DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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MERIKE JÕESAAR

Diversity of key catabolic genes at degradation of phenol and *p*-cresol in pseudomonads

TARTU UNIVERSITY
PRESS

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

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Microbiology on April 30, 2010 by the Council of the Institute of Molecular and Cell Biology, University of Tartu

Supervisor: Ain Heinaru, PhD, Professor, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Opponent: Hermann J Heipieper, PhD, Senior Scientist, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany

Commencement: Room No 217, Riia 23, Tartu, on June 21, 2010, at 10.00

Publication of this dissertation is granted by the Graduate School of Biomedicine and Biotechnology, University of Tartu

ISSN 1024–6479
ISBN 978–9949–19–372–1 (trükis)
ISBN 978–9949–19–373–8 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr 265

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following original papers that will be referred in the text by Roman numerals I–IV.

I Heinaru E, Viggor S, Vedler E, Truu J, **Merimaa M**, Heinaru A (2001) Reversible accumulation of *p*-hydroxybenzoate and catechol determines the sequential decomposition of phenolic compounds in mixed substrate cultivations in pseudomonads. *FEMS Microbiol Ecol* 37: 79–89.

II Heinaru E, **Merimaa M,** Viggor S, Lehiste M, Leito I, Truu J, Heinaru A (2005) Biodegradation efficiency of functionally important population selected for bioaugmentation in phenol- and oil-polluted area. *FEMS Microbiol Ecol* 51: 363–373.

III **Merimaa M,** Heinaru E, Liivak M, Vedler E, Heinaru A (2006) Grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes among phenol- and *p*-cresol-degrading *Pseudomonas* species and biotypes. *Arch Microbiol* 186: 287–296.

IV **Jõesaar M,** Heinaru E, Viggor S, Vedler E, Heinaru A (2010) Diversity of the transcriptional regulation of the *pch* gene cluster in two indigenous *p*-cresol-degradative strains of *Pseudomonas fluorescens*. *FEMS Microbiol Ecol* 72: 464–475.

Author's contributions:

Ref. I, II and III participated in study disain, contributed to experiments, analysed the data (catabolic genes, phylogenetic and DGGE analyses) and participated in preparation of the paper.

Ref. IV designed and performed experiments and wrote the paper.

ABBREVIATIONS

carA – carbamoylphosphate synthase gene
CAA – casamino acids
C12O – catechol 1,2-dioxygenase, catechol *ortho* cleavage enzyme
C23O – catechol 2,3-dioxygenase, catechol *meta* cleavage enzyme
DGGE – denaturing gradient gel electrophoresis
DMP – dimethylphenol
G12O – gentisate 1,2-dioxygenase
Km – kanamycin
K_S – apparent half-saturation constant
K_I – apparent inhibition constant
LmPH – large subunit of multicomponent phenol hydroxylase
mPH – multicomponent phenol hydroxylase
ORF – open reading frame
$ortho^{prot}$ – protocatechuate *ortho* pathway
PCMH – *p*-cresol methylhydroxylase
PC34O – protocatechuate 3,4-dioxygenase, protocatechuate *ortho* cleavage enzyme
PC45O – protocatechuate 4,5-dioxygenase, protocatechuate *meta* cleavage enzyme
PH – phenol hydroxylase
Pmen – *Pseudomonas mendocina*
PfF – *Pseudomonas fluorescens* biotype F
PfB – *P. fluorescens* biotype B
Ppu – *Pseudomonas putida*
POB – *p*-hydroxybenzoate
POBH – *p*-hydroxybenzoate hydroxylase
RBS – ribosomal binding site
sPH – single component phenol hydroxylase
TGGE – temperature gradient gel electrophoresis
μ_{max} – maximum specific growth rate

I. INTRODUCTION

Aromatic compounds are widely distributed class of organic compounds in nature, but they are also major concern because of their environmental persistence and toxicity. Although some of these compounds are recalcitrant or toxic for the vast majority of the microorganisms, bacteria usually have evolved biochemical and genetic information that allows them to use aromatic compounds as sole carbon and energy sources (Widdel and Rabus, 2001; Lovely, 2003). Albeit a wide phylogenetic diversity of microorganisms capable of aerobic degradation of contaminants, *Pseudomonas* species and closely related bacteria have been most intensively investigated owing to their ability to degrade so many different contaminants (Lovely, 2003). So, mainly pseudomonads were isolated in mid-nineties from water samples taken from channels surrounding semicoke mounds, and from the Kohtla and Purtse Rivers polluted with phenolic compounds in Northeast Estonia (Heinaru *et al*., 2000). The pollution is caused by solid wastes and leachate from semi-coke mounds, formed from flushing water and precipitations. These wastes are rich in several organic and inorganic compounds and have a high pH. In water contaminated with phenolic leachate the concentrations of phenol and *p*-cresol are higher than that of dimethylphenols, other cresols, resorcinols and polycyclic aromatic hydrocarbons (PAHs) (Ideon, 2007).

It has been shown that mixed cultures have a potential for broad metabolic activity, and that interaction of two or more strains is often a prerequisite for growth and biodegradation (van Hamme and Ward, 2001). Many xenobiotic compounds require the action of a bacterial consortium and they are not degraded by pure culture (Møller *et al*., 1998). To characterise functional activity and structural fluctuations of bacterial consortia many catabolic key genes have been studied (Watanabe *et al*., 1998; Futamata *et al*., 2003; Mesarch *et al*., 2000; Junca and Pieper, 2003; Merimaa *et al*., 2006). For practical purposes the biodegradative bacterial strains can be added to local microbial community to enhance bioremediation in polluted areas (bioaugmentation) (Dejonghe *et al*., 2001).

Aromatic catabolic pathways have to function efficiently within the context of the host and should be regulated in order to avoid detrimental energy fluxes that would otherwise compromise production, host fitness and survival (Shingler, 2003). The success of a particular catabolic pathway depends on two major elements: the catabolic enzymes catalysing mineralisation of the compound; and the regulatory elements (de Lorenzo and Pérez-Martín, 1996). Transcriptional regulators lie at the top of the hierarchy of events that lead to expression of the genes and operons that encode specialised suites of pathway enzymes for the catabolism of aromatic compounds (Shingler, 2003).

The main aim of the thesis was to genetically characterise *Pseudomonas* strains isolated from polluted area with phenolic compounds, to clarify genetic background of *p*-cresol catabolism in *Pseudomonas fluorescens* strains PC18 and PC24 by characterising the *pch* operons, their transcriptional regulation and enzyme induction.

2. REVIEW OF LITERATURE

2.1. Catabolic pathways of aromatic compounds

The constituents of fossil fuels and lignin, aromatic amino acids and different synthetic aromatic compounds can be either fully or partly degraded by micro-organisms. Their degradation depends on the number of aromatic rings and, especially, on the type of substituents. The substituents (e.g. halogen atoms, methyl and nitro groups) may remain intact or can be transformed or eliminated before the ring cleavage, and outcome of the reaction depends on bacterial species (Harwood and Parales, 1996). Regardless of specific strategy (anaerobic or aerobic), the catabolic pathways include two key steps: the activation of thermodynamically stable benzene ring, and its subsequent cleavage. In aerobic microbial degradation, oxygenases play a key role in both steps. They comprise monooxygenases and dioxygenases, which respectively insert one or both atoms of oxygen of O_2 into organic substrate (Gibson and Parales, 2000; Ferraro *et al*., 2005). The major reactions catalysed by dioxygenases include cleavage of the aromatic ring bond, which may be located: i) between two hydroxylated carbon atoms – *ortho* (β-ketoadipate) pathway; ii) adjacent to a hydroxylated carbon atom – *meta* pathway or iii) in an indole ring (Hayaishi and Nozaki, 1969; Vaillancourt *et al*., 2006). Three intermediates are common to all aerobic metabolic pathways of aromatic compounds: catechol, protocatechuate and gentisic acid (Fig. 1). These central compounds are broken down in similar pathways to simple acids and aldehydes which are readily used for cell synthesis and energy (Harwood and Parales, 1996).

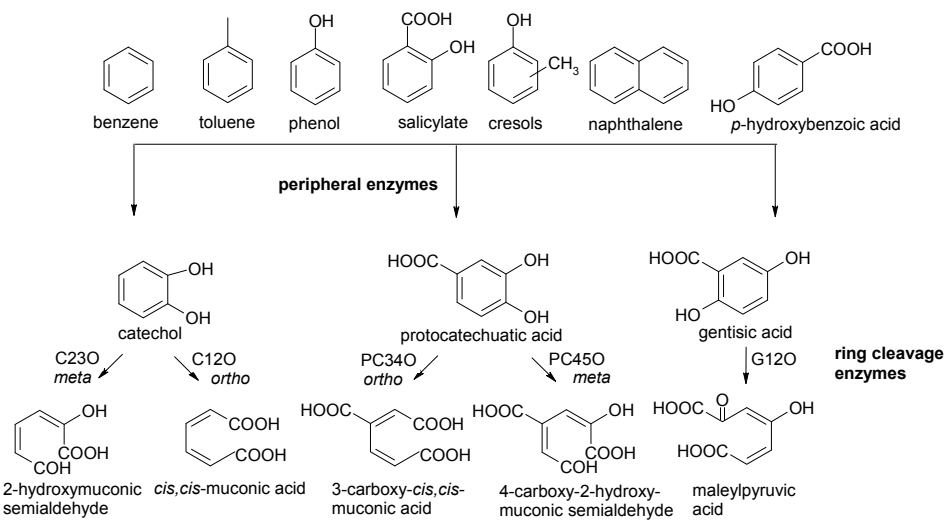

Figure 1. Aerobic routes of aromatic ring cleavage (Harwood and Parales, 1996).

o-dihydroxylated aromatic compounds are cleaved in *ortho* pathway by intradiol dioxygenases, such as catechol 1,2-dioxygenase (C12O, EC 1.13.11.1) and protocatechuate 3,4-dioxygenase (PC34O, EC 1.13.11.3), which contain Fe^{3+} in catalytic centre. *Meta*-fission pathway enzymes differ from those of the *ortho* pathway in their ability to catalyse also degradation of methylated catecholic substrates. From these alternative aromatic ring cleavage pathways the β-ketoadipate pathway is not suited for degradation of methylcatechol, whereas chlorosubstituted and unsubstituted catechols are normally cleaved by *ortho* pathway (Müller *et al*., 1996). During degradation of methylaromatics via *ortho* pathway 4-methylmuconolactone as a dead-end product is accumulating, since muconolactone isomerases require a proton at the C-4 carbon atom to catalyse the isomerisation to enol-lactone (Knackmuss *et al*., 1976; Chari *et al*., 1987). Metabolic route for 4-methylcatechol have been proposed only for *Cupriavidus necator* JMP134, *Rhodococcus rhodochrous* N75 and *Pseudomonas reinekei* MT1 (Pieper *et al*., 1985; Bruce and Cain, 1988; Marín *et al*., 2010). Modified *ortho*-cleavage pathways include enzymes that are closely related to those of the β-ketoadipate pathway but have evolved to handle also chlorinated substrates. The modified *ortho*-cleavage pathways are encoded usually on catabolic plasmids.

Meta-cleavage pathways specifying degradation of phenol, toluene and naphthalene are mostly plasmid-encoded (Harwood and Parales, 1996). *Meta*-cleavage of the aromatic ring is catalysed by extradiol dioxygenases, such as catechol 2,3-dioxygenase (C23O, EC 1.13.11.2) and protocatechuate 4,5-dioxygenase (PC45O, EC 1.13.1.18), which contain Fe^{2+} in catalytic centre (Dagley *et al*., 1960; Nogales *et al*., 2005). The majority of C23Os are phylogenetically closely related, belonging to the subfamily 1.2.A of the 1.2 extradiol dioxygenase family, and are of particular importance in degradation of monocyclic aromatic compounds (Eltis and Bolin, 1996). The best characterised extradiol dioxygenase is C23O, encoded by *xylE* gene which is located on TOL plasmid, pWW0. This enzyme consists of four identical subunits and contains one catalytically essential Fe^{2+} per subunit. The substrate range of this enzyme is relatively broad: 3-methyl-, 3-ethyl-, 4-methyl-, and 4-chlorocatechol (Harayama and Rekik, 1989; 1990). Much less is known about the protocatechuate 4,5-cleavage pathway. Genes of this pathway were observed only in *Comamonas testosteroni* BR6020, *Sphingomonas pausimobilis* SYK-6, *Arthrobacter keyseri* 12B and *Pseudomonas ochraceae* (Eaton, 2001; Providenti *et al*., 2001; Maruyama *et al*., 2001; Hara *et al*., 2003).

Binuclear compounds such as naphthalene are attacked twice. In the first step, one of the rings is cleaved and partially removed, leading to the formation of salicylate, which is further catabolised via catechol or gentisate by dioxygenases (Cerniglia, 1992; Peng *et al*., 2008). Degradation of gentisate is initiated by gentisate 1,2-dioxygenase (G12O, EC 1.13.11.4), which cleaves the aromatic ring between the carboxyl and proximal hydroxyl group to form maleylpyruvate (Lack, 1959) employing Fe^{2+} as cofactor (Harpel and Lipscomb, 1990). The maleylpyruvate can be converted to central metabolites either by

cleavage to pyruvate and maleate (Bayly *et al*., 1980) or by isomerisation to fumarylpyruvate and subsequent cleavage to fumarate and pyruvate (Lack, 1961). All isolated G12Os have very low similarity to other known ring-cleaving dioxygenases and the oxidative cleavage of *p*-dihydroxylated aromatic ring is probably less common route for bacterial degradation of aromatic compounds than either of the more extensively studied pathways through *o*-dihydroxybenzenes (Zhou *et al*., 2001).

In anaerobic catabolism of aromatic substrates, the peripheral pathways converge to benzoyl-CoA (occasionally to resorcinol and phloroglucinol), which becomes dearomatised by a specific multicomponent reductase that requires energy in the form of ATP (Gibson and Harwood, 2002).

2.2. Aerobic biodegradation of phenol

During first step of the aerobic pathway of phenol biodegradation, molecular oxygen is used by the enzyme phenol hydroxylase (PH, phenol 2-mono-oxygenase, EC 1.14.13.7) to add a second hydroxyl group in *ortho*-position to the one already present. Aromatic monooxygenases are divided into two groups: activated-ring monooxygenases (single-component) and nonactivated-ring enzymes (multicomponent). In latter case, the active site must contain a strong hydroxylgenerating unit, i.e. a dinuclear iron centre in which an oxygen atom is complexed with two iron atoms Fe-O-Fe. In former case, the enzyme is a simple flavoprotein (Neujahr and Gaal, 1973; Enroth *et al*., 1994) that uses NAD(P)H and O_2 as co-substrates and is composed of either one (single-component phenol hydroxylase, sPH) or several (multicomponent phenol hydroxylase, mPH) components (Pessione *et al*., 1999). The resulting catechol (1,2-dihydroxybenzene) molecule can then be degraded via two alternative pathways, depending on an organism. In *ortho* pathway, aromatic ring is cleaved between the catechol hydroxyls by a catechol 1,2-dioxygenase (C12O) (Harwood and Parales, 1996). The resulting *cis*,*cis*-muconate is further metabolised, via β-ketoadipate to Krebs cycle intermediates. In *meta* pathway the enzyme catechol 2,3-dioxygenase (C23O) transforms catechol to 2-hydroxymuconic semialdehyde. This intermediate can be channelled into the Krebs cycle (Mason and Cammack, 1992; Harwood and Parales, 1996) (Fig. 1).

Watanabe *et al*. (1996) have analysed the kinetics of phenol-oxygenating activity in several phenol-degrading bacteria, and suggest that phenol-degrading bacteria can be classified into three distinct groups based on K_S, (the apparent half-saturation constant in Haldane's equation) and K_{SI} (the apparent inhibition constant) values of these activities. Group 1, represented by *Comamonas testosteroni* R5 is characterised by low K_S and low K_{SI} values. Group 2, which is characterised by moderate K_S and moderate K_{SI} values, includes *Pseudomonas pickettii* (other names: *Burkholderia pickettii*, *Ralstonia pickettii*) PKO1 and *Acinetobacter calcoaceticus* AH; and group 3, which is characterised by high K_S and high K_{SI} values, includes *Pseudomans* sp. CF600 and *P. putida* BH. In

group 3, PHs from *Pseudomonas* sp. CF600 (Nordlund *et al*., 1990) and *P. putida* BH (Takeo *et al*., 1995) are of multicomponent type, while PH from *P. pickettii* PKO1 in group 2 shows characteristics of a single-component type. It was shown by our study group that K_S values for phenol in strains harbouring mPH were by almost four orders of magnitude lower than in strains having sPH (Viggor *et al*., 2008).

2.2.1. Single component phenol hydroxylases

Genes encoding flavoprotein hydroxylases (monooxygenases) induced by growth on phenol have been identified and characterised in a number of microorganisms, including *P. pickettii* PKO1 (*tbuD*) (Kukor and Olsen, 1992), *Pseudomonas* sp. EST1001 (*pheA*) (Nurk *et al.,* 1991) and the yeast *Trichosporon cutaneum* (Kälin *et al.,* 1992). The *tbuD* gene is co-transcribed with the C23O (*tbuE*) (Kukor and Olsen, 1991) and the *pheA* gene of *Pseudomonas* sp. EST1001 shares the operon with *pheB* gene encoding C12O (Kivisaar *et al*., 1991).

The *pheBA* cluster is flanked by two IS elements (IS*1472* and IS*1411*) (Fig. 2), which could facilitate movement of these genes from one DNA molecule to another (Kasak *et al*., 1993; Kallastu *et al*., 1998).

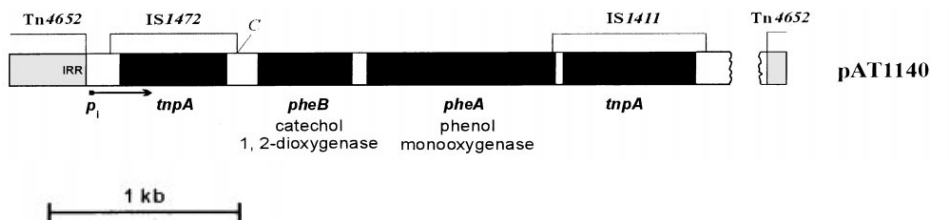

Figure 2. Organisation of *pheBA* operon in plasmid pAT1140 (Kasak *et al*., 1993).

The promoter of the operon is located upstream of IS*1472* and shows homology to chromosomal *catBC* promoter region which is recognised by CatR (Kasak *et al*., 1993; Parsek *et al*., 1995; Tover *et al*., 2000). After release of the laboratory *P. putida* strain carrying the *pheBA* genes on a plasmid into phenol-contaminated mining area in Estonia, horizontal transfer of the *pheBA* operon and its expression in different soil bacteria was observed (Peters *et al*., 1997).

The *tbuD* gene, encoding PH in *P. pickettii* PKO1 (Kaphammer *et al*., 1990) is located in an operon separate from *tbuEFGKIHJ*, which encodes the enzymes of the *meta* cleavage pathway. The *tbuD* gene and its promoter shows homology to 2-monooxygenases for which both phenol and alkyl-substituted benzenes are the substrates. All *tbu* operons in PKO1 are under control of the transcriptional activator TbuT (Olsen *et al*. 1997; Kahng *et al*. 2000).

2.2.2. Multicomponent phenol hydroxylases

Bacterial multicomponent monooxygenases comprise a family of nonheme, di-iron enzymes capable of using molecular oxygen to hydroxylate a variety of organic compounds (Notomista *et al*., 2003). Multicomponent aromatic monooxygenases contain at least two components, one responsible for hydroxylation (the oxygenase that binds substrate and oxygen, and catalyses the monohydroxylation of substrates), the other component is responsible for electron transfer from NAD(P)H to the oxygenase (the reductase that binds NAD(P)H).

It has been shown that initial conversion of phenol into catechol by a thermophilic microorganism *Bacillus thermoglucosidasius* A7 is carried out by two proteins, – a flavin reductase and a flavin-dependent monooxygenase, encoded by *pheA1* and *pheA2* genes, respectively. *Bacillus thermoleovorans* strain A2 degrades phenol and cresols via *meta* cleavage pathway. The first two enzymes involved in this process, the phenol hydroxylase and catechol 2,3-dioxygenase, are encoded by the *pheA* and *pheB* genes respectively (Duffner and Müller, 1998; Duffner *et al*., 2000; Kirchner *et al*., 2003).

Phenol hydroxylase that catalyses the conversion of phenol to catechol in *Rhodococcus erythropolis* UPV-1 was also identified as a two-component flavin-dependent monooxygenase. The two proteins are encoded by the genes *pheA1* and *pheA*2, the deduced amino acid sequences of both genes showed a high homology with several two-component aromatic hydroxylases. The phenol hydroxylase activity required the presence of both, PheA1 and PheA2 components, as well as redox coenzymes NADH and FAD (Saa *et al*., 2010).

In *Pseudomonas* sp. CF600 and *Acinetobacter calcoaceticus* NCIB8250 PHs, a third component (the size about 10 kDa) is present and regulates the functional interaction of the other two components (Powlowski and Shingler, 1990; Ehrt *et al*., 1995). Regulative component was found to be strictly necessary for the phenol to catechol conversion (Griva *et al*., 2003). All known bacterial multicomponent monooxygenases are transcribed from single operons that code for four to six polypeptides.

Multicomponent phenol hydroxylase (mPH) is considered as major enzyme in the natural environment (Peters *et al*., 1997; Watanabe *et al*., 1998; Futamata *et al*., 2001; Merimaa *et al*., 2006). All sequenced genes coding for mPHs encode similar enzyme structure; they comprise six subunits, among which the catabolic site exists within the largest (approx. 60 kDa) subunit. Some of these enzymes have different substrate specificity for substituted phenols (Teramoto *et al*., 1999). The DNA fragment encoding the largest subunit (catabolic site) of the mPH (LmPH) has been used as a molecular marker to assess functional and genetic diversity of phenol-degrading bacteria in the environment (Watanabe *et al*., 1998, 2002; Zhang *et al*., 2004; Merimaa *et al*., 2006).

The multicomponent phenol hydroxylases are classified into two types according to genetic organisation of the operons (Hino *et al*., 1998), i.e. i) the ***dmp*** type followed by the genes for a ferredoxin-like protein and a catechol 2,3-dioxygenase (Shingler *et al*., 1989), and ii) the ***mop*** type followed by a gene for

catechol 1,2-dioxygenase without a gene for a ferredoxin-like protein (Ehrt *et al.,* 1995). This may reflect the preference of these mPHs to catabolise different substituted phenols.

2.2.2.1. *dmp*-type multicomponent phenol hydroxylases coupled with the C23O gene

The mPH was first identified in phenol and (di)methylphenol (dmp) degradation pathways in *Pseudomonas* sp. CF600 by Shingler *et al*. (1992). *Pseudomonas* sp. CF600 can catabolise phenol and some of its methylated derivatives, *o*-, *m*-, and *p*-cresol and 3,4-dimethylphenol as sole carbon and energy source. The phenol-dimethylphenol *meta*-cleavage pathway of this strain is encoded on a large IncP-2 plasmid designated pVI150 (Shingler *et al*., 1989). The *dmp* mPH is encoded by six genes in the order of *dmpKLMNOP*. The genetic and biochemical studies showed that DmpP is FAD/[2Fe2S] reductase component, whereas a dimer of DmpLNO is an oxygenase component that contains a carboxylated-bridged di-iron centre at the DmpN active site (Powlowski and Shingler, 1994). DmpM is an activator for the catalysis, and DmpK may function in assembling iron at the active site (Powlowski *et al*. 1997).

The product of *pox* operon (*poxRABCDEFG*) of *Ralstonia eutropha* E2 is a multicomponent enzyme that is structurally similar to well-characterised *dmp* products (Shingler *et al*., 1992), although the deduced amino acid sequences of the *pox* products were unexpectedly different from those of the *dmp* products. The *pox* genes belong to the *dmp* type, although it is the only known example of the gene which is not highly homologous to equivalent genes of the other members of the *dmp* group (Hino *et al*., 1998). The chromosomally encoded PH of *Cupriavidus eutropha* JMP134 (formerly *Alcaligenes eutrophus, Ralstonia eutropha*) has been shown to be also a multicomponent system encoded by the gene cluster *phlKLMNOP* (Ayoubi and Harker, 1998).

The strain *Comamonas testosteroni* TA441 has a catabolic gene cluster (*aph* genes) similar to other multicomponent phenol hydroxylases. The *aph* genes are probably located on chromosome, because attempts to isolate an *aph*-gene-containing plasmid, failed. The structural genes encoding mPH and C23O (*aphKLMNOPQB*) and a regulatory gene of the NtrC family (*aphR*), were located in a divergent transcriptional unit (Arai *et al*., 1998).

2.2.2.2. *mop*-type multicomponent phenol hydroxylases coupled with the C12O gene

In *mop*-type strains contrary to the *dmp*-type strains, phenol is degraded by mPH to catechol, but catechol is degraded via the *ortho* pathway using C12O. *Mop*-type mPHs have been described mostly in genera *Acinetobacter* and *Pseudomonas*: in *Pseudomonas* sp. strain ADP (Neumann *et al*., 2004), in many

different pseudomonads (Merimaa *et al*., 2006) and *Acinetobacter radioresistens* S13 (Griva *et al*., 2003).

The strain *Pseudomonas* sp. M1 was isolated from the Rhine River (Iurescia *et al*., 1999). It is able to utilise several toxic and/or recalcitrant compounds as sole carbon and energy sources, including phenol (Santos *et al*., 2002). Phenol catabolism in strain M1 also involves an upper pathway, including the *phc* gene cluster required for the formation of catechol and *cis*,*cis*-muconate. The phenol/benzene upper pathway in *Pseudomonas* sp. M1 includes two different σ^{54}-dependent catabolic promoters, *Pa* and *Pk*, that independently control the expression of C12O (PhcA) and of phenol/benzene hydroxylase (PhcKLMNOP), respectively. As for the *dmp* model of *Pseudomonas* sp. CF600, the catabolic enzyme responsible for the initial oxidation of phenol or benzene in M1 is a mPH (*phcKLMNOP*) with a high degree of similarity (ranging from 56 to 85%, depending on the subunit) to the *dmpKLMNOP* hydroxylase. Furthermore, the *phc* gene cluster is σ^{54}-dependent and controlled by PhcR, a transcription factor 65% homologous to the DmpR regulator of *Pseudomonas* sp. CF600 (Shingler and Moore, 1994). The *phcA* and *phcR* genes are located upstream of the *phcKLMNOP* operon and their transcription proceeding in the opposite direction compared to *phcKLMNOP* (Santos and Sá-Correia, 2007).

The sole gene cluster described so far for phenol catabolism in *Acinetobacter calcoaceticus* NCIB8250, includes a multicomponent phenol hydroxylase (*mopKLMNOP*) and the *catA* gene encoding a C12O (Fig. 3). However, differently from the cluster of *Pseudomonas* sp. M1, these genes are organised in one operonic structure, being controlled from a single σ^{54}-dependent promoter (*Pmop*) regulated by MopR (Schirmer *et al*., 1997). *A. calcoaceticus* NCIB8250 is able to grow on phenol as sole carbon and energy source by virtue of a chromosomally encoded mPH (Ehrt *et al*., 1995).

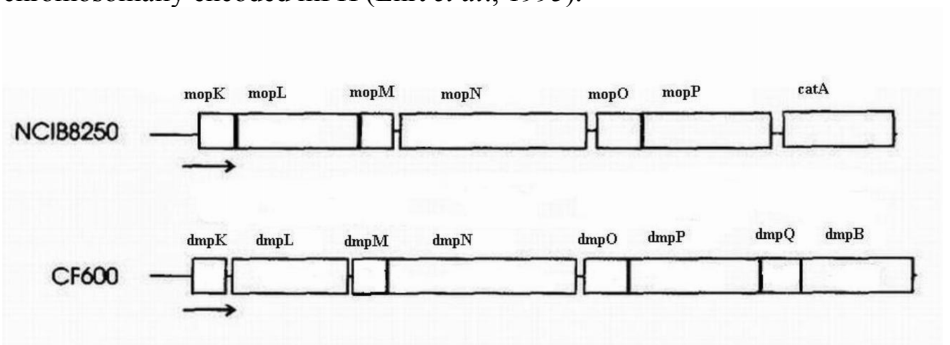

Figure 3. Comparison of the genetic organisation of mPH genes from *A. calcoaceticus* NCIB8250 and *Pseudomonas* sp. CF600 (Ehrt *et al*., 1995).

In contrast to *Pseudomonas* sp. CF600, *A. calcoaceticus* NCIB8250 is unable to grow on 3,4-dimethylphenol as sole carbon source (Shingler and Moore, 1994). The sequence similarity of the *mop* and *dmp* operons is confined to the region

encoding subunits of PH. Only *dmpQ* has no similarity with *mop* operon, because this gene is present in sequences encoding the reactivation of C23O (Ng *et al*., 1994).

Acinetobacter calcoaceticus PHEA-2 was isolated by enrichment for growth on phenol and benzoate from wastewater of an oil refinery (Xu *et al*., 2000). This strain can utilise phenol and benzoate as sole carbon and energy sources via the same catechol branch of β-ketoadipate pathway. The upper pathways involve the *mph* gene cluster (*mphKLMNOP*) encoding a multicomponent phenol hydroxylase homologous to DmpKLMNOP of *Pseudomonas* sp. CF600 and MopKLMNOP of *A. calcoaceticus* NCIB8250, sharing 38–72% and 58.5–93.5% amino acid identity, respectively. The upper pathways involve also the transcriptional regulator MphR, *ben* gene cluster (*benMABCDEKP*) encoding a benzoate 1,2-dioxygenase and the transcriptional regulator BenM (Zhan *et al*., 2008). An unknown gene directly follows the operon coding for PH and then are genes encoding transcriptional activator of *benABC* and benzoate dioxygenase downstream, which indicates that the arrangement of the functional genes of phenol and benzoate degradation in *A. calcoaceticus* PHEA-2 is different from that in *A. calcoaceticus* NCIB8250, in which a gene similar to C12O is located directly downstream of the *mopKLMNOP* (Ehrt *et al*., 1995). In PHEA-2, benzoate and phenol are converted to catechol by enzymes encoded by *mph* and *ben* operon independently; then catechol is further catalysed by enzymes encoded by the *cat* genes located downstream of the *ben* operon.

2.3. Aerobic biodegradation of *p*-cresol

Methylphenols (cresols) are produced in large quantities as constituents of resins, solvents, disinfectants, and wood preserving chemicals in petrochemical processes; but they are also products of anaerobic tyrosine fermentation via *p*-hydroxyphenylacetate (Yu *et al*., 2006). Luckily, these compounds are degraded in nature quite easily both by aerobic (Hopper, 1976) and anaerobic bacteria (Bossert and Young, 1986).

Two main catabolic routes have been described for *p*-cresol (4-methylphenol) (Fig. 4). In one of the pathways, a hydroxyl-group is added to *p*-cresol and the resulting 4-methylcatechol is then cleaved by catechol *meta* pathway enzymes (Bayly *et al*., 1966). In the second pathway the first enzyme that degrades *p*-cresol via the *ortho* pathway is *p*-cresol methylhydroxylase (PCMH, EC 1.17.99.1) (Hopper, 1976). PCMH converts *p*-cresol to *p*-hydroxybenzyl alcohol and later to *p*-hydroxybenzaldehyde (Hopper, 1976; Cronin *et al*., 1999; Cunane *et al*., 2000) which is subsequently oxidised to *p*-hydroxybenzoate (POB) by *p*-hydroxybenzaldehyde dehydrogenase (Fig. 4). The formation of protocatechuate from POB is catalysed by POB hydroxylase.

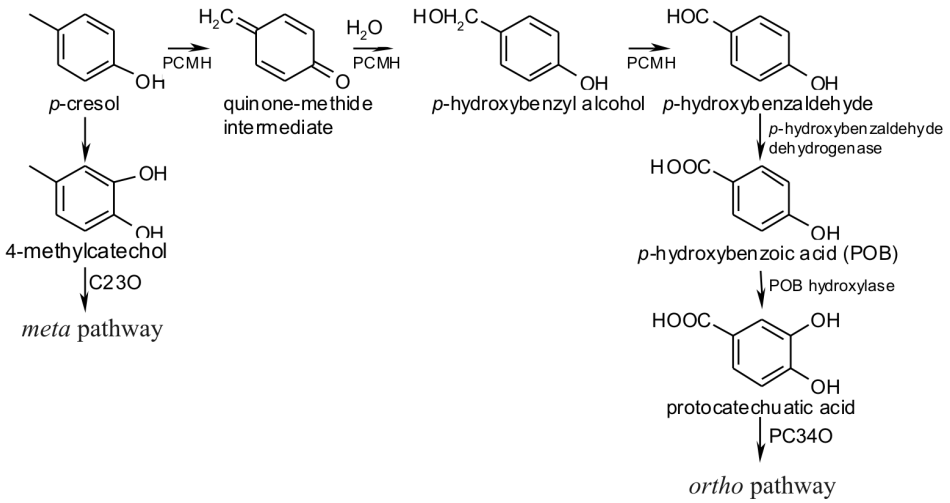

Figure 4. Two distinct catabolic routes for degradation of *p*-cresol (Dagley and Patel, 1957; Bayly *et al*., 1966).

The PCMH consists of two subunits forming an $\alpha_2\beta_2$ complex: the α subunits contain an active site flavin adenine dinucleotide (FAD) covalently linked to a tyrosine residue, whereas the β subunit is a *c*-type cytochrome (McIntire *et al*., 1981; McIntire *et al*., 1985). The natural electron acceptor for this periplasmic enzyme (Hopper *et al*., 1985) is azurin (Causer *et al*., 1984).

Despite studies showing that several *Pseudomonas* species and other microorganisms possess PCMH (Hopper, 1983; Hopper *et al*., 1991; Lovely and Lonergan, 1990; O'Reilly and Crawford, 1989; Rudolphi *et al*., 1991; Wright and Olsen, 1994; Heinaru *et al*., 2000; Peters *et al*., 2007), the genetics of the corresponding metabolic pathways has been studied in sufficient detail only in three *Pseudomonas* strains: *P. putida* NCIMB 9866, *P. putida* NCIMB 9869 (Kim *et al*., 1994), and *P. mendocina* KR1 (Wright and Olsen, 1994).

The genes encoding PCMH and *p*-hydroxybenzaldehyde dehydrogenase are organised as an operon (*pchACXF* in *P. putida* NCIMB 9866 and *pcuCAXB* in *P. mendocina* KR1) with *pchA*/*pcuC* encoding the second and *pchCF*/*pcuAB* encoding the first enzyme of the *p*-cresol metabolic pathway (Burlage, *et al*., 1989; Wright and Olsen, 1994). The gene designated as *pchX*/*pcuX* encodes a protein of unknown function (Cronin *et al*., 1999; Wright and Olsen, 1994). The *pcuR* gene, transcribed divergently from the *pcuCAXB* operon encodes the σ^{54}-dependent transcriptional regulator of this operon (Ramos-González *et al*., 2002).

The best-characterised PCMH is the plasmid-encoded $PCMH_{69A}$ from *P. putida* NCIMB 9869, which is expressed when the organism is grown on *p*-cresol, 3,5-dimethylphenol, glutamate or succinate as the carbon source. Chromosomally encoded $PCMH_{69B}$ genes of *P. putida* NCIMB 9869 are induced

only when the organism is grown on *p*-cresol (Kim *et al*., 1994). In *P. mendocina* KR1, these enzymes are involved in *p*-cresol metabolism; however, only this substrate was experimentally used as an inducer in this case (Wright and Olsen, 1994).

Also, a third route of *p*-cresol degradation is proposed. In some *Bacillus* strains *p*-cresol is converted into POB, which is further metabolised through gentisate pathway (Crawford, 1976; Tallur *et al*., 2006).

2.4. Sigma 54-dependent regulators, XylR/NtrC-type

Regulatory proteins and regulated promoters are key elements that control the transcription of catabolic operons to assure an adequate metabolic return when a particular substrate serves as the nutrient source (Díaz and Prieto, 2000). Regulation of aromatic-compound degradation is very often mediated by σ^{54}-dependent NtrC-type regulators, serving an efficient transcriptional control system (de Lorenzo and Pérez-Martín, 1996).

The family name of the σ^{54}-dependent regulators comes from recognition that all members of this group act in concert with alternative sigma factor σ^{54} (also called sigma N) encoded by *rpoN* (*ntrA*) and its homologues (Merrick, 1993). Sigma-54 confers on the core enzyme the ability to recognise and initiate transcription from a distinct class of -24/-12 bacterial promoters that differ considerably from the more usual -35/-10-type of promoters which are recognised by $E\sigma^{70}$ utilising the 'housekeeping' sigma factor encoded by *rpoD*. The consensus sequence of -24/-12 promoters, TGGCAC-N5-TTGC, contains an invariant -24 GG motif, an almost universally conserved -12 GC motif, and two or more T residues, the number of which appears to modulate the stability of $E\sigma^{54}$/promoter complex (Buck and Cannon, 1992). All sigma 54-dependent promoters analysed so far are positively regulated by transcriptional activators that usually bind to specific DNA sequences located unusually far (between 100 and 200 bp) upstream of the promoter. Their binding sequences are often inverted repeats that can be moved away by more than 1 kb without losing their ability to activate the transcription (Kustu *et al*., 1991; Morett and Segovia, 1993).

Most σ^{54}-dependent activators are constitutively produced, but their activity is controlled in response to environmental signals. Each regulator is activated by the aromatic substrate of the catabolic pathways it controls (Shingler, 1996). The best studied examples of these regulators are the XylR and the DmpR proteins from *Pseudomonas* (Table 1).

Table 1. Representative sigma 54-dependent regulatory proteins involved in biodegradation of aromatics (Shingler, 2003)

Family	Pathway	Host (plasmid)	Reference
DmpR	(Methyl)phenols	*Pseudomona*s sp. CF600 (pVI150)	Shingler *et al*. (1993)
HbpR	2-Hydroxybiphenyl	*P. azelaica* HBP1	Jaspers *et al*. (2000)
MopR	Phenol	*Acinetobacter calcoaceticus* NCIB8250	Schirmer *et al*. (1997)
PhlR	Phenol	*P. putida* H (pPGH1)	Burchardt *et al*. (1997)
PhnR	Phenanthrene/ naphthalene	*Burkholderia* sp. RP007	Laurie and Lloyd-Jones (1999)
TbuT	Toluene	*Ralstonia pickettii* PKO1	Byrne and Olsen (1996)
TouR	Toluene	*P. stutzeri* OX1	Arenghi *et al*. (1999)
XylR	Toluene/xylene (upper)	*P. putida* mt-2 (TOL pWW0)	Inoye *et al*. (1988)

Members of the σ^{54}-dependent family of regulators are composed of three distinct functional domains involved in signal reception, transcriptional activation, and DNA binding (see Fig. 4, reviewed by North *et al*., 1993; Morett and Segovia, 1993).

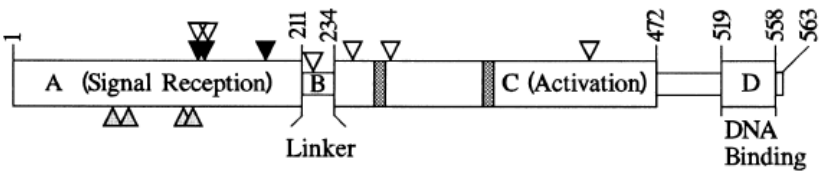

Figure 4. Schematic representation of the functional regions of sigma 54-dependent regulator (Shingler, 1996).

The A-domain acts as receiver module involved in recognition of cognate environmental signals. This domain is poorly conserved and is most variable in length. The A-domain accommodates at least three functions: the repression of transcriptional activity via binding to the central domain; the specific binding to the effector; and the release of the repression of the domain C upon this specific interaction with the effector (Devos *et al*., 2002).

The A-domain of DmpR and XylR (211 amino acids long) was shown to interact directly with an inducing aromatic compound, and various effector specificity mutations have been generated in this region of the protein (Pavel *et al*., 1994; Delgado *et al*., 1995; Fernández *et al*., 1995; Shingler and Pavel, 1995; Pérez-Martín and de Lorenzo, 1996a; Salto *et al*., 1998; Skärfstad *et al*., 2000; Wise and Kuske, 2000; O´Neill *et al*., 2001; Sarand *et al*., 2001; Solera *et al*., 2004; Galvão and de Lorenzo, 2006). Delgado and Ramos (1994) have shown that due to single amino acid change at the N-terminal end of XylR, the

protein acquired the ability to bind a new effector not recognised by wild-type protein. By DNA shuffling between the A-domains of DmpR and XylR, a subregion was identified as being primarily responsible for determining the distinct effector profiles of the two regulators (Skärfstad *et al*., 2000).

The central C-domains (~240 residues) of regulators, involved in transcriptional activation, are the most highly conserved and appear to have a common ancestral origin. This domain is involved in binding and hydrolysis of ATP, which forms the basis of the activation of σ^{54} promoters. Short carboxy-terminal D-domains of all σ^{54}-dependent regulators contain a helix-turn-helix DNA-binding motif analogous to those found in a number of transcriptional activators and repressors. Typically the A- and C-domains of the XylR-family proteins are connected with flexible Q-linker or „hinge" region, which is a short hydrophobic region of the protein (Fernandez *et al*., 1995). The number of residues separating domains C and D is highly variable within this family, and these regions bear little sequence identity.

XylR and DmpR are two mechanistically related sigma 54-dependent regulators that respond to distinct sets of aromatic effectors. XylR which activates the Pu promoter of the upper operon of pWW0 for the conversion of toluene and *m*/*p*-xylene to benzoate and corresponding alkyl-benzoates, also responds to some quite structurally dissimilar compounds such as chloro- and alkyl-substituted benzyl aldehydes and benzyl alcohols (Abril *et al*., 1989). For the activation of transcription from Pu promoter, XylR protein binds to two sites called UAS (upstream activating sequence) locating distant to the Pu promoter (Pérez-Martín and de Lorenzo, 1996). The activation of the transcription from the Pu promoter requires formation of the DNA loop between the UAS region and the Pu promoter sequence. The looping of the DNA is assisted by the DNA-bending proteins such as IHF (integration host factor) and HU (Histone like protein) (Pérez-Martín and de Lorenzo, 1995). Interaction between XylR bound to the UAS elements and RNA polymerase results in initiation of the transcription from the Pu promoter. This step needs ATP hydrolysis, stimulated by binding of the effector molecule to the XylR protein. ATP hydrolysis is required also for XylR multimerisation that makes possible the interaction between the XylR and the σ^{54} subunit of RNA polymerase (Pérez-Martín and de Lorenzo, 1996). DmpR regulates the expression from the P_o promoter, which drives transcription from one single large operon (*dmpKLMNOPQBCDEFGHI*) for phenol degradation that is present on the pVI150 plasmid in *Pseudomonas* sp. strain CF600. A large number of regulators highly similar to DmpR controlling phenol degradation operons have been found in bacteria (Table 1) (Shingler *et al*., 1993).

2.5. Degradation of mixed aromatic substrates

One of major hindrances in use of microorganisms for bioremediation is their preference for a simple carbon source over complex aromatic compounds. In nature, both complex and simple carbon sources are available. When two carbon sources are present, microbes first utilise the simple one, then followed by the complex carbon source. Different degradation patterns are observed in metabolism of multiple substrates: diauxic type, simultaneous utilisation, competitive inhibition and synergistic interactions between the substrates. During diauxic growth, depletion of the first substrate is followed by a lag period in which the microorganisms adapt to the second substrate. After this lag phase, exponential growth on second substrate starts. The length of the intermediate lag period depends on pre-culturing conditions as well as nature and relative concentrations of the substrates (Harder and Dijkhuizen, 1982). During the first exponential growth phase, the preferred substrate is utilised and the genes encoding catabolic enzymes required for the utilisation of the second carbon source are repressed, despite the continued presence of the second carbon source (Stülke and Hillen, 2000; Phale *et al*., 2007). If two cleaving enzymes are induced and if their substrate specificity allows the attack of both compounds in the mixture, substrates may be channeled into a wrong pathway and an accumulation of dead-end metabolites can result (Hollender *et al*., 1994).

Researchers have noted that microbial degradation of a compound in a mixture can be strongly impacted by other substituents of the mixture (Egli, 1995; Saéz and Rittman, 1993). To understand the mixture effects, one must consider the metabolic role of each compound of the mixture for the microorganism. More commonly, negative interactions are reported. Reasons for decreased biodegradation rates include competitive inhibition (Bielefeldt and Stensel, 1999; Chang *et al*., 1993; Oh *et al*., 1994), toxicity (Haigler *et al*., 1992) and formation of toxic intermediates by nonspecific enzymes (Bartels *et al*., 1984).

From a mixture of aromatic compounds, microbes utilise simple aromatic compounds or compounds that consume low energy to metabolise (i.e. compounds with higher oxidation level) over complex ones. For example *P. putida* and *Acinetobacter* strains utilise benzoate over 4-hydroxybenzoate (Nichols and Harwood, 1995) while *Rhodococcus* prefers benzoate over phthalate when supplied as a mixture (Choi *et al*., 2007; Patrauchan *et al*., 2005). Although certain mixtures are degraded more rapidly than compounds present individually (Jahnke *et al*., 1993), the biodegradation rate of aromatic mixtures may be low under natural conditions, mostly due to catabolite repression, but also due to insufficient concentration of other nutrients or a limited bioavailability of substrates as a result of binding to soil particles or low water solubility (Holtel *et al*., 1994; Duetz *et al*., 1996). Repression of the catabolism of aromatic pollutants by alternative carbon sources often contributes to the recalcitrant nature of such pollutants within conventional bioremediation processes.

Repression of the catabolism of aromatic growth substrates by acetate, succinate and other organic acids is documented in *Acinetobacter*, *Pseudomo-*

nas and *Ralstonia* species (Ampe *et al*., 1997, 1998; Dal *et al*., 2002; McFall *et al*., 1997). Ampe *et al*. (1996) observed that in *Ralstonia eutropha* 335, acetate represses the catabolism of aromatic compounds degraded via the *meta* pathway (i.e. phenol) and those of the catechol branch of the *ortho* pathway supporting growth rates and yields lower than acetate itself. The same order of substrate preference, benzoate > acetate > phenol, is shown for *R. eutropha* 335 and *Acinetobacter radioresistens* S13 (Mazzoli *et al*., 2007). Besides similarties, two main differences between these two strains exist: i) *A. radioresistens* S13 degrades phenol through the *ortho* pathway (while *R. eutropha* 335 utilises the *meta* route for the same compound) with a growth rate and yield similar to that on acetate; ii) both *R. eutropha* 335 and *A. radioresistens* S13 catabolise benzoate through the *ortho* pathway, but in *R. eutropha* 335 benzoate supports faster and more efficient growth than acetate while the opposite is true for *A. radioresistens* S13. Furthermore, catechol postulated to repress acetate metabolism in *R. eutropha* 335, was also observed in growth medium during both phenol catabolism and benzoate catabolism in *A. radioresistens* S13 suggesting that although substrate preference of these two bacteria is similar, the regulation phenomena controlling this cascade must differ.

The *ortho* and *meta* pathways are alternatives, whereby simultaneous operation of these pathways in degradation of mixed substrates yields toxic intermediates and causes accumulation of dead-end metabolites which may increase the degradation time and cause a sequential degradation of substrates (Hollender *et al*., 1994; Pieper *et al*., 1995; Erb *et al*., 1997). When the strain PC20 of *P. fluorescens* was grown on the mixture of phenol and salicylate, the consumption of both substrates occurred simultaneously despite the fact that two alternative pathways (*ortho* and *meta*) were used. In this strain catechol *ortho* and *meta* degradation pathways are encoded by different plasmids pPHE20 and pNAH20, respectively (Heinaru *et al*., 2009)

During batch growth of *Ralstonia eutropha* on benzoate-phenol mixture, benzoate (induces the catechol *ortho* pathway) completely inhibited phenol degradation (induces the catechol *meta* pathway, respectively) causing diauxic growth, and repression of phenol utilisation was attributed to the presence of benzoate (Ampe *et al*., 1998). It was also shown that *P. putida* cells degrade benzoate in preference to *p*-hydroxybenzoate (POB) by repressing POB transport by transcriptional downregulation of *pcaK*, the gene encoding POB permease (Nichols and Harwood, 1995; Cowles *et al*., 2000).

Hamed *et al*. (2003) observed that *P. putida* strain F1 degrades benzene, toluene and phenol mixture and investigated interactions between these substrates during their aerobic biodegradation. This strain was able to consume these three substrates completely. Toluene and benzene were better growth substrates than phenol, resulting in faster growth. Toluene was biodegraded slightly faster than benzene, and both benzene and toluene were biodegraded faster than phenol. The effect of toluene on biodegradation of phenol was more positive than the effect of benzene. Benzoate was also preferred to phenol in *A. radioresistens* S13 (Mazzoli *et al*., 2007), in which both compounds are

degraded through the same branch of the *ortho* pathway. Furthermore *cis,cis*-muconate and catechol, postulated to be molecular effectors of benzoate repression of the catabolism of other aromatic compounds in *Acinetobacter* sp. ADP1 and *Pseudomonas* strains (Gaines *et al*., 1996; Heinaru *et al*., 2001), accumulated during the degradation of each compound in *A. radioresistens* S13. *A. radioresistens* S13 seems to be strongly adapted to selectively degrade benzoate in environment containing alternative carbon sources. Furthermore, growth of bacteria on benzoate leads to the expression of additional genes encoding other enzymes necessary for the degradation of alternative aromatic compounds. It is generally accepted that inherent properties of each aromatic compound do not dictate the order of their consumption (Brzostowicz *et al*., 2003).

2.6. Degradation of aromatic compounds by mixed cultures of bacteria

In various natural and engineered environments, many species of microorganisms stably coexist by interacting and cooperating with each other. Microbial communities are fundamental components of ecosystems, playing critical roles in metabolism of organic matter. They are predominantly involved in detoxification of contaminated sites and organisms degrading a wide range of pollutants have been described. In most situations, microbial reactions drive natural attenuation or bioremediation processes (Brennerova *et al*., 2009). Bacteria of different genera existing in close proximity, are thought to aid each other in growth and survival via gene transfer and metabolic cross-feeding. The latter case has been relatively well studied with bacteria that provide amino acids or vitamins to other strains with biosynthetic deficiencies (de Souza *et al*., 1998).

It is essential to clarify the behaviour of microbial populations responsible for degradation of target pollutants. It is also important to fully understand the ecology of whole microbial community, including microbial populations that are not responsible for the degradation, because they may affect the behaviour of the degrading bacteria through microbial interactions (Sei *et al*., 2004). In nature bacteria do not exist as pure cultures, and significant proportions of microorganisms are associated with surfaces forming complex multispecies communities. During degradation of many xenobiotic compounds, such as chlorinated herbicides, nitrate esters, naphthalene derivatives and alkylbenzene sulfonates, the combined action of several species present in bacterial communities enhances or is required for complete mineralisation of the compounds. Processes which cannot be performed efficiently or which cannot be performed at all by a pure culture depending on joint action of two or more bacterial species are termed community level processes (Møller *et al*., 1998). In these microbial communities a wider spectrum of metabolic properties and processes exists, including synthesis of growth factors, removal of toxic substances and enabling cometabolism. Cometabolism, in particular, has been identified as a beneficial process for metabolism of xenobiotics (Singleton, 1994).

Pelz *et al*. (1999) disclosed an intricate network of carbon sharing in the community, defined the ecological roles of its three dominant members – two different *Pseudomonas* spp. (MT1 and MT4), an *Alcaligenes* sp. (MT3) and an *Empedobacter* sp. (MT2), and revealed that the substrate (chlorosalicylate) is catabolised by two completely different parallel routes, one of which is novel and involves protoanemonin as a critical intermediate, a toxic substance not previously found as a pathway intermediate in microbial world (Blasco *et al*.,1995; 1997). The community seemed to be so stable because each member played a crucial role, by either providing carbon skeletons for the others (MT1) or by scavenging toxic metabolites that inhibit the primary degrader if they accumulate.

Cordova-Rosa *et al*. (2009) observed a mixed culture from a coal wastewater treatment plant containing a high concentration of phenolic compounds and showed the ability of community to degrade phenol in both continuous and batch systems. The strains from the community were identified as *Pseudomonas alcaligenes,* mesophilic *Pseudomonas* and *A. calcoaceticus* var. *anitratus*. The mixed culture was able to survive in the presence of phenol concentration as high as 1200 mg L^{-1} and promote its degradation. In an environmental application of the activated sludge from an industrial coal wastewater treatment plant it was observed that inoculated bacterial consortium survived in a new environment, and after 15 days of microbiological degradation, they showed a decrease in phenol concentration from 19.48 to 3.19 mg kg^{-1}, and to 1.13 mg kg^{-1} after 20 days of microbiological treatment.

Elimination of aromatic compounds from industrial effluents and sewage by microbial degradation is usually not very effective because of chemical heterogeneity that leads to biochemical incompatibility of native microorganisms. The intermediary metabolite of the chlorobenzoate degradation pathway, chlorocatechol, irreversibly inhibits the *meta* cleaving enzyme C23O and also decelerates the reaction of the *ortho* pathway enzyme, C12O. Hence, simultaneous degradation of chlorobenzoates and phenol is usually not very effective. However, mixed cultures containing strains that can degrade either one or the other component of the mixture have been effective in eliminating both chemicals, but only when these substrates are present in suitable proportions. The concentration of the chloroaromatic compound should be equal or less than that of the nonchloroaromatic substrate (Jayachandran and Kunhi, 2009).

However, the use of mixed cultures in degradation may be less effective than use of pure culture of a genetically manipulated organism as Haugland *et al*. (1990) showed in case of degradation of chlorinated phenoxyacetate herbicides.

3. AIMS OF THE STUDY

General objective of this thesis was to characterise key catabolic genes for phenol and *p*-cresol degradation in pseudomonads isolated from area polluted with phenolic compounds.

The specific aims were:

1. To study diversity and phylogenetic grouping of catabolic genes coding phenol hydroxylase and catechol 2,3-dioxygenase among phenol/*p*-cresol degraders.
2. To evaluate kinetic parameters of phenol and *p*-cresol-degrading pseudomonads possessing different catabolic pathways for phenol and *p*-cresol degradation.
3. To study microbial activities under mixed-substrate growth conditions and specify the mechanisms regulating degradation of phenol and *p*-cresol from the mixtures.
4. To characterise the *pch* operons of *Pseudomonas fluorescens* strains PC18 and PC24 and transcriptional regulation of these operons in *p*-cresol degradation.
5. To investigate biodegradation efficiency of mixed phenol/*p*-cresol degraders in laboratory microcosms.

4. PHENOL/*p*-CRESOL DEGRADING BACTERIAL STRAINS

The 38 *Pseudomonas* strains used in this study (Table 2) were isolated in mid nineties as phenol/*p*-cresol degrading bacteria from water samples of the channels surrounding semicoke mounds in Kohtla-Järve, and of the Kohtla and Purtse Rivers (Heinaru *et al*., 2000). Importantly this watershed was continuously polluted with phenolic compounds. Based on specification of ring-cleavage dioxygenases, three main catabolic types of phenol and *p*-cresol degradation were revealed among these strains: i) *meta-meta* catabolic type strains use *meta* cleavage of catechol by C23O for both phenol and *p*-cresol; ii) *ortho-ortho*^{prot} catabolic type strains degrade phenol through *ortho* fission of catechol by C12O and *p*-cresol through *ortho* cleavage of protocatechuic acid by PC34O; iii) *meta-ortho*^{prot} catabolic type strains degrade phenol by using C23O, and *p*-cresol via the protocatechuate *ortho* pathway by PC34O.

Microbial strains used in current study are deposited in the Collection of Environmental and Laboratory Strains of Tartu University (CELMS, http://www.miccol.ut.ee).

Table 2. Description of studied strains.

Species and biotypes identified by *carA*	Strain designation (PC)	Catabolic type of phenol–*p*-cresol degradation	LmPH group[a]	C23O group[a]	*pheBA* operon[a,b]
P. fluorescens B	18, 21–23, 32–34, 37, 38	*meta-ortho*^{prot}	I	I	–
P. mendocina	1, 3, 4, 8, 10, 11	*meta-meta*	IIa	IIa	–
		meta-meta	IIb	IIb	–
	5–7, 9, 19	*meta-meta*	similar to 12	similar to PhhB	–
	2	*meta-ortho*^{prot}	similar to 2	similar to IIa	–
	12				
P. putida B	14, 15	*ortho-ortho*^{prot}	IIIa	–	–
	16	*ortho-ortho*^{prot}	IIIa	–	+
	30	*ortho-ortho*^{prot}	IIIa	–	d
	13, 35, 39	*ortho-ortho*^{prot}	–	–	+
	36	*meta-meta*	IIb	similar to XylE	–
P. fluorescens F	17	*ortho-ortho*^{prot}	IIIb	–	d
	P69	*ortho*[c]	IIIb	–	+
	20	*ortho-meta*	–	similar to NahH	+
P. fluorescens C	24–26, 28, 31	*ortho-ortho*^{prot}	–	–	+

[a]-, *pheBA* operon, genes for LmPH or C23O are absent
[b]+, *pheBA* operon is present
ortho[c], the strain does not degrade *p*-cresol
d, *pheA* gene from *pheBA* operon has been eliminated

5. RESULTS AND DISCUSSION

5.1. Species- and biotype-specific phylogenetic grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes (Ref. III)

To understand functional diversity of isolated strains, the evolutionary relationships of key catabolic genes was examined. PH and C23O are essential proteins in degradation of a wide range of aromatic pollutants. Although PH occurs in both single- and multicomponent variants in phenol-degrading consortia, mPHs are predominant in bacteria isolated from phenol-polluted areas (Peters *et al*., 1997). The sequence of LmPH has been used to evaluate the diversity of functionally dominant populations in trichloroethylene-contaminated aquifer soil (Watanabe *et al*., 1998; 2002). The C23O subfamily 1.2.A genes (Eltis and Bolin, 1996) have been analysed in studies of diverse environments (Wikström *et al*., 1996; Okuta *et al*., 1998; Mesarch *et al*., 2000; Junca and Pieper, 2003).

In order to assess the diversity between the catabolic genes of phenol/*p*-cresol-degraders, we analysed partial sequences of LmPH and C23O genes of 38 bacterial strains (Table 2). Respective PCR amplifications resulted in 29 LmPH- and 24 C23O-positive strains. The deduced amino acid sequences of these PCR products were aligned against the protein sequences of reference strains. The resulting phylogenetic tree of the proteins deduced from the sequences revealed four main groups (I, IIa, IIb, IIIa + IIIb) of LmPH genes (Fig. 5). Six strains (Ppu PC14-PC16, Ppu PC30, PfF PC17, PfF P69) harbouring group IIIa + IIIb LmPHs did not contain C23O genes. The phylogenetic tree for the C23O genes indicated the presence of three distinct groups (I, IIa, IIb) with the exception of strains Pmen PC2, Ppu PC36 and PfF PC20. Notably, the LmPH gene was absent in the PfF PC20 strain.

Comparison of the clustering data (Fig. 5) of ten strains belonging to the *meta–ortho*^{prot} degradation type of phenol and *p*-cresol shows that all nine *P. fluorescens* biotype B strains analysed (PC18, PC21-PC23, PC32-PC34, PC37, PC38) form a unique set within the group I LmPH and the group I C23O genes. We suppose that it may reflect selective pressure of phenolic pollutants in the environment on *P. fluorescens* biotype B bacteria. *P. mendocina* strains degrade phenol and *p*-cresol through the *meta* pathway. Two main clusters of strains were revealed: six strains (PC1, PC3, PC4, PC8, PC10, PC11) form group IIa and five strains (PC5-PC7, PC9, PC19) form group IIb, according to sequences of LmPH and C23O. LmPHs from group IIb are closely related to those of reference strains and belong to the Dmp family (DmpN, PhlD, PhhN and PheA4) (Fig. 5). The C23O genes of group IIb are similar to DmpB from *Pseudomonas* sp. CF600. Our analysis concerning catabolic genes revealed genetic heterogeneity of *P. mendocina* strains that may reflect adaptation of these bacteria to the presence of phenolic pollutants in the environment.

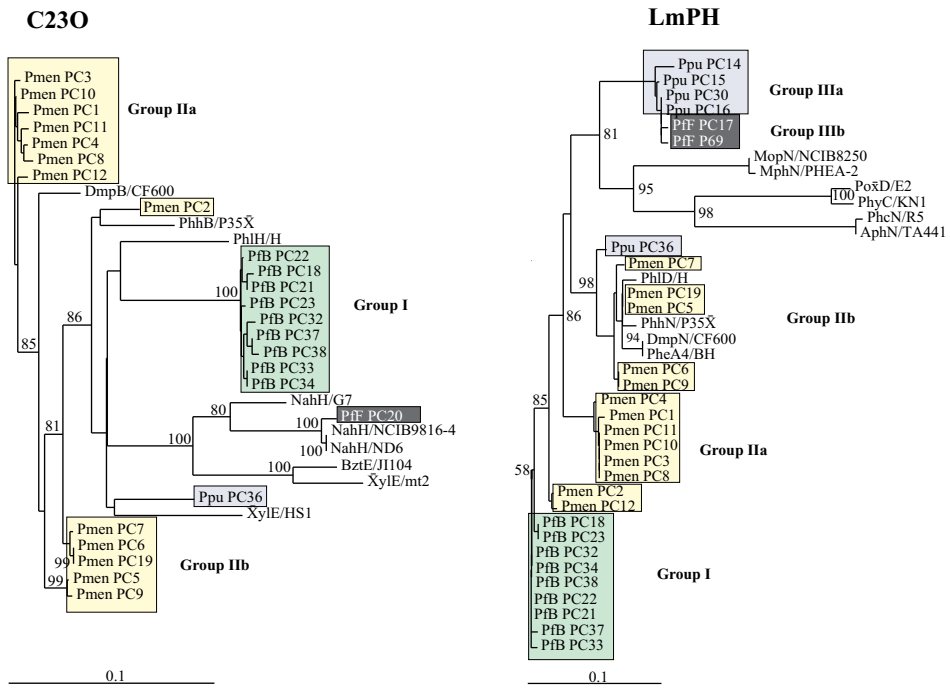

Figure 5. Neighbour-joining trees based on deduced amino acid sequences of the LmPHs (580-bp) and C23Os (924-bp) of the phenol/*p*-cresol degraders and reference strains. **Pmen** – *P. mendocina*, **PfF** – *P. fluorescens* biotype F, **PfB** – *P. fluorescens* biotype B, **Ppu** – *P. putida*. The sequences obtained in this study were aligned with the known LmPHs: MopN from *Acinetobacter calcoaceticus* NCIB8250 (Z36909), MphN from *A. calcoaceticus* PHEA-2 (AJ564846), PoxD from *Ralstonia* sp. E2 (AF026065), PhyC from *Ralstonia* sp. KN1 (AB031996), PhcN from *Comamonas testosteroni* R5 (AB024741), AphN from *C. teststeroni* TA441 (AB006479), PhlD from *P. putida* H (X80765), PhhN from *P. putida* P35X (X79063), DmpN from *Pseudomonas* sp. CF600 (M60276), PheA4 from *P. putida* BH (D28864), and C23Os: DmpB from *P. putida* CF600 (M33263), PhlH from *P. putida* H (X80765), NahH *P. putida* G7 (P08127), NahH from *P. putida* NCIB9816-4 (AA064305), NahH from *Pseudomonas* sp. ND6 (NP-943120), BztE from *P. aeruginosa* JI104 (X60740), XylE from *P. putida* mt2 (V01161), XylE from *P. putida* HS1 (M65205), PhhB from *P. putida* P35X (X77856). Bootstrap values (per 1000 trials) higher than 50% are indicated at the nodes. The scale bars represent 0.1 substitutions per amino acid site (LmPH, C23O). The phylogenetic groups of different species and biotypes revealed according to the *carA* gene sequence analysis are designated using boxes with different colours.

We also analysed the presence of the *pheBA* operon (sPH) in studied strains. The *pheBA* operon determines the synthesis of sPH (encoded by the *pheA*) and C12O (encoded by the *pheB*), it is plasmid-borne and there is strong evidence of horizontal transfer of this operon in nature (Peters *et al*., 1997). The *pheBA* operon is flanked by two IS elements, IS*1472* and IS*1411*. The promoter of the *pheBA* operon is located upstream of IS*1472* (Kasak *et al*., 1993). IS*1411* was

discovered as a consequence of insertional activation of the promoterless *pheBA* genes in *P. putida* due to the presence of outward-directed promoters at the left end of IS*1411* (Kallastu *et al*., 1998). We found this operon in 13 strains belonging to the *ortho–ortho*^{prot} type of degradation of phenol and *p*-cresol (Table 2). However, these strains belong to different *Pseudomonas* species and biotypes: *P. fluorescens* biotype C (PC strains 24–26, 28, 31), *P. putida* biotype B (PC strains 13, 16, 35, 39, 30) and *P. fluorescens* biotype F (PC strains 20, 17 and P69) (Table 2). In four strains (Ppu PC16, Ppu PC30, PfF PC17, PfF P69) *pheBA* operon and mPH were detected (Table 2). Our results indicate elimination of the gene coding for sPH from the *pheBA* operon in strains PfF PC17 and Ppu PC30. We assume that these strains have acquired the full-length *pheBA* operon through horizontal gene transfer, and further genetic rearrangements have led to the loss of the *pheA* gene. In strains Ppu PC16 and PfF P69 possessing genes for both sPH and mPH, the plasmid-encoded *pheA* gene is functional and expresses sPH activity but LmPH gene is probably not expressed as shown by northern analysis. According to our analysis, the *pheBA* operon from strain PfF P69 is similar to that of pAT1140 (Kasak *et al*., 1993), whereas in Ppu PC16 it lacks IS*1411* (Fig. 3, Ref. III). It is known that some bacteria employ more than one pathway to degrade hydrocarbons, which allows formation of novel mixed metabolic pathways and may explain why bacterial strains capable of growing on contaminants emerge so quickly (Notomista *et al*., 2003). DNA fragments can move into new hosts creating new mosaic genetic structures (van der Meer and Sentchilo, 2003).

In conclusion, LmPHs and C23Os clustered similarly in *P. fluorescens* biotype B, whereas in *P. mendocina* strains strong genetic heterogeneity became evident. *P. fluorescens* strains from biotypes C and F were shown to possess the *pheBA* operon, which was also detected in the majority of *P. putida* biotype B strains. Two strains possessed the genes for both single and multicomponent PHs, and two had genetic rearrangements in the *pheBA* operon leading to the deletion of the *pheA* gene.

5.2. Growth characteristics of representative strains on phenol and *p*-cresol (Ref. I and IV)

For further studies one representative strain from each catabolic type was chosen: *Pseudomonas mendocina* PC1 (*meta-meta*), *Pseudomonas fluorescens* biotype C PC24 (*ortho-ortho*^{prot}) and *P. fluorescens* biotype B PC18 (*meta-ortho*^{prot}) (Heinaru *et al*., 2000). The strains PC1 and PC18 degraded phenol by using mPH and C23O whereas the strain PC24 contains the *pheBA* operon determining C12O and single component PH. PCMH as the first enzyme for the degradation of *p*-cresol via protocatechuate branch of β-ketoadipate pathway was active in strains PC18 and PC24. Surprisingly, induction of this enzyme by phenol in strain PC18 was also revealed. In strain PC1 degradation of both phenol and *p*-cresol is catalysed by catechol *meta* pathway enzymes.

The PH-harbouring *Pseudomonas* strains were evaluated by kinetic parameters for phenol degradation by Viggor *et al*. (2008). It was shown that the values of apparent half-saturation constant for phenol-oxygenating activity (K_S) of strains possessing mPH were almost an order of magnitude lower than of strains having sPH. The mPH-possessing strains exhibiting high affinity towards phenol had also more than twise higher maximum specific growth rates (μ_{max}) compared to sPH-possessing strains (Table 3). The sPH strain PC24 with functional *pheBA* operon had lowest growth yield ($Y_{X/S}$) among the studied strains. This phenomenon could be explained by non-productive exogeneous accumulation of the metabolic intermediate *cis,cis*-muconate, which can be used for growth only by permeable mutants (Williams and Shaw, 1997).

Table 3. Comparison of kinetic parameters[a] for specific phenol-oxygenating activity and growth parameters of studied strains (Viggor *et al*., 2008)

Strain	Apparent constants in Michaelis-Menten equation for specific phenol-oxygenating activity	Apparent kinetic constants in Aiba-Edwards equation for growth		Yield factor
	K_s (μM)	μ_{max} (h^{-1})	K_I (mM)	$Y_{X/S}$[b] (g DW g^{-1} PHE)
PC1	1.7 ± 0.2	0.596 ± 0.042	9.41 ± 1.20	0.669
PC18	1.4 ± 0.5	0.535 ± 0.075	2.52 ± 0.18	0.700
PC24	21.4 ± 5.1	0.231 ± 0.010	7.44 ± 0.30	0.469

[a] Values are means of three independent experiments ± standard derivations of the mean
[b] The standard deviations for $Y_{X/S}$ was about 10%

It was concluded that the types of PH and catechol cleavage (*ortho* or *meta*) of different phenol-degrading strains do not affect the rate of phenol degradation and tolerance to phenol but may be strain-specific (Viggor *et al*., 2008). Indeed, the strain PC18 has lower K_I value than the strain PC1. The bacteria have certain protective mechanisms to survive at phenol concentrations that are otherwise bactericidal. One opportunity and possible mechanism for that is isomerisation of *cis*-unsaturated fatty acids to *trans*-configuration in bacterial membrane after the exposure to phenol (Heipieper *et al*., 1992).

However, the growth rates of strain PC1 on phenol and *p*-cresol were almost similar (0.60–0.63 h^{-1}). The strains PC18 and PC24 have higher growth rates on *p*-cresol than on phenol (Table 3; Fig. 2, Ref. IV). We found that the μ_{max} values on *p*-cresol for strains PC18 and PC24 were 1.12 and 0.71 h^{-1}, respectively (Fig. 2, Ref. IV). The specific growth rate, μ_{max}, was determined using the Richards model from absorbance values of cultures during batch cultivation experiments. It was also shown that in addition to almost two-fold higher μ_{max} values of PC18 for *p*-cresol growth, the length of the lag phase was also about three-fold shorter compared to PC24. The K_S values for *p*-cresol-oxygenating activity were

calculated from data obtained using a Clark-type oxygen electrode by measuring the oxygen consumption rate dependence of strains PC18 and PC24 on *p*-cresol concentration (Fig. 3A, Ref. IV). The K_S value for the strain PC18 (3.8 μM) was almost five-fold lower compared to the strain PC24 (17.3 μM). A low K_S value of PC18 reflects a high affinity of the strain for *p*-cresol which is in agreement with values of *p*-cresol maximum inhibitory concentration at which no oxygen consumption was observed, (S_m), determined from respiration measurements. Namely, S_m of strain PC18 was lower than that of strain PC24 (Fig. 3B, Ref. IV). These results are in accordance with chemical composition of the habitat of the strains – PC24 was isolated from the ditch surrounding the oil shale semi-coke mounds where concentration of aromatic compounds is much higher compared to downstream-located Kohtla river from where PC18 was isolated (Heinaru *et al*., 2000). Thus, according to kinetic analysis of *p*-cresol degradation, strain PC24 which has a high substrate tolerance expresses a high K_S and low specific growth rate, while PC18 which has a low substrate tolerance and a low K_S grows rapidly on *p*-cresol.

We suppose that differences in whole-cell kinetic parameters between the strains PC18 and PC24 revealed in this study are mainly caused by differences in structure and regulation of expression of respective PCMH genes. This statement is also supported by different growth yields of these strains on *p*-cresol (Fig. 2, Ref. IV), despite the fact that both strains gave the same growth yields on POB (Ref. I). However, different growth yields of these strains may also be caused by different catechol cleavage enzymes. Namely, strain PC18 harbours only C23O, but strain PC24 degrades aromatic compounds only via C12O. Although both strains catabolised *p*-cresol via the protocatechuate *ortho* pathway, minor C12O activity (Ref. I) causes lactone accumulation and thus the low growth yield of the strain PC24 can be explained. It is known that *ortho* fission reactions are rarely used by bacteria growing on methyl-substituted phenols due to the non-productive accumulation of a non-metabolisable methyl-substituted lactone.

In summary, we can conclude that although the strains PC18 and PC24 catabolise *p*-cresol using PCMH, clear differences in whole-cell kinetic parameters for this compound were revealed. Namely, affinity for the substrate and specific growth rate were higher in PC18 whereas maximum *p*-cresol tolerance was higher in PC24.

5.3. Phenol and *p*-cresol degradation in mixed substrate cultivations (Ref. I)

It is well known that microorganisms are able to degrade substrates from the mixtures either simultaneously or sequentially. Simultaneous utilisation happens when the pathways involved in degradation of both substrates are almost identical or at least similar, when the enzyme induction system is non-specific or catabolic pathways converge (Hutchinson and Robinson, 1988). However, it

is known that high concentrations of aromatic compounds can induce substrate-derived inhibitory effects and accumulation of toxic intermediates (Leveau *et al*., 1999; Bordel *et al*., 2007; Muñoz *et al*., 2007). Accumulation of (dead-end) intermediates favors sequential degradation of compounds (Gaines *et al*., 1996).

The fact that the presence of a methyl group on phenol ring changes the kinetic parameters of substrate utilisation by representative strains can be due to differences in catabolic pathways. The experiments were performed to specify the degradation pattern of phenol and *p*-cresol from mixtures. In two-substrate cultivations the initial concentrations of phenol and *p*-cresol in the growth medium were 2.5 and 1.3 mM, respectively (Ref. I). It was revealed that phenol and *p*-cresol were utilised simultaneously during the growth of *P. mendocina* PC1 and *P. fluorescens* C PC24 on phenol and *p*-cresol mixture (Fig. 4 A,C, Ref. I). Simultaneous utilisation of these compounds can be explained by the absence of metabolic conflict: strain PC1 degraded the above mentioned compounds via the same *meta* pathway by C23O, and strain PC24 via the β-ketoadipate pathway whereas the ring cleavage enzymes for phenol and *p*-cresol were C12O and PC34O, respectively. In contrast, during the growth of the strain PC18 on phenol and *p*-cresol mixture, typical diauxic growth was observed with *p*-cresol being the preferred substrate and degraded first (Fig. 4B, Ref. I). As phenol and *p*-cresol are biodegraded in this strain by alternative catabolic pathways (catechol *meta* and protocatechuate *ortho*, respectively), sequential consumption of substrates (diauxic growth) was observed. During growth on benzoate-*p*-cresol mixture the strain PC18 preferred *p*-cresol to benzoate (Fig. 9A, Ref. I). In this case both substrates were catabolised via β-ketoadipate pathway but using different ring cleavage enzymes, i.e. for benzoate by C12O and for *p*-cresol by PC34O. The activities of key enzymes of catabolic pathways in each exponential growth phase in different substrate mixtures were also monitored (Table 1, Ref. I). The enzymes specific for benzoate catabolism showed a more pronounced increase in activity in second growth phase. In case of preferential utilisation of *p*-cresol accumulation of the intermediate, *p*-hydroxybenzoate (POB), in growth medium was detected. If POB accumulation represses the expression of phenol *meta* pathway at growth of PC18 on phenol-*p*-cresol mixture, then diauxie should also occur at growth of this strain in POB-phenol mixture. Indeed, in this case diauxic growth was observed and degradation of phenol took place only after consumption of POB (Fig. 5A, Ref. I). Thus we showed that inability of the strain PC18 to degrade phenol and *p*-cresol simultaneously is caused by reversible accumulation of POB.

No diauxie was observed during the growth of the strain PC24 on phenol and POB mixture (Fig. 5B, Ref. I). This strain does not accumulate significant amounts of POB in the growth medium (Fig. 4C, Ref. I). High specific activities of *p*-hydroxybenzoate hydroxylase (POBH) in the first exponential growth phase of strain PC18 showed that accumulation of POB was not caused by a pathway bottleneck at the POBH step (Table 1, Ref. I). Our earlier work showed that in PC18 phenol also induced the *p*-cresol protocatechuate pathway by the induction of PCMH. Enzymological assays revealed that PCMH activity

was two-fold higher during the growth on phenol than on *p*-cresol in PC18 (Fig. 8, Ref. IV).

We assume that induction of PCMH simultaneously by *p*-cresol and phenol causes accumulation of the intermediate (POB), which at high concentration represses the activity of the catechol *meta* pathway and thereby coordinates utilisation of these two incompatible metabolic substrates.

5.4. Diversity of *p*-cresol methylhydroxylase genes and their transcriptional activation in strains *P. fluorescens* PC18 and PC24 (Ref. IV)

PCMH was used as a tool to characterise catabolic differences between phenol- and *p*-cresol-degrading *P. fluorescens* strains PC18 and PC24 in Paper IV. Although both strains catabolise *p*-cresol using PCMH, the whole-cell kinetic parameters for this compound and induction profiles during growth on phenol were different. We supposed that these differences between the strains PC18 and PC24 were caused mainly by differences in sequence structure and regulation of expression of respective PCMH genes (Ref. IV). In both strains the *pchACXF* operon, which encodes *p*-hydroxybenzaldehyde dehydrogenase and PCMH, was sequenced.

5.4.1. Characterisation of the *pch* gene cluster

Five putative ORFs of *pch* operon were identified (Fig. 4A, Ref. IV) based on comparison of nucleotide and deduced amino acid sequences with corresponding genes of *P. putida* NCIMB 9866 (Burlage *et al*., 1989; Cronin *et al*., 1999) and *P. mendocina* KR1 (Ramos-González *et al*., 2002; Wright and Olsen, 1994). The genes encoding PCMH and *p*-hydroxybenzaldehyde dehydrogenase are organised as an operon (*pchACXF*) with *pchA* encoding the second and *pchCF* encoding the first enzyme of the *p*-cresol metabolic pathway. The gene designated as *pchX* encodes a protein of unknown function. The *pchR* gene, transcribed divergently from the *pchACXF* operon encodes the sigma 54-dependent transcriptional regulator of this operon. The structure of the promoter, containing the consensus sequence (TGGCAC-N_5-TTGCW) (Merrick, 1993) of sigma 54-dependent promoters was identified upstream of the *pchA* gene of both strains (Fig. 4B, Ref. IV). However, these promoter sequences differ from the consensus sequence, having T instead of C at position -12 (TGGCAC-N_5-TTGTT). The -12 element, with the central consensus sequence TTGCW contributes to binding affinity. The latter element may play a more complex role in RNA synthesis, beyond simply assisting in promoter recognition (Buck and Cannon, 1989; Tintut *et al*., 1995; Wang *et al*., 1997; Wang and Gralla, 1998). Prior studies have suggested that the -12 region sequences

contribute to establishment of the basal transcription level (Wang *et al*., 1997). The -12 region can contribute not only to transcription specificity but also to its regulatory response. Thus, a common feature of deregulated promoters is a loss of C at -12, thereby this nucleotide would appear as critical for fork junction binding (Wang *et al*., 1999). Transcription starts at thymine located 261 bases upstream of the *pchA* translational start site in PC24, and at guanine located 143 bases upstream of the translational start in PC18 (Fig. 4B, Ref. IV). Transcriptional regulation of these operons by PchR, a putative σ^{54}-dependent regulator, was shown. PchR is a member of the sigma 54-dependent NtrC/XylR family of positive transcriptional activators (Morrett and Segovia, 1993; Shingler, 1996). PchR proteins of PC18 and PC24 have all of the specific sequence characteristics of this family (Fig. 5, Ref. IV). This type of regulator has a conserved four-part structure that includes an amino-terminal (A-domain) region linked to a central activation C-domain by a short B-domain, and a carboxyl-terminal DNA binding D-domain (Helix-Turn-Helix). The number of residues separating the C and D domains is highly variable within this family, and these regions bear little sequence identity (Shingler *et al*., 1993). An A-domain of sigma 54-dependent regulator acts as the receiver module involved in recognition of cognate environmental signals. This domain is poorly conserved and is most variable in length. Sequence identity in the effector-binding domain A of PchR proteins from PC18 and PC24 is 92% (19 amino acid differences) (Fig. 6, Ref. IV). Fig. 6 shows the positions in XylR and DmpR where mutations affected inducer binding and which were closest to our amino acid differences. In XylR172, Glu (E) was mutated to Lys (K) (Delgado and Ramos, 1994); in XylR85, Pro (P) was mutated to Ser (S) (Delgado *et al*., 1995), and in DmpR184, Arg (R) was mutated to Trp (W) (Shingler and Pavel, 1995).

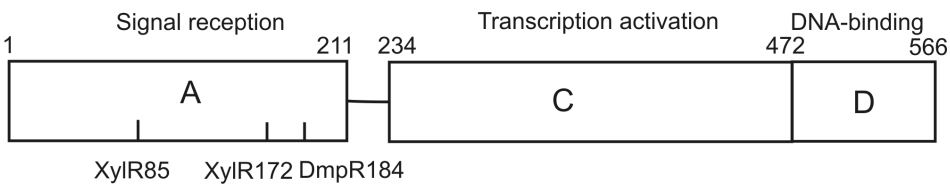

Figure 6. Domains A, C and D of the XylR/DmpR regulators and locations of point mutations, which were responsible for interactions with effectors (Delgado and Ramos, 1994; Delgado *et al*., 1995; Shingler and Pavel, 1995).

Comparison of *pchR* sequences with translated nucleotide sequence entries in the GenBank database revealed highest homology of PchR proteins with PcuR of *P. mendocina* KR1 (76–78% identity). TbuT of *Ralstonia pickettii* PKO1 (Byrne and Olsen, 1996), EugR of *Pseudomonas* sp. OPS1 (Brandt *et al*., 2001), XylR of *P. putida* mt-2 (Inouye *et al*., 1988), PhlR of *P. putida* H (Burchhardt *et al*., 1997), MopR of *Acinetobacter calcoaceticus* NCIB8250 (Schirmer *et al*.,

1997), TouR of *P. stutzeri* OX1 (Arenghi *et al*., 1999), and DmpR of *Pseudomonas* sp. strain CF600 (Shingler *et al*., 1993) showed 37 to 46% identity to PchR of PC18 and PC24. Among these sequences, TbuT and EugR are more similar to PchR. TbuT and EugR regulate operons involved in catabolism of toluene and eugenol, respectively. Eugenol hydroxylase genes *ehyA* and *ehyB* have strong sequence similarities to the PCMH genes *pchC* and *pchF* from *P. putida* NCIMB 9866, respectively (Brandt *et al*., 2001). Our analysis indicated that PchR regulators of PC18 and PC24 and PcuR of KR1, which are all activated in response to the aromatic substrate *p*-cresol, constitute a separate cluster in the tree. Thus, they are far more similar to each other than to other well-known sigma 54-dependent regulators (Fig. 7, Ref. IV).

5.4.2. Diversity of transcriptional regulation of the *pch* gene cluster

To investigate whether different induction patterns of the PchR regulators of PC18 and PC24 observed are caused by some specific structural features of these two proteins, a complementation assay in which *pchR* (entire *pchR* and the upstream promoter-operator area) (Fig. 7A) of PC18 was introduced into PC24 was constructed (PC24+18RBS) and inducibility of the *pch* operon was analysed in the resulting strain.

As shown in Fig. 7B, expression of *pch* operon was induced by phenol in PC24 bearing PchR of PC18. At the same time, in wild-type PC24 growing in phenol-containing medium as well as in CAA (uninduced conditions), PCMH activity was not found (Fig. 7B). As a control, strain PC24 was complemented with plasmid containing the deficient *pchR* of PC18 (most of *pchR* was deleted, Fig. 7A) and the induction of PCMH activity with phenol was not found.

In addition, complementation assay was conducted in which the native *pchR* regulator in strain PC18 was inactivated by Km^r gene insertion. As expected, after complementation of PC18*pchR*$^-$ with *pchR* (entire *pchR* and the upstream promoter-operator area) of PC24 (Fig. 7A), PCMH activity was induced only with *p*-cresol (Fig. 7B). Based on these results, we conclude that differences in amino acid sequences of PchR regulators of the two studied strains lead to different effector-binding capabilities of these proteins. Phenol is a more efficient effector molecule for PchR of PC18 than *p*-cresol, but it does not activate the regulator of PC24. At the same time, both regulators respond similarly to *p*-cresol. The capability of bacteria to adapt to certain contaminated environments by enhancing degradative capacities has been shown to be caused by mutational change within the effector binding subregion of DmpR (Sarand *et al*., 2001). Also, Delgado and Ramos (1994) have shown that due to a single amino acid change at the N-terminal end of XylR, the protein acquired the ability to bind a new effector not recognised by the wild-type protein (Fig. 6). This and other studies (Pavel *et al*., 1994; Delgado *et al*., 1995; Fernández *et al*., 1995; Shingler and Pavel, 1995; Pérez-Martín and Lorenzo, 1996a; Salto *et al*., 1998;

Skärfstad *et al*., 2000; Wise and Kuske, 2000; O'Neill *et al*., 2001; Sarand *et al*., 2001; Solera *et al*., 2004; Galvão and de Lorenzo, 2006) support the regulatory noise hypothesis to describe how transcriptional regulators may evolve competence to deal with novel environmental signals (de Lorenzo and Pérez-Martín, 1996; Garmendia *et al*., 2001).

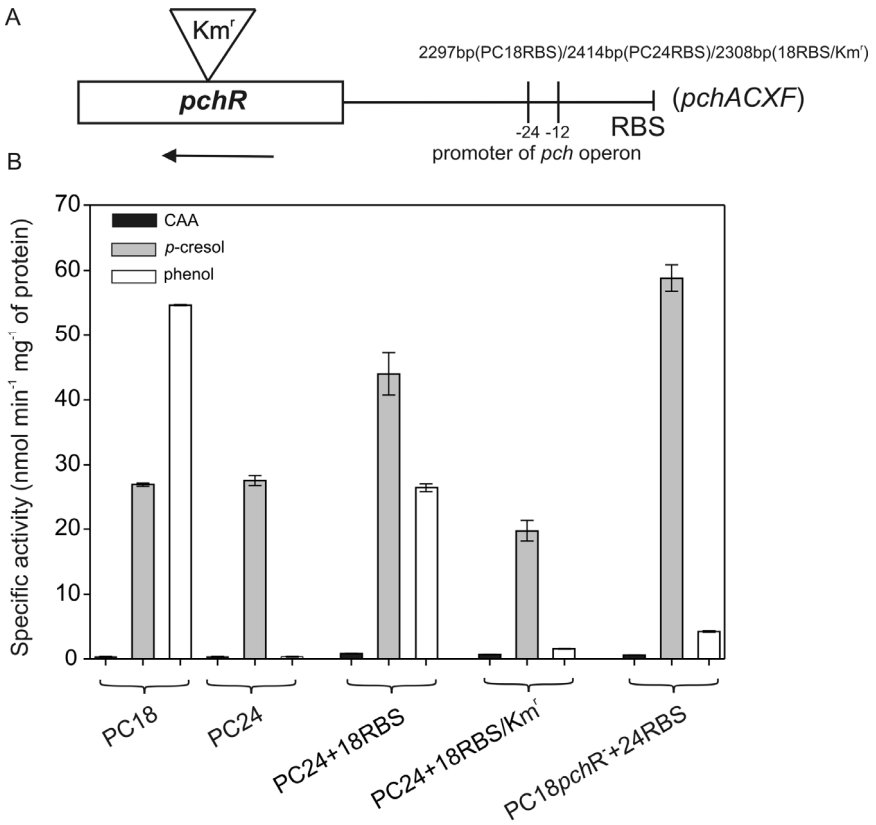

Figure 7. A – Schematic representation of inserts 18RBS/24RBS/18RBS-Km^r for complementation assays. **B** – Expression of PCMH activities (nmoles min^{-1}mg^{-1} of protein) in cell-free extracts of strains PC18 and PC24; and constructs PC24+18RBS, PC24+18RBS/Km^r and PC18*pchR*$^-$+24RBS during growth on *p*-cresol, phenol and casamino acids (CAA). Mean values for independent cultures are shown with standard deviation.

Thus in the cases of PC18 and PC24, changes in *pchR* regulatory gene structures have led to different expression patterns of catabolic routes, probably for overcoming potential metabolic conflicts during degradation of phenol and *p*-cresol mixtures.

5.5. Biodegradation efficiency of mixed bacterial populations (Ref. II)

Catabolic pathways operating in natural communities reflect interactions between microbial species under mixed culture conditions where extensive sharing of nutritional resources is common (Pelz *et al*., 1999) and interaction of two or several strains is often a prerequisite for growth and biodegradation (Van Hamme and Ward, 2001).

To examine biodegradation efficiency of mixed culture in leachate and oil-amended microcosms, phenol- and oil-degrading mixed culture was used comprised of four phenol and *p*-cresol degrading strains (PC1, PC18, PC20, PC24) with known complementary degradative capabilities (defined consortia). Among these, the strain PC18 is able also to degrade salicylate and strain PC20 salicylate and naphthalene. To understand interactions within a pollutant-degrading bacterial consortium *in situ*, we identified functionally dominant populations using analysis of the DNA isolated from laboratory microcosm samples. TGGE and DGGE analyses of 16S rRNA- and LmPH encoding genes have been used to study functional activity and structural fluctuations of bacterial consortia in a microcosm (Futamata *et al*., 2003). The same kind of molecular techniques are used in bioremediation studies. So, Watanabe and coworkers (Watanabe *et al*., 1998) used a combination of molecular and microbiological methods to detect and characterise dominant phenol-degrading bacteria in activated sludge. In our study, DGGE analysis of PCR products of 16S rRNA genes and of the gene encoding LmPH showed a shift in composition of bacterial population during incubation for 30 days on phenolic leachate and crude oil.

DGGE analysis of PCR products of 16S rRNA genes showed that *P. mendocina* PC1 became dominant and *P. fluorescens* PC20 disappeared after incubation of mixed population in leachate microcosms for 30 days (Fig. 1(a) and (b), Ref. II). Phenol and benzoate were removed by mixed cultures during the first day, cresols after 10 days, and from dimethylphenols 3,4-DMP in two days (Fig. 3, Ref. II). Disappearance of a particular population (PC20) from the leachate microcosm and maintenance of the same population in the naphthalene-contaminated oil microcosms throughout the entire experiment indicates that changes in bacterial consortia largely depend on substrate properties. In contrast, no dominant population was detected in oil-amended microcosms by DGGE. The catabolic significance of LmPH-possessing strains (PC1 and PC18) in biodegradation of pollutant mixtures revealed dominance of the strain PC1 in leachate and that of the strain PC18 in oil-amended microcosms (Fig. 2, Ref. II). The ability to degrade salicylate, an intermediate of naphthalene degradation, may be the reason why the LmPH-possessing strain PC18 dominated over PC1 through the first 10 days in oil-amended microcosms. Molecular monitoring of genes coding for catabolic enzymes of pollutant degradation pathways can show which population has major importance in a specific polluted ecosystem. In leachate microcosms, relative proportions of

bacteria having *meta* (PC1) and *ortho* (PC24) pathways for degradation of phenol and *p*-cresol changed alternately (Fig. 7, Ref. II). It is well known that *meta* and *ortho* ring fission of phenolic compounds are alternative pathways for a single strain and that pollutants are degraded faster under mixed culture conditions. Intermediates of a catabolic pathway of one strain (e.g., strain PC18) may be further degraded by another strain (e.g., strain PC24) possessing suitable catabolic pathway. The shifts in composition of mixed population indicated that different pathways of metabolism of aromatic compounds dominated and that this process is optimised response to contaminants present in microcosms.

6. CONCLUSIONS

Bioavailability of pollutants as well as survival and catabolic activity of introduced microorganisms play important roles in bioremediation technologies. Therefore, before applying the bioaugmentation, it is necessary to isolate, identify and characterise pollutant-degrading indigenous bacterial strains and analyse their activity *in situ*.

Accordingly, the first scope of the current thesis was to analyse the structure and regulation of expression of the key enzymes in catabolic pathways of phenol and *p*-cresol degradation in pseudomonads isolated from river water continuously polluted with phenolic compounds. The second aim of the thesis was to characterise catabolic activity of the specific types of strains in single as well as mixed substrate cultivations, and longevity of these strains in mixed populations.

Based on results presented in the thesis, following conclusions were made:

1. In the studied natural consortium of phenol and *p*-cresol degraders the strains bearing multicomponent phenol hydroxylases were most abundant. The strains described belong to three species – *Pseudomonas mendocina*, *Pseudomonas putida* and different biotypes of *Pseudomonas fluorescens*. In most of these strains, aromatic ring is opened by the action of catechol 2,3-dioxygenase. Multicomponent phenol hydroxylases and catechol 2,3-dioxygenases clustered in similar phylogenetic groups case of *P. fluorescens* biotype B strains. In *P. mendocina* strains, strong heterogeneity of these enzymes appeared. In case of six strains possessing multicomponent phenol hydroxylase, aromatic ring is cleaved by catechol 1,2-dioxygenase.
2. *P. fluorescens* strains from biotypes C and F were shown to possess the *pheBA* operon, which was also detected in the majority of *P. putida* biotype B strains. The strains *P. putida* PC16 and *P. fluorescence* P69 possessed both, single and multicomponent phenol hydroxylases, but only single component phenol hydroxylase activity was expressed.
3. The strains *P. mendocina* PC1, *P. fluorescens* PC18 and PC24, representing different catabolic types, have different whole-cell kinetic parameters. Expectedly, the growth rates of PC1 on phenol and *p*-cresol were similar, as both these substrates are degraded via catechol *meta* pathway. Albeit in both strains, PC18 and PC24, *p*-cresol is degraded via protocatechuate *ortho* pathway, affinity for the substrate and specific growth rate were higher for PC18 whereas maximum *p*-cresol tolerance was higher for PC24.
4. The degradation of phenol and *p*-cresol from their mixtures by bacteria occurred simultaneously in cases where these substrates were degraded via the same pathway (strains PC1 and PC24); and via diauxie if different degradative pathways were involved (PC18). In case of sequential consumption of phenol and *p*-cresol, the catabolism of phenol becomes inhibited by the reversible accumulation of *p*-hydroxybenzoate (an intermediate of *p*-cresol degradation pathway) due to the induction by phenol of the first enzyme of *p*-cresol catabolism – *p*-cresol methylhydroxylase.

5. Albeit in strains PC18 and PC24 the expression of *p*-cresol methylhydroxylase genes was induced differently, the deduced amino acid sequences of the corresponding genes, *pchC* and *pchF*, revealed high identity value (97%). Complementation assays confirmed that differences in amino acid sequences of the PchR regulators between the two studied strains led to different effector-binding capabilities of these proteins.
6. The microcosm experiments using mixed bacterial cultures on phenolic leachate, and oil-amended microcosms to investigate biodegradation efficiency indicated that specific bacterial populations were selected in both microcosms. In mixed populations, relative proportions of bacteria having *meta* (PC1) and *ortho* (PC24) pathways for degradation of phenol and *p*-cresol changed alternately throughout the experiment.

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SUMMARY IN ESTONIAN

Kataboolseid võtmeensüüme kodeerivate geenide mitmekesisus fenooli ja *p*-kresooli lagundamisel pseudomonaadides

Aromaatsed ühendid satuvad keskkonda peamiselt tööstustootmise tulemusena ning need on toksilised enamikele elusorganismidele. Eestis põhjustab olulisi keskkonnaprobleeme põlevkivi kaevandamine ning põlevkivikeemia- ja energiatööstused. Kirde-Eesti jõgedesse juhitavad kaevandus- ja leoveed (põlevkivi termilise töötlemise tagajärjel tekkinud poolkoksi ladestustest) sisaldavad kõrges kontsentratsioonis sulfaate, raskemetalle, õliprodukte ja mitmesugust toksilist aromaatikat. Erinevate asendustega fenoolide kui olulisema leovete reostuse lagundamisega saavad hakkama bakterid. Sellise tugeva fenoolse reostusega keskkond on selektiivseks survefaktoriks jõgede looduslikule mikroobikooslusele, kujundades välja erinevate katabolismiradadega mikroobid, kes on võimelised edukalt funktsioneerima mitmesugustes keskkonnatingimustes. Teadmised sellistes tingimustes väljakujunenud mikroobikonsortsiumidest on kas üldised või puuduvad. Biodegradatsiooniradade tuvastamine ja nendes osalevaid võtmeensüüme kodeerivate geenide uurimine lubab välja selekteerida ekstreemsetes tingimustes vastupidavad ja kõrgema biodegradatsiooni efektiivsusega tüved, kasutamaks neid bioremediatsioonis.

Sellest tulenevalt oli käesoleva töö eesmärkideks uurida pideva fenoolse reostusega veest eraldatud geneetiliselt erinevate pseudomonaadide kui enimlevinud biodegradatiivsete bakterite fenooli ja *p*-kresooli katabolismi võtmeensüüme kodeerivate geenide struktuuri ja avaldumise regulatsiooni, iseloomustada valitud tüüptüvede kataboolset aktiivsust nii üksiksubstraatidel kui ka substraatide segudes ning nende vastupidavust segapopulatsioonidena.

Töös esitatud tulemused võib kokku võtta järgnevalt:

1. Uuritud fenooli ja *p*-kresooli lagundajate konsortsiumis on enimlevinud multikomponentset fenooli hüdroksülaasi omavad bakteritüved, kes määrati liigiliselt *Pseudomonas mendocina, Pseudomonas putida* ja erinevate biotüüpidega *Pseudomonas fluorescens* esindajateks. Enamikel multikomponentset fenooli hüdroksülaasi omavatel tüvedel toimub aromaatse rõnga avamine katehhooli 2,3-dioksügenaasi abil, kusjuures *P. fluorescens* biotüüp B tüvedes klasterduvad mõlemad ensüümid fülogeneetiliselt sarnastesse ning *P. mendocina* tüvedes erinevatesse gruppidesse. Kuus tüve rühmituvad multikomponentse fenooli hüdroksülaasi järgi gruppi, kus aromaatset tuuma lagundatakse katehhooli 1,2-dioksügenaasi abil. Sellise lagundamisraja ehk Mop-tüübi olemasolu pole varasemalt pseudomonaadides kirjeldatud.
2. *P. fluorescens* biotüübis C ja F ning paljudes *P. putida* tüvedes toimub esmane fenooli lagundamine ühekomponentse fenooli hüdroksülaasi ja katehhooli 1,2-dioksügenaasi abil (kodeeritud *pheBA* operoni poolt). Selle kataboolse tüübiga bakterite hulgas on kaks tüve (*P. putida* PC16 ning *P. fluorescens* P69), kes omavad nii ühe- kui ka multikomponentset fenooli

hüdroksülaasi, kusjuures ekspresseerub vaid ühekomponentne fenooli hüdroksülaas.

3. Erineva katabolismitüübiga fenooli ja *p*-kresoolilagundajaid baktereid *P. mendocina* PC1, *P. fluorescens* PC18 ja PC24 iseloomustavad oluliselt erinevad kineetilised kasvuparameetrid. Tüvel PC1 on kasvukiirused nii fenoolil kui *p*-kresoolil sarnased, sest mõlemad substraadid lagundatakse katehhooli *meta* rada mööda. Kuigi tüvedes PC18 ja PC24 toimub *p*-kresooli lagundamine üle protokatehhuaadi *ortho* raja, omab tüvi PC18 suuremat kasvukiirust ning afiinsust *p*-kresoolil kui selle substraadi suhtes tolerantsem tüvi PC24.
4. Fenooli ja *p*-kresooli lagundatakse nende substraatide segudest samaaegselt juhul, kui lagundamine toimub samades degradatsiooniradades (tüved PC1 ja PC24), ja üle diauksia, kui lagundamisrajad on erinevad (tüvi PC18). Fenooli lagundamine teises logaritmilises kasvufaasis tüve PC18 puhul toimub *p*-kresooli katabolismi vaheprodukti *p*-hüdroksübensoaadi pöörduva kogunemise tõttu, sest *p*-kresooli lagundamisraja esimest ensüümi (*p*-kresooli metüülhüdroksülaas) indutseerib ka fenool.
5. Kuigi tüve PC18 *p*-kresooli methüülhüdroksülaasi süntees on indutseeritud lisaks *p*-kresoolile ka fenooliga, on seda ensüümi kodeerivate geenide *pchF* ja *pchC* ennustatav aminohappelise järjestuse sarnasus tüvedel PC18 ja PC24 väga suur (97%). PC24-s indutseerib selle ensüümi sünteesi ainult *p*-kresool. Märksa olulisemad erinevused leiti nende kahe tüve regulaatorgeeni *pchR* nukleotiidjärjestustes. Selle geeni produkt kuulub NtrC/XylR perekonda, on sigma 54-sõltuv ja reguleerib *pch* kataboolse operoni transkriptsiooni. Komplementatsioonikatsed tõestasid oletust, et regulaatorvalkude erinevast järjestusest tuleneb ka nende seondumine erinevate efektormolekulidega.
6. Biodegradatiivse efektiivsuse uurimine poolkoksi leovett ning põlevkivi toorõli sisaldavates mikrokosmkatsetes tüüptüvedest koosneva segapopulatsiooniga näitas, et erinevates mikrokosmides domineerivad kindlate aromaatsete ühendite lagundamisradadega populatsioonid. Fenooli ja *p*-kresooli lagundamine nendest segudest toimub ainult *meta* (PC1) ja ainult *ortho* (PC24) rada omavatel tüvedel alternatiivselt, s.t. kataboolne aktiivsus ning suhteline arvukus on segapopulatsioonis ajaliselt koordineeritud.

Käesoleva töö tulemused näitavad, et uuritud bakteritüved on efektiivsed ja sobilikud kasutamiseks fenoolse reostusega alade ja leovete bioremediatsioonil. Tänu antud uurimusele on võimalik välja valida ning kombineerida erinevaid kataboolseid võtmeensüüme ning kineetilisi parameetreid omavaid tüvesid bioremediatsiooni efektiivsuse suurendamiseks.

ACKNOWLEDGEMENTS

I thank my supervisors Eeva and Ain Heinaru for guidance, support and helping to set the goals. I have been lucky to have excellent advisors ready to help and to inspire me.

I whish to thank all my previous and present colleagues for help and advice. I would also like to thank all the co-authors of the publications presented in this thesis. Special thanks go to Signe Viggor, Eve Vedler, Merit Aava, Jaak Truu, Heili Ilves, Viia Kõiv and Eve Laasik for their contribution to the present study, and to Tiina Alamäe for critical reading of the manuscript of this thesis.

I am thankful to Anneli Kukk, Ene Põldroos, Milvi Siniroht, Tiiu Rootslane and Sulev Ingerpuu for technical or administrative help.

I am very grateful to my friends and family for all their support.

PUBLICATIONS

CURRICULUM VITAE

I. General data

Name:	Merike Jõesaar (Merimaa)
Date of birth:	15.06.1977. Voka, Ida-Virumaa
Citizenship:	Estonian
Address at work	University of Tartu, Institute of Molecular and Cell Biology, Riia Street, 51010 Tartu, Estonia
Phone:	+372 737 6038
E-post:	merike.joesaar@ut.ee
Current position:	University of Tartu, Institute of Molecular and Cell Biology, Researcher

Education

1999–	University of Tartu, teacher of natural science
2000–	University of Tartu, BSc in molecularbiology and genetics
2002–	University of Tartu, MSc in microbiology
2002–2007	University of Tartu, Institute of Molecular and Cell Biology, PhD student

Language skills

Estonian, English, Germany, Russian

Working experience

2003 – ... University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology, University of Tartu, Chair of Genetics; Researcher

II. Scientific activities

1. Main research interest: Diversity of catabolic genes in phenol -and *p*-cresol degrading bacteria. Catabolic performance of microbial communities in oil-contaminated Baltic Sea water.
2. List of publications:
   1) E. Heinaru, S. Viggor, E. Vedler, J.Truu, M. Merimaa, A. Heinaru (2001). Reversible accumulation of *p*-hydroxybenzoate and catechol determines the sequential decomposition of phenolic compounds in mixed substrate cultivations in pseudomonads. *FEMS Microbiol Ecol* 37: 79-89.
   2) S. Viggor, E. Heinaru, J. Loponen, M. Merimaa, T. Tenno, A. Heinaru (2002). Biodegradation of dimethylphenols by bacteria with different ring-cleavage pathways of phenolic compounds. *Environ Sci Pollut Res* 1: 19-26.

3) E. Heinaru, M. Merimaa, S. Viggor, M. Lehiste, I. Leito, J. Truu, A. Heinaru (2005). Biodegradation efficiency of functionally important populations selected for bioaugmentation in phenol- and oil-polluted area. *FEMS Microbiol Ecol* 51: 363-373.
4) M. Merimaa, E. Heinaru, M. Liivak, E. Vedler, A. Heinaru (2006). Grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes among phenol- and *p*-cresol degrading *Pseudomonas* species and biotypes. *Arch Microbiol* 186: 287-296.
5) M. Jõesaar, E. Heinaru, S. Viggor, E. Vedler, A. Heinaru (2010). Diversity of the transcriptional regulation of the *pch* gene cluster in two indigenous *p*-cresol-degradative strains of *Pseudomonas fluorescens*. *FEMS Microbiol Ecol* 72: 464-475.

3. Fellowships: I have received FEMS stipends for participation on different international congresses and meetings
4. Other organizational and professional activities: Member of Estonian Society for Microbiologists

ELULOOKIRJELDUS

I. Üldandmed

Ees- ja perekonnanimi:	Merike Jõesaar (Merimaa)
Sünniaeg ja koht:	15.06.1977, Voka, Ida-Virumaa
Kodakondsus:	Eesti
Aadress:	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut
Telefon:	+372 737 6038
E-post	merike.joesaar@ut.ee
Praegune töökoht, amet:	TÜ MRI teadur

Haridus

1999	TÜ, diplom loodusteaduste õpetaja põhikoolis
2000	TÜ, BSc molekulaarbioloogia ja geneetika erialal
2002	TÜ, MSc mikrobioloogia erialal
2002–2007	TÜ, doktorant

Keelteoskus

eesti, inglise, saksa, vene keel

Töökogemus

2003–	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Tartu Ülikooli Molekulaar- ja Rakubioloogia Instituut, Geneetika õppetool; teadur

II. Teaduslik ja arendustegevus

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2. Publikatsioonide loetelu:
   1) E. Heinaru, S. Viggor, E. Vedler, J.Truu, M. Merimaa, A. Heinaru (2001). Reversible accumulation of *p*-hydroxybenzoate and catechol determines the sequential decomposition of phenolic compounds in mixed substrate cultivations in pseudomonads. *FEMS Microbiol Ecol* 37: 79-89.
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3. Saadud uurimistoetused ja stipendiumid: FEMS stipendiumid osalemiseks erinevatel rahvusvahelistel konverentsidel
4. Muu teaduslik organisatsiooniline ja erialane tegevus (konverentside ettekanded, osalemine erialastes seltsides, seadusloome jms.): Olen Eesti Mikrobioloogide Ühenduse liige

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