomonas* sp. strain BCNU the trehalose levels increase in the presence of toluene due to the induction of trehalose biosynthetic genes (Park *et al.*, 2007).

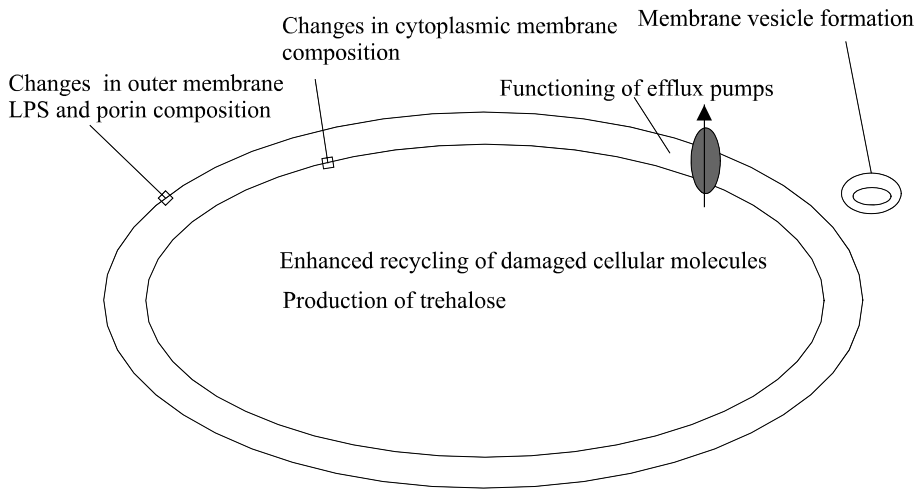

Figure 2. Principal defence strategies of solvent tolerance of bacteria.

Different solvent tolerance mechanisms have been studied using *P. putida* strains like DOT-T1E, S12 and F1 (Heipieper & de Bont, 1994; Huertas *et al.*, 1998; Mosqueda & Ramos, 2000). Bacteria, which are often living in an

environment where concentrations of toxic compounds are constantly changing, usually respond to the changes at the level of gene expression, modulating thereby the levels of relevant proteins. Therefore, vast amount of studies have focused on a bacterial transcription regulation. For instance, it has been shown that bacteria respond to the noxious chemicals by induction of heat shock proteins and by up-regulation of various cellular transporters (Bott & Love, 2001; Muller *et al.*, 2007; Santos *et al.*, 2004). The results of quantitative proteomics have revealed that *P. putida* KT2440 responds to phenol-induced stress by up-regulation of many proteins, e.g., those involved in the oxidative stress response and transport of small molecules (Santos *et al.*, 2004). It is also interesting to note that there is a trade-off between stress response and metabolic capacity. Namely, it has been shown that stress genes are always up-regulated first and only later the genes responsible for biodegradation of stress-inducing compound are induced (Dominguez-Cuevas *et al.*, 2006; Santos *et al.*, 2004; Velazquez *et al.*, 2006).

1.3.1.1.1 Changes in membrane lipid composition

Solvents destabilize the cell membrane and make it more permeable. Therefore, to counteract the effect of solvent different reorganizations occur in the membrane. When bacteria are in contact with membrane-dissolving aromatic compounds it is important to maintain the membrane fluidity within certain limits in order to preserve its functioning. Optimal membrane fluidity is maintained by changing the quantity and composition of lipids, which means changes in phospholipid head groups and linked fatty acids (Heipieper *et al.*, 1992; Sinensky, 1974; Suutari & Laakso, 1994). The first solvent response mechanism is isomerisation of *cis*-unsaturated fatty acids to *trans* isomers (Weber *et al.*, 1994). Unsaturated fatty acids with *cis*-double bond have a kink in the hydrocarbon chains and that results in a relatively high fluidity of a membrane (Keweloh & Heipieper, 1996). The *trans* isomers with their long linear structures, behave more like saturated fatty acids, taking up less volume and creating a more ordered membrane. The regulation of unsaturated fatty acids synthesis in response to the membrane fluidity-affecting hydrocarbons seems to be very complex. For example, proteome analysis has shown that the FabB protein, catalysing the production of unsaturated fatty acids, was over-expressed when *P. putida* cells were exposed to phenol (Santos *et al.*, 2004). Contrary to that Heipieper and co-authors showed that the percentage of unsaturated fatty acids decreases under phenol stress (Heipieper *et al.*, 1992). Besides of *cis-trans* isomerisation of unsaturated fatty acids the second mechanism involves *de novo* biosynthesis of saturated fatty acids and results in replacement of unsaturated fatty acids with saturated ones that pack tightly side-by-side forming a rigid membrane. The third mechanism for changing the fatty acid composition is the synthesis of cyclopropane fatty acids from *cis-*

unsaturated fatty acids, which also makes the cell membrane more rigid (Grogan & Cronan, 1997).

An additional resistance mechanism is changing the head groups of membrane lipids (Weber & de Bont, 1996). In Pseudomonads there are three main phospholipids, the ratio of which is influenced by the growth conditions. Phosphatidylethanolamine (PE) constitutes about 75% of the total phospholipids content and the rest is composed of phosphatidylglycerol (PG) and cardiolipin (CL) (Schweizer & Hofmann, 2004). PG dominates over CL in logarithmic-phase cells whereas CL accumulates in stationary-phase cells (Bernal *et al.*, 2007a; Ramos *et al.*, 1997a). There is evidence that changes in phospholipid head groups are taking place when bacteria are exposed to membrane-damaging compounds. It has been shown that in the presence of toluene or o-xylene the amount of zwitterionic PE molecules decreases and the amount of acidic PG and CL levels increase. These changes compensate the action of solvent to the cell membrane through lowering the membrane fluidity (Pinkart & White, 1997; Ramos *et al.*, 1997a; Ramos *et al.*, 2002).

The combinatory effect of disruption of *cls* and *cti* genes encoding cardiolipin synthase and *cis*/*trans*-isomerase, respectively, has been studied on solvent tolerance in *P. putida* DOT-T1E strain (Bernal *et al.*, 2007b). Mutations in *cls* and *cit* genes have opposite effects on cell membrane fluidity and *cti/cls* double mutant has similar membrane rigidity as the wild-type strain. However, the double mutant is more sensitive to solvents than the wild-type strain because of the impaired functioning of efflux drug transporters, indicating to the complexity and dependency of different resistance mechanisms (Bernal *et al.*, 2007b).

1.3.1.1.2 Extrusion of toxic compound from the cell

Presence of RND (resistance-nodulation-cell division) family efflux pumps has been considered as a main cause of solvent tolerance of bacteria (Poole, 2004; Ramos *et al.*, 1998; Ramos *et al.*, 2002; Segura *et al.*, 2004a). RND family efflux pumps are of great interest also because of their wide substrate range that includes besides aromatic compounds several antibiotics and therefore can be involved in emergency of multidrug resistant pathogens (Poole, 2001). Although there are many efflux pumps that have been studied in detail, there is still no consensus of their real biological function (Lewis, 2001; Schweizer, 2003). For example, in *Escherichia coli* the overexpression of AcrAB-TolC efflux pump caused the cells to reach lower culture density supporting a model in which the natural function of this pump could be the export of some cell-cell communication signal (Yang *et al.*, 2006). At the same time, it has been shown that AcrAB-TolC efflux pump participates in tolerance to the wide variety of lipophilic and amphiphilic compounds, including dyes, detergents, and anti-microbial agents such as ethidium bromide, crystal violet, sodium dodecyl sulfate, bile acids, tetracycline, chloramphenicol, fluoroquinolones, ß-lactams, erythromycin, and fusidic acid (Nikaido, 1998; Nikaido & Zgurskaya, 1999).

The involvement of RND family efflux pumps in solvent tolerance has been documented in many cases. For example, transcription of MexAB-OprM efflux pump-encoding genes is induced when *P. aeruginosa* cells are exposed to pentachlorophenol (PCF). In accordance with that, the PCF MIC for efflux pump deficient strain was fourfold lower than MIC for wild-type (Muller *et al.*, 2007). In *P. putida* strain DOT-T1E three efflux pumps TtgABC, TtgDEF and TtgGHI are responsible for its high resistance to organic solvents (Mosqueda & Ramos, 2000; Ramos *et al.*, 1998; Rojas *et al.*, 2001). For one of the efflux pumps, TtgABC, it has been proposed that its biological function could be the promotion of plant roots colonisation by bacterium as it can expel several plant-produced antimicrobials like phloretin, quercetin, naringenin or coumestrol (Teran *et al.*, 2006).

Besides efflux pumps, another interesting possibility for getting rid of toxic hydrocarbons has been described. Gram-negative bacteria are able to form membrane vesicles that are composed of outer membrane components such as phospholipids, proteins and lipopolysaccharides, but contain also periplasmic components (Beveridge, 1999). In *P. putida* IH-2000, membrane vesicles were found to be released from the outer membrane when toluene was added to the culture (Kobayashi *et al.*, 2000). Importantly, these membrane vesicles contained a higher concentration of toluene than the cell membrane indicating that release of vesicles may be the mechanism for getting rid of a noxious compound (Kobayashi *et al.*, 2000). *E. coli* cells are also able to form membrane vesicles to remove damaged parts of the membrane. While the exact mechanism of membrane vesicles formation is not known, the precipitation of membrane vesicles and analysis of their content suggests that the release of vesicles may be used in different processes like delivery of bacterial toxins, proteins and DNA as well as interspecies communication molecules (Mashburn-Warren & Whiteley, 2006).

1.3.1.2 Induction of stress response by aromatic compounds

Ubiquitous microorganisms such as pseudomonads are able to modulate their gene expression in response to a wide range of environmental stressors enabling successful physiological/biochemical adaptation. While stress response mechanisms in bacteria are well studied, the primary sensors of the stress remain to be elucidated in many cases. It has been shown that when bacteria are exposed to toxic substrates then a long lag phase precedes biodegradation (Balfanz & Rehm, 1991; Li *et al.*, 1991). During lag phase bacteria adapt to the toxic compound and these mechanisms are of great interest for rational design of bacteria for biodegradation. The question is how bacteria sense the presence of noxious compounds and how these signals are integrated into the global transcriptional network for optimal performance of the cell?

The bacterial cell membrane, fluidity and integrity of which is affected by many environmental stressors, is proposed to be a likely candidate to sense and

transduce different stress signals (Baysse & O'Gara, 2007). There are several signal transduction devices situated in the cell membrane that can pass the stress signal further to the cytoplasm. First, several systems have been described that recognize the stressor molecule directly. Secondly, changes in the membrane or periplasmic space properties can be sensed as well.

Direct recognition of toxic molecules occurs, for example, during induction of efflux pumps. Solvent tolerant *P. putida* strain DOT-T1E has three RND family efflux pumps TtgABC, TtgDEF and TtgGHI (Rojas *et al.*, 2001). Expression of these efflux pumps is controlled by repressor proteins TtgR, TtgT and TtgV, respectively (Segura *et al.*, 2004a). The general activation mechanism is mediated by the dissociation of the repressor protein from the promoter of respective efflux pump genes after binding with specific effector molecule. Importantly, not all substrates of these efflux pumps act as effector molecules for transcription induction and *vice versa* (Segura *et al.*, 2004a).

It is clear, that bacteria cannot possess specific recognition devices for every toxic aromatic compound that can be faced in the environment. Therefore, the stress response can be induced not only by the direct recognition of a molecule but also by sensing the harm that the molecule has done. The latter signals can arise, for example, from the changed membrane fluidity, accumulation of damaged proteins or drop in energy charge of cytoplasmic membrane (Darwin, 2005; Raivio, 2005).

It has been previously shown that transcription of *cis-trans* isomerase Cti encoding gene is not induced by membrane stress, indicating that fatty acid isomerisation process activating stress-signal should operate at post-transcriptional level (Junker & Ramos, 1999). Nevertheless, in a recent study moderate up-regulation of *cti* gene expression in response to toluene has been shown (Bernal *et al.*, 2007b). Actually, the primary stress-sensor could be the enzyme itself. For instance, it has been suggested that Cti protein can reach its substrate, the double bond, only when fluidity of the membrane increases because this situation enables deeper penetration of the enzyme into the membrane (Segura *et al.*, 2004a). As described below this kind of universal regulatory mechanism that is independent of recognition of exact stress molecules is also used in cases when signal receiver regulates multiple genes.

The term "extracytoplasmic or envelope stress response" is used to describe stress systems that respond to perturbations in bacterial cell membranes or periplasm. In *E. coli* the response to several extracytoplasmic stresses is mediated by the alternative sigma factor RpoE (Rouviere *et al.*, 1995). The activity of RpoE, as most of ECF sigma factors, is controlled by its cognate membrane-bound anti-sigma factor RseA, which binds RpoE, rendering it inactive (Rowley *et al.*, 2006). The presence of misfolded outer-membrane proteins, that expose the specific motif in their carboxyl end, induce the regulatory cascade which results in degradation of anti-sigma factor RseA (Rowley *et al.*, 2006). The released sigma factor RpoE subsequently binds to the core RNA polymerase and triggers transcription of genes that encode

proteins involved in the folding or degradation of polypeptides in the periplasm as well as genes involved in lipopolysaccharide (LPS) biogenesis and/or modification, and several genes of unknown function (Rowley *et al.*, 2006). This kind of strategy is proposed to prevail in regulation of ECF sigma factors because they are often adjacent to genes encoding for probable anti-sigma factor with an extracytoplasmic sensory domain and an intracellular inhibiting domain (Raivio & Silhavy, 2001). This means that membrane-bound anti-sigma factors are good possible sensors for aromatic compound induced membrane stress.

P. putida encodes altogether 19 ECF sigma factors, of which 13 are potentially involved in iron acquisition (Martinez-Bueno *et al.*, 2002). The function of remaining six ECF sigma factors is unknown, but some of them have been shown to be involved in response to membrane stress. At least one of these ECF sigma factors-encoding genes, PP4553, has been shown to be upregulated in *P. putida* after exposure to *o*-xylene (Dominguez-Cuevas *et al.*, 2006).

The counterpart of *E. coli* ECF sigma factor RpoE in *P. aeruginosa* is AlgT (AlgU or sigma 24) that is regulated analogously by membrane bound anti-sigma factor and respective protease like in *E. coli* (Hershberger *et al.*, 1995; Wood & Ohman, 2006). Amount of AlgU in Pseudomonads is increased in response to various cell envelope stresses as shown by responses of AlgU regulated promoters. For example, an inhibition of peptidoglycan synthesis induces transcription from AlgU controlled *algD* promoter in *P. aeruginosa* (Wood *et al.*, 2006). In *P. putida* the expression of *algD* has shown to be induced by low water availability, but not by osmotic stress (Chang *et al.*, 2007).

Another sigma factor, which is involved in regulation of multiple stress-responsive genes, is RpoH. Besides controlling expression of heat shock genes, it has been shown to enhance transcription from promoters of solvent catabolic genes, such as Pm promoter of toluene degradation "lower" pathway genes on TOL plasmid (Marques *et al.*, 1999). Interestingly, *in silico* analysis of upstream regions of genes encoding proteins that were up-regulated in response to phenol in *P. putida* KT2440 revealed a motif, which matches with the part of RpoH consensus sequence (Mendes *et al.*, 2006). Therefore, it is possible that response to phenol-induced stress might be at least partially controlled by RpoH. Expression of *rpoH* gene itself in *P. aeruginosa* is under the control of AlgU (Potvin *et al.*, 2008). Typical sigma(24)-consensus sequence is found upstream of *rpoH* also in *P. putida*, yet, transcription initiation of the *rpoH* gene did not change in response to sudden heat shock or after the exposure to aromatic compounds (Manzanera *et al.*, 2001). Nevertheless, it has been suggested that the amount of RpoH could be regulated at the level of translation efficiency and/or mRNA half-life (Manzanera *et al.*, 2001).

Recently, involvement of ECF sigma factor RpoT (encoded by PP3007) in solvent tolerance of *P. putida* has been shown (Duque *et al.*, 2007). While RpoT regulon includes about 1% of *P. putida* genes, the main reason of

hypersensitivity of the *rpoT*-deficient strain to organic solvents can be attributed to the several fold lower expression of the toluene efflux pump TtgGHI (Duque *et al.*, 2007). Two flanking open reading frames of *rpoT*, PP3005 encoding for probable anti-sigma factor and PP3007 encoding a periplasmic protein, are the most likely candidates for sensing solvent-stress (Duque *et al.*, 2007).

Among two-component signal transduction systems there is a diverse group of sensor kinases which have the transmembrane helices that appear to play a central role in stimulus perception (Mascher *et al.*, 2006). For example, the activity of CorS sensor kinase is controlled by the membrane fluidity (Smirnova & Ullrich, 2004). Therefore changes in membrane fluidity after the expose to aromatic compounds could possibly be sensed also by some two-component systems.

Other potential stress-mediators are diguanylate cyclases and c-di-GMP phosphodiesterases that are responsible for regulation of the amount of signalling molecule c-di-GMP. Arr protein with two transmembrane domains, a periplasmic domain that could recognize an environmental stimulus, and a cytoplasmic domain having c-di-GMP phosphodiesterase activity has been studied in *E. coli*. It has been shown that antibiotic tobramycine enhances by unknown mechanism phosphodiesterase activity of the Arr leading to c-di-GMP degradation and augmented biofilm formation (Hoffman *et al.*, 2005). The fact that *P. putida* encodes totally 39 enzymes involved in synthesis or degradation of c-di-GMP of which many are membrane-bound (Galperin, 2005) makes them likely but so far unexplored candidates for sensing the aromatic compounds-triggered stress.

1.3.2 Aromatics as growth substrates

The biodegradation of aromatic compounds can actually be seen also as one of the resistance mechanisms because most of the aromatic compounds can be degraded to the non-harmful end products, thus lowering the concentration of toxicant in the environment. Nevertheless, generally biodegradation capacity has been considered as the least important tolerance mechanism. For example, *P. putida* DOT-T1E is a toluene-tolerant strain that degrades toluene via the toluene-dioxygenase (Tod) pathway. However, mutants unable to metabolise toluene are as tolerant as wild-type to that compound (Mosqueda *et al.*, 1999).

The most difficult step in degradation of aromatic compounds is the cleavage of benzene ring. In aerobic degradation at first the benzene ring is oxygenated resulting in formation of dihydroxylated benzene ring. The next step is ring fission that is catalysed by dioxygenases. If the benzene ring is cleaved between the hydroxyl groups, then the reaction is part of *ortho*-pathway. In case of *meta*-pathways the ring cleavage occurs adjacent to one of the hydroxyl groups (Harwood & Parales, 1996). Usually the *ortho*- and *meta*-

pathways do not co-exist to avoid the unwanted toxic degradation products (Timmis *et al.*, 1994).

P. putida has been a widely used model organism in biodegradation studies because of its ability to degrade a wide variety of pollutants (van der Meer, 2006). The parental strain of *P. putida* KT2440, the strain mt-2 was isolated from soil. Therefore, its capacity to degrade several plant-derived aromatic hydrocarbons is not surprising. The ability to degrade different compounds depends not only on chromosomal pathways but also on biodegradation genes present on extrachromosomal DNA (Timmis, 2002; van der Meer, 2006).

1.3.2.1 Biodegradation pathways

The catabolism of aromatic compounds can be divided into two stages. First, the diverse substrates are channelled through peripheral pathways into a limited number of common intermediates. Secondly, these structurally simple intermediates are further processed by a few central pathways to Krebs cycle compounds (Harwood & Parales, 1996; Jimenez *et al.*, 2002).

P. putida strain KT2440 encodes three central pathways for the catabolism of aromatic compounds (Jimenez *et al.*, 2004). Most important is the *β*-ketoadipate pathway, which has protocatechuate and catechol branch (Harwood & Parales, 1996). In addition, there are also the phenylacetate-CoA and the homogentisate pathways. Protocatechuate, for example, is degradation intermediate of 4-hydroxybenzoate and several lignin monomers while catechol is common intermediate in benzoate or phenol degradation (Fig. 3). It is important to note that the ability to degrade aromatic compounds is strain-specific. For example, *P. putida* strain U encodes homoprotocatechuate pathway which is absent in KT2440 (Jimenez *et al.*, 2004).

The catabolic capacity is significantly widened by the catabolic operons present on extrachromosomal DNA. One well-known example of expanded catabolic capacity is toluene degradation by plasmid-encoded operons. The TOL plasmid pWW0 carries *xyl* operons encoding enzymes for aerobic degradation of *m*-, *p*-xylene and toluene as well as regulatory proteins (Assinder & Williams, 1990). The *xyl* genes are clustered into two operons named upper and *meta* pathway operons, the expression of which is controlled by XylR and XylS regulatory proteins, respectively (Ramos *et al.*, 1997b). Other characterized TOL plasmids have conserved gene order and regulatory proteins, yet, the number of operons and regulatory genes may vary (Sentchilo *et al.*, 2000).

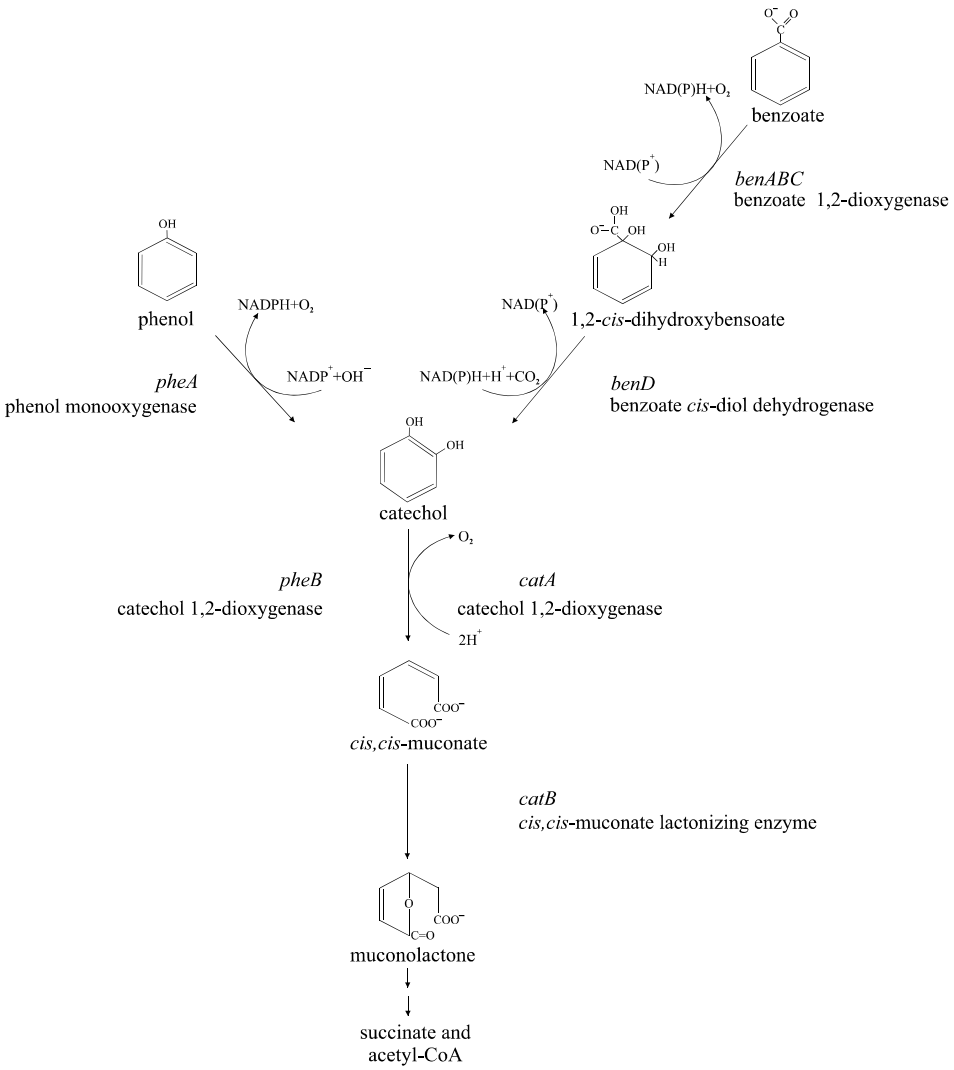

Figure 3. The β-ketoadipate pathway by which phenol and benzoate are converted to catechol and to the common metabolic intermediates succinate and acetyl-CoA.

The biodegradation operons have served as research objects not only because of the biodegradation capacity they encode but also as model systems for understanding the evolution of catabolic pathways (van der Meer & Sentchilo, 2003). For example, even if the exposure of bacteria to compounds like benzene, toluene, ethylbenzene and xylenes has disturbed many microbial communities, it has probably also selected for bacteria with more efficient metabolic properties (Diaz, 2004; van der Meer, 2006).

1.3.2.2 Specific regulation of transcription of biodegradation operons

The expression of catabolic operons is mostly regulated by so called one-component signal transduction systems. It is common that the gene located next to the biodegradation operon encodes the regulatory protein. Often, the regulatory and catabolic genes are divergently transcribed. The transcriptional activation of biodegradation operons usually needs a specific inducer molecule (the substrate or intermediate of the pathway) that binds to the regulator (Parsek *et al.*, 1996).

The regulatory proteins of biodegradation operons can be divided by their mode of action to repressors or activators. Most commonly, the transcription of biodegradation genes is activated by LysR-type transcriptional regulators (LTTR), which comprise the largest family of prokaryotic regulatory proteins identified so far (Schell, 1993). Due to their tetrameric form, LTTRs interact with several sites on the DNA of the promoter region (Tropel & van der Meer, 2004). Usually there are two distinct binding sites, RBS (recognition binding site) and ABS (activation binding site), which have different binding affinities for regulatory protein. Contacts of the regulator to the ABS are supposed to be necessary for mediating interactions with RNA polymerase (McFall *et al.*, 1998).

Besides LysR-type regulators, regulatory proteins of IclR and AraC/XylS family are also frequently involved in expression of catabolic operons. Expression of the XylS-type regulator encoding genes is controlled in many cases by other activators or cascades. For instance, in case of well-studied XylS the expression of *xylS* gene is strongly influenced by another regulatory protein, XylR. The regulator of *xyl* upper pathway XylR stimulates *xylS* transcription from a sigma 54-dependent promoter when cells are grown on xylenes, while in the absence of suitable aromatic inducers, the *xylS* gene is expressed at low constitutive level from another, sigma 70-dependent promoter (Gallegos *et al.*, 1996). This kind of regulation enables to coordinate the expression of lower and upper pathway of xylene and toluene degradation genes.

Another quite common type of regulation includes proteins from XylR/NtrC family. Differently from the above-described regulatory proteins which mostly control transcription from sigma 70-dependent promoters, XylR/NtrC-type regulators activate the transcription of RNAP containing the alternative sigma factor sigma 54. Another difference is that interaction between sigma 54-bound RNAP and the regulatory protein is often facilitated by a bend in the intervening DNA, which can be created by binding of integration host factor (Perez-Martin *et al.*, 1994).

Some of transcriptional regulators of catabolic operons can also act as repressors. For example, the PaaX protein is a repressor of operon coding for enzymes for degradation of phenylacetic acid, which is an intermediate of styrene degradation (Ferrandez *et al.*, 2000). Another examples of GntR-family repressor proteins are PhcS that represses transcription from genes coding for phenol metabolism in *Comamonas testosteroni* (Teramoto *et al.*, 2001) and BphS that regulates expression of biphenyl degradation operon in *Ralstonia*

eutropha (Mouz *et al.*, 1999). Importantly, such kind of regulation is in most cases accompanied also with another regulatory protein capable of activating the transcription (Tropel & van der Meer, 2004).

Additionally, a number of two-component regulatory systems are described that control the expression of catabolic pathways. For example, in *P. putida* TodST controls the expression of genes involved in the degradation of toluene, benzene, and ethylbenzene via the toluene dioxygenase pathway. The catabolic genes of the toluene dioxygenase pathway are transcribed from a single promoter called PtodX once the response regulator TodT is phosphorylated by the TodS sensor kinase in response to pathway substrates (Busch *et al.*, 2007). StyRS TCS that is found in different *Pseudomonas* species regulates operon that is responsible for degradation of styrene to phenylacetate. Transcription from respective promoter is induced by styrene and repressed by phenylacetate (Leoni *et al.*, 2003; Leoni *et al.*, 2005).

1.3.2.3 Optimal performance of biodegradation operons

The presence of biodegradation operons and respective substrate compound itself does not mean that these enzymes are expressed as well and that biodegradation takes place. There are many limiting factors like a) a low concentration of inducer compound, b) the toxicity of a compound or its degradation products, c) the availability of suitable terminal electron acceptors, d) a presence of alternative carbon sources (Cases & de Lorenzo, 2005; Shingler, 2003). Thus, bacteria have to sense different environmental as well as intracellular parameters and to integrate these signals to optimise metabolic performance (Fig. 4).

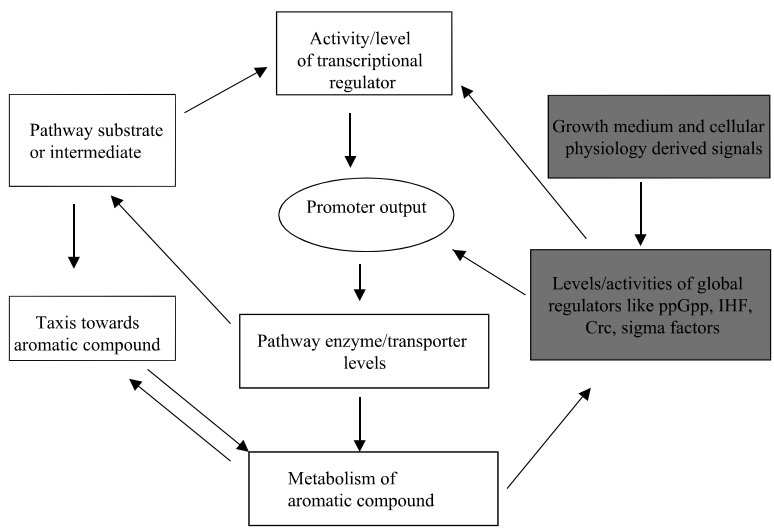

Figure 4. Specific (white boxes) and superimposed (grey boxes) regulation of transcription of biodegradation operons (adapted from Shingler, 2003).

1.3.2.3.1 Taxis towards aromatic compounds

The efficient use of aromatic compounds as carbon and energy sources requires also the ability of bacteria to move towards the catabolizable compound. Taxis towards aromatic compounds is not extensively studied but few examples, discussed below, show that it is a complex process and different mechanisms are involved in case of different compounds.

The ability to sense the concentration and to move in a chemical gradient is called chemotaxis. Movement (swimming) towards the desired concentration of a compound is achieved by changes in flagella rotation – the cells tumble less frequently when they are moving towards higher concentrations of attractants or lower concentrations of repellents, and conversely, they tumble more frequently when moving in disadvantageous directions. The well-described mechanism of signal sensing in *E. coli* is a variant of the TCS and involves a specific ligand-binding transmembrane receptor MCP (methyl-accepting chemotaxis protein). The signal is thereafter transmitted across the membrane to the histidine kinase CheA and further via the phosphorylation to the cognate response regulator CheY that controls the functioning of bacterial flagella motor (Bren & Eisenbach, 2000). The chemotaxis of soil bacteria towards aromatic compounds has been described in several studies. *P. putida* chemotaxis to naphthalene is mediated by MCP-like NahY protein that is encoded along with naphthalene degradation *nah* operon (Grimm, Harwood 1999). In some cases the MCP has not been identified. For example, besides toluene *P. putida* F1 is chemotactic also for non-metabolizable substrates like trichloroethylene by an unidentified mechanism that requires regulators of toluene degradation pathway, TodST TCS (Parales *et al.*, 2000). There have also been studies indicating that taxis depends on a transport of specific aromatic hydrocarbon. For instance, chemotaxis towards 4-hydroxybenzoate requires PcaK, a major facilitator superfamily transporter protein (Harwood *et al.*, 1994).

In contrast to the chemotaxis when the metabolism of the specific compound is not needed, there are also types of taxis which require metabolism of the attractant (Alexandre & Zhulin, 2001). It is clear that if the range of different metabolizable substrates is wide then it is expensive for bacteria to have a specific chemotaxis sensor for each potential carbon source. Therefore, it is not surprising that the mechanisms, where bacteria do not recognize the substrate *per se* but rather the change in cellular energy level resulting from metabolism of the compound, have been described. This type of chemotaxis is similar to aero-, photo- or redox-taxis in which the cognate signal transduction systems detect changes in the rate of electron transport or related parameters and is also known as energy taxis (Alexandre & Zhulin, 2001). It is important to stress that in case of aerobically degradable substrate the energy taxis composes of both chemotaxis and aerotaxis allowing bacteria to move to the location where the concentration of both oxygen and carbon source is optimal. In *E. coli* there are two redundant receptors Aer and Tsr that monitor intracellular energy levels. Aer protein mediates the response to oxygen as well as metabolism-

dependent taxis towards succinate, glycerol, ribose, galactose, maltose, malate, proline, alanine and some of the substrates of the phosphotransferase system (Bibikov *et al.*, 1997; Greer-Phillips *et al.*, 2003; Zhulin *et al.*, 1997). In a recent study Sarand and co-workers have shown that *P. putida* exhibits energy taxis towards different (methyl)phenols. This taxis is shown to be metabolism-dependent and includes only those compounds that can be degraded by the *P. putida* strain KT2440 carrying a plasmid expressing the dimethylphenols degradation *dmp* pathway (Sarand *et al.*, 2008). Loss of PP2111, one of the four genes of *P. putida* that possesses similarity to the *E. coli aer* gene results in reduced taxis towards (methyl)phenols and inactivation of histidine autokinase *cheA* gene (PP4338) abolishes the taxis completely (Sarand *et al.*, 2008).

In my opinion the described energy taxis mechanism could be most useful in case of potentially toxic carbon source like phenol and serve also as a stress response mechanism. Namely, at higher concentrations phenol can be so toxic that it lowers the energetic status of the cells and energy taxis could enable bacteria to move to the more optimal concentrations.

1.3.2.3.2 Superimposed regulation of transcription of biodegradation operons

From human point of view the degradation of aromatic compounds is always favoured. For bacteria the strong expression of biodegradation genes is not always optimal for their fitness and therefore the expression of biodegradation genes is often abolished despite the presence of pathway substrate. The phenomenon when the presence of one carbon source represses utilization of another has been called "catabolite repression". Catabolite repression has been thoroughly studied in enterobacteria where it is mediated by signal molecule cAMP and the preferred carbon source is glucose (Collier *et al.*, 1996). In *Pseudomonas* species the preferred carbon sources are different organic acids rather than glucose. Another difference from enterobacteria is the fact that there is no single signal molecule that mediates catabolite repression in *Pseudomonas* (Collier *et al.*, 1996). Importantly, in *Pseudomonas* the term "catabolite repression" is used also when simultaneous consumption of two alternative carbon sources takes place. For example, glucose has slight repressive effect on the expression of toluene degradation pathway and at the same time toluene represses somewhat glucose metabolism, which means that both carbon sources elicit catabolite repression over each other (del Castillo & Ramos, 2007). Another term that has been used to describe regulation of biodegradation operons by alternative growth substrates is "exponential silencing", which is defined as a lack of activity of promoters of biodegradation operons when cells grow exponentially in a rich medium (Cases *et al.*, 1996). It has been shown that regulatory mechanisms controlling transcription from Pu promoter of toluene degradation upper pathway are different in the presence of alternative carbon source glucose in minimal medium (catabolite repression) or during exponential growth phase at rich medium (Cases & de Lorenzo, 2000). However, it is possible that in some cases the carbon source and

growth phase dependent repression of biodegradation operons is achieved through same mechanisms. Collectively the absence of induction of biodegradation operon in the presence of alternative carbon source can be referred to as superimposed regulation.

Optimal expression of biodegradation operons is mostly acquired at the level of gene transcription. As elegantly stated by Cases and de Lorenzo there is a white cat/black cat principle meaning that regulatory mechanisms controlling optimal expression of biodegradation operons may be diverse but importantly they all fulfil their function (Cases & de Lorenzo, 2001). As evolution of biodegradation operons has taken place in different ways then it is logical that the novel biodegradation genes are connected to the physiological and metabolic state of the cell in different ways. Therefore, there are superimposed regulation mechanisms that are common to different type of promoters of biodegradation operons and also unique mechanisms, described at least so far only for some specific operons. An example of very unusual mechanism of catabolite repression is regulation of *clc* operon where TCA intermediate fumarate directly inhibits transcriptional activation of *clc* promoter by competing with inducer 2-chloromuconate for binding to the ClcR activator (McFall *et al.*, 1997).

Several global factors such as the Crc protein (Hester *et al.*, 2000b; Morales *et al.*, 2004; Yuste & Rojo, 2001), the CyoB (Dinamarca *et al.*, 2002; Petruschka *et al.*, 2001), the FtsH protease (Carmona *et al.*, 1999; Sze *et al.*, 2002) and signalling molecule ppGpp (Sze & Shingler, 1999; Sze *et al.*, 2002) have been related to superimposed regulation of more than one biodegradation pathway in *P. putida*.

While generally it is hard to predict the superimposed regulation mechanisms for an unstudied promoter, still some assumptions can be made for promoters that are transcribed by alternative sigma factors. The impact of a particular sigma factor on the expression level of genes belonging to its regulon depends not only on the amount of specific sigma factor but also on the level of other sigma factors as they compete over the core RNA polymerase. Therefore, catabolic operons can be connected to the global transcriptional network through sigma factors (Cases & de Lorenzo, 2001). For example, it has been shown that overexpression of sigma 54 (RpoN) completely abolished superimposed regulation of toluene degradation upper pathway *xyl* genes (Cases *et al.*, 1996). Expression of lower pathway of toluene degradation is also controlled by alternative sigma factors. In exponential growth phase the expression of *xyl meta*-pathway is controlled by heat shock sigma factor RpoH and in stationary phase by RpoS (Marques *et al.*, 1999). An important player in sigma factor competition is alarmone ppGpp because it is able to bind to the core RNA polymerase and enhance its binding with different alternative sigma factors like RpoS and RpoN (Jishage *et al.*, 2002; Laurie *et al.*, 2003). The level of ppGpp, which is controlled by RelA and SpoT proteins, is low during exponential growth phase in rich medium but increases significantly when the amount of amino acids becomes limiting (Schreiber *et al.*,

1991). It has been shown that the exponential silencing of RpoN-controlled phenol degradation *dmp* operon is strongly relieved by artificial overexpression of ppGpp (Sze & Shingler, 1999).

Signal molecule ppGpp has also other effects. It has been shown that binding of ppGpp to the core RNA polymerase affects open complex stability and therefore transcription from specific promoters (Toulokhonov *et al.*, 2001). Overexpression of ppGpp has a positive effect on the level of IHF and this in turn influences positively the transcription from most RpoN-dependent promoters (Bertoni *et al.*, 1998; Carmona *et al.*, 2000). Additionally, positive effect of ppGpp on the level of RpoS has also been reported (Bertani *et al.*, 2003).

As described in previous section, the changes in energetic status of the cell are monitored and used as a signal. This kind of mechanism is probably used also for transcriptional regulation of catabolic operons. For instance, inactivation of cytochrome *o* ubiquinole oxidase (the main oxidase under high-energy conditions) protein CyoB in *P. putida* relieved succinate-caused catabolite repression of phenol degradation *phl* operon (Petruschka *et al.*, 2001). Also, the exponential silencing of alkane degradation *alk* operon was relieved in the absence of functional CyoB (Dinamarca *et al.,* 2001).

Another interesting mechanism, mediating catabolite repression in *P. putida*, involves PtsN and PtsO proteins, whose exact biological role is still unclear (Cases *et al.*, 1999; Cases *et al.*, 2001). PtsN seems to somehow regulate metabolic flux as a *ptsN*-deficient *P. putida* was observed to accumulate polyhydroxyalkanoates which are typical products of carbon overflow (Velazquez *et al.*, 2007). Although the exact mechanisms are unknown, the repression of toluene degradation *xyl* operon by glucose is signalised by 2-dehydro-3-deoxygluconate-6-phosphate and the effective repression requires a functional PtsN protein (del Castillo & Ramos, 2007).

One of the best-studied global regulatory proteins implicated in regulation of several biodegradation operons is Crc (catabolite repression control). Crc has been shown to modulate negatively expression of several chromosomal *P. putida* genes involved in aromatic compound degradation like *hpd* and *hmgA* from the homogentisate pathway, *benA* and *catBCA* from catechol pathway and *pobA* and *pcaHG* from the protocatechuate pathway (Morales *et al.*, 2004). In addition, Crc regulates negatively also TOL plasmid-encoded *xyl* pathway (Aranda-Olmedo *et al.*, 2005). Crc also exerts a strong repression on the induction of the *P. putida bkd* operon for branched-chain keto acid degradation (Hester *et al.*, 2000a; Hester *et al.*, 2000b) and on the alkane degradation pathway *alk* genes encoded by the OCT plasmid (Yuste & Rojo, 2001). The effect of Crc on the transcription of catabolic operon is indirect because it is not a DNA binding protein. The exact mechanism of Crc in the regulation of expression of catabolic operons has been verified only in case of *alk* and *ben* operons (Moreno *et al.*, 2007; Moreno & Rojo, 2008). Crc affects the amount of regulatory proteins AlkS and BenR by binding to their mRNA in a way that hinders the access of ribosomes to the RBS, therefore impeding translation

initiation (Moreno *et al.*, 2007; Moreno & Rojo, 2008). Similar mechanism is proposed also for the regulation of *bkd* operon where Crc affects negatively the amount of regulatory protein BkdR (Hester *et al.*, 2000b). The effect of Crc can be indirect as well. For example, during repression of *ben* operon, which encodes peripheral pathway of benzoate degradation, also the central pathway *cat* genes are repressed because of the decreased concentration of respective transcription inducer molecule (Moreno & Rojo, 2008). The negative effect of Crc protein on the expression of toluene degradation *xyl* pathway in rich growth medium is also probably indirect (Aranda-Olmedo *et al.*, 2005).

It has been shown that the level of Crc is higher under the conditions where its repressive effect has been reported (Ruiz-Manzano *et al.*, 2005). However, in case of artificial expression of Crc protein over the certain level the inhibitory effect of Crc is reduced or even lost (Ruiz-Manzano *et al.*, 2005). Therefore, additional mechanisms must influence the inhibitory effect of Crc protein in addition to its cellular levels. The mechanism by which activity of Crc protein is modulated is currently unknown, but involvement of small regulatory RNA-s has been suggested (Moreno & Rojo, 2008). So, even for some well-studied promoters of biodegradation operons there are still much to discover in the field of superimposed regulation.

1.3.2.4 Regulation of expression of *pheBA* and *catBCA* operons

The phenol degradation genes *pheBA* originate from the multiplasmid *Pseudomonas sp.* strain EST1001 (Kivisaar *et al.*, 1989). Coordinated expression of the introduced/plasmid encoded *pheBA* genes and the chromosomal catechol degradation *ortho*-pathway genes *catBCA* enables *P. putida* strain PaW85 to degrade phenol and use it as a growth substrate (Kasak *et al.*, 1993; Parsek *et al.*, 1995). It has been shown that *pheB* encodes for catechol 1,2-dioxygenase and *pheA* for phenol monooxygenase (Kivisaar *et al.*, 1991; Nurk *et al.*, 1991). Catechol degradation is a part of *β*-ketoadipate pathway and carried out by *catBCA* operon encoded catechol 1,2-dioxygenase (C12O), *cis,cis*-muconate lactonizing enzyme and muconolactone isomerase (Houghton *et al.*, 1995). The transcription of the *catBCA* operon is induced by the LysR family transcriptional activator CatR and an inducer molecule *cis,cis*-muconate (CCM) that is intermediate of the catechol degradation (Rothmel *et al.*, 1990; Rothmel *et al.*, 1991). Expression of *pheBA* operon is also controlled by the chromosomally encoded CatR protein (Kasak *et al.*, 1993; Parsek *et al.*, 1995; Tover *et al.*, 2000).

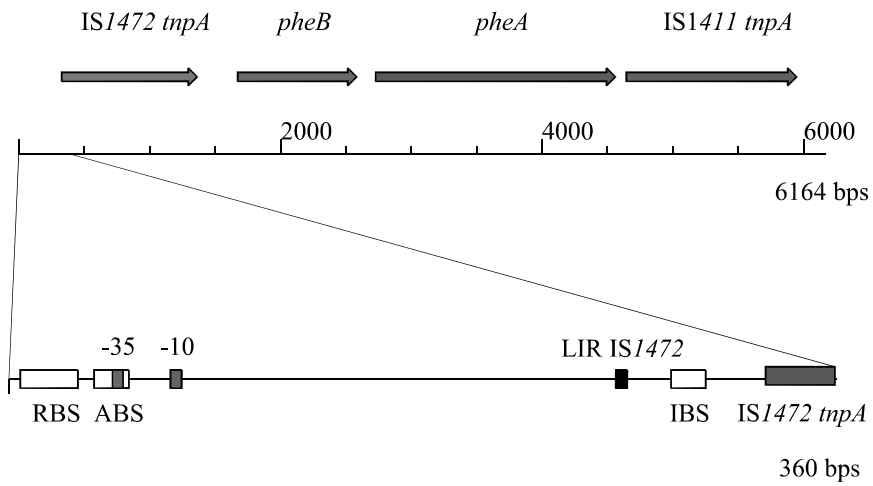

Figure 5. Organization of *pheBA* operon and its promoter region. The genes *pheB* and *pheA* are flanked by two IS elements, IS*1472* and IS*1411*. The promoter of the *pheBA* operon is located upstream of IS*1472* and shown in detail in lower graph. The open boxes designate CatR binding sites RBS, ABS and IBS. The sigma 70 recognized -35 and -10 hexamers of the promoter are indicated with grey boxes. The left inverted repeat (LIR) of IS*1472* is marked by black box.

The binding of CatR to the *pheBA* and *catBCA* promoters is very similar (Chugani *et al.*, 1997; Parsek *et al.*, 1995). Both operons have three CatR binding sites, two of them upstream and one downstream of the transcription start site (Chugani *et al.*, 1998; Tover *et al.*, 2000) (Fig. 5). In the absence of inducer CCM, CatR binds to the high affinity recognition (or repression) binding site (RBS). CatR binding to the second, activation binding site (ABS), is cooperative, requiring the presence of an intact RBS and takes place only in the presence of CCM (Kasak *et al.*, 1993; Parsek *et al.*, 1992; Parsek *et al.*, 1995). The ABS overlaps with –35 hexamer of RNA polymerase sigma 70 subunit recognition sequence and the transcription initiation from the *pheBA* and *catBCA* promoters is mediated by interaction of ABS-bound CatR protein with C-terminus of RNA polymerase α subunit (Chugani *et al.*, 1997; Parsek *et al.*, 1992; Parsek *et al.*, 1995). The third CatR binding site IBS (internal binding site) acts as a repressor region (Chugani *et al.*, 1998; Tover *et al.*, 2000). In case of the *catBCA* operon the IBS is located in the open reading frame of *catB* (Chugani *et al.*, 1998). The IBS of the *pheBA* operon is located between the left inverted repeat (LIR) and the transposase gene *tnpA* of the IS element IS*1472* which is located between the *pheBA* genes and their promoter (Parsek *et al.*, 1996; Tover *et al.*, 2000) (Fig. 5). It has been proposed that IBS occupation by CatR occurs only at high CCM levels, therefore enabling to lower the expression levels of *pheBA* and *catBCA* operons after the optimal enzyme levels have been reached (Chugani *et al.*, 1998; Tover *et al.*, 2000). It has been

previously shown that besides specific regulation by the presence of inducing carbon source also growth medium composition regulates expression of the *pheBA* and *catBCA* operons (Tover *et al.*, 2001). While addition of benzoate, which is metabolised to the CCM, into minimal growth medium of *P. putida* leads to rapid induction of transcription from the *pheBA* and *catBCA* promoters, then transcription was severely impaired in exponentially growing bacteria when amino acids were present in the growth medium (Tover *et al.*, 2001). Although the general regulation mechanisms are identical for the *pheBA* and *catBCA* promoters there are also some differences. The RBS sequence of the *catBCA* promoter contains G-N_{11}-A motif instead of the consensus T-N_{11}-A of the LysR family binding motifs that is present in *pheBA* RBS. Therefore, CatR binds to the *pheBA* promoter with higher affinity than to the *catBCA* promoter (Parsek *et al.*, 1995). The differences are also in the RNA polymerase sigma factor recruitment. *In vivo* transcription from *catBCA* promoter is mediated by sigma 70 (RpoD) and is not affected by sigma S (RpoS) (Tover *et al.*, 2001). At the same time, the activity of *pheBA* promoter remains approximately threefold lower in the RpoS-deficient strain than in the wild-type strain in stationary phase cultures (Tover *et al.*, 2001). Also, the exponential silencing of the *pheBA* promoter in the presence of amino acids is stronger than that of the *catBCA* promoter (Tover *et al.*, 2001). These slight differences most probably reflect evolutionary optimisation of promoter regions to enable most advantageous enzyme levels for biodegradation.

It is remarkable that the *pheBA* gene cluster is flanked by transposable elements IS*1472* and IS*1411* (Fig. 5) which could facilitate the movement of these genes from one DNA molecule to another (Kallastu *et al.*, 1998; Kasak *et al.*, 1993). Indeed, it has been shown that after the release of the laboratory *P. putida* strain carrying the *pheBA* genes on a plasmid into a phenol-contaminated mining area in Estonia, a horizontal transfer of the *pheBA* operon and its expression in different soil bacteria was observed (Peters *et al.*, 1997). In most cases, additional mobile elements or genes encoding transposases were found nearby to the acquired *pheBA* operon, indicating that transposition-mediated transfer is the most probable distribution mechanism of the *pheBA* operon (Peters *et al.*, 1997; Peters *et al.*, 2004). During the horizontal transfer, the *pheBA* genes were either integrated into other plasmids or chromosome but despite of several rearrangements, still, in eight cases out of nine the original promoter was present upstream of *pheBA* genes (Peters *et al.*, 1997; Peters *et al.*, 2004). Thus, soil bacteria carrying the *ortho*-pathway genes regulated by CatR may easily expand their substrate range via horizontal transfer of the *pheBA* gene cluster which carries regulatory elements for activation of the *pheBA* operon as well as for silencing of it in the presence of more favourable carbon source.

2 AIMS OF THE STUDY

Biodegradable aromatic compounds are of interest because they can elicit different types of signals simultaneously. In bacteria that are able to metabolise these compounds, they signal for the presence of a carbon source. At the same time, depending on the exact structure of the chemical and its local concentration, many aromatic compounds can be deleterious to microorganisms and therefore they can induce stress response as well. In my studies I have used *P. putida* strain PaW85 as a model organism and focused on investigation of its responses to signals caused by phenol.

P. putida is able to use phenol as a sole carbon and energy source after introduction of the *pheBA* operon. The molecular mechanisms of positive control of the plasmid-originated *pheBA* operon have been studied in detail (Kasak *et al.*, 1993; Parsek *et al.*, 1995; Tover *et al.*, 2000; Tover *et al.*, 2001). Nevertheless, the mechanism by which the expression of phenol degradation genes is abolished in bacteria growing exponentially in rich medium (Tover *et al.*, 2001) has remained unclear.

Phenol dissolves easily in bacterial cell membrane and is therefore toxic for *P. putida*. Certain changes in membrane lipid composition reduce membrane fluidity and this is one of the ways allowing bacteria to adapt and to tolerate higher concentrations of phenol (Heipieper *et al.*, 1992; Ramos *et al.*, 1998). However, much less is known about other mechanisms of phenol tolerance, about how the toxicity of this aromatic compound is recognized and how the tolerance mechanisms are induced. Potential link between the mutational processes in bacteria in the presence of phenol and two-component signal transduction system ColRS has been previously proposed (Hõrak *et al.*, 2004; Ilves, 2006). Therefore, investigation of the ColRS system seemed to be promising also in respect of understanding of how the response to phenol is evoked in *P. putida*.

Consequently, the main aims of my study were as follows:

1. To identify the factors that affect negatively transcription initiation from the promoter of the phenol degradation operon *pheBA* in *P. putida*.
2. To further elucidate the mechanisms by which transcription from the *pheBA* promoter is impeded during exponential growth phase of bacteria grown in the presence of amino acids.
3. To unravel the role of two-component system ColRS in *P. putida* by characterizing different phenotypes of *colR*-deficient mutant.
4. To investigate the mechanisms of involvement of ColRS system in phenol tolerance of *P. putida*.

3 RESULTS AND DISCUSSION

3.1 Expression of the phenol degradation operon *pheBA* in *Pseudomonas putida* is affected negatively by several factors (Ref. I)

In the first part of my thesis, I have concentrated on mechanisms, which negatively affect transcription of the phenol degradation operon *pheBA*. Transcriptional regulation of the *pheBA* operon is described in detail in "Review of the Literature" section 1.3.2.4. Previous studies have revealed that the transcriptional activation from the *pheBA* and *catBCA* promoters is repressed in *P. putida* cells growing exponentially in minimal medium in the presence of amino acids (Tover *et al.*, 2001). In order to study the regulation of the *pheBA* promoter under different growth conditions and in the presence of two carbon sources, phenol or benzoate, which both can serve as precursors of the inducer of the *pheBA* promoter, CCM, I constructed a new *P. putida* strain PaWpheBA-lux. This strain harbours the *pheBA* genes under the control of their natural promoter Pi (enabling the bacteria to degrade phenol to CCM) and a Pi-luxAB reporter system (the *pheBA* promoter cloned upstream of the *luxAB* reporter) in the chromosome. Strain PaWpheBA-lux enabled to induce the transcription from *pheBA* promoter with phenol as well as to measure the level of transcription with sensitive *luxAB* reporter encoding luciferase.

3.1.1 High phenol concentrations inhibit transcription initiation from *pheBA* promoter

Transcription from the *pheBA* promoter in a PaWpheBA-lux strain can be induced both by benzoate or phenol. However, whilst addition of 2.5 mM benzoate into the growth medium allowed rapid activation of transcription from the *pheBA* promoter during the first hour of growth then 2.5 mM phenol did not (Fig. 2, Ref. I). Only later, after 2 and 3 hours, the level of transcription from the *pheBA* promoter remarkably increased in the presence of phenol. These results raised the question whether addition of 2.5 mM phenol into the growth medium could somehow hinder transcription initiation from the *pheBA* promoter.

To test the possibility that 2.5 mM phenol is non-optimal for the induction of transcription from the *pheBA* promoter, the strain PaWpheBA-lux was cultivated in glucose minimal medium supplemented with different concentrations of phenol. I observed about 30-fold higher level of transcription from the *pheBA* promoter after one hour of induction if the concentration of phenol was reduced 10- to 100-fold from the usual 2.5 mM (Fig. 3a, Ref. I). I also confirmed that the exposure of bacteria to a high concentration of phenol influences specifically transcription from the *pheBA* promoter but not the expression of the *luxAB*

reporter or transcription from the Ptac promoter, activation of which is unrelated to phenol catabolism (Fig. 3b, Ref. I). The fact that transcription from the *pheBA* promoter is rapidly activated in the presence of 0.025–0.25 mM phenol but is significantly delayed when bacteria are exposed to 2.5 mM phenol indicates that induction of phenol detoxification mechanisms are superimposed over induction of phenol metabolism. It has been shown in *P. fluorescens* bio-reporter strain that transcription from promoter of toluene-degradation operon *tbu* was decreased when the concentrations of effectors like trichloroethylene or ethylenebenzene were over 600 μM and 1024 μM, respectively (Stiner & Halverson, 2002). Similar phenomenon has been described also for toluene catabolism *xyl* operon when after exposure of bacteria to crude oil residues, stress response genes were induced at first and only thereafter the *xyl* genes necessary for the degradation of carbon sources available in this otherwise toxic mixture (Velazquez *et al.*, 2006). The mechanism of silencing of catabolic genes by elevated concentration of pathway substrate with possible toxic impact on bacterial cells is not clear. Nevertheless, it has been suggested that the genetic networks that make stress responses to prevail over metabolic programs have been selected because of the evolutionary benefit they brought (Cases & de Lorenzo, 2005).

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

The main goal of Ref. I was to find out why amino acids in the bacterial growth medium inhibit the induction of transcription from the *pheBA* promoter despite of the presence of phenol or benzoate. There are basically two possibilities how transcription from a catabolic operon can be repressed regardless of the presence of respective carbon source. First, a specific negative regulator can bind to DNA and repress transcription. Secondly, some components of a positive performance are perturbed. According to literature data the latter variant seems to prevail in the case of operons involved in aromatic compound degradation (Shingler, 2003). Therefore, we decided to artificially manipulate the amount of factors that are involved in the activation of transcription from the *pheBA* promoter – the inducer molecule CCM and CatR activator protein. Analysis of transcriptional activation of the *pheBA* promoter in a CatR-overexpressing strain demonstrated that more than 100-fold higher level of the *pheBA* promoter activity could be recorded in the amino acid-containing growth medium (Fig. 4a, Ref. I). Up to 20-fold positive effect of the CatR overexpression could be detected in minimal medium-grown cells as well (Fig. 4a, Ref. I). This indicates that the intracellular amount of CatR is limiting in the transcriptional activation

from the *pheBA* promoter not only in the presence of amino acids, but also in bacteria grown in minimal medium.

Next, I wanted to manipulate the amount of other player of specific transcription initiation complex of the *pheBA* promoter – the inducer molecule CCM. Two approaches for increasing the level of CCM were exploited. First, the *catB*-mutant strain deficient in CCM degradation was constructed. Secondly, a strain carrying medium copy plasmid pAT1142 with inducible *pheBA* genes was used to increase the amount of CCM in cells. If the transcription from the *pheBA* promoter was measured in the *catB*-deficient mutant, the promoter was rapidly induced by phenol even in this case when amino acids were added into the growth medium (Fig. 4b, Ref. I). This indicates that CCM is highly limiting in amino acids-rich medium. Importantly, our results show that CCM almost always limits the transcription from the CatR-regulated promoters: even if bacteria were grown in a nutritionally poor environment, transcription from the *pheBA* promoter was more than fivefold higher with excess of CCM compared with that measured in the wild-type strain (compare Fig. 3a with Fig 4b, Ref. I). In agreement with the results of *catB*-deficient strain also the artificial overexpression of PheA and PheB enzymes on the plasmid pAT1142 enabled rapid activation of the *pheBA* promoter in both the presence and absence of amino acids in the growth medium (Fig. 4c, Ref. I).

The fact that overexpression of CatR protein as well as increasing the amount of CCM had positive effect on transcription initiation from *pheBA* promoter suggests that the amount of CatR-CCM complex is limiting. In the light of the data with *catB* mutant and PheBA-overexpression strains, there are two possibilities how to interpret the positive effect of CatR overproduction on the *pheBA* promoter activation in the presence of amino acids. First, we cannot rule out the possibility that similarly to the other biodegradation operons the downregulation of the *pheBA* operon transcription operates through the lowered level of a transcription activator protein (Müller *et al.*, 1996; Sze *et al.*, 1996; Yuste *et al.*, 1998). However, it is more plausible that intracellular amounts of CCM primarily limit the transcription initiation from the *pheBA* promoter and if there is more CatR protein, then a small number of CCM molecules available at an early stage of induction of the phenol degradation pathway are more likely bound by CatR to activate transcription.

3.1.3 Repression of transcription from the *pheBA* promoter in the presence of amino acids is partially caused by a Crc protein that affects expression of PMO and C12O

The above-presented results suggest that increasing the amount of CatR-CCM complex can significantly alleviate the negative effect of amino acids on transcription from the *pheBA* promoter. This led us to hypothesise that the limiting amount of CCM could be the main mechanism for the delayed induction of the

pheBA promoter when bacteria are growing exponentially in the presence of amino acids.

Several possible reasons can be considered why the intracellular levels of CCM may be reduced in amino acids-rich medium. First, the intracellular amounts of precursor molecule phenol may be too low for sufficient production of CCM. Secondly, the degradation of CCM may be faster in the presence of amino acids and therefore the amounts of CCM become limiting for the induction of the *pheBA* promoter. Also, the activity of CCM-producing enzymes could be inhibited or the levels of these enzymes could be reduced. To test the latter possibility we assessed the amounts of PheB and PheA proteins with the aid of specific antibodies. Interestingly, about four- and eightfold lower amounts of PheA and PheB proteins, respectively, were detected in bacteria grown in the presence of amino acids compared to those grown in minimal medium (Fig. 5a, Ref. I). Importantly, the cell extracts analysed were prepared from CatR-overexpressing strain and the samples for Western blot analysis were prepared from such amounts of bacteria that expressed equal luciferase activity from the *pheBA* promoter. Thus, our results indicated that the cellular amounts of PheA and PheB enzymes are negatively controlled by amino acids at the post-transcriptional level.

Next, I wanted to find out by which mechanism the amounts of PheB and PheA are downregulated in amino acids-rich medium. As a hint we knew that Crc (catabolite repression control) protein inhibits the expression of *P. putida* chromosomal genes for the homogentisate, catechol and protocatechuate pathways when cells grow in a complete medium (Morales *et al.*, 2004). The precise mechanism of Crc-mediated regulation was unknown at that time, but it was suggested that Crc modulates the expression of target genes post-transcriptionally (Hester *et al.*, 2000b; Rojo & Dinamarca, 2004). Therefore, we decided to compare the amount of PheA and PheB enzymes in *P. putida* wild-type and *crc*-deficient strain. Western blot analysis showed that the abundance of PheA and PheB proteins was not any more affected by the growth medium composition in the Crc mutant strain (Fig. 5A, Ref. I).

Based on these results, which indicated that Crc controls negatively the intracellular amounts of the PheA and PheB proteins in bacteria growing in the presence of amino acids, the disruption of *crc* should influence the level of pathway inducer CCM as well. Therefore, I expected to see that transcription from the *pheBA* promoter should be elevated in the *crc*-deficient background. Indeed, data presented in Figure 4d (Ref. I) showed that transcription from the *pheBA* promoter in the *crc*-deficient *P. putida* strain grown in amino acids-containing medium was up to 150 times higher than in wild-type strain. As higher intracellular amounts of phenol degradation enzymes in Crc-deficient background leads to increased production of the pathway inducer CCM, this is the most probable mechanism by which Crc indirectly interferes with the transcription of the *pheBA* genes.

The target and molecular mechanism of Crc action has been established only in a few cases. First, it has been shown that in *P. putida* the Crc inhibits the expression of the transcriptional activator of the alkane degradation pathway, the AlkS protein, by binding to the 5´ end of the corresponding mRNA and inhibiting its translation (Moreno *et al.*, 2007). Also, in the case of benzoate degradation operon the regulator protein is the target of Crc that binds to the 5´ end of the *benR* mRNA, which means that the downregulation of *benABCD* expression is achieved directly by decreasing the levels of the transcriptional activator BenR (Moreno & Rojo, 2008). Importantly, through that mechanism the genes contributing to the further steps of benzoate degradation, the *cat* genes, are not induced because the pathway inducer CCM is not produced (Moreno & Rojo, 2008). The possibility that Crc protein binds also to the PheB and PheA mRNA-s should be verified in the future.

In case of complex biodegradation pathways consisting of several operons, it is clearly not reasonable to repress the expression of lower pathway genes independently from upper pathway, to avoid accumulation of pathway intermediates. It must be noticed that the accumulation of high levels of CCM is toxic to the cells (Gaines *et al.*, 1996). In *Acinetobacter baylyi* ADP1 a complex regulatory circuit involving two LysR-family transcriptional regulators BenM and CatM has evolved to allow optimal expression of benzoate and catechol degradation genes, while concentration of potentially harmful CCM has been kept sufficiently low (Ezezika *et al.*, 2006). In the case of phenol degradation, the coordinate expression of the *pheBA* and *catBCA* operons is also needed. In *P. putida* the regulatory protein of *pheBA* operon CatR, which is shared with the *catBCA* operon encoding a part of the phenol catabolism pathway is not subjected to posttranscriptional regulation by rich growth medium (Moreno & Rojo, 2008). So the regulation of expression of the *pheBA* genes through the regulation of the amounts of respective enzymes at post-transcriptional level could be reasonable, even if this kind of regulation is unusual. It has been suggested that every mechanism of transcriptional regulation by different physiological conditions is good as long as it works (Cases & de Lorenzo, 2001).

Nevertheless, the negative effect of amino acids on the induction of the phenol degradation pathway cannot be explained only by the action of the Crc protein, as the expression level of the *pheBA* promoter in *crc*-deficient strain was still about four times lower in rich growth medium compared with that when bacteria were grown in minimal medium (Fig. 4d, Ref. I). Therefore, some additional control mechanisms operating on transcriptional activation of the *pheBA* promoter should exist. Competition between different sigma factors for free RNA polymerase has shown to be a mechanism affecting expression of several biodegradation operons (Marques *et al.*, 1999; Sze & Shingler, 1999). Thus, it is possible that in the presence of amino acids the transcription machinery is recruited to promoters whose expression is greatly needed with an excess of nutrients (e.g. stable RNA promoters) and therefore some other promoters, like the *pheBA* promoter, could be less efficiently transcribed.

Importantly, the inhibitory effects of high phenol concentrations on the transcriptional activation of the *pheBA* promoter were not decreased in the *crc* mutant (Fig. 4d, Ref. I). This indicates that reduced activation of the transcription from *pheBA* promoter in the presence of amino acids and high phenol concentration could operate through different mechanisms. The question why 2.5 mM phenol does not allow efficient induction of the transcription from the *pheBA* promoter cannot be answered satisfactory in this work. However, one may speculate that higher phenol concentrations may enhance rigidity of cell membrane as well as activate efflux systems extruding phenol from the cells, thereby making intracellular amounts of phenol limiting for the production of CCM, which is needed for the activation of the *pheBA* operon. Also, catechol or CCM could be extruded from the cells as well. During degradation of benzoate and phenol by different *Pseudomonas* isolates, accumulation of catechol and benzoate into the bacterial growth medium has been detected (Heinaru *et al.*, 2001). The possibility of extrusion of phenol degradation pathway compounds is illustrated also by finding that several efflux pumps are specifically induced by the deleterious compounds like toluene and other aromatics (Ramos *et al.*, 2002; Santos *et al.*, 2004). In *P. putida* F1 transcription of *sepABC* operon encoding RND family efflux pump has shown to be induced among other aromatic compounds also by phenol (Phoenix *et al.*, 2003). Nevertheless, the possibility that some of the at least 11 *sepABC* homologues in *P. putida* KT2440 are involved in phenol, catechol or CCM efflux remains to be elucidated.

3.2 Phenol sensitivity of *P. putida* in the absence of functional ColRS two-component system (Ref. II, III and IV)

Second part of my studies is devoted to the ColRS two-component system and to its role in phenol tolerance of *P. putida*. As already mentioned, *P. putida* strain PaW85 is able to use phenol as a sole carbon and energy source if only a single gene, phenol monooxygenase-encoding *pheA*, is expressed in the cells. Therefore, *pheA* has been used for construction of several test systems (measuring point mutations or transposition of Tn*4652*) utilizing selection of *P. putida* on phenol as a sole carbon source. Importantly, commonly used phenol concentration (2.5 mM) had no impact on wild-type *P. putida* in long-term carbon starvation experiments measuring mutation frequency. However, both point mutations and transposition of Tn*4652* were repressed in starving *colS*- and *colR*-knockout *P. putida* at 2.5 mM phenol (Hõrak *et al.*, 2004). As the frequency of mutations was partially restored by lowering the phenol concentration in the selective medium (Ilves, 2006), this suggested that the ColRS two-component system may be somehow implicated in phenol tolerance of *P. putida*.

3.2.1 Some of phenol-enhanced phenotypes of the *colR* mutant become evident only on glucose media and in the presence of glucose porin OprB1 (Ref. II and III)

In the following part of my thesis I show that in addition to phenol sensitivity, the *colR*-deficient strain experiences serious stress on glucose medium but not on other carbon sources. Several glucose-caused phenotypes of *colR*-deficient strain are abolished by the inactivation of glucose porin-encoding *oprB1*. Interestingly, many of phenotypes characteristic to glucose-stress are significantly enhanced by phenol suggesting that there may be link between glucose and phenol-caused stress.

3.2.1.1 Activation of specific promoters in response to phenol in *colR*-deficient strain (Ref. II)

Search for ColR-controlled promoters helped us not only to discover the genes regulated by the ColRS system but it also revealed a phenol sensitive phenotype of the *colR*-deficient *P. putida*. To discover the ColRS-regulated genes the promoter library from total chromosomal DNA of *P. putida* PaW85 was constructed (Fig. 1, Ref. II). As a primary reporter, the phenol monooxygenase-encoding *pheA* gene was used to enable selection of promoter-containing library clones on phenol minimal plates. As a second reporter *β*-glucuronidase-encoding *gusA* was employed enabling quantitative measuring of promoter activities. The promoter library was introduced into a *P. putida colR*-deficient strain harbouring an extra copy of a mutated *colR* gene, *colR(D51A)*, in the chromosome under the control of the IPTG-inducible Ptac promoter. The choice of reporter strain was based on our previous observation that the induction of phosphorylation-deficient ColR(D51A) caused a stronger effect on Tn*4652* transposition than did overexpression of wild-type ColR (Hõrak *et al.*, 2004). Library screen in the presence and absence of IPTG identified four promoters whose expression was affected by the level of ColR(D51A) protein. Next we carried out more specific inspection of promoters, which were identified as ColR-regulated, in wild-type and *colR*-deficient strain. It emerged that in the absence of phenol in the growth medium the transcription was altered by ColR-minus background only in the case of two promoters. Thus, our results demonstrated that only promoters of *oprQ* and *algD* were controlled directly by the ColRS system whereas *csuB* and *ompA* (PP0773) promoters were not affected by ColR and responded merely to phenol (Fig. 2, Ref II). Actually, all four investigated promoters were slightly affected by phenol also in wild-type background but the effect became more prominent in the absence of the functional ColRS system (Fig. 2, Ref II). This strongly suggested that in the absence of the ColRS system bacteria might become somehow sensitive to phenol.

While ColR protein is specific repressor of *algD* promoter, the absence of ColR has also an indirect effect on transcription from the *algD* promoter. Namely, in the presence of phenol transcription from the *algD* promoter is strongly (more that 10 times) induced in *colR*-mutant but not in wild-type bacteria. It has been shown that activation of transcription from the *algD* promoter in *P. aeruginosa* is strictly controlled by alternative sigma factor AlgT (AlgU), which is analogous to *E. coli* sigma E (RpoE) (Wood *et al.*, 2006). Among the others, inhibition of peptidoglycan synthesis is a stress that induces transcription from the *algD* promoter in *P. aeruginosa* (Wood *et al.*, 2006). In wild-type *P. putida* the expression of *algD* is induced by low water availability, but not by osmotic stress (Chang *et al.*, 2007). Therefore, it is most likely that in the absence of ColR bacteria experience phenol-induced cell wall stress which results in the activation of the *algD* expression.

Surprisingly, none of the studied promoters were affected by phenol in *colR*-deficient background on other carbon sources than glucose (Fig. 4, Ref. II and data not shown). Moreover, after inactivation of glucose porin-encoding *oprB1* the transcription from these promoters was not influenced by phenol any more even on glucose medium (Fig. 4, Ref. II and data not shown). The role of OprB1 in transcriptional regulation of specific promoters in *colR*-deficient strain by phenol is obscure. However, there is a report where solvent-tolerant *P. putida* strain S12 has been used for bioproduction of phenol from glucose (Wierckx *et al.*, 2005). As disruption of the *oprB1* gene caused enhanced phenol production, the authors suggested that phenol may re-enter the cell via OprB1 porin (Wierckx *et al.*, 2005). Therefore, our initial interpretation was also that phenol might enter the cells through OprB1 porin. We supposed that the membrane permeability of the *colR* mutant to phenol is intrinsically increased and that additional phenol uptake through OprB1 porin may elevate the concentration of phenol in *colR* mutant bacteria over a threshold level, increasing physiological stress and enhancing the phenol response of the *colR* mutant strain. Nevertheless, our next studies (presented in the following chapter) indicated that not phenol but rather glucose influx through OprB1 is a stress factor for the *colR*-deficient strain, and the presence phenol just enhances this phenomenon.

3.2.1.2 Phenol enhances glucose-dependent cell membrane leakiness and cell lysis of *colR*-deficient *P. putida* (Ref. III)

The results presented in previous chapter suggested that besides phenol-caused stress the *colR*-defective strain might experience additional stress caused by glucose. Careful inspection of bacteria grown on different carbon sources revealed a new glucose-related phenotype of the *colR* mutant. Namely, we observed that unlike the wild-type, the colonies of the glucose-grown *colR* mutant were sticky indicating increased aggregation of cells. In addition, the presence of phenol in the growth medium substantially enhanced the

aggregation of the *colR*-deficient cells but phenol did not affect the nonaggregative phenotype of wild-type cells. Aggregating bacterial cells usually stain with Congo Red dye that binds to different surface exopolymers (Collinson *et al.*, 1993; Zogaj *et al.*, 2001; Ude *et al.*, 2006). As shown in Figure 1 (Ref. III), stickiness of *colR*-deficient bacteria is accompanied with staining of bacteria with Congo Red while wild-type strain stains at much lesser extent. As expected from severe stickiness the staining of *colR*-deficient cells with Congo Red was enhanced in the presence of phenol (Fig. 1, Ref. III). The staining and aggregation phenotype of *colRoprB1* double mutant was similar to that of the wild-type and *oprB1*-deficient strain (Ref. III, Fig. 1) suggesting that the absence of glucose porin OprB1 can compensate the ColR deficiency. Notably, none of the studied strains stained significantly with Congo Red when grown on citrate minimal medium (Fig. 1, Ref. III) indicating that phenol-enhanced production of Congo Red-binding substance (CBS) was characteristic to glucose-grown *colR*-mutant strain.

Congo Red binding was strongly enhanced by phenol which is known to harm the cell envelope and permeabilize the membrane (Heipieper *et al.*, 1991). Therefore, we suspected that the membrane permeability of the *colR*-deficient strain could be changed. The membrane permeability of different *P. putida* strains was assessed with the aid of *β*-galactosidase assay. Only about 4% of total *β*-galactosidase activity was measurable in non-permeabilized wild-type cells regardless of the presence of phenol in the growth medium (Fig. 3, Ref. III). At the same time, up to 15% of total *β*-galactosidase activity was detected from *colR*-deficient cells grown on glucose minimal plates, and about 35% when cells were grown on glucose medium supplemented with 1 mM phenol (Fig. 3, Ref. III). As in previous experiments, the disruption of the glucose porin-encoding *oprB1* in the *colR*-deficient background restored the wild-type phenotype by totally eliminating the higher membrane permeability of the *colR* mutant (Fig. 3, Ref. III).

To examine the possibility whether the cytoplasmic enzyme *β*-galactosidase could be released from the cells the activity of *β*-galactosidase was assessed also from the supernatants of bacterial cells suspended in a buffer. As shown in Fig. 4A (Ref. III), *β*-galactosidase activity was barely detectable in supernatants of the wild-type and *oprB1*-deficient strains. At the same time, the supernatant of the glucose-grown *colR*-deficient strain contained 4% of the total *β*-galactosidase activity of the initial cell suspension and up to 15% when phenol was added to the growth medium (Fig. 4A, Ref. III). These results suggested that cell lysis might occur in the culture of *colR*-deficient strain. To verify that hypothesis the amount of chromosomal DNA was also evaluated from supernatants. This experiment showed that over 10 times higher level of chromosomal DNA was present in the supernatants of the *colR*-mutant compared to that of the wild-type and *oprB1*-deficient strains (Fig. 4B, Ref. III). The occurrence of cell lysis was also supported by closer inspection of *colR*-mutant colonies on glucose media. Specifically, after two days of growth, visible concavities developed in

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the centre of ageing colonies of *colR*-deficient bacteria whereas colonies of the wild-type as well as both *oprB1*-deficient strains stayed smooth (Fig. 5, Ref. III). It is most likely that the concavities in the centre of colonies of the *colR* mutant originated from the autolysis of a subpopulation of bacteria. It is known that cell lysis is often regulated by cell density-dependent signals (Allesen-Holm *et al.*, 2006; D'Argenio *et al.*, 2002; Kolodkin-Gal *et al.*, 2007; Steinmoen *et al.*, 2002). Therefore, it is not surprising that concavities can be seen only in the centre of the *colR* mutant colonies suggesting that cell lysis-proficient population may develop under some specific conditions, for example, at high cell densities or at certain physiological states.

The above-discussed results led us to analyse the population heterogeneity of the *colR*-deficient strain. The heterogeneity was assessed by flow cytometry and staining of bacterial cells with fluorescent dyes. Flow cytometric analysis of bacteria stained with the mixture of SYTO9 and propidium iodide (PI) demonstrated that the population of the *colR*-deficient strain grown on glucose minimal medium was heterogeneous: in addition to wild-type-like cells we detected a subpopulation of cells with the membrane permeable to PI (Fig. 6A and B, Ref. III). Addition of phenol to the growth medium did not change the profile of wild-type cells whereas in case of *colR*-deficient strain the subpopulation with PI-permeable membrane was increased. As expected, no differences in populations' profiles between the wild-type and *colR*-deficient strains grown on citrate were observed (Fig. 6E and F, Ref. III). Thus, the results of flow cytometric analysis suggest that not all cells of *colR*-deficient population have compromised membrane but only a portion of them, most probably those that are in a specific physiological state.

One interesting question that rises from the above-described results is why cell lysis is detected only on glucose medium and not even on gluconate (data not shown), the substrate that is also degraded trough Entner-Doudoroff pathway. It has been shown that the transcriptional as well as metabolic profile differs between *P. putida* cells grown either on glucose or on gluconate (van der Werf *et al.*, 2006; van der Werf *et al.*, 2008). One important difference was that glucose-grown cells seemed to be energetically at a lower state compared to the cells grown on other carbon sources including gluconate (van der Werf *et al.*, 2008). So, the cell lysis on glucose medium could be related to the reduced energetic status of the cells.

The mechanism that leads to the lysis of *colR* mutant cells on glucose medium remains obscure. Hopefully, this mechanism will be resolved after identification and closer analysis of ColR target genes. So far, we know that genes, most strongly induced by ColRS system, are the ones locating just downstream of the *colRS* operon – PP0903, PP0904 and PP0905 (Kivistik *et al.*, Manuscript). The exact function of PP0903, PP0904 and PP0905 is not known, but by genome annotation they encode for hypothetical methyltransferase, probable lipopolysaccharide kinase InaA and small hypothetical protein, respectively (www.pseudomonas.com). Interestingly, it has been shown that orthologous genes to PP0903 and PP0904 in

P. aeruginosa are induced by antibiotic ceftazidime, which inhibits the penicillin-binding protein 3 (FtsI) (Blazquez *et al.*, 2006). FtsI is a cytoplasmic membrane-locating protein that regulates cell division by directing the cell wall peptidoglycan synthesis (Errington *et al.*, 2003; Nakamura *et al.*, 1983). In the study by Blazquez and co-workers the induction of orthologs of PP0903 and PP0904 was seen at low antibiotic concentrations when the growth and division of bacterial cells was not visibly affected (Blazquez *et al.*, 2006). This means that even a slight disturbance in cell division might significantly induce the expression of genes which are ColR-regulated in *P. putida* and *P. fluorescens* (de Weert *et al.*, 2006; Kivistik *et al.*, Manuscript). Interestingly, at the same time when phenol enhances cell lysis of *colR*-deficient bacteria, it also enhances transcription from the PP0903-PP0905 operon in wild-type cells while in the *colR* mutant bacteria the transcription from the promoter of these genes is completely abolished (Kivistik *et al.*, Manuscript). In the future it would be interesting to study whether these genes can affect the lysis as well as phenol tolerance of *colR*-deficient cells.

The remarkable glucose sensitivity and the lowered phenol tolerance of the *colR*-deficient strain do not overlap because, as shown in the next chapter, OprB1-deficiency, which abolishes cell lysis does not restore phenol tolerance of *colR*-deficient strain to wild-type level. The lowered phenol tolerance and the glucose-induced cell lysis of the *colR*-deficient strain are most probably caused by different mechanisms, though compromised cell membrane of the *colR* mutant seems to be implicated in both cases.

3.2.2 The ColR-deficient strain is less tolerant to phenol only under growth-permitting conditions (Ref. II, III and IV)

Since phenol enhanced several phenotypes of glucose-grown *colR*-deficient strain then it was reasonable to test whether there could be any differences between the wild-type and *colR*-deficient strains in respect of their phenol tolerance. First I assessed the growth of the *P. putida* wild-type and *colR* mutant strains on glucose and citrate plates supplemented with different concentrations of phenol. Results clearly demonstrated that compared to wild-type the *colR* mutant was significantly more sensitive to higher phenol concentrations on both tested carbon sources (Fig. 5, Ref. II). However, the MIC of phenol for the *colR* mutant was 8 mM on glucose medium and 6 mM on citrate medium (Fig. 5, Ref. II). It should be emphasized that while *oprB1* knockout completely abolished the glucose-induced cell lysis (Fig. 3 and 4, Ref. III) then it only slightly alleviated the phenol sensitivity of the *colR* mutant. In fact, disruption of *oprB1* improved phenol tolerance of the *colR*-deficient strain but compared to the wild-type and *oprB1* mutant, the *colR oprB1* double mutant was significantly less tolerant to 8 mM phenol on glucose medium (Fig. 5, Ref. II). This was the first hint that ColR-dependent glucose and phenol stress responses may originate from different reasons.

In previous phenol tolerance plate-assay only the ability of cells to form colonies could be measured. It does not tell whether bacteria die because of phenol toxicity or they just receive some kind of growth inhibiting signal. In order to clarify whether the elevated phenol entrance would abolish growth of the *colR* mutant at higher phenol concentrations or kill bacteria, I measured the viability of bacteria that were exposed to higher phenol concentrations over the short time period. In that experiment I used bacteria pre-grown on solid glucose medium stimulating the appearance of a subpopulation of *colR*-deficient cells with propidium iodide-permeable cell membrane (Ref. III). I expected that if phenol entry into the glucose-grown *colR* mutant is facilitated then the *colR*-deficient cells should die faster than wild-type cells. Interestingly, sudden exposure of glucose-grown cells to 50 mM phenol in liquid M9 minimal medium lacking carbon source revealed identical phenol tolerance levels of the wild-type and the *colR* mutant as both strains died with equal rates (Fig. 1, Ref. IV). These results indicated that the membrane of the *colR* mutant is not more permeable to phenol than the membrane of the wild-type strain.

I also performed long-term experiments in which bacteria were incubated in a liquid medium supplemented with different concentrations of phenol for 24 hours. Optical density of the culture as well as count of viable cells was assessed after 24 hours. The experiment was carried out either without carbon source or in the presence of glucose or citrate. Interestingly, the differences between wild-type and *colR*-deficient strain in phenol tolerance became evident only when cells had growth-enabling carbon source in the medium and not under starvation conditions (Fig. 2, Ref. IV). These results clearly show that non-growing *colR*-deficient cells are not vulnerable to phenol and confirm that the cell membrane of the *colR*-deficient bacteria is not more permeable to phenol than the membrane of the wild-type cells.

As already mentioned, I also monitored the growth and viability of bacteria after 24 hours of exposure to different phenol concentrations in the presence of either glucose or citrate. Several energy-demanding mechanisms are implicated in tolerance to the aromatic compounds in *P. putida* (Ramos *et al.*, 2002). Therefore, as expected, the presence of carbon and energy source clearly improved the survival of phenol-treated wild-type bacteria compared to that of starving ones (Fig. 2, Ref. IV). At the same time, addition of carbon source had the opposite effect on phenol tolerance of the *colR*-deficient strain. 50% of the initially inoculated *colR*-deficient cells survived in carbon-free medium at the presence of 8 mM phenol over 24 hours while only about 10% of the cells survived if carbon source was added (Fig. 2, Ref. IV). At lower phenol concentrations the phenol tolerance of *colR*-deficient strain was reduced more strongly in the presence of glucose than in the presence of citrate, which suggests that the additional glucose-caused stress could be involved as well. Here, it is important to notice that *colR* mutant bacteria are more susceptible to phenol at lower concentrations (4-6 mM) than at higher (10 mM) concentrations when grown on glucose medium (Fig. 2c, Ref. IV).

The lowered phenol-tolerance of the *colR*-deficient strain in the presence of carbon source could be connected either with cell division deficiencies or problems in induction or functioning of energy-demanding tolerance mechanisms. As we know from the previous study, a subpopulation of *colR*-deficient cells lyses even on pure glucose medium while phenol can just enhance this phenotype (Fig. 3 and Fig. 4, Ref. III). Therefore, it seems that *colR*-deficient cells have membrane integrity-related problems on glucose medium which are most probably independent from phenol tolerance mechanisms. Importantly, single cell analysis indicated the heterogeneity of *colR* deficient cells in respect to membrane permeability to propidium iodide (Fig. 6, Ref III). So, the hypothesis that *colR*-deficient cells are more vulnerable to phenol during the cell division seems to be more favourable. In this case it would be also logical that increasing phenol concentration eventually inhibits the cell division totally regardless of the presence of carbon source and therefore the differences in survival of wild-type and *colR*-deficient strain would reduce or disappear at higher phenol concentrations. Parallels can be drawn with the fact that susceptibility to antibiotics can be enhanced for bacterial cells undergoing particular sensitising events like DNA replication or cell division. For example, sensitivity to β-lactams is strongly correlated with the amount of peptidoglycan biosynthesis (Gilbert *et al.*, 1990).

3.2.3 Enhanced phenol tolerance of the *colR*-deficient strain acquired by additional disruption of *gacS*, *ttgC* or *argD* gene cannot alleviate the effect of phenol on facilitating the glucose medium-related autolysis of the *colR* mutant (Ref. IV)

Phenol-tolerance experiment on solid glucose medium revealed that the *colR*-defective strain is able to accumulate phenol tolerant mutants with very high frequency (approximately 10^{-4} mutants per plated cell) at 8 mM phenol (Fig. 3a, Ref. IV). This prompted us to carry out the transposon mutagenesis to find genes whose disruption could elevate the level of phenol tolerance of *colR*-deficient bacteria. Sequencing of transposon mutants that tolerated higher concentrations of phenol revealed that the phenol sensitive phenotype of the *colR*-deficient strain was suppressed by disruption of genes dispersed between different functional classes (Table 1, Ref IV). Three representative genes were chosen for further studies and they were disrupted by homologous recombination in wild-type and *colR*-deficient background. The first one was *ttgC* gene that encodes for outer membrane protein of the TtgABC efflux pump, reported to be involved in toluene efflux (Rojas *et al.*, 2001). Secondly, I chose *gacS* gene encoding for sensor kinase of a two-component signal transduction system GacAS as a representative of regulatory genes (Heeb & Haas, 2001). The third gene was *argD* encoding for acetylornithine aminotransferase. This is the first gene of an operon that is probably responsible for conversion of arginine to

glutamate, and this gene was chosen to represent genes linked to central metabolism. Disruption of *gacS*, *ttgC* or *argD* genes resulted in enhanced phenol tolerance in *colR*-deficient as well as in wild-type background (Fig. 3b, Ref. IV) indicating that these genes were not exclusively involved in complementation of the *colR* negative phenotype but rather generally increased the phenol tolerance of *P. putida*.

I have shown in previous chapters that all glucose-medium related phenotypes of *colR*-deficient bacteria became more prominent if phenol was added to the growth medium. For instance, the glucose-induced release of cytoplasmic β-galactosidase into the growth medium occurring due to the autolysis of *colR* mutant bacteria was significantly enhanced if the medium was supplied with phenol (Fig. 3, Ref. III). Therefore, I tested these newly constructed *gacS*, *ttgC* or *argD*-deficient strains also in assay measuring the glucose-induced release of cytoplasmic *β*-galactosidase occurring due to the autolysis of *colR*-deficient bacteria. I expected that enhanced phenol tolerance could reduce the effect of phenol to membrane leakiness and autolysis of *colR*-deficient cells. Interestingly, both *colRgacS* and *colRttgC* double mutants behaved in *β*-galactosidase assay exactly like their *colR*-deficient ancestor (Fig. 4, Ref. IV). Only disruption of *argD* in *colR*-deficient background complemented the lack of ColR by reducing the *β*-galactosidase release when cells were grown on glucose minimal medium (Fig. 4, Ref. IV). However, the release of the cytoplasmic enzyme was still enhanced by phenol in the case of *colRargD* double mutant. Hence, it is possible that not elevated phenol tolerance of the *colRargD* strain is responsible for decreased cell lysis but rather the *colRargD* strain can better resist the glucose-caused stress. Thus, the enhanced phenol tolerance of the *colR*-deficient strain acquired by additional disruption of *gacS*, *ttgC* or *argD* gene cannot alleviate the effect of phenol on facilitating the glucose medium-related autolysis of the *colR* mutant.

Coming back to my results about mechanisms of the transcription initiation from the *pheBA* promoter (Ref. I), it seems that phenol can elicit different signals. Data demonstrated that the level of transcriptional activation from this promoter depends on the concentration of phenol. Only at higher concentrations (over 1 mM) it acts as a transcription repressor and probably as a stress-inducing factor (Fig. 3a, Ref. I). Studies of transcription initiation from the *pheBA* promoter also showed that bacteria are able to adapt to the high phenol concentrations over a few hours enabling full level of transcription of the *pheBA* genes (Fig. 2, Ref. I). Thus, clearly the cellular responses to the phenol depend on its concentration as well as on time of exposure to it. Importantly, 1 mM concentration of phenol, which significantly enhanced autolysis of *colR*-deficient cells, was strongly below MIC for the *colR*-mutant as this mutant was able to form colonies also on glucose minimal medium supplemented with 6 mM phenol (data not shown). Our *β*-galactosidase measurement data suggest that at lower concentrations (e.g., 1 mM) phenol most probably acts like signal molecule because its effect on cell lysis does not depend on overall tolerance

level of particular strain (Fig. 4, Ref IV). This means that phenol could act as a signal leading to the cell death rather than being a killing factor itself. It must be noted that there is a growing evidence demonstrating that bacteria respond specifically to the antibiotics at subinhibitory concentrations (Goh *et al.*, 2002; Hoffman *et al.*, 2005; Linares *et al.*, 2006). Actually, it has been suggested that at certain concentrations (usually at ten times lower concentrations than MIC) the antibiotics serve as signalling molecules (Linares *et al.*, 2006; Yim *et al.*, 2007). Though phenol is not strictly xenobiotic compound, it is found at high quantities in the environment mostly due to the pollution. Nevertheless, the benzene ring is common for several natural compounds and therefore at low concentrations when cellular functions are not disturbed, phenol might be recognised as some kind of signalling molecule. Further work with ColRS system and its regulon will hopefully shed the light to this interesting possibility that depending on the environmental conditions and concentration of phenol, this aromatic compound can provoke additional processes besides inducing specific biodegradation genes or stress response.

CONCLUSIONS

From the first part of my thesis concerning regulation of transcription of the *pheBA* genes in *P. putida*, the following conclusions can be drawn:

1. The inducer molecule CCM (*cis,cis*-muconate) is a limiting factor in transcription initiation from the promoter of the phenol degradation *pheBA* operon in *P. putida*.
2. In the presence of amino acids in the growth medium the amount of CCM is decreased due to the downregulation of PheB and PheA proteins, which are responsible for the synthesis of CCM.
3. The levels of PheB and PheA proteins are downregulated at posttranscriptional level by the Crc protein.
4. High (over 1 mM) phenol concentrations inhibit transcription induction from the *pheBA* promoter in exponentially growing bacteria. Inhibition of the *pheBA* promoter by high phenol concentrations operates through different mechanisms than inhibition by amino acids.

The second part of my thesis was dedicated to the role of ColRS signal transduction system in *P. putida*. The results of this study indicated that the phenotypes of phenol sensitivity and glucose-caused cell lysis in the absence of ColRS system are most probably caused by different mechanisms, though compromised cell membrane of the *colR* mutant seems to be implicated in both cases. More specifically, the following conclusions can be drawn:

1. The ColRS system is implicated in phenol tolerance of *P. putida*. Importantly, the *colR*-deficient strain is less tolerant to phenol than the wild-type only under growth-permitting conditions indicating that particularly cell growth and/or cell division-related processes are sensitive to phenol in *colR*-deficient bacteria.
2. In the absence of functional ColRS system a cell lysis takes place among portion of glucose-grown *P. putida* cells. Cell lysis is abolished by disruption of glucose porin OprB1 in *colR*-deficient strain indicating that glucose metabolism may be involved in generating signals for cell lysis. It is also possible that the presence of a high amount of OprB1 in the outer membrane of glucose-grown *colR* mutant may somehow destabilise intrinsically fragile membrane of *colR*-deficient cells causing the lysis.
3. OprB1-deficiency, which abolishes cell lysis, does not eliminate phenol sensitivity of *colR*-deficient strain. Therefore it seems that the lowered phenol tolerance and the remarkable glucose sensitivity of the *colR*-deficient strain do not overlap.
4. Enhancement of phenol tolerance of the *colR*-deficient strain by disruption of *ttgC*, *gacS* or *argD* genes does not influence the phenol-enhanced cell lysis on glucose medium. This shows that low phenol tolerance *per se* is not the reason why phenol enhances glucose-induced cell lysis of *colR*-deficient cells. Hence, phenol might act as a cell lysis-enhancing signal rather than a killing factor itself.

Altogether my studies have shown that the nature of the phenol-induced signals, which are sensed by *P. putida*, strongly depend on the concentration of phenol in the medium as well as other growth conditions. Our current knowledge of phenol-induced signals is far from complete and subsequent studies are needed to reveal additional phenol-evoked processes in *P. putida* besides inducing specific biodegradation or stress response genes. Discovery that signal transduction system ColRS participates in the development of phenol tolerance of *P. putida* is valuable for unravelling the mechanisms of bacterial tolerance to this aromatic compound as well as for understanding of signalling components involved.

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SUMMARY IN ESTONIAN

Pseudomonas putida vastused fenoolist tulenevatele metabolismi- ja stressisignaalidele

Käesolev uurimistöö keskendub erinevatele signaalidele, mida tajub mullabakter *P. putida* kokkupuutes sellise aromaatse süsinikuühendiga nagu fenool.

Kirjanduse ülevaates tutvustan esmalt bakteriperekonda *Pseudomonas*, kuhu kuulub ka uurimisobjektiks olev mullabakter *Pseudomonas putida*. Perekond *Pseudomonas* on tuntud selle poolest, et sinna kuuluvad liigid on võimelised kasutama süsinikuallikana äärmiselt erinevaid ühendeid ja suuresti just seetõttu asustavad nad ka väga erinevaid keskkondi. Eriti suurt huvi on pälvinud nende bakterite võime lagundada erinevaid aromaatseid süsinikuühendeid, mis võivad olla päritolult nii looduslikud kui ka tööstuslikud. Benseeni tuuma sisaldavad aromaatsed süsinukuühendid on äärmiselt stabiilsed ega lagune looduses iseeneslikult. Samas on paljud aromaatsed süsinikuühendid väga toksilised, sest oma keemiliste omaduste tõttu lahustuvad nad kergesti rakumembraanis, häirides sellega membraani funktsioone. Perekonna *Pseudomonas* bakterite puhul on kirjeldatud erinevaid mehhanisme, mis aitavad neil toksilisi aromaatseid ühendeid taluda, võimaldades seega bakteritel elada keskkonnas, mis on paljudele teistele organismidele kahjulik. Teatav tolerantsuse tase on vajalik ka selleks, et bakterid oleksid võimelised neid aromaatseid süsinikuühendeid lagundama juhul, kui neil on olemas vastavad metabolismigeenid.

Üks huvitav kuid väheuuritud küsimus on see, kuidas bakterid toksilise ühendi olemasolust keskkonnas aru saavad. Bakterite levinuimaks keskkonna tunnetamise vahendiks on kahekomponendilised signaaliülekande süsteemid, mis koosnevad membraanis paiknevast signaali vastuvõtvast valgust ja tsütoplasmaatilisest vastust täidesaatvast valgust. Meie labori varasemad uurimistulemused viitasid, et *P. putida* kahekomponendiline signaalsüsteem ColRS võib mõjutada bakterite elutegevust fenooli sisaldavas keskkonnas (Hõrak *et al.*, 2004; Ilves, 2006). Selle hüpoteesi kontrollimiseks seadsin endale üheks töö eesmärgiks uurida ColRS süsteemi rolli bakteris *P. putida*.

Inimeste huvides oleks rakendada baktereid keskkonna puhastamiseks sinna sattunud toksilistest ühenditest. Selleks on aga vajalikud teadmised, kuidas ja miks bakterid neile ühenditele erinevates tingimustes reageerivad. Näiteks on teada, et vaatamata aromaatse süsinikuühendi olemasolule ei ekspresseerita bakterites vastavaid metabolismiradasid juhul, kui keskkonnas esineb ka mõni teine, energeetiliselt kasulikum süsinikuallikas. Bakteris *P. putida* on uuritud plasmiidse päritoluga fenooli lagundamist võimaldavate *pheBA* geenide ekspressiooni. Transkriptsioon *pheBA* operoni promootorilt on kontrollitud kromosomaalselt kodeeritud regulaatorvalgu CatR ja fenooli lagundamisel tekkiva vaheühendi *cis,cis*-mukonaadi poolt (Kasak *et al.*, 1993). Eelnevalt oli teada, et vaatamata induktori tekkimiseks vajaliku substraadi olemasolule ei aktiveerita transkriptsiooni *pheBA* promootorilt, kui bakterid kasvavad

eksponentsiaalselt aminohappeid sisaldavas söötmes (Tover *et al.*, 2001). Sellest tulenevalt oli üheks minu töö eesmärgiks selgitada välja, milliste mehhanismide abil toimub *pheBA* promootorilt lähtuva transkriptsiooni pärssimine.

Oma doktoritöö tulemuste ja arutelu osa, mis puudutab fenooli lagundamist võimaldavate *pheBA* geenide ekspressiooni bakteris *P. putida,* võtaksin kokku järgmiselt:

1. *pheBA* promootorilt lähtuva transkriptsiooni aktivatsioonil on limiteerivaks faktoriks bakterirakkudes tekkiva induktormolekuli *cis,cis*-mukonaadi hulk. Aminohappeid sisaldavas söötmes kasvanud bakterites tekib *cis,cis*-mukonaati vähem kui minimaalsöötmes kasvanud rakkudes.
2. Induktormolekuli *cis,cis*-mukonaadi madalama taseme põhjustab *cis,cis*-mukonaadi sünteesil osalevate PheA ja PheB ensüümide ekspressioonitaseme langus aminohapeterikkas söötmes kasvanud rakkudes.
3. PheA ja PheB ensüümide hulka mõjutab negatiivselt Crc valk, mis toimib postranskriptsioonilisel tasemel.
4. Lisaks aminohapetele pärsib ka söötmesse lisatud kõrge fenooli kogus (üle 1 mM) transkriptsiooni initsiatsiooni *pheBA* promootorilt. Sealjuures ei vähenda suuremad fenooli kontsentratsioonid Crc-sõltuvalt PheB ja PheA hulka, pigem toimub induktormolekuli koguse vähendamine kas fenooli või selle lagundamise vaheühendite suurenenud rakust väljapumpamise tõttu.

Minu doktoritöö tulemuste ja arutelu teine osa puudutab kahekomponendilise signaaliülekande süsteemi ColRS rolli bakteris *P. putida.* Töös on uuritud ColRS süsteemi rolli nii üldiselt, kui ka selle seost rakkude fenoolitolerantsusega, kusjuures esitatud katsetes on fenool vaid stressifaktor, mitte süsinikuallikas, sest bakteritel puuduvad fenooli lagundamist võimaldavad geenid. Funktsionaalse ColRS süsteemi olemasolu ei ole bakterile *P. putida* küll hädavajalik, kuid teatud kasvutingimustel ilmnevad *colR*-defektse ja algse tüve vahel erinevused. Saadud tulemused võib võtta kokku järgmiselt:

1. Algse *P. putida* tüve ja *colR*-defektsete rakkude kasv glükoosil on erinev. Funktsionaalse ColRS süsteemi puudumisel lüüsub osa glükoosi tardsöötmel kasvanud rakkudest. Samuti on osadel *colR*-defektsetel rakkudel suurenenud membraani läbilaskvus propiidium jodiidile, mis siseneb tervetesse rakkudesse halvasti. Huvitaval kombel suudab fenooli lisamine kasvukeskkonda (seega lisastressi tekitamine) neid fenotüüpe veelgi võimendada. Lisaks mõjutab fenool ka glükoosil kasvanud *colR*-defektsetes bakterites mitmete geenide transkriptsiooni, samas kui teistel süsinukuallikatel fenooli mõju neile geenidele ei ilmne.
2. Huvitaval kombel kaovad kõik *colR*-defektse tüve glükoosistressiga seotud fenotüübid, kui rakkudes puudub glükoosi poriin OprB1. Täpne mehhanism ei ole praeguseks teada, kuid võib oletada, et glükoosi poriini puudumine mõjutab erinevate glükoosi lagundamist võimaldavate radade osakaalu glükoosi metabolismil ja seega kogu raku metabolismi. Siiski ei saa välistada ka võimalust, et *colR*-defektsete rakkude membraan on spetsiifiliselt tundlik OprB1 poriini paigutumisele välismembraani.

3. Fenool küll võimendab glükoosistressi, kuid ometi on fenoolil ka glükoosist sõltumatu mõju *colR*-defektsetele rakkudele. Nimelt on *colR*-defektse tüve rakud fenoolile tundlikud ka glükoosist erinevatel süsinikuallikatel kasvades. Teiseks on glükoosil kasvanud *colR*-defektse tüve rakud fenoolile tundlikud ka siis, kui rakkudes puudub funktsionaalne glükoosi poriin OprB1. Seega viitavad need tulemused sellele, et *colR* geeni puudumisel on häiritud mitmed erinevad raku funktsioonid, millest ühed põhjustavad eelkõige vähenenud fenoolitaluvust ja teised suurenenud glükoositundlikkust.
4. ColRS süsteem kaitseb bakterirakke fenooli eest, kuid seda ainult rakkude kasvu võimaldavates tingimustes. Suur fenooli kogus (üle 16 mM), mis blokeerib bakterite kasvu täielikult, tapab *P. putida* rakke ColRS süsteemist sõltumatult. Samuti ei ole ColRS süsteem oluline *P. putida* rakkude fenoolitaluvuses, kui bakterite kasvukeskkonnas puudub süsinikuallikas ja rakud ei jagune.
5. Minu uurimistöö tulemused ja kirjanduse andmed viitavad selle, et ColRS süsteem reguleerib bakterirakkude membraani läbilaskvust. Kuna *colR*-defektse tüve membraani läbilaskvus ei erine algse tüve omast aga sugugi mitte kõigis tingimustes, siis võib oletada, et ColRS süsteem muutub rakkudele oluliseks teatud füsioloogilises seisundis. Saadud tulemused lubavad oletada, et ColRS signaalirada osaleb rakkude kasvamise ja/või jagunemisega seotud membraanikomponentide sünteesis, reguleerides membraani läbilaskvust rakkude jagunemisel.

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PUBLICATIONS

CURRICULUM VITAE

I. General data

1. Name: Marta Putrinš
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4. Address, telephone, e-mail: Kungla 46, Tartu; tel. 5232938; marta.putrinsh@ut.ee
5. Current position: University of Tartu, Institute of Molecular and Cell Biology; Extraordinary Researcher
6. Education: 1998 – Tallinn Secondary Science School
   2002 – University of Tartu,BSc
   2004 – University of Tartu, MSc
7. Language skills: Estonian. Latvian, English, Russian
8. Working experience: from September 2008 – University of Tartu, Institute of Molecular and Cell Biology; Extraordinary Researcher

II. Scientific activities

1. Main research interests: First I have studied the regulation of transcription of the phenol degradation *pheBA* operon in *Pseudomonas putida*. My second research interest has been the role of two-component signal transduction system ColRS in *P. putida*.
2. List of publications:
   1. Kivistik PA, Putrinš M, Püvi K, Ilves H, Kivisaar M, Hõrak R: The ColRS two-component system regulates membrane functions and protects *Pseudomonas putida* against phenol. J Bacteriol 2006, 188:8109–8117.
   2. Putrinš M, Ilves H, Kivisaar M, Hõrak R: ColRS two-component system prevents lysis of subpopulation of glucose-grown *Pseudomonas putida*. Environ Microbiol 2008, 10:2886–2893.
   3. Putrinš M, Tover A, Tegova R, Saks U, Kivisaar M: Study of factors which negatively affect expression of the phenol degradation operon *pheBA* in *Pseudomonas putida*. Microbiology 2007, 153:1860–1871.
3. Fellowships: I have received FEMS Research Fellowship for studies in Karolinska Institute, Stockholm, Sweden, 4–31 January 2009.
4. Other organizational and professional activities:
   Member of Estonian Society for Microbiologists
   Teaching the practical course in genetics in The University of Tartu and also in The Gifted and Talented Development Centre
5. International courses attended:
   Workshop for PhD students "Microbial Activity at Biogeochemical Gradients" 3.–6. April 2006, Leipzig, Germany

CURRICULUM VITAE

I. Üldandmed

1.	Ees- ja perekonnanimi:	Marta Putrinš
2.	Sünniaeg ja koht:	24.04.1980. Tallinn
3.	Kodakondsus:	Eesti
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5.	Praegune töökoht, amet:	Tartu Ülikool, erakorraline teadus
6.	Haridus:	1998 – Tallinna Reaalkool, keskharidus 2002 – Tartu Ülikool,BSc 2004 – Tartu Ülikool, MSc
7.	Keelteoskus:	eesti. läti, inglise, vene
8.	Töökogemus (teenistuskäik):	alates septembrist 2008 – Tartu Ülikooli erakorraline teadur

II. Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad

Üheks uurimisvaldkonnaks on olnud fenooli lagundamist määravate geenide *pheBA* ekspressiooni bakteris *P. putida*. Teiseks olen uurinud *P. putida* kahekomponendilise signaaliülekande valkude ColR ja ColS rolli erinevates bakterite kasvutingimustes, sealhulgas ka fenooli stressi korral

2. Publikatsioonide loetelu
    1. Kivistik PA, Putrinš M, Püvi K, Ilves H, Kivisaar M, Hõrak R: The ColRS two-component system regulates membrane functions and protects *Pseudomonas putida* against phenol. J Bacteriol 2006, 188: 8109–8117.
    2. Putrinš M, Ilves H, Kivisaar M, Hõrak R: ColRS two-component system prevents lysis of subpopulation of glucose-grown *Pseudomonas putida*. Environ Microbiol 2008, 10:2886–2893.
    3. Putrinš M, Tover A, Tegova R, Saks U, Kivisaar M: Study of factors which negatively affect expression of the phenol degradation operon *pheBA* in *Pseudomonas putida*. Microbiology 2007, 153:1860–1871.
3. Saadud uurimistoetused ja stipendiumid: Olen saanud "FEMS Research Fellowship" stipendium uurimistöö läbiviimiseks Karolinska Instituudis, Stokholmis 4–31 jaanuar 2009.
4. Muu teaduslik organisatsiooniline ja erialane tegevus:
   Olen läbi viinud geneetika praktikumi Tartu Ülikooli Teaduskoolis
   Kuulun Eesti Mikobioloogide Ühendusse
5. Erialane enesetäiendus:
   Osalesin doktorantidele suunatud kevadkoolis "Microbial Activity at Biogeochemical Gradients" 3.–6. aprillini 2006 Leipzigis, Saksamaal.

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