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Plasmids and aromatic degradation in *Sphingomonas* for bioremediation - Aromatic ring cleavage genes in soil and rhizosphere

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ACADEMIC DISSERTATION

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Contents

List of original publications

This thesis is based on the following publications:

I Sipilä TP., Väisänen P., Paulin L. & Yrjälä K 2009: *Sphingobium* sp. HV3 degrades both herbicides and polyaromatic hydrocarbons using *ortho*- and *meta*-pathways with differential expression shown by RT-PCR. Submitted manuscript.

II Sipilä TP., Riisiö H. & Yrjälä K 2006: Novel upper *meta*-pathway extradiol dioxygenase gene diversity in polluted soils. FEMS Microbiology Ecology, 58:134-144.

III Sipilä TP., Keskinen A-K., Åkerman M-L., Fortelius C., Haahtela K. & Yrjälä K 2008: High aromatic ring-cleavage diversity in birch rhizosphere: PAH treatment-specific changes of I.E.3 group extradiol dioxygenases and 16S rRNA bacterial communities in soil. The ISME Journal, 2:968-981.

IV Sipilä TP., Paulin L. & Yrjälä K 2009: Mobility of aromatic degradation pathway genes in *Sphingomonas* - complete sequence of pSKY4 plasmid. Manuscript.

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Author's contributions

	I	II	III	IV
Study design	TS, KY	TS, KY	TS, MÅ, CF, KH, KY	TS, LP, KY,
DNA/RNA analysis	TS, PV	TS, HR	TS, AK	TS
Other analysis	TS, PV	TS, HR	MÅ, CF	TS
Data handling	TS, PV	TS, HR	TS, AK	TS
Writing the manuscript	TS, KY	TS, KY	TS, KY, CF	TS

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Abstract

Microbial degradation pathways play a key role in the detoxification and the mineralization of polyaromatic hydrocarbons (PAHs), which are widespread pollutants in soil and constituents of petroleum hydrocarbons. In microbiology the aromatic degradation pathways are traditionally studied from single bacterial strain with capacity to degrade certain pollutant. In soil the degradation of aromatics is performed by a diverse community of micro-organisms. The aim of this thesis was to study biodegradation on different levels starting from a versatile aromatic degrader *Sphingobium* sp. HV3 and its megaplasmid, extending to revelation of diversity of key catabolic enzymes in the environment and finally studying birch rhizoremediation in PAH-polluted soil.

To understand biodegradation of aromatics on bacterial species level, the aromatic degradation capacity of *Sphingobium* sp. HV3 and the role of the plasmid pSKY4, was studied. Toluene, *m*-xylene, biphenyl, fluorene, phenanthrene were detected as carbon and energy sources of the HV3 strain. Tn5 transposon mutagenesis linked the degradation capacity of toluene, *m*-xylene, biphenyl and naphthalene to the pSKY4 plasmid and qPCR expression analysis showed that plasmid extradiol dioxygenases genes (*bphC* and *xylE*) are inducted by phenanthrene, *m*-xylene and biphenyl whereas the 2,4-dichlorophenoxyacetic acid herbicide induced the chlorocatechol 1,2-dioxygenase gene (*tfdC*) from the *ortho*-pathway.

A method to study upper *meta*-pathway extradiol dioxygenase gene diversity in soil was developed. The extradiol dioxygenases catalyse cleavage of the aromatic ring between a hydroxylated carbon and an adjacent non-hydroxylated carbon (*meta*-cleavage). A high diversity of extradiol dioxygenases were detected from polluted soils. The detected extradiol dioxygenases showed sequence similarity to known catabolic genes of Alpha-, Beta-, and Gammaproteobacteria. Five groups of extradiol dioxygenases contained sequences with no close homologues in the database, representing novel genes.

In rhizoremediation experiment with birch (*Betula pendula*) treatment specific changes of extradiol dioxygenase communities were shown. PAH pollution changed the bulk soil extradiol dioxygenase community structure and birch rhizosphere contained a more diverse extradiol dioxygenase community than the bulk soil showing a rhizosphere effect. The degradation of pyrene was enhanced in soil with birch seedlings compared to soil without birch.

The complete 280,923 bp nucleotide sequence of pSKY4 plasmid was determined. The open reading frames of pSKY4 were divided into putative conjugative transfer, aromatic degradation, replication/maintaining and transposition/integration function-encoding proteins. Aromatic degradation orfs shared high similarity to corresponding genes in pNL1, a plasmid from the deep subsurface strain *Novosphingobium aromaticivorans* F199. The plasmid backbones were considerably more divergent with lower similarity, which suggests that the aromatic pathway has functioned as a plasmid independent mobile genetic element.

The functional diversity of microbial communities in soil is still largely unknown. Several novel clusters of extradiol dioxygenases representing catabolic bacteria, whose function, biodegradation pathways and phylogenetic position is not known were amplified

with single primer pair from polluted soils. These extradiol dioxygenase communities were shown to change upon PAH pollution, which indicates that their hosts function in PAH biodegradation in soil. Although the degradation pathways of specific bacterial species are substantially better depicted than pathways *in situ*, the evolution of degradation pathways for the xenobiotic compounds is largely unknown. The pSKY4 plasmid contains aromatic degradation genes in putative mobile genetic element causing flexibility/instability to the pathway. The localisation of the aromatic biodegradation pathway in mobile genetic elements suggests that gene transfer and rearrangements are a competetive advantage for *Sphingomona*s bacteria in the environment.

Abbreviations

16S rRNA	16S ribosomal RNA encoding gene
2,4-D	2,4-dichlorophenoxyacetic acid herbicide
bphC	2,3-dihydroxybiphenyl-1,2-dioxygenase encoding gene
BTEX	Benzene, toluene, ethylbenzene, and xylenes
C23O	Catechol 2,3-dioxygenase
DGGE	Denaturing gradient gel electrophoresis
EDO	Extradiol dioxygenase
HPLC	High pressure liquid chromatography
MCPA	2- methyl-4-chlorophenoxyacetic acid herbicide
nahC	1,2-dihydroxynaphthalene-dioxygenase encoding gene
nahH	Catechol 2,3-dioxygenase encoding gene in naphthalene degradation pathway in *Pseudomonas* NAH7 plasmid
Orf	Open reading frame
OTU	Operational taxonomic unit in this thesis defined by *Hha*I restriction enzyme pattern
PAH	Polyaromatic hydrocarbon
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RHD	Ring hydroxylating dioxygenases
RuBisCo	Ribulose bis-phosphate carboxylase
SSCP	Single-strand conformation polymorphism
T-RFLP	Terminal restriction fragment length polymorphism
xylE	Catechol 2,3-dioxygenase encoding gene

1. Introduction

Microbial degradation pathways play a key role in detoxification and mineralization of polyaromatic hydrocarbons (PAHs) and benzene, toluene, ethylbenzene, and xylenes (BTEX) compounds that are widespread pollutants in soil and constituents of petroleum hydrocarbons. In microbiology the aromatic degradation pathways are traditionally studied as a potential of a single bacteria strain to degrade pollutants. In soil the degradation of aromatics is typically performed by a community of micro-organism. In this thesis biodegradation genes were studied in the single bacterial strain *Sphingobium* sp. HV3 as well as in bacterial communities in soil.

1.1. *Sphingomonas*

The genus *Sphingomonas* was separated from *Pseudomonas* by Yabuuchi *et al*. 1990 with the type species, *Sphingomonas paucimobilis*, previously named as *Pseudomonas paucimobilis* (Holmes *et al.* 1977). The genus *Sphingomonas* has further been classified to the subclass 4-alphaproteobacteria (Takeuchi *et al.* 1994). *Sphingomonas* are yellow-pigmented, motile rods with single polar flagella and nonmotile, nonfermentative, gram-negative rods. All sphingomonads contain in their outer membranes glycosphingolipids in place of lipopolysaccharides (LPS) which are present in the outer membranes of most other Gram-negative bacteria. The *Sphingomona*s was divided into four genera *Sphingobium*, *Novosphingobium*, *Sphingopyxis* and *Sphingomonas sensu stricto* on the basis of 16S ribosomal RNA gene (16S rRNA) phylogeny and polyamine profiles (Takeuchi *et al.* 2001) but the division was later on rejected by Yabuuchi *et al.* 2002. The *Sphingosinicella* genus with close similarity to *Sphingobium*, *Novosphingobium*, *Sphingopyxis* and *Sphingomonas* was later on proposed (Maruyama *et al.* 2006). The family Sphingomonadaceae currently contains ten genera *Blastomonas*, *Erythromonas*, *Novosphingobium*, *Sandaracinobacter, Sandarakinorhabdus*, *Sphingobium*, *Sphingomonas*, *Sphingopyxis*, *Sphingosinicella* and *Zymomonas* (http://www.bacterio.cict.fr/). The position of the genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* are currently under debate and most *Sphingomonas* are not yet classified accordingly. In this thesis to evade naming issues of the *Sphingomonas* genus *Sphingomonas* will be defined *sensu latu* (including *Sphingobium*, *Novosphingobium, Sphingomonas* and *Sphingopyxis)* and the term sphingomonads will be used to cover all ten genera belonging to this group. The more specific genus names are used when the classification is available.

1.1.1. Aromatic degradation

Sphingomonas are well known for their exceptionally diverse degradation capacity of natural and xenobiotic compounds, such as biphenyl, (substituted) naphthalene(s),

fluorene, (substituted) phenanthrene(s), pyrene, (chlorinated) diphenyl ether(s), (chlorinated) furan(s), (chlorinated) dibenzo-p-dioxin(s), carbazole, estradiol, polyethylene glycols, chlorinated phenols and different herbicides and pesticides (Stolz 2009, Basta *et al.* 2005) and recently *Sphingomonas* CHY-1 was isolated with remarkable ability to grow on four-ring PAH chrysene as its sole carbon and energy source (Willison 2004). It has been also observed that a high proportion of the PAH degrading isolates in soil belong to the sphingomonads (Peng *et al.* 2008) and their presence in polluted soil has been confirmed by cultivation independent methods (Kleinsteuber *et al.* 2006, Leys *et al.* 2004).

Biochemically the naphthalene and biphenyl degradation pathways of *Sphingomonas* seem to resemble those in well known *Pseudomonas* and other gram negative bacteria with identical intermediates (Stolz 2009, Pinyakong *et al.* 2003b). Intermediates in upper *meta*-pathway naphthalene degradation are: *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, 1,2-dihydroxynaphthalene, 2-hydroxychromene-2-carboxylate, *trans-o*-hydroxybenzylidene-pyruvate, salicylaldehyde, salicylate and catechol (Eaton and Chapman 1992) (Figure 1). Biphenyl intermediates are: cis-2,3-dihydro- 2,3-dihydroxybiphenyl, 2,3-dihydroxybiphenyl, 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, cis-2-Hydroxypenta-2,4-dienoate and benzoate (Seeger *et al.* 1995) (Figure 1).

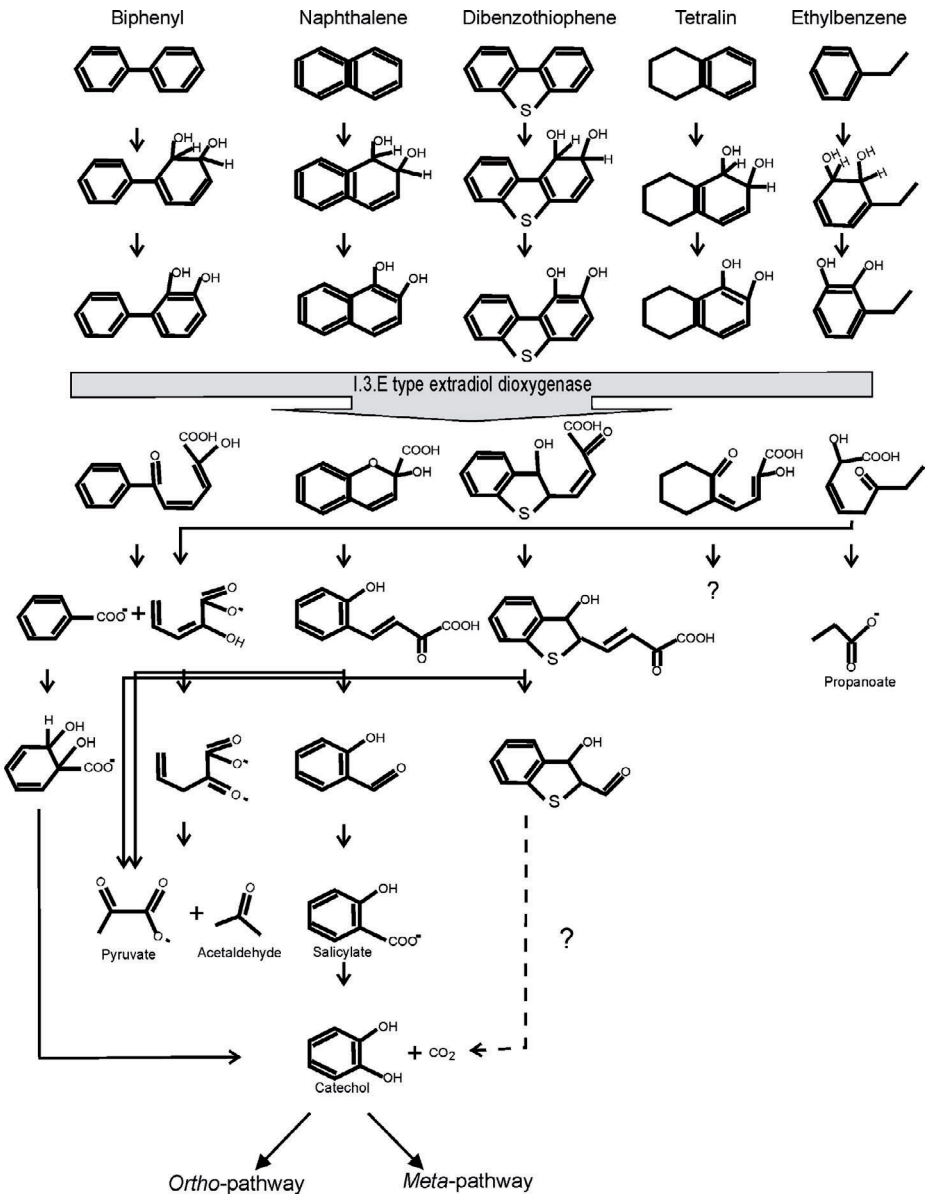

Figure 1 *Biochemical reaction in upper meta-pathway in degradation of biphenyl, naphthalene, dibenzothiophene, tetralin and ethylbenzene. The drawing is modified from Romine et al. (1999), Di Gregorio et al. (2004), Andujar et al. (2000) and Masai et al. (1997). The I.3.E extradiol dioxygenase studied in this thesis is shown.*

Sphingomonas aromatic degradation pathways genes are differently organised than in other well known degraders belonging to the *Pseudomonas*, *Burkholderia* and *Ralstonia* genera (Romine *et al.* 1999, Kim and Zylstra 1999). The aromatic degradation genes of biphenyl, naphthalene, *m*-xylene, and *p*-cresol in pNL1 plasmid are predicted to be distributed among 15 gene clusters in a 71 kb gene sequence. The gene organizations in operons do not follow the biochemical degradation pathway. For example the genes encoding naphthalene degradation are distributed into five different putative operons. The regulation of these operons is not known (Stolz 2009) although catabolic enzymes are shown to be induced by their substrates (Furukawa *et al.* 1983). Another peculiarity is that *Sphingomonas* aromatic degradation pathway seems to contain multiple genes encoding initial aromatic ring hydroxylation dioxygenase subunits, but only one set of electron transfer subunits and only one dihydrodiol dehydrogenase (*bphB*) and upper *meta*-pathway extradiol dioxygenase (*bphC)* gene for degradation of naphthalene, biphenyl and phenanthrene (Stolz 2009). *Sphingomonas* upper *meta*-pathway enzymes have high substrate range like the PAH dihydrodiol dehydrogenase, encoded by *bphB* gene, which is able to dehydrogenate 2,3-dihydroxy-2,3-dihydrobiphenyl, 1,2-dihydroxy-1,2-dihydronaphthalene, 3,4-dihydroxy-3,4-dihydrophenanthrene, 1,2-dihydroxy-1,2-dihydro-anthracene, 3,4-dihydroxy-3,4-dihydrochrysene, 4,5-dihydroxy-4,5-dihydropyrene, 2,3-dihydroxy-2,3-dihydrofluoranthene, 1,2-dihydroxy-1,2-dihydrobenz[a]anthracene and 9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene (Jouanneau and Meyer 2006).

1.1.2. Plasmids in *Sphingomonas*

Sphingomonas frequently contain large circular plasmids in size range 50-500 kb (Basta *et al.* 2004) but only two complete megaplasmid sequences from *Sphingomonas* bacteria i.e. larger than 100 kb, have been published like the pNL1 (Romine *et al.* 1999) and the pCAR3 (Shintani *et al.* 2007). The 184 kb pNL1 plasmid was isolated from *Novosphingobium aromaticivorans* F119 that contains a complex degradation pathway that serves in breakdown of both mono- and polyaromatic compounds. The 255 kb pCAR3 encodes degradation genes for the mineralization of carbazole into tricarboxylic acid cycle intermediates. pNL1 and pCAR3 are suggested to belong to same incompatibility group on the basis of sequence similarity (Shintani *et al.* 2007).

Degradation genes of heterocyclic compounds in *Sphingomonas* are also located in plasmids shown by hybridization studies (Basta *et al.* 2004). Large plasmids are suggested to encode carbofuran (Ogram *et al.* 2000), mecoprop (Lim *et al.* 2004), lindane (Ceremonie *et al.* 2006, Nagata *et al.* 2006) and polymeric xenobiotic compound degradation (Tani *et al.* 2007, Hu *et al.* 2008) enzymes in *Sphingomonas* strains.

Some evidence of *Sphingomonas* plasmid conjugation in natural sites exists. Identification of *Sphingomonas* plasmids with a similar *repA* gene in different bacterial strains isolated from same polluted surface soils suggests that plasmids similar to pNL1 are conjugative among different *Sphingomonas* strains in soil (Basta *et al.* 2004). Highly conserved pentachlorophenol-4-monooxygenase *pcpB* genes have been detected in different *Sphingomonas* strains isolated from polluted groundwater (Tiirola *et al.* 2002)

suggesting horizontal gene transfer. Conjugation of *Sphingomonas* plasmids in laboratory conditions has also been demonstrated (Romine *et al.* 1999, Basta *et al.* 2004). In the study of Basta *et al.* (2004) the plasmids did not conjugate outside the *Sphingomonas* genus suggesting that the plasmid conjugation ability is restricted specifically to *Sphingomonas* bacteria.

1.1.2.1. pNL1 plasmid

The pNL1 comprises 184,457 bp and was isolated from the *Novosphingobium aromaticivorans* F199 (Romine *et al.* 1999). The host bacterium of pNL1 was isolated from deep subsurface sediment (410 m below ground) and it is capable of degrading several aromatic compounds like, *p*-cresol, naphthalene, biphenyl, dibenzothiophene, fluorene, salicylate, benzoate, and all isomers of xylene (Fredrickson *et al.* 1995). The pNL1 containing kanamycin resistant marker (F199 tn349) was successfully conjugated to *Sphingomonas* sp. S88 (Romine *et al.* 1999). The degradation properties of the exconjugant were identical to the host *N. aromaticivorans* F199 tn349, except for the degradation capacity of *p*-cresol suggesting that either none or only portions of the *p*-cresol degradation pathway are encoded by pNL1. The conjugation experiment showed that the degradation capacity of *m*-xylene, salicylate, and benzoate were linked to pNL1 plasmid.

The aromatic degradation genes of biphenyl, naphthalene, *m*-xylene, and *p*-cresol in pNL1 plasmid are predicted to be distributed among 15 gene clusters in a 71 kb sequence (Romine *et al.* 1999). The gene organizations in operons did not follow the biochemical degradation pathways. The genes encoding naphthalene degradation are distributed in to five different putative operons. Seven orfs encoding aromatic-ring-hydroxylating dioxygenases (RHD) were located in pNL1 aromatic pathway, but only one ferredoxin and ferredoxin reductase mediating the electron transfer to initial dioxygenases. In pNL1 the large (α) and small (β) subunits of RHDs are located in gene pairs probably reflecting their function as a multi-component dioxygenase. One peculiarity in the pNL1 sequence was that the aromatic degradation region did not contain any orfs encoding a salicylate hydroxylase enzyme catalyzing mono oxygenation and decarboxylation of salicylate to catechol in naphthalene biodegradation (White-Stevens and Kamin 1972). RHDs of *Sphingomonas* sp. CHY-1, *Sphingomonas yanoikuyae* B1 and *Sphingobium* sp. strain P2 has been shown to mediate salicylate hydroxylase activity (Jouanneau *et al.* 2007, Cho *et al.* 2005, Pinyakong *et al.* 2003a).

1.1.2.2. pCAR3 plasmid

The pCAR3 plasmid (254,797 bp) was isolated from the *Sphingomonas* sp. KA1 strain (Shintani *et al.* 2007). The KA1 strain originates from activated sludge and is able to grow on carbazole as a sole carbon, nitrogen, and energy source and the genes encoding carbazole degradation enzymes are localised in the pCAR3 (Habe *et al.* 2002). The

complete sequence of pCAR3 identified putative orfs of anthranilate, catechol, 2-hydroxypenta-2,4-dienoate, dibenzofuran/fluorene, protocatechuate, and phthalate degradation. The replication and conjugation genes in pCAR3 are similar to those in pNL1 but the conjugation experiments with pCAR3 failed. pCAR3 plasmid was, however, successfully cured from strain. The cured KA1W strain could not grow on benzoate or carbazole demonstrating that these abilities of *Sphingomonas* sp. KA1 are linked to the pCAR3 plasmid.

1.1.3. *Sphingobium* sp. HV3

Sphingobium sp. strain HV3 was isolated as a 2- methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D) metabolizing strain from Finnish agricultural field soil (Kilpi *et al.* 1980). In the beginning the strain was characterized as *Pseudomonas* sp. HV3 but later on classified as *Sphingomonas* sp. HV3. The actual species name could not be assigned because no close relatives of the HV3 strain were at that time available (Yrjälä *et al.* 1998). In current *Sphingomonas* taxonomy based on 16S rRNA phylogeny, the strain belongs to the S*phingobium* genus and therefore in this thesis the strain is called *Sphingobium* sp. HV3 (Figure 2).

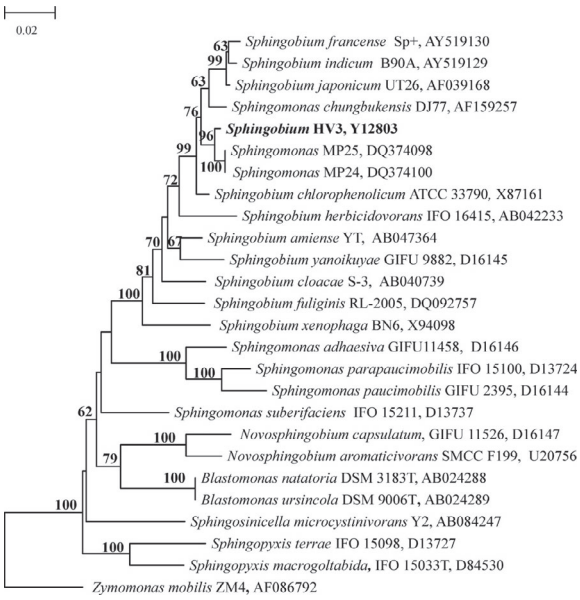

Figure 2 *Neighbor-joining tree of Sphingobium sp. HV3 16S rRNA gene sequence and related sequences representing Sphingomonas, Sphingobium, Novosphingobium and Sphingopyxis. Bootstrap values of 100 replicates are shown.*

Sphingobium sp. HV3 was found to harbour the pSKY4 plasmid with a catechol *meta*-pathway (Yrjälä *et al.* 1994). Catechol *meta*-pathway genes have been cloned and characterised (Yrjälä *et al.* 1997). These genes share a high similarity to corresponding genes in pNL1. *Sphingobium* sp. HV3 grows on 2,4-dichlorophenoxy acetic acid, 2-methyl-4-clorophenoxyacetic acid, 3-chlorobenzoate, 4-chlorobenzoate 3-methyl-salicylate, 3-methyl-salicylate, benzoate, *m*-toluate, naphthalene, *p*-toluate and salicylate (Kilpi 1980, Kilpi *et al.* 1988, Kilpi *et al.* 1983).

1.2. Diversity of micro-organism in soil

Soil contains a mosaic of microsites and gradients that are potential niches or habitats for one or more microbial species up keeping the immense microbial diversity (O'Donnell *et al.* 2007). One gram of soil may contain more than 10 billion prokaryote organisms enumerated by direct epifluorescence microscopy, but cultivation methods results in 100- to 1000-fold lower number of cells (Rossello-Mora and Amann 2001). The phenomenon is known as the great plate count anomaly. Cultivation methods are biased towards fast growing species belonging mostly to four bacterial phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Hugenholtz 2002). More importantly these bacteria rarely represent numerically dominant species in environmental communities. Although biased the culturing of bacteria is important part of environmental microbiology allowing more detailed physiological and genomic studies or biotechnological applications of isolated bacterial strains.

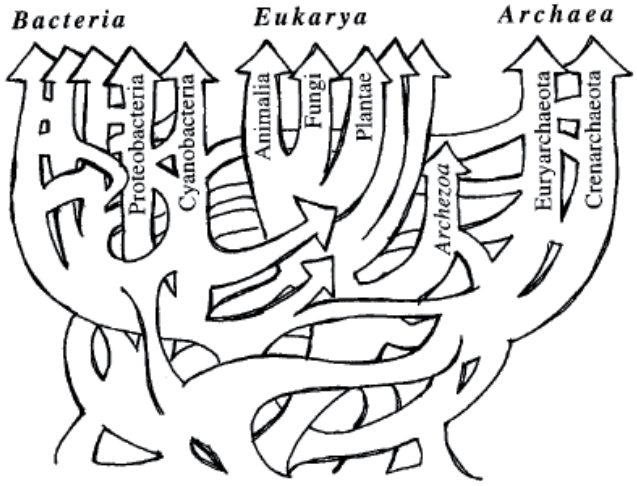

Figure 3 *Dendrogram or net of life which represents life's history taking into account the lateral gene transfer. From Doolittle WF (1999) Phylogenetic Classification and the Universal Tree. Science 284:2124-2128. Reprinted with permission from AAAS.*

Currently 52 bacterial phyla are recognized on the basis of their 16S rRNA sequence similarity (Rappe and Giovannoni 2003). Approximately half of the known phyla's are detected only with cultivation independent approaches and therefore contain no cultured members. Phylogenetic dendrograms describing bacterial diversity are frequently drawn on the basis of the 16S rRNA gene. The benefits of using the 16S rRNA as a phylogenetic tool and marker gene are undeniable. It is a ubiquitously spread ancient gene with essential fundamental function and interactions in the cell, contains extreme sequence conservation and a domain structure with variable evolutionary rates allowing phylogenetic comparison of both distant and closely related species (Woese 1987, Green and Noller 1997). Although the 16S rRNA is highly useful in taxonomic and phylogentic studies it displays stagnant picture of evolution and does not take account the lateral gene transfer with noteworthy effect on tree of life (Figure 3) (Doolittle 1999). The 16S rRNA approach in environmental community analysis is limited to taxonomical identification of the species and resolving the shifts in community composition but reveals little about the functional diversity of micro-organisms in the community (Torsvik and Øvreås 2002).

The culture independent approaches have revealed a detailed picture of microbial communities in the environment (Cardenas and Tiedje 2008). Some highlights of results acquired using 16S rRNA approach in environmental community analysis are listed in following.

1. The acknowledgement that most microbial species are still uncultured (Rappe and Giovannoni 2003).
2. Ubiquitous and abundant nature of Acidobacteria phyla in terrestrial environment (Hugenholtz *et al.* 1998, Barns *et al.* 1999).
3. Ubiquity of Verrucomicrobia, Planctomycetes and Chloroflexi phyla and their presence in various environments (Cardenas and Tiedje 2008).
4. The emergence of several candidate phyla of uncultured micro-organisms (Fuhrman *et al.* 1993, Liesack and Stackebrandt 1992, Stackebrandt *et al.* 1993).
5. The presence and diversity of archaea in non extremophilic environments (Fuhrman *et al.* 1992, Ueda *et al.* 1995, Jurgens *et al.* 1997).

Discovery of new microbial diversity shows that traditional cultivation techniques are insufficient to describe the bacterial species in different environments. Isolation of bacterial species done in cooperation with molecular methods is exemplified in study to isolate pathogen suppressive species (Benitez *et al.* 2008). Development of new cultivation methods demonstrate that culturing larger amount of species is often possible if niche conditions are mimicked (Cardenas and Tiedje 2008). Novel cultivation approaches have resulted in isolation of bacterial species from previously uncultured groups (Janssen *et al.* 2002, Stott *et al.* 2008, Eichorst *et al.* 2007, Bollmann *et al.* 2007). The ability to cultivate isolates of environmentally abundant micro-organism will greatly benefit the understanding of functional capacity of micro-organisms keeping up key processes in ecosystems. Recently the molecular tools in environmental microbial community analysis have enlarged from 16S rRNA gene fingerprinting and sequencing to study of collective genomes in present in environment with metagenomics approach. In metagenomic

approach total DNA is extracted from enviromental sample and studied by functional screening of clones (Handelsman *et al*. 1998) or by sequencing (Venter *et al.* 2004).

1.3. Functional diversity of micro-organism

Functional diversity is an aspect of the overall microbial diversity in soil, and encompasses a range of activities (Torsvik and Øvreås 2002). The relationship between microbial diversity and function in soil is largely unknown. The study of the collective genomes in an environmental sample, the metagenome, has shed some light on the functional diversity of micro-organism in communities.

The first published metagenome was from the mirco-organisms within filtered water of the Sargasso Sea (Venter *et al.* 2004). The metagenome of the Sargasso Sea contained 1.045 billion base pairs from at least 1800 different species. The metagenome revealed peculiar features of the gene diversity in sea water communities like surprisingly low amount of ribulose bis-phosphate carboxylase (RuBisCo) encoding gene (37 hits) the key enzyme in carbon dioxide assimilation in Calvin cycle but more than 650 proteorhodopsin homologs from 13 different subfamilies. The presence of proteorhodopsin in marine environment is also confirmed by other genomic analysis (Beja *et al.* 2000). These proteorhodopsins have challenged the notion that solar energy can only enter marine ecosystems by chlorophyll-based photosynthesis although a proteorhodopsins function in marine ecosystem is still under debate (Fuhrman *et al.* 2008). The Sargasso Sea study demonstrated several difficulties in metagenome analysis of complex community like, assembling sequences across species, low coverage of most species and inability to assign function to most predicted gene. Most open reading frames (orfs) in the metagenome were identified as conserved hypothetical proteins emphasising how little is known about the functional diversity of the microbial ecosystem.

Complex microbial communities, such as in soil ecosystem will demand enormous sequencing expenditure for the genome assembly of even the most predominant members (Venter *et al.* 2004), and most microbial communities are extremely complex and thus not amenable to genome assembly (Torsvik *et al.* 2002). Although the genome assembly form diverse communities seems to be almost impossible to obtain these can still be analyzed using gene-centric comparative analysis (Tringe *et al.* 2005). In this type of study the aim is to identify genes and compare the gene content of different communities. Most known orthologous gene groups from each environment could be resolved and each environment displayed individual “functional” profile. Interestingly very few orthologous groups are exclusively occurring in a particular environment.

Microbial communities associated with a certain ecological function have been assayed by fingerprinting genes for key enzymes of the process such as nitrate reductase (*nirS* and *nirK*) (Braker *et al.* 2000), ammonia monooxygenase (*amoA*) (Rotthauwe *et al.* 1997) methane monooxygenase (*pmoA*) (McDonald and Murrell 1997) methyl-coenzyme M reductase α-subunit (*mcrA*) (Galand *et al.* 2002). The use of functional primers as community marker genes has enlarged the knowledge of micro-organism of important key ecosystem functions like denitrification, ammonia oxidation, methanotrophy and

methanogenesis. These functional marker gene studies have shown that environmental communities are much more diverse than the known cultured representatives. The important benefit of functional gene analysis compared to the 16S rRNA gene analysis is that the community is linked to a certain function. The major pitfall is that detected novel functional groups cannot easily be assigned to a certain microbial taxonomic group. The functional genes are often less conserved than the 16S rRNA gene encoding structural RNA (Woese 1987) making the primer design more challenging. The metagenome studies have shown (Venter *et al.* 2004, Tringe *et al.* 2005) that the functional diversity of microbial communities is largely unknown, making it difficult to evaluate how well the used functional primers encompass the true diversity.

1.3.1. Diversity of aromatic catabolic genes

Low-molecular-weight (MW) PAHs, such as naphthalene, phenanthrene and anthracene, are usually readily degraded by bacteria in soil and under laboratory conditions (Cerniglia 1984, Cerniglia 1992). Over 300 catabolic genes involved in catabolism of aromatics have been cloned and identified from cultured strains (Widada *et al.* 2002a, Widada *et al.* 2002b) and the number is constantly increasing. A more detailed knowledge on catabolic genes in microbial communities and from isolates, can improve our understanding of microbial functioning and degradation processes in the environment. This can benefit the development of new and innovative bioremediation strategies of organic pollutants. Several studies have been focused on development of primers and probes to monitor catabolic bacteria in the environment or characterize isolated bacterial strains (Table 1, Table 2 and Table 3).

Compounds with aromatic ring structures are under oxic conditions degraded by multicomponent enzymes through initial mono- or dihydroxylation (Eaton and Chapman 1992, Barnsley 1976) (Figure 1). Hydroxylated rings are then channelled into central *ortho*- and *meta*-cleavage pathways by specific ring cleavage (Harayama *et al.* 1992, Reineke 1998). Extradiol dioxygenases (EDOs) are catalysing the *meta*-cleavage of the aromatic ring and intradiol dioxygenases the *ortho*-cleavage of the aromatic ring. EDOs belong to at least three evolutionarily independent families (Eltis and Bolin 1996, Vaillancourt *et al.* 2006). Type I extradiol dioxygenases discussed in this thesis belongs to the vicinal oxygen chelate superfamily and includes two-domain and one-domain enzymes (Gerlt and Babbitt 2001). The enzymes of this family have one subunit with two domains and have been shown to function in different oligomeric states. In catabolism of two aromatic ring-containing polyaromatic hydrocarbons (PAH) (Peng *et al.* 2008) the first aromatic ring structure is cleaved by the upper *meta*-pathway EDOs and the second ring by lower *meta*-pathway EDOs (Williams and Sayers 1994, van der Meer 1997, Lloyd-Jones *et al.* 1999).

Genes encoding catechol 2,3-dioxygenases (C23O) in the lower *meta*-pathway has been most frequently used as target for primer design (Mesarch *et al.* 2000, Wikström *et al.* 1996, Meyer *et al.* 1999, Sei *et al.* 1999, Hendrickx *et al.* 2005, Junca and Pieper 2004, Junca and Pieper 2003) (Table 1.). These studies have generally focused on I.2.A group of

catechol 2,3-dioxygenase most frequently isolated from the genus *Pseudomonas* (Eltis and Bolin 1996) but also primers with more broader specificity have been designed (Wikström *et al.* 1996, Sei *et al.* 1999). Catechol 2,3-dioxygenases have a key activity in lower *meta*-pathway catalyzing the ring cleavage of central intermediate (catechols) in degradation of aromatics. Initially the primers designed were mostly used for characterization of isolated bacterial strains with aromatic degradation capacity but later on the catabolic genes were monitored directly from the environment. The diversity I.2.A catechol 2,3-dioxygenases in the environment seems to correspond well with the C230 genes found from cultivated bacterial isolates (Junca and Pieper 2004, Hendrickx *et al.* 2006) indicating either high cultivability of I.2.A C23O genes containing bacteria or that the primers designed to this cluster amplify only the cultivated members of I.2.A group. Very recently a study using C23O primers (Sei *et al.* 1999) that amplified the subfamilies I.2.A, I.2.B and I.2.C from microcosms of pristine eutrophic lake water enriched with dissolved organic matter from different natural sources showed that I.2.A group contains a novel group of C23O genes (Kasuga *et al.* 2007). The first functional metagenome study of C230 genes showed, that most environmental extradiol dioxygenases have a low amino acid similarity to those of cultured micro-organism (Suenaga *et al.* 2007). Four new EDO subfamilies were identified in the study on the basis of environmental sequences suggesting that dominant catechol *meta*-pathways from the polluted environment are still unknown.

Table 1. *Published primers pairs designed and applied for analysis of extradiol dioxygenase genes in environmental samples and bacterial isolates. Nucleotide letter codes are those proposed by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.*

Primer name	Primer sequences (5´->3´)	Enzyme	Target taxa	Product size (bp)	Reference
23CAT-F 23CAT-R	CGACCTGATCTCCATGACCGA TCAGGTCAGCACGGTCA	catechol 2,3-dioxygenase I.2.A[a]	Gamma[b]	238	(Mesarch *et al.* 2000)
DEG-F DEG-R	CGACCTGATCWSCATGACCGA TYAGGTCAKMACGGTCA	catechol 2,3-dioxygenase I.2.A	Gamma	238	(Mesarch *et al.* 2000)
23DOF 23DOR	ATGGATITIATGGGITTCAAGGT ACIGTCAIGAAICGITCGTTGAG	catechol 2,3-dioxygenase I.2.A/B	Gamma, Alpha[c]	721	(Wikström *et al.* 1996)
PSCA23St PSCA23End	ATGAAMAAAGGHGTWATGCG DGTCADGAADCGDTCGTTGAG	catechol 2,3-dioxygenase I.2.A	Gamma	900	(Meyer *et al.* 1999)
SPHCA23St 23OR	ATGAAMAAAGGHGTWATGCG ACIGTCAIGAAICGITCGTTGAG	catechol 2,3-dioxygenase I.2.B	Alpha	900	(Meyer *et al.* 1999)
C230f C230r	AAGAGGCATGGGGGCGCACCGGTTCGATCA CCAGCAAACACCTCGTTGCGGTTGCC	catechol 2,3-dioxygenase I.2.A	Gamma, Beta[d], Alpha	390	(Sei *et al.* 1999)
XYLE1-F XYLE1-R	CCGCCGACCTGATCWSCATG TCAGGTCAKCACGGTCAKGA	catechol 2,3-dioxygenase I.2.A	Gamma	242	(Hendrickx *et al.* 2006)
XYLE2-F XYLE2-R	GTAATTCGCCCTGGCTAYGTICA GGTGTTCACCGTCATGAAGCGBTC	catechol 2,3-dioxygenase I.2.B	Alpha	906	(Hendrickx *et al.* 2006)
CDO-F CDO-R	CATGTCAACATGCGCGTAATG CATGTCTGTGTTGAAGCCGTA	catechol 2,3-dioxygenase I.2.C	Gamma	225	(Hendrickx *et al.* 2006)
TBUE-F TBUE-R	CTGGATCATGCCCTGTTGATG CCACAGCTTGTCTTCACTCCA	catechol 2,3-dioxygenase I.2.C	Beta	444	(Hendrickx *et al.* 2006)
TODE-F TODE-R	GGATTTCAAACTGGAGACCAG GCCATTAGCTTGCAGCATGAA	catechol 2,3-dioxygenase I.3.B	Gamma	246	(Hendrickx *et al.* 2006)
BP-F BP-R	TCTAYCTVCGNATGGAYHDBTGGCA TGVTSNCGNBCRTTGCARTGCATGAA	Extradiol dioxygenase I.3.E	Alpha, Gamma, Beta, Actino[e]	467	Article II
193C230 719C23O	ATGGATTTYATGGSBTTCA TCGATVGAKGTRTCGGTCATG	catechol 2,3-dioxygenase I.2.A	Gamma	527	(Junca and Pieper 2004)
10C23O 924C23O	AGGTGWCGTSATGAAMAAAGG TYAGGTSAKMACGGTCAKGAA	catechol 2,3-dioxygenase I.2.A	Gamma	934	(Junca and Pieper 2004)
C23O-F C23O-R	TGWCGTSATGAAMAAAGG VTYAGGTSAKMACGGTCAKGAA	catechol 2,3-dioxygenase I.2.A	Gamma	934	(Junca and Pieper 2004)

a) Extradiol dioxygenase subfamily according to Eltis and Bolin (1996) b) Gammaproteobacteria, c) Alphaproteobacteria, d) Betaproteobacteria, e) Actinobacteria.

Ring-hydroxylating dioxygenases (RHD) encoding genes are frequently used as marker genes for bacterial communities able to degrade aromatic compounds (Meyer *et al.* 1999, Hendrickx *et al.* 2005, Yeates *et al.* 2000, Ni Chadhain *et al.* 2006, Bordenave *et al.* 2008, Witzig *et al.* 2006) (Table 2). Ring-hydroxylating dioxygenases catalyze stereo specific dioxygenation of aromatic compounds to arene *cis*-diols (Gibson and Parales 2000). They are multicomponent enzymes which consist of an electron transport chain containing a ferredoxin, ferredoxin reductase and a terminal dioxygenase. The terminal dioxygenase contains two subunits large (α) and small (β) either in homo- (αn) or hetero-oligomer (αnβn) composition. The degradation in large portion known aromatic pathways is initiated through the introduction of two hydroxyl groups into the benzene ring by ring-

hydroxylating dioxygenases (Butler and Mason 1997). Yeates *et al*. (2000) showed for the first time that novel forms of ring-hydroxylating dioxygenases are widespread in pristine and contaminated soils and that the diversity of environmental aromatic degradation genes extends beyond that found in isolated bacterial strains. Primers targeted to all known neutral RHDs were designed in study of Ni Chadhain *et al*. (2006) where PAHs specific RHD and 16S rRNA communities were studied in enrichment cultures. High diversity of RHD genes were obtained in enrichments, but the PCR product length was only 78 bp that will limit the primer applicability in fingerprinting and phylogenetic analysis in future studies.

Table 2. *Published primer pairs designed and applied for analysis of ring-hydroxylating dioxygenases (RHDs) gene diversity in environmental samples and bacterial isolates. Nucleotide letter codes are those proposed by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.*

Primer name	Primer sequences	Enzyme	Target taxa	Product size (bp)	Reference
ISPGRLE1B ISPGRRI1B	AAAGATCTGTACGGCG TAAGCCCGGTAGAAACCACG	α-subunits of aromatic dioxygenases	Gamma[b]	900	(Meyer *et al.* 1999)
TODC1-F TODC1-R	CAGTGCCGCCAYCGTGGYATG GCCACTTCCATGYCCRCCCCA	α-subunits of aromatic dioxygenases	Gamma, Beta, Actino	510	(Hendrickx *et al.* 2006)
BPDOXF BPDOXR	ATHCCNTGTAAYTGGAARTTYGC CCARTTYTCNCCRTCRTCYTGYTC	α-subunits of aromatic dioxygenases	Alpha[c], Gamma, Beta[d], Actino[e],	540	(Yeates *et al.* 2000)
Rieske_f Rieske_r	TGYMGICAYMGIGG CCANCCRTGRTANSWRCA	Dioxygenases targeting non polar substrates	Alpha, Gamma, Beta, Actino	78	(Ni Chadhain *et al.* 2006)
FRT5A FRT6A	TYRARGCYAACTGGAA TACCACGTBGGTTGGAC	α-subunits of aromatic dioxygenases	Beta, Gamma	491	(Bordenave *et al.* 2008)
FRT3B FRT4B	CATGTCTTTTTCKACVATGGC GWHDCYGTYTCCATRTTGTC	α-subunits of aromatic dioxygenases	Beta, Gamma	437	(Bordenave *et al.* 2008)
NAPH-1F NAPH-1R	TGGCTTTTCYTSACBCATG DGRCATSTCTTTTTCBAC	α-subunits of aromatic dioxygenases	Gamma, Beta	896	(Gomes *et al.* 2007)
adoF1 adoB1	GTGTTCCTGAACCAGTGCCGSCACCG TGGTACATGTCRCTGCARAACTGCTC	α-subunits of aromatic dioxygenases	Gamma, Beta, Actino	384	(Taylor *et al.* 2002)
bphAf668-3 bphAr1153-2	GTTCCGTGTAACTGGAARTWYGC CCAGTTCTCGCCRTCRTCYTGHTC	α-subunits of aromatic dioxygenases	Alpha, Gamma, Beta, Actino	540	(Witzig *et al.* 2006)
Ac149f Ac1014r	CCCYGGCGACTATGT CTCRGGCATGTCTTTTTC	α-subunits of aromatic dioxygenases	Gamma, Beta	866	(Ferrero *et al.* 2002)

b) Gammaproteobacteria, c) Alphaproteobacteria, d) Betaproteobacteria, e) Actinobacteria

In addition to extradiol dioxygenases (EDOs) and ring-hydroxylating dioxygenases (RHDs) genes several other aromatic degradation genes have been used as marker genes for bacterial catabolic communities (Table 3). Four primers have been designed to monitor the modified *ortho*/*ortho*-pathway (Sei *et al.* 1999, Leander *et al.* 1998, Vallaeys *et al.* 1996). Monooxygenases have also been targeted in primer design to monitor benzene, toluene, ethylbenzene, and xylene (BTEX) degradation genes in isolated bacteria and bacterial communities (Hendrickx *et al.* 2006, Baldwin *et al.* 2003). Recently two primers targeted to a known anaerobic benzoate degradation pathway were designed (Song and Ward 2005, Kuntze *et al.* 2008). The primers targeted to the α-subunits of benzoyl-CoA reductase showed broad specificity by amplification of the diversity of denitrifying bacteria in estuarine sediment communities with several novel clusters of α-subunits of

benzoyl-CoA reductases (Song and Ward 2005). This study demonstrated that even anaerobic aromatic degradation pathways are diverse in the environment.

Table 3. *Published primer pairs designed and applied for analysis of aromatic catabolism that target genes other than extradiol dioxygenases and ring-hydroxylation genes. Nucleotide letter codes are those proposed by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.*

Primer name	Primer sequences	Enzyme	Target taxa	Product size (bp)	Reference
fwdSP9	CAGTACAAYTCCTACACVACBG	6-Oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolases	Beta[d], Delta[f], Alpha[c]	300	(Kuntze *et al.* 2008)
revASP1	CMATGCCGATYTCCTGRC				
C12Of	GCCAACGTCGACGTCTGGCA	catechol 1,2-dioxygenase	Gamma[b], Actino[e], Beta	288	(Sei *et al.* 1999)
C12Or	CGCCTTCAAAGTTGATCTGCGTGGT				
TBMD-F	GCCTGACCATGGATGCSTACTGG	α-subunits of multi-component mono-oxygenases (Subfamily R.1)	Gamma, Beta	640	(Hendrickx *et al.* 2006)
TBMD-R	CGCCAGAACCACTTGTCRRTCCA				
TMOA-F	CGAAACCGGCTTYACCAAYATG	α-subunits of multi-component mono-oxygenases (Subfamily R.3)	Gamma, Beta	505	(Hendrickx *et al.* 2006)
TMOA-R	ACCGGGATATTTYTCTTCSAGCCA				
TOL-F	TGAGGCTGAAACTTTACGTAGA	alkyl group-hydroxylating monooxygenases (Subfamily T)	Gamma	475	(Baldwin *et al.* 2003)
TOL-R	CTCACCTGGAGTTGCGTAC				
XYLA-F	CCAGGTGGAATTTTCAGTGGTTGG	Electron transfer component of mono-oxygenases	Gamma	510	(Hendrickx *et al.* 2006)
XYLA-R	AATTAACTCGAAGCGCCCACCCCA				
bzAQ4F	GTGGGCACCGGNTAYGGNMG	α-subunits of benzoyl-CoA reductase	Alpha, Beta, Gamma	484	(Song and Ward 2005)
bzAQ4R	GGTTCTTGGCGAYNCCNCCNGT				
CCDb	GTITGGCAYTCIACICCIGAYGG	chlorocatechol 1,2-dioxygenase	Alpha and Beta	270	(Leander *et al.* 1998)
CCDe	CCICCYTCGAAGTAGTAYTGIGT				
tfdAf	ACGCAGCGRTTRTCCCA	alpha-ketoglutarate dependent dioxygenase	Beta	360	(Vallaeys *et al.* 1996)
tfdAr	ACGGAGTTCTGYGAYATG				
tfdBf	ATAGCGCTGRTTCATYTC	2,4-dichlorophenolhydroxylase	Beta	1100	(Vallaeys *et al.* 1996)
tfdBR	CGCAYATCACCAAYCARC				
PcpB-G	GGSTTCACSTTCAAYTTCGA	pentachlorophenol-4-monooxygenase	Alpha	700	(Beaulieu *et al.* 2000)
PcpB-D2	TCCTGCATSCCSACRTTCAT				

b) Gammaproteobacteria, c) Alphaproteobacteria, d) Betaproteobacteria, e) Actinobacteria, f) Deltaproteobacteria

1.3.2. I.3.E group extradiol dioxygenases

I.3.E group EDOs are frequently found in *Pseudomonas* that are part of naphthalene degradation pathway (Ferrero *et al.* 2002, Takizawa *et al.* 1999, Boronin *et al.* 1989, Harayama and Rekik 1989, Li *et al.* 2004, Bosch *et al.* 1999) but in *Pseudomonas* sp. C18 DoxG an extradiol dioxygenase of this group mediates also ring cleavage of dibenzothiophene and phenanthrene as well (Table 4). In *Sphingomonas* I.3.E group EDOs seems to mediate ring cleavage of biphenyls, naphthalene's, phenanthrene, anthracene, fluoranthene, chrysene, naphthalenesulfonates, 6-dimethyldibenzothiophene and tetralin. (Romine *et al.* 1999, Furukawa *et al.* 1983, Pinyakong *et al.* 2003a, Demaneche *et al.* 2004, Kim *et al.* 1999, Story *et al.* 2001, Kuhm *et al.* 1991, Andujar *et al.* 2000, Lu *et al.* 2000, Keck *et al.* 2006, Kim and Zylstra 1995, Zylstra and Kim 1997). I.3.E EDOs are also found from marine *Cycloclasticus* sp. A5 (Kasai *et al.* 2003), dibenzothiophene degrading *Burkholderia* sp. DBT1 (Di Gregorio *et al.* 2004),

naphthalene degrading Betaproteobacteria *Polaromonas naphthalenivorans* and *Ralstonia* sp. U2 (Jeon *et al.* 2006, Fuenmayor *et al.* 1998). Six extradiol dioxygenases have been isolated from *Rhodococcus jostii* (Masai *et al.* 1997, Sakai *et al.* 2002) and the complete genome of the strain revealed altogether ten genes annotated as extradiol dioxygenase (McLeod *et al.* 2006). One extradiol dixygenase of *Rhodococcus jostii* encoded by *etbC* gene belongs to I.3.E group and mediates biphenyl and ethylbenzene degradation.

Table 4. *Aromatic degradation pathways containing I.3.E group extradiol dioxygenases*

Gene name	Bacterial strain	Putative aromatic target compouds in pathway containing I.3.E group EDOs	Reference
dbtC	*Burkholderia* sp. DBT1	Dibenzothiophene	(Di Gregorio *et al.* 2004)
phnC	*Cycloclasticus sp.* A5	Naphthalene, Phenanthrene, Biphenyl	(Kasai *et al.* 2003)
nagC	*Polaromonas naphthalenivorans* CJ2	Naphthalene	(Jeon *et al.* 2006)
pahC	*Pseudomonas aeruginosa*	Naphthalene	(Takizawa *et al.* 1999)
nahC	*Pseudomonas fluorescens*		Unpublished
nahC1	*Pseudomonas putida* 5IIIASal	Naphthalene	(Ferrero *et al.* 2002)
nahA1	*Pseudomonas putida* BS202	Naphthalene	(Boronin *et al.* 1989)
nahC	*Pseudomonas putida* G7	Naphthalene	(Harayama and Rekik 1989)
nahC	*Pseudomonas putida* OUS82	Naphthalene	(Takizawa *et al.* 1999)
doxG	*Pseudomonas sp. C18*	Dibenzothiophene, Naphthalene, Phenanthrene	(Denome *et al.* 1993)
nahC	*Pseudomonas sp.* ND6	Naphthalene	(Li *et al.* 2004)
nahC	*Pseudomonas stutzeri* AN10	Naphthalene	(Bosch *et al.* 1999)
nagC	*Ralstonia sp.* U2	Naphthalene	(Fuenmayor *et al.* 1998)
etbC	*Rhodococcus jostii* RHA1	Ethylbenzene, Biphenyl	(Masai *et al.* 1997)
bphC	*Sphingomonas aromaticivorans*	Biphenyl, Naphthalene, Phenanthrene	(Romine *et al.* 1999)
phnC	*Sphingomonas so. CHY-1*	Chrysene, Naphthalene, Phenanthrene, Anthracene	(Demaneche *et al.* 2004)
phnQ	*Sphingomonas chungbukensis*	Biphenyl, Naphthalene, Phenanthrene	(Kim *et al.* 1999)
-	*Sphingomonas paucimobilis* EPA505	Fuoranthene, Naphthalene, Anthracene, Phenanthrene	(Story *et al.* 2001)
-	*Sphingomonas paucimobilis* Q1	Biphenyls (substituted), Naphthalene	(Furukawa *et al.* 1983, Kuhm *et al.* 1991)
dmdC	*Sphingomonas paucimobilis* TZS-7	6-dimethyldibenzothiophene	(Lu *et al.* 2000)
nsaC	*Sphingomonas sp.* BN6	Naphthalenesulfonates(substituted)	(Keck *et al.* 2006)
xylK	*Sphingomonas sp.* P2	Phenanthrene	(Pinyakong *et al.* 2003a)
bphC	*Sphingomonas yanoikuyae* B1	Biphenyl, Naphthalene, Phenanthrene	(Kim and Zylstra 1999)
thnC	*Sphingopyxis macrogoltabida*	Tetralin	(Andujar *et al.* 2000)

1.4. Molecular methods to assay microbial diversity

The most detailed microbial community composition - a list of who is there, is obtained by analysis of DNA/RNA extracts isolated directly from environmental samples (Little *et al.* 2008). The complicated analysis methodology with different benefits and pitfalls can be reduced in to three crucial steps.

1. DNA/RNA extraction from environmental sample.
2. Amplification of marker genes.
3. Analysis of marker gene composition.

Each step may be a source of bias leading changes in biological data obtained from the microbial community.

1.4.1. DNA/RNA extraction

DNA-based molecular microbiological studies are depended on DNA/RNA extraction methods from environmental samples with complex composition. Several methods have been developed to extract DNA (Bürgmann *et al.* 2001, Ogram *et al.* 1987, Liles *et al.* 2008, Zhou *et al.* 1996) and also commercial kits are available. There are two main strategies that are used to extract DNA/RNA from soil samples. DNA can directly be extracted *in situ* from soil matrix and cells in soil are disrupted by mechanical and chemical lysis (Ogram *et al.* 1987, Zhou *et al.* 1996). In another strategy the bacterial cells are first isolated from soil and the DNA is extracted from cells (Liles *et al.* 2008, Torsvik 1980, Holben *et al.* 1988). In the direct approach the DNA yield is generally large and represents well the micro-organisms in soil (Kozdroj and van Elsas 2001) but the DNA is frequently contaminated with humic acids, metals and organics from soil and DNA is severely fragmented. The cell extraction method yields high purity DNA containing also high molecular weight DNA fractions. The yields of DNA from cell extraction method are generally smaller than in the direct approach and the representativeness of soil bacterial community is lower due to the bias in cell isolation. Generally if high molecular weight and high purity DNA is needed like in metagenome studies (Liles *et al.* 2008) the cell extraction method seems to be more beneficial, but if more representative communities are aimed at, like in diversity studies, the direct extraction seems to be the more appropriate method. Methods for simultaneous RNA and DNA extraction are developed with both approaches (Hurt *et al.* 2001, Korkama-Rajala *et al.* 2008).

1.4.2. PCR amplification of marker genes

Several types of marker genes can be amplified from soil DNA extracts (Section 1.2 and 1.3) using the revolutionary polymerase chain reaction technique (Kleppe *et al.* 1971). The most commonly used marker gene is the 16S rRNA gene present in not only in all bacterial species, but in Archaea as well. It is relatively easy to amplify from soil DNA due to its abundance in all bacterial and Archaea cells, usually several copies per bacterial cell. 16S rRNA is even more abundant in RNA extracts because its high transcription rates in active cells. There are several 16S rRNA primers targeted to specific taxonomic groups (Heuer *et al.* 1997, Gomes *et al.* 2001, Mühling *et al.* 2008). The benefit of universal bacterial primers is the possibility to amplify almost all bacterial species simultaneously, but on the other hand the immense diversity can lead to difficulties in following fingerprinting step. Lack of the resolution of fingerprints and the huge amount of sequencing needed to comprehensively analyse complex communities complicate these studies.

Although marker gene amplification is the most commonly applied technique to characterize uncultured micro-organisms from environmental sample the PCR step is complicated and biased by several factors of the process like template secondary structures, G+C differences, primer annealing, competition within degenerated primer

pools, chimera and heteroduplex formation, polymerase errors and differences in annealing temperature, cycle number, product length or even template concentrations (Reysenbach *et al.* 1992, Farrelly *et al.* 1995, Suzuki and Giovannoni 1996, Thompson *et al.* 2002, Sipos *et al.* 2007, Huber *et al.* 2009). In comparison to the bias caused by the cultivation of micro-organism (Rossello-Mora and Amann 2001, Hugenholtz 2002) the PCR bias is, however, small and manageable by using reduced cycle numbers, equal amplification of each sample, analysis of chimeras from sequences and taking in account that small differences in marker gene sequences might be polymerase errors which should not be taken as biologically significant diversity (Eckert and Kunkel 1991).

1.4.3. Fingerprinting microbial community structure

Several methods have been developed for analysis of amplified marker gene composition that reflects to the microbial community in the environment in frames of used marker gene/genes and DNA extraction and PCR biases. These methods can be divided into direct fingerprinting and clone library screening.

In direct fingerprinting methods the amplified PCR product is fingerprinted using assays based on different chemical properties of DNA molecules with dissimilar sequences like denaturation (DGGE, TGGE) (Muyzer *et al.* 1993) or different tendency to form single stranded secondary structures (SSCP) (Lee *et al.* 1996). Direct fingerprinting methods can be also based on sequence specific digestion of the community product by restriction enzymes, resulting in characteristic restriction patterns (T-RFLP, RFLP) (Avaniss-Aghajani *et al.* 1994). The benefit of direct fingerprinting methods is that they are relatively fast in comparison to clone library based method and larger amount of samples can conveniently be analysed, but many times the assay has to be experimentally optimized for each sample type and for each marker gene. The optimization of DGGE and SSCP is laborious and demanding work and the sequencing of the individual bands can be cumbersome because of overlapping bands that many times lead to over lapping sequences. In T-RFLP identification of individual terminal restriction fragments in electropherograms will require cloning and further T-RFLP analysis of clonal amplicons.

In clone library based methods the marker genes from bacterial community are ligated to a suitable vector (plasmid) and transferred in to a host micro-organism (Usually *E. coli* bacteria) (Sambrook *et al.* 1989). The marker gene composition in clone libraries is studied by clone sequencing, restriction fragment length polymorphism (RFLP) or other fingerprinting methods. The benefit of clone library based analysis is that the individual marker gene sequences are easily obtained from the selected or randomly chosen clones using basic molecular methods and the community diversity can be studied in detail. The down side in clone library based methods is that the cloning cause's additional bias and analysis of composition single community is laborious including screening of large amount of clones.

In selection of fingerprinting method it is important to consider the aims and resources of the study. How detailed should the microbial community analysis be to answer the questions in the study and how many samples are needed. In Figure 3 the community

analysis methods are plotted in sense of expenses and resolution. Generally methods with high resolution are connected to high costs although the new pyrosequencing technique holds a promise as a high sensitivity low cost method for microbial community analysis. If 6 – 16 samples are sufficient for the study and a detailed community analysis is the aim of the study, then the clone library based methods are more convenient than direct fingerprinting methods. Direct fingerprinting methods are again more suitable when larger amount of samples are analysed.

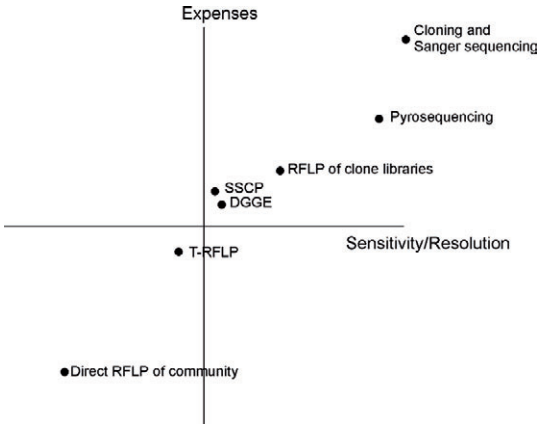

Figure 4 *Rough coordination plot of fingerprinting and sequencing methods for microbial community analysis in sense of expenses and sensitivity in the analysis. The plot is based on to the review of literature and candidates own practical experience.*

1.5. Rhizosphere environment

The rhizosphere, the soil surrounding a plant roots contains highest microbial biomass and activity and greatest complexity within the soil environment (Prosser *et al.* 2006). Plant photosynthesis produces carbohydrates which in the course of plant metabolism become carbon sources for rhizobacteria in the form of root exudates (Da Silva *et al.* 2006). Plants release 7 to 27% of the total plant mass annually as rhizodeposition (Whipps and Lynch 1986). The rhizodeposition from plant root system varies depending on plant species, age, temperature stress response, light, nutrition and micro-organisms (Bertin *et al.* 2003, Rovira 1969). The microbial metabolism of root exudates and other soil organic and inorganic compounds releases nutrients and improves the soil structure benefitting the plant. Micro-organisms in rhizosphere may also be exploited to benefit plant growth and have therefore economical value. Specific interactions with the plant root system like nodular nitrogen fixation is found from 10 angiosperm plant families and performed by endosympionts bacteria belonging to Betaproteobacteria, Alphaproteobacteria, and Actinobacteria (Soltis *et al.* 1995, Sprent 2007). Well know mycorrhizal interactions between plant and fungi can benefit the plant (Bolan 1991, Harley and Smith 1983) and

many rhizosphere bacteria mediate antagonistic interactions that inhibit the growth of plant pathogens on root surface.

1.5.1. Rhizoremediation of organic pollutants

The introduction of man made toxic chemicals, and the massive relocation of natural materials (petroleum hydrocarbons) to different environments soils, ground water, and atmosphere has laid pressure on the self-cleansing capacity of ecosystems (Susarla *et al.* 2002). Several strategies have been developed to enhance the environments self-purification capacity. Three strategies of remediation of soil are widely used. They are immobilization or retention of toxicant within a confined area, removal of contaminants from soil and thirdly destruction of organic pollutant by chemical, physical or biological means (Mackova *et al.* 2006). In several studies plants are demonstrated to enhance the removal and/or transformation of a pollutant (Singer *et al.* 2003). Plants promote the remediation of a wide organic range of chemicals in soil by several mechanisms (Chang and Corapcioglu 1998). They can be listed as follows:

1. Modifying the physical and chemical properties of contaminated soils (Miller *et al.* 1990).
2. Releasing root exudates, thereby increasing organic carbon and active microbial populations (Foster 1986).
3. Improving aeration by directly releasing oxygen to the root zone, as well as increasing the porosity of the upper soil (Schnoor *et al.* 1995).
4. Intercepting and retarding the movement of chemicals by root uptake mechanisms (Nair *et al.* 1993).
5. Stimulating co-metabolic microbial and plant enzymatic transformations of recalcitrant chemicals (Nair *et al.* 1993, Aprill and Sims 1990).
6. Decreasing vertical and lateral migration of pollutants to ground water by extracting available water and reversing the hydraulic gradient (Schnoor *et al.* 1995).

Rhizoremediation is a biological treatment of a contaminant by enhanced bacterial and fungal activity in the rhizosphere of plants (Kuiper *et al.* 2004). In some cases, rhizosphere microbes are the main contributors to the degradation process. There are also limitations in plant mediated remediation of soil. The rhizoremediation is a time-consuming process and for most sites with purification decision the results are needed in a shorter time scale (Khan *et al.* 2000). The limited depth of the root system and slow growth rate of plants may hamper the purification. Plants are also sensitive to some pollutants preventing the use of rhizoremediation techniques. Plants belong to ecosystem food chain usually eaten by several herbivores which may lead to the spread of pollutant. A universal plant rhizoremediation system is impossible to obtain because climate variation and also the winter season hinders the remediation. The main benefit of rhizoremediation is that it is a low cost method (installation and maintenance) and its applicable to large surface soil land areas, without immediate land use, are needed to remediate.

2. Aims of the study

The biodegradation process of aromatics in the environment is mediated by complex communities of micro-organisms. The aim of this thesis was study biodegradation genes in bacterial cell and additionally as entity of genes in the community in soil and rhizosphere. As a long term goal this knowledge should benefit the development of biological bioremediation methods like bioaugmentation, natural attenuation and rhizoremediation. The specific aims were

- Re-evaluation of the degradation capacities of *Sphingobium* sp. HV3 chlorobenzoate and phenoxy herbicide degrader.

- The sequencing of pSKY4 plasmid to study the evolution and mechanism of aromatic degradation in *Sphingomonas.*

- Development of research method to describe the diversity of aromatic (like PAHs) degradation genes in the environment and in polluted soils with the aim of monitoring bioremediation of aromatic compounds.

- To evaluate the effects of PAHs on bacterial communities in soil and rhizosphere by a rhizoremediation model study using extradiol dioxygenase encoding genes as a functional genetic markers.

3. Materials and methods

3.1. Study sites and sampling

Three different polluted environmental soils: a mineral oil polluted landfill site (Troll-oil), a mineral oil land farming site (Sköld-oil) and an artificially PAH-polluted birch rhizosphere-associated soil (Rhiz-PAH) were used in study to test the functionality of designed primers to amplify environmental extradiol dioxygenases (EDOs) (Article I). Troll-oil soil was from Trollberget, Southern Finland (N 59º 51´53´´, E 23º 01´41´´), an abandoned dumping ground contaminated with lubricated oil and lightweight fuel, which is a monitored natural attenuation site (Salminen *et al.* 2004). The naphthalene concentration at the site was below 6.2 mg kg^{-1} dry-weight soil and the Troll-oil sample was taken from surface soil. The Sköld-oil soil sample was from a land farming test site of oil refinery wastes in Sköldvik (N 60º 18´44´´, E 25º 32´80´´). Agricultural soils in Sköldvik have been used as oily waste dump site from 1980 to 2005 (Mutku conference 2009). The waste consists mostly of oil wastewater sludge, container sediments and oil polluted soil from oil refining industry. Total petroleum hydrocarbons in field soil exceeded the approved 900-2700 mg kg^{-1} (C10-C21) and 1900-4900 mg kg^{-1} (C21-C41). Main portion of petroleum hydrocarbon pollution in fields were constituted of aliphatic- (C16-C35) and aromatic fraction (C21-C35). The Rhiz-PAH sample is from greenhouse experiment described in the next section.

3.2. Greenhouse microcosm experiment

Greenhouse microcosm experiment was set up to study the rhizoremediation of PAHs using two micropropagated birch clones *Betula pendula* W008 and *Betula pubescens* Ha02 (Tervahauta *et al.* 2008). W008 birch is from spoil heaps of disused Pb/Zn mine near Aberystwyth, Wales and Ha02 is from Cu/Ni smelter area in Harjavalta, Finland. After four week micropropagation period the plantlets were transferred to soil which consisted of fertilized *Sphagnum* peat, sand and steam-sterilized garden soil, 1:1:1 (v/v/v), where the plants were grown for 2 months. Birches were individually exposed to PAH in greenhouse pot experiment (Article III).

The pots contained 500 g of sand (<1.2 mm granules) and unfertilized peat (Kekkilä Oyj, Tuusula, Finland) in ratio 1:2 or 1:4. Two levels of PAHs (anthracene, phenanthrene, fluoranthene and pyrene) in acetone, 300 mg kg^{-1} and 50 mg kg^{-1} each were amended to the pots giving a PAH concentration of 1200 mg kg^{-1} and 200 mg kg^{-1} respectively. Spiking was performed in hermetical glass bins by adding PAHs in 100 ml acetone to 500 g soil and then mixing in a rotating pot mill for 30 min. The soil was then transferred to a plant pot and vented in a fume chamber for 72 h before planting of birches. Control pots with soil containing 100 ml acetone (without PAH addition) were made. The temperature

in the greenhouse was 18 °C and light/dark cycle 16/8 h (colors 77 and 965, Osram Fluora and Biolux) to simulate the typical northern conditions in summer temperature.

Destructive sampling and PAH degradation analysis were performed after three month growth period in the greenhouse. Composite soil samples (20 g) were taken from each pot to microbial community analysis and rhizosphere soils were manually sampled from by shaking the roots. Microbial communities were analysed by 16S rRNA terminal restriction fragment length polymorphism (T-RFLP) and RFLP analysis EDO clone libraries. PAHs were extracted analysed from total soil remaining in the pot (480 g). Soils with peat/sand ratio 1:4 were used in Article II and soils 1:2 ratio in Article III.

3.3. Primer design

Primers targeted to genes encoding I.3.E EDOs were designed on the basis of genes found in database [e.g. I.3.E designates type (I), family (3) and subfamily (E)] (Eltis and Bolin 1996) (Article II).The hosts of the EDOs that belong to Alpha- Beta- Gamma-proteobacteria and Actinobacteria are shown in Figure 5 and 6. Most hosts are well known PAH degraders in genus *Pseudomonas* and *Sphingomonas*. The primers amplify a 469 bp fragment of the *nahC* gene (from 131 to 600) found from NAH7 plasmid of *Pseudomonas putida.*

Bacterium	Gene		Gene bank ac.
Burkholderia sp. DBT1	*dbtC*	T C T A T T T G C G C C T C G A T T A T C A G C A	AAK96189
Cycloclasticus sp. A5	*phnC*	T T T A C C T A A G A A T G G A T G G T C A G C A	AB102786
Polaromonas naphthalenivorans CJ2	*NagC*	T C T A T C T G C G A A T G G A C A A T T G G C A	DQ167474
Pseudomonas aeruginosa	*pahC*	T C T A T C T G C G G A T G G A T T A C T G G C A	D84146
Pseudomonas fluorescens	*nahC*	T C T A T C T G C G G A T G G A T T A C T G G C A	AY048760
Pseudomonas putida 5IIIASal	*nahC1*	- - - - - - T A C G T A T G G A T T A C T G G C A	AF320640
Pseudomonas putida BS202	*nahC*	T C T A T C T G C G G A T G G A T T A C T G G C A	AF010471
Pseudomonas putida G7	*nahC*	T C T A T C T G C G G A T G G A T T A C T G G C A	J04994
Pseudomonas putida OUS82	*nahC*	T C T A T C T G C G G A T G G A T T A C T G G C A	AB004059
Pseudomonas sp. C18	*doxG*	T C T A T C T G C G G A T G G A T T A C T G G C A	M60405
Pseudomonas sp. ND6	*nahC*	T C T A T C T G C G G A T G G A T T A C T G G C A	AY208917
Pseudomonas stutzeri AN10	*nahC*	T C T A T C T A C G T A T G G A T T A C T G G C A	AF039533
Ralstonia sp. U2	*nagC*	T C T A T C T G C G A A T G G A C A A T T G G C A	AF036940
Rhodococcus sp. RHA1	*etbC*	T C T A T C T C C G G A T G G A C A G G T G G C A	AB120955
Sphingomonas aromaticivorans	*bphC*	T C T A T C T G C G A A T G G A C C T T T G G C A	AF079317
Sphingomonas chungbukensis	*phnQ*	T C T A T C T G C G G A T G G A C C T T T G G C A	AF061802
Sphingomonas paucimobilis EPA505	-	T C T A C C T G C G C A T G G A C C A G T G G C A	AF259397
Sphingomonas paucimobilis Q1	-	T C T A C C T G C G C A T G G A C C A G T G G C A	M20640
Sphingomonas paucimobilis TZS-7	*dmdC*	T C T A T C T G C G G A T G G A C C T T T G G C A	AB035677
Sphingomonas xenophaga BN6	*nsaC*	T C T A T C T C C G G A T G G A T C T G T G G C A	U65001
Sphingomonas sp. P2	*xylK*	T C T A C C T G C G C A T G G A C C A G T G G C A	AB091692
Sphingomonas yanoikuyae B1	*bphC*	T C T A C C T G C G C A T G G A C C A G T G G C A	U23374
Sphingopyxis macrogoltabida	*thnC*	T C T A T C T C A G G A T G G A C G G C T G G C A	AF157565
BP-f sequence 5´-->3´		T C T A Y C T V C G N A T G G A Y H D B T G G C A	

Figure 5 *The design of BP-f primer targeting genes encoding I.3.E extradiol dioxygenases. The variations in the sequence are visualized in gray shading. The base pairs with inverted colors are mismatches in the primer sequence. Nucleotide letter codes are those proposed by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.*

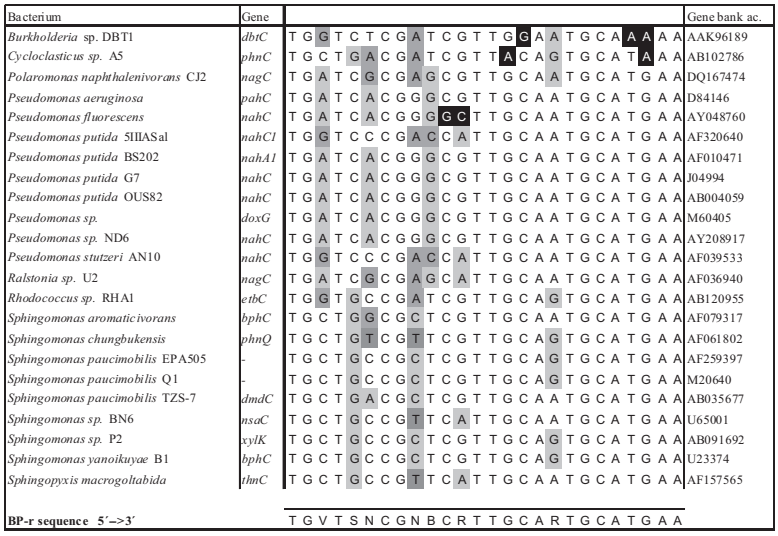

Figure 6 *The design of BP-r primer targeting genes encoding I.3.E extradiol dioxygenases. The variations in the sequence are displayed in gray shading. The base pairs with inverted colors are mismatches in the primer sequence. Nucleotide letter codes are those proposed by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.*

Designed primers amplify genes from different type of genetic *meta*-pathways. The target aromatic pathways containing I.3.E EDOs are displayed in table 4. The I.3.E extradiol dioxygenase genes were chosen as marker gene because the group contains genes from several different biochemical degradation pathways like naphthalene, biphenyl, phenanthrene, dibenzothiophene, tetralin and ethylbenzene (Figure 1) and it is characterized from several bacterial groups including Alpha-, Beta-, Gammaproteobacteria and Actinobacteria. Other important factors in the choice of marker gene were that the aligment of I.3.E dioxygenase genes showed relatively well conserved regions for primer design with variable regions between the primer sites and that several I.3.E extradiol dioxygenase genes where found at the genbank from the genus *Sphingomonas* and *Pseudomonas* a well known degrader of aromatic compounds.

3.4. Experimental methods

An overview of experimental methods of the thesis is presented in table 5. A more detailed description of materials and methods is found in each Article cited in the table 5.

Table 5. *The experimental methods used in the publications of this thesis.*

Methods	Aim	Article
High through-put sequencing	pSKY4 sequence	I, IV
Cloning	pSKY4 plasmid library or environmental clone library	I, II, III, IV
Plasmid isolation	pSKY4 sequence	I, IV
Generation of sequencing templates using transposons	pSKY4 sequence	I, IV
Polymerase chain reaction (PCR)	Amplification of gene fragments	I, II, III, IV
Restriction analysis	Size selection and analysis of library inserts	I, IV
Isolation of environmental DNA	Template to marker gene amplification and library construction	II, III
Isolation of total RNA from bacteria	Quantification of mRNA expression	I
Designing primers using Genbank sequences	Amplification of functional marker genes	II
RFLP fingerprinting	Dividing marker genes in to operational taxonomic units	II, III
T-RFLP fingerprinting	Analysis of 16S rRNA communities	III
Phylogentic analysis	Classification of operational taxonomic units/ 16S rRNA genes in to phylogenetic groups	I, II, III
Real time PCR	Quantification of mRNA expression	I
454 Pyrosequencing	pSKY4 sequence	IV
High pressure liquid chromatography (HPLC)	Analysis of PAH dissipation	III

4. Results

The results of this thesis are presented in four articles with figures and tables. In this chapter a synthesis of the key findings are presented.

4.1. *Sphingobium* sp. HV3 and its aromatic degradation pathways

The degradation potential of aromatic compounds of *Sphingobium* sp. HV3 were re-evaluated on the basis of sequence data of aromatic *meta*-pathway obtained from pSKY4 plasmid of the strain. Degradation tests were made both by BTEX compounds, biphenyl and polyaromatics. Toluene, *m*-xylene, biphenyl, fluorene and phenanthrene were found to be carbon and energy sources of the strain (Article I). *Sphingobium* sp. HV3 degradation capacity of naphthalene, phenanthrene and biphenyl was connected to pSKY4 megaplasmid encoded *meta*-pathway by Tn5 mutagenesis. The cloning of transposition sites displayed in total 15.7 kb plasmid catabolic pathway sequence. The disrupted genes encoded, reductase component of a dioxygenase, *cis*-biphenyl dihydrodiol dehydrogenase and large subunit of a ring hydroxylating dioxygenase.

The sequencing project of the pSKY4 plasmid revealed no catabolic genes connected with chloroaromatic degradation (Article IV). To learn about the bases for degradation of chloroaromatics a PCR-based study was conducted to find putative *ortho*-pathways of the strain (Article I). The modified *ortho*-pathway was detected in the strain using the Leander *et al*. (1998) primers. The sequence analysis of HV3 strain chlorocatechol 1,2-dioxygenase showed 100% similarity to *tfdC* gene in *Sphingomonas* sp. TFD44.

The versatile degradation capacities of *Sphingobium* sp. HV3 with several aromatic degradation pathways targeted on biodegradation of several types of aromatic compounds prompted a study of regulation of gene expression in the strain (Article I). A qPCR was used for analysis of ring cleavage gene expression of the upper and lower *meta*-pathway and of the modified *ortho*-pathway. The expression of plasmid-encoded *xylE* and *bphC* extradiol dioxygenase genes of *meta*-pathways were induced by *m*-xylene, phenanthrene and biphenyl, but the chromosomal chlorocatechol dioxygenase *tfdC* was induced by 2,4-dichlorophenoxyacetic acid in timely fashion.

4.2. Extradiol dioxygenase diversity in soil

A PCR-method was developed to simultaneously detect several extradiol dioxygenases (EDOs) genes from the bacterial community. The method is based on PCR amplification, cloning and RFLP of I.3.E group EDOs from clone libraries representing polluted soil EDO communities (Article II). The I.3.E extradiol dioxygenase genes were chosen as marker gene because the group contains genes from several different biochemical

degradation pathways like naphthalene, biphenyl, phenanthrene, dibenzothiophene, tetralin and ethylbenzene (Figure 1) and it is characterized from several bacterial groups including Alpha-, Beta-, Gammaproteobacteria and Actinobacteria. Other important factors in the choice of marker gene were that the aligment of I.3.E dioxygenase genes showed relatively well conserved regions for primer design with variable regions between the primer sites and that several I.3.E extradiol dioxygenase genes where found at the genbank from the genus *Sphingomonas* and *Pseudomonas* a well known degrader of aromatic compounds.

A high diversity of the I.3.E EDOs genes was found from different polluted soils (Figure 7). The natural polluted soils displayed different EDO profiles with distinct characteristics showing different bacterial communities able to degrade aromatics. The phylogenetic analysis displayed several distinct EDO clusters with relatively low similarity to each other. The I.3.E group EDOs characterized from cultivated *Pseudomonas* and *Sphingomonas* strains seemed to be a minority in studied soils although operational taxonomic units (OTUs) with highly similar sequences were identified. OTU highly similar to the *Sphingomonas xenophaga nsaC* gene from the naphthalenesulfonic acid catabolic pathway (Keck *et al*. 2006, Heiss *et al*. 1995) was identified from abandoned dumping ground at Trollberget in very south of Finland. Minor OTU from Rhiz-PAH soil displayed high similarity to *nahC* gene encoding 2-dihydroxynaphthalene dioxygenase from *Pseudomonas putida* naphthalene biodegradation pathway (Eaton 1994). The several major OTUs in polluted soil displayed low similarity to EDOs from known aromatic biodegradation pathways.

4.3. Bacterial aromatic ring-cleavage communities in birch rhizoremediation

The effects of PAH and birch (*Betula pendula*) in rhizoremediation of soil was studied in microcosm experiment. The I.3.E extradiol dioxygenase (EDO) gene composition in soil was analysed to evaluate the catabolic potential in soil with woody plant (Tervahauta *et al.* 2008). Rhizosphere associated soil contained higher diversity of bacterial EDOs than bulk soil (Article III). Several OTUs specific to rhizosphere associated soil were detected. PAH pollution changed the extradiol dioxygenase community in bulk soil. PAH polluted bulk soil was strongly dominated by OTU 72 that was most similar to the *thnC* gene from *Sphingopyxis macrogoltabida* TFA strain (70% amino acid similarity). THNC enzyme is known to catalyse ring cleavage of tetralin an aromatic compound composed of one aromatic ring structure fused to a six-carbon aliphatic ring structure (Andujar *et al.* 2000). Interestingly the PAH addition had only modest effect on catabolic bacterial community composition in birch rhizosphere associated soil.

Treatment specific changes were also detected in structural diversity analysed by terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of bacterial 16S rRNA gene communities (Article III). PAH changed the bacterial communities in bulk soil and rhizosphere associated soil. Rhizosphere effect in structural community was modest but notable, probably partly masked by the high diversity of the total 49 terminal

fragments. Birch tree enhanced the PAH dissipation in pots with 1200 mg kg^{-1} pollution showing a potential for rhizoremediation.

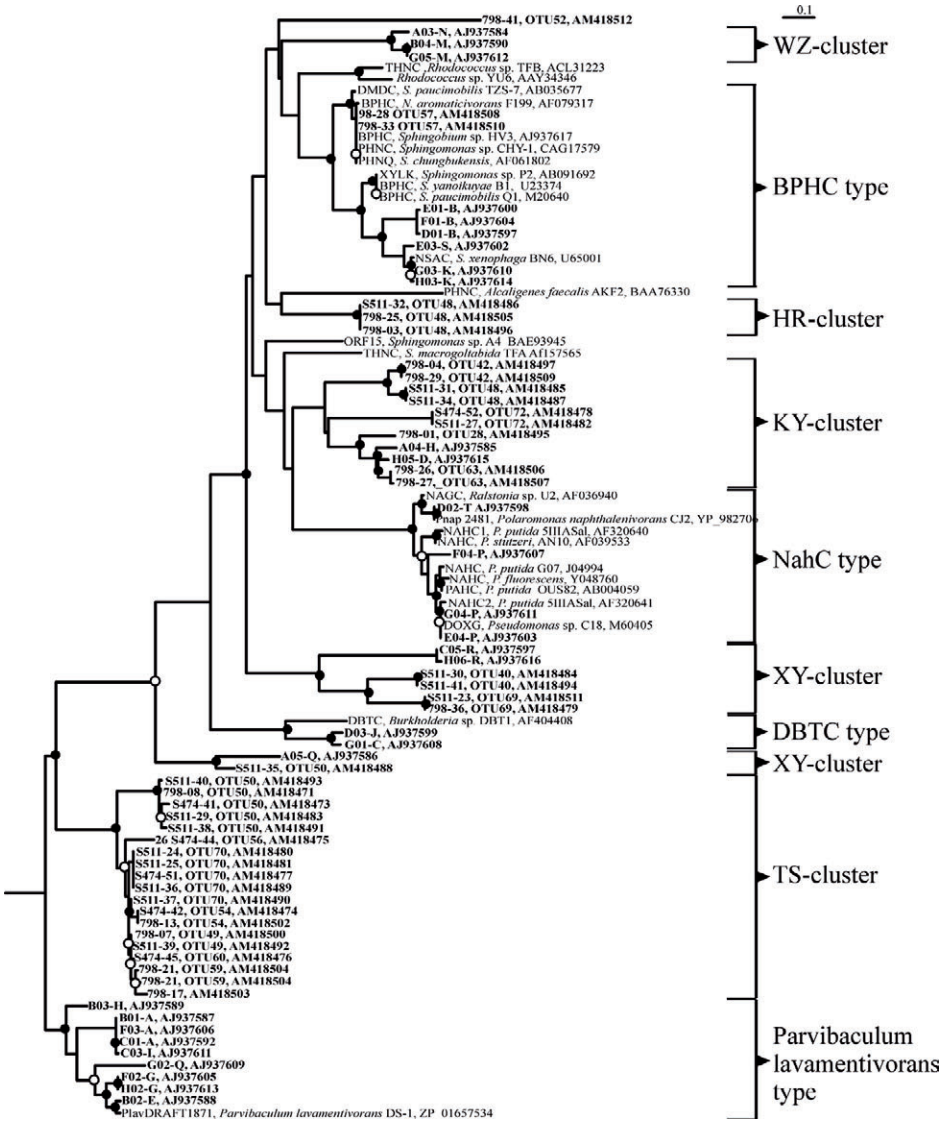

Figure 7 *Maximum likelihood tree of I.3.E group extradiol dioxygenases sequenced in this thesis (boldface) and reference sequences from the genebank. Bootstrap values (100 replicates) with 100-70% support are labelled with black circle in the node, 75-50% support with open circle and nodes without circle less than 50% support. The genbank accession numbers are marked after the sequence name.*

4.4. Complete sequence of pSKY4 plasmid

The total length of pSKY4 plasmid was 280,923 bp, where 265 putative open reading frames (orfs) were identified (Article IV). The plasmid orfs could be divided into transposition/integration (54 orfs), conjugative transfer (16 orfs), aromatic degradative (52 orfs), plasmid stabilization (10 orfs), DNA/RNA processing/plasmid maintaining (19 orfs), transport/segregation (14 orfs), other orfs (30) and hypothetical proteins (70) on the basis of putative function of genes (Figure 8).

The pSKY4 partition locus was of novel type with different gene order than in pNL1 and pCAR3 suggesting that pSKY4 belongs to new incompatibility group. Sixteen orfs were identified to be involved in conjugative transfer of pSKY4 plasmid. These orfs encode proteins similar to TraG/TrwB/VirD4 family of coupling proteins mediating conjugation via the type IV secretion system. The conjugation and replication genes had a low 39-75% amino acid similarity to corresponding genes in pNL1 and pCAR3.

The aromatic degradation genes in pSKY4 share high similarity to corresponding genes in pNL1, most genes are 90-99% identical. The different similarity levels of plasmid backbone and catabolic pathway of the two plasmids points out that the catabolic pathway has functioned as an independent mobile genetic element. The regions flanking the aromatic degradation pathways are a hot spot of DNA recombination and transposition related genes (26 orfs), which suggests that several mechanisms of aromatic pathway transfer might exists. Thirteen putative IS elements were located in the pSKY4 with size range 1092-7094 bp containing inverted repeats of 18-34 bp. The plasmid back bone was stabilized with four pairs of different type toxin/antitoxin proteins putatively causing cell death upon plasmid disappearance from host.

A comparison with the pNL1 aromatic *meta*-pathway revealed that a 10 kb deletion in pNL1 aromatic *meta*-pathway with orfs encoding one putative CoA-transferases, four acyl-CoA dehydrogenases, two (R)-hydratases, one short-chain dehydrogenase and one acetoacetyl-CoA thiolase. The presence of these orfs in aromatic *meta*-pathway indicates that they might have role in aromatic catabolism.

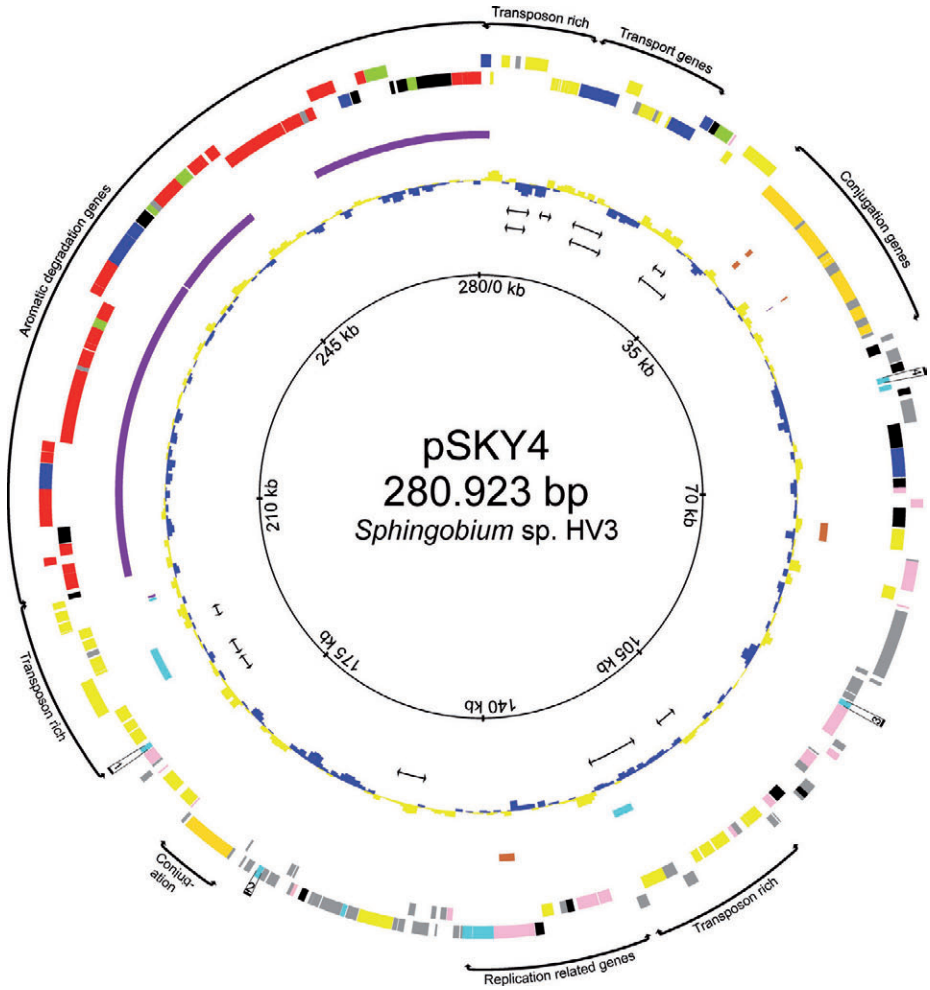

Figure 8 *Circular gene map of pSKY4 plasmid. Genes outside the circle are coded clockwise, and those inside are coded counterclockwise. The color codes of the orfs in the first circle are, Red; aromatic degradation, Blue; transport, Green; coenzyme and vitamin synthesis, Black; regulation of gene expression, Yellow; transposition and integration, Gold; plasmid conjugation, Pink; Replication and RNA/DNA processing, Turquoise; Plasmid stabilization and antirestriction and Gray; conserved hypothetical proteins and predicted orfs. Second circle shows plasmids DNA similarity to pNL1 plasmid from Novosphingobium aromaticivorans F199 (outside the circle) and to pCAR3 from Sphingomonas sp. KA1 (inside the circle). 90-100% DNA similarity is displayed as purple line, 80-90 as blue and 70-80 as brown line. The GC skew is displayed in third circle. Identified IS elements are shown as arrows in fourth circle and the plasmid size ruler in the fifth circle. The eight antidote and toxin orfs are marked with numbering 1-4.*

5. Discussion

A research method to analyse genetic potential in soil for degradation of aromatic compounds was developed taking advantage of PCR and genetic fingerprinting analysis. The utilisation of this method revealed novel aromatic degradation gene diversity in polluted soils. In birch rhizoremediation the rhizosphere displayed higher diversity of aromatic degradation genes than bulk soil supporting the idea that plants can be used to enhance remediation of organic pollutant. A novel type of megaplasmid was completely sequenced from the *Sphingobium* genus containing aromatic degradation pathway in putative mobile element demonstrating that different levels of gene transfer is involved in development aromatic degradation capacities in the *Sphingomonas* genus. The study of the expression of upper and lower *meta*-pathways genes in *Sphingobium* sp. HV3 showed these plasmid encoced pathways are differently induced than the chlorocatechol *ortho*-pathway which most likely is of chromosomal origin.

5.1. *Sphingobium* sp. HV3 a versatile aromatic degrader from agricultural soil

Sphingomonas are well known for their exceptionally diverse degradation capacity of natural and xenobiotic compounds (Stolz 2009, Basta *et al.* 2005). The HV3 strain contained the modified *ortho*-pathway most likely located in the chromosome (Article I) whereas the upper and lower *meta*-pathway genes were located in the pSKY4 plasmid (Article IV). The degradation capacities of the strain were shown to be diverse and the strain can degrade chlorinated aromatics like herbicides, BTEX compounds, biphenyl and polyaromatics. In comparison to other *Sphingomonas* the degradation capacity of HV3 is versatile. *N. aromaticivorans* F199 and *S. yanoikuyae* B1 degrade BTEX compounds and polyaromatics, but these strains have not been reported to degrade chlorinated aromatics or herbicides (Romine *et al.* 1999, Kim and Zylstra 1999). *S. herbicidovorans* MH can degrade various herbicides like 2,4-dichlorophenoxy acetic acid (2,4-D) and 2-(4-chloro-2-methylphenoxy) propanoic acid (Mecoprop), but is not reported to degrade polyaromatics or BTEX compounds (Kohler 1999). *Sphingomonas* sp. strain CHY-1 degrades polyaromatic and mono aromatic compounds and even high molecular weight PAHs like four aromatic ring chrysene (Demaneche *et al.* 2004, Willison 2004). However, there are no reports about *S.* CHY-1 capacity to degrade herbicides. The wide degradation capacity of the HV3 strain can be partly due to the origin of the strain. In pristine environment it can be beneficial for bacteria to maintain various aromatic degradation capacities (generalist) to be able to assimilate carbon from different low abundance sources present in the environment.

It is a well known fact that aromatic catalytic activity in *Sphingomonas* is induced by biphenyl, xylene/toluene, and salicylate (Furukawa *et al.* 1983). The whole cell enzymatic assay cannot separate, however, the activity of upper and lower *meta*-pathway extradiol dioxygenases because of the partially overlapping substrate ranges of these enzymes. The

XylE and BphC enzymes in *Sphingobium yanoikuyae* B1 still have higher specificity to catechol and 2,3-dihydroxybiphenyl respectively (Kim and Zylstra 1995). *Sphingobium* sp. HV3 growth on *m*-toluate, *p*-toluate, *o*-cresotate and salicylic acid induced enzymatic activity of the catechol 2,3-dioxygase, but the growth on benzoate does not show significant C23O induction (Kilpi *et al.* 1983). In Article I the expression of *bphC*, *xylE* EDO genes and *tfdC* intradiol dioxygenase gene were studied with biphenyl, *m*-xylene, phenanthrene and 2,4-D. The different pathways in *Sphingobium* sp. HV3 were activated in aromatic compound specific manner: The *ortho*-pathway is induced by the 2,4-D herbicide and *meta*-pathways seemed to be repressed by the 2,4-D, especially the *bphC* gene expression was down regulated by the 2,4-D herbicide. *m*-xylene was the strongest inducer of both *meta*-pathways genes followed by biphenyl and phenanthrene. The induction of the *xylE* gene seemed to be constitutive although partly repressed by the 2,4-D. The expression of the *tfdC* operon was timely regulated and the induction lasted only about 20 h after which the expression started to decline.

5.2. Novel aromatic degradation potential in polluted soils

The discovery of new microbes and characterizing their functions are major goals in the study of microbial diversity (Cardenas and Tiedje 2008). In Article II a research method was developed to study the diversity of I.3.E group extradiol dioxygenases directly from soil. The targeted genes are known from *Sphingomonas meta*-pathways for at least phenanthrene, naphthalene and biphenyl degradation (Romine *et al.* 1999) and *Pseudomonas meta*-pathway in NAH plasmid encoding enzymes for naphthalene degradation (Yen and Serdar 1988), but also mediating dibenzothiophene and phenanthrene degradation in *Pseudomonas* sp. C18 (Denome *et al.* 1993) (Table 4). A large diversity of extradiol dioxygenases was found from polluted soil in the thesis work. Few of the retrieved environmental EDO sequences were similar to target genes from known degradation pathways. *Pseudomonas* type *nahC* genes were detected in artificially polluted birch rhizosphere and *nsaC* type *Sphingomonas g*enes were found from a surface soil of a monitored natural attenuation site. The great majority of the environmental EDOs directly amplified from soil displayed low similarity to those from known pathways. This is in accordance with metagenomic analysis of C230 genes from activated sludge (Suenaga *et al.* 2007) where most (37 out of 43) extradiol dioxygenases had a low amino acid similarity (< 70%) to those sequenced from cultured micro-organism. The catalytic activity of these EDOs towards catechol, 3-methylcatechol, 4-methylcatechol, 4-chlorocatechol and 2,3-dihydroxybiphenyl were shown demonstrating that these low similarity enzymes still catalyses the aromatic *meta*-ring-cleavage. Novel types of extradiol dioxygenases have been also found from pristine eutrophic lake waters enriched with dissolved organic matter from different natural sources (Kasuga *et al.* 2007). The diversity of EDOs in polluted and pristine soil (Article II), activated sludge (Suenaga *et al.* 2007) and lake waters (Kasuga *et al.* 2007) demonstrates that a large proportion of the environmental aromatic degradation capacity is to be found in unknown bacteria, that evidently are not easily cultivated. Knowledge of these environmentally abundant EDOs

and their host bacteria could facilitate development of more efficient bioremediation strategies for petroleum hydrocarbons.

5.3. Birch rhizospere a hot spot for extradiol dioxygenase genes

The rhizosphere is the site of highest microbial biomass and activity and the area of greatest complexity within the soil environment (Prosser *et al.* 2006). Rhizoremediation of PAH compounds is enhanced by plant root exudates and improved aeration of soil, that increase the active microbial populations in rhizosphere associated soil (Foster 1986, Schnoor *et al.* 1995). It has been demonstrated that cultivated bacteria isolated from rhizosphere with PAH degrading capacity are diverse (Daane *et al.* 2001). In Article III the I.3.E EDOs and 16S rRNA bacterial communities were studied in birch (*Betula pendula*) rhizoremediation. Birch rhizosphere contained higher diversity of EDOs than bulk soil emphasising the rhizosphere effect to bacterial community. The higher diversity might be explained by the effect of aromatic compounds from plant secondary metabolism or lignin derived aromatic compounds enrich aromatic degraders. It has been hypothesised that xenobiotic biodegradation genes in bacteria are partly evolved as a response to plant secondary metabolites resembling different types of xenobiotics (Singer *et al.* 2003). Flavonoids have been shown to support the growth of PCB degrading micro-organism (Donnelly *et al.* 1994) and amendment of terpenes-containing plant tissues to PCB-contaminated soil increased number of biphenyl degrading bacterial isolates and the degradation rate of PCBs (Hernandez *et al.* 1997). The plant secondary metabolites (Terpene, carvone) have been shown to induce the expression of extradiol dioxygenase (*bphC*) in *Alcaligenes eutrophus* H850 (Park *et al.* 1999).

The birch tree rhizosphere soil sustains a stable diversity of EDOs where PAH amendment exerts only minor effects on the bacterial ring-cleavage community. Birch trees accumulate numerous secondary metabolites, such as phenolics and terpenoids (Julkunen-Tiitto *et al.* 1996) and the biodegradation of these compounds might favour the detected EDO diversity. Opposing results have been obtained from willow tree (*Salix viminalis* x *schwerinii*) in PCB rhizoremediation experiment (de Cárcer *et al.* 2007) where RHDs diversity was lower in rhizosphere associated soil than in the bulk soil. The willow rhizosphere was dominated by *Pseudomonas* sp. IC type RHDs. De Cárcer *et al*. (2007) studied PCB-polluted willow rhizosphere and bulk soil without non polluted control leaving the pollution effect to the RHD diversity in rhizosphere unanswered. NahC type I.3.E EDOs (Figure 7.), frequently isolated from *Pseudomonas,* were not found from the birch rhizosphere. The presence of *Pseudomonas* IC type biphenyl pathway in birch rhizosphere remains open because in *Pseudomonas* biphenyls are subjected to *meta*-cleavage catalyzed by I.3.A group EDOs (Eltis and Bolin 1996, Hofer *et al.* 1994). This group is not amplified by the BP-primers targeted explicitly to the I.3.E group. The discrepancy of results from willow and birch rhizospheres can also be related to substantial difference in the secondary metabolites composition of the tree species (Palo 1984). The findings in Article III show that the presence of aromatic degradation genes with corresponding pathways in the environment can be manipulated by different

treatments. To identify the most efficient strategies for stimulating *in situ* biodegradation of pollutants more knowledge is needed about degrading bacteria (also not yet cultivated ones) and their catabolic pathways.

5.4. Plasmids and dispersal of aromatic degradation pathways in *Sphingomonas*

Sphingomonas that degrade xenobiotic compounds frequently contain large circular plasmid in size range 50-500 kb (Basta *et al.* 2004) but only two complete megaplasmid-sequences (larger than 100 kb) have been published (Romine *et al.* 1999; Shintani *et al.* 2007). It has been suggested that plasmids with pNL1 and pCAR3 type backbone have important role in spread of catabolic genes in *Sphingomonas* (Vedler 2008). In the thesis the pSKY4 plasmid was completely sequenced displaying novel type of plasmid backbone, and an aromatic degradation pathway, demonstrating that aromatic degrading genes are spread in *Sphingomonas* by at least two different types of large plasmids. The aromatic degradation pathway is located in a putative mobile genetic element within the pSKY4 plasmid. This was inferred by the contradiction of similarities in plasmid backbone and aromatic degradation pathway to pNL1. The aromatic degradation region was flanked with several mobile genetic elements indicating several possible transposition mechanisms. Large catabolic gene clusters are frequently found on mobile elements integrated into bacterial chromosomes as genomic islands or conjugative transposons (Burrus *et al.* 2002, Gaillard *et al.* 2006, Toussaint *et al.* 2003) They have also been found from plasmids of *Pseudomonas* (Sota *et al.* 2006, Maeda *et al.* 2003, Sota *et al.* 2003, Yano *et al.* 2007, Tsuda and Iino 1990). Not much is known however of mobile genetic elements in *Sphingomonas* plasmids, although it has been shown that massive gene rearrangement occurs among plasmids and the phenomena has been described in carbofuran-degrading isolates (Ogram *et al.* 2000, Feng *et al.* 1997) and during the conjugation of pNL1 (Basta *et al.* 2004). Large amount of transposition/integration genes (54 orfs) in pSKY4 suggest that some regions in *Sphingomonas* plasmids are hot spots of mobile genetic element that could explain the detected gene arrangements. Rapid gene arrangement in plasmid borne genes not encoding essential enzymes for cell upkeep, could give evolutionary advantage in competition for natural and manmade carbon sources.

The catabolic pathway comparison between pNL1 and pSKY4 revealed a large 10 kb deletion in the pNL1 plasmid aromatic degradation region. Orfs putatively involved in Beta-oxidation related metabolism were identified from pSKY4, but not in pNL1. The CoA-transferases in pSKY4 aromatic pathway belonged to recently identified family III of CoA-transferases known to be involved the metabolism of oxalate, carnitine, toluene, bile acid, autotrophic 3-hydroxypropionate cycle and also in Stickland fermentation of (R)-phenyllactate (Heider 2001, Friedmann *et al.* 2006). Two orfs encoding the hotdog fold superfamily proteins are known to have dehydratase, thiolase and regulatory activity (Dillon and Bateman 2005). Hotdog fold proteins have functions in aromatic catabolism of phenylacetate and in dehalogenation of chlorobenzoate releasing the CoA-moiety from

pathway intermediates (Song *et al.* 2006, Zhuang *et al.* 2003) but also in polyhydroxyalkanoates synthesis where they encode (R)-specific enoyl-CoA hydratase (Park and Lee 2003). Another gene involved in polyhydroxyalkanoate synthesis acetoacetyl-CoA thiolase, was also identified from pSKY4. It mediates the synthesis of Acetoacetyl-CoA from two acetyl-CoA. We could not identify any genes that would encode medium chain polyhydroxyalkanoate synthase suggesting that the detected orfs do not catalyse polyhydroxyalkanoate synthesis, or that the lacking genes are of chromosomal origin.

Four different genes encoding different types of Acyl-CoA dehydrogenases were identified in the deletion area. Acyl-CoA dehydrogenases are generally linked to Beta-oxidation of fatty acid where they introduce C-C double pond into in to their fatty acyl-CoA substrates (Thorpe and Kim 1995, Ghisla and Thorpe 2004) but the Acyl-CoA dehydrogenases have shown to be involved in various metabolic reactions like antibiotic biosynthesis (Zhang *et al.* 1999), denitrification of nitroalkanes (Daubner *et al.* 2002), aromatic dibenzothiophene desulfurization (Denome *et al.* 1994) and in phenylalkanoate Beta-oxidation (McMahon and Mayhew 2007). Recently it has been shown that a novel acyl-CoA dehydrogenase domain containing the *idoA* gene catalyses the oxygenation of indole to indigo in *Pseudomonas alcaligenes* PA-10 that is capapable to degrade four ring PAHs (Alemayehu *et al.* 2004). The disruption of the *idoA* gene leads to loss of ability to metabolize fluoranthene and to oxygenate indole suggesting that acyl-CoA dehydrogenases might have a role in higher PAH metabolism. Interestingly also *Sphingobium* sp. HV3 is capable to co-metabolize fluoranthene (Article I), a property that is not reported for *Novosphingobium aromaticivorans* F119 (Pinyakong *et al.* 2003b, Fredrickson *et al.* 1995, Fredrickson *et al.* 1991). It is tempting to speculate that co-metabolism of fluoranthene by the HV3 strain could be related to the presence of a beta-oxidation operon in the pSKY4 plasmid, but further studies are needed to confirm the function of these genes. The complete sequence of the pSKY4 plasmid revealed new genes putatively involved in aromatic metabolism of *Sphingomonas* and demonstrated that catabolic plasmid diversity in *Sphingomonas* goes beyond the single incompatibility group of pNL1 and pCAR3.

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References

Alemayehu D Gordon L O'Mahony M O'Leary N & Dobson A (2004) Cloning and functional analysis by gene disruption of a novel gene involved in indigo production and fluoranthene metabolism in *Pseudomonas alcaligenes* PA-10. *FEMS Microbiology Letters* 239:285-293.

Andujar E Hernaez MJ Kaschabek SR Reineke W & Santero E (2000) Identification of an extradiol dioxygenase involved in tetralin biodegradation: gene sequence analysis and purification and characterization of the gene product. *Journal of Bacteriology* 182:789-795.

Aprill W & Sims R (1990) Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. *Chemosphere(Oxford)* 20:253-265.

Avaniss-Aghajani E Jones K Chapman D & Brunk C (1994) A molecular technique for identification of bacteria using small subunit ribosomal RNA sequences. *BioTechniques* 17:144-149.

Baldwin BR Nakatsu CH & Nies L (2003) Detection and Enumeration of Aromatic Oxygenase Genes by Multiplex and Real-Time PCR. *Applied and Environmental Microbiology* 69:3350-3358.

Barns SM Takala SL & Kuske CR (1999) Wide Distribution and Diversity of Members of the Bacterial Kingdom Acidobacterium in the Environment. *Applied and Environmental Microbiology* 65:1731-1737.

Barnsley EA (1976) Naphthalene metabolism by pseudomonads: the oxidation of 1,2-dihydroxynaphthalene to 2-hydroxychromene-2-carboxylic acid and the formation of 2'-hydroxybenzalpyruvate. *Biochemical and Biophysical Research Communications* 72:1116-1121.

Basta T Buerger S & Stolz A (2005) Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics. *Microbiology* 151:2025-2037.

Basta T Keck A Klein J & Stolz A (2004) Detection and characterization of conjugative degradative plasmids in xenobiotic-degrading *Sphingomonas* strains. *Journal of Bacteriology* 186:3862-3872.

Beaulieu M Becaert V Deschenes L & Villemur R (2000) Evolution of bacterial diversity during enrichment of PCP-degrading activated soils. *Microbial ecology* 40:345-355.

Beja O Aravind L Koonin EV Suzuki MT Hadd A Nguyen LP Jovanovich SB Gates CM Feldman RA & Spudich JL (2000) Bacterial Rhodopsin: Evidence for a New Type of Phototrophy in the Sea. *Science* 289:1902-1906.

Benitez MS Gardener MS & Brian B (2009) Linking sequence to function in soil: Sequence-directed isolation of novel bacteria contributing to soilborne plant disease suppression. *Applied and Environmental Microbiology*. 75:915-924.

Bertin C Yang X & Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil* 256:67-83.

Bolan N (1991) A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and Soil* 134:189-207.

Bollmann A Lewis K & Epstein SS (2007) Incubation of Environmental Samples in a Diffusion Chamber Increases the Diversity of Recovered Isolates. *Applied and Environmental Microbiology* 73:6386-6390.

Bordenave S Goni-urriza M Vilette C Blanchard S Caumette P & Duran R (2008) Diversity of ring-hydroxylating dioxygenases in pristine and oil contaminated microbial mats at genomic and transcriptomic levels. *Environmental Microbiology* 10:3201-3211.

Boronin AM Tsoi TV Kosheleva IA Arinbasarov MU & Adanin VM (1989) Cloning of *Pseudomonas putida* genes responsible for the primary stages of oxidation of naphthalene in *Escherichia coli* cells. *Genetika* 25:226-237.

Bosch R García-Valdés E & Moore ERB (1999) Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. *Gene* 236:149-157.

Braker G Zhou J Wu L Devol AH & Tiedje JM (2000) Nitrite reductase genes (nirK and nirS) as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. *Applied and Environmental Microbiology* 66:2096-2104.

Bürgmann H Pesaro M Widmer F & Zeyer J (2001) A strategy for optimizing quality and quantity of DNA extracted from soil. *Journal of Microbiological Methods* 45:7-20.

Burrus V Pavlovic G Decaris B & Guedon G (2002) Conjugative transposons: the tip of the iceberg. *Molecular microbiology* 46:601-610.

Butler CS & Mason JR (1997) Structure-function analysis of the bacterial aromatic ring-hydroxylating dioxygenases. *Advances in Microbial Physiology* 38:47-84.

Cardenas E & Tiedje JM (2008) New tools for discovering and characterizing microbial diversity. *Current opinion in biotechnology* 19:544-549.
Ceremonie H Boubakri H Mavingui P Simonet P & Vogel TM (2006) Plasmid-encoded γ-hexachlorocyclohexane degradation genes and insertion sequences in *Sphingobium francen*se (ex-*Sphingomonas paucimobilis* Sp). *FEMS Microbiology Letters* 257:243-252.
Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3:351-368.
Cerniglia C (1984) Microbial metabolism of polycyclic aromatic hydrocarbons. *Advances in Applied Microbiology* 30:31-71.
Chang YY & Corapcioglu MY (1998) Plant-enhanced subsurface bioremediation of nonvolatile hydrocarbons. *Journal of Environmental Engineering* 124:162-169.
Cho O Choi KY Zylstra GJ Kim YS Kim SK Lee JH Sohn HY Kwon GS Kim YM & Kim E (2005) Catabolic role of a three-component salicylate oxygenase from *Sphingomonas yanoikuyae* B1 in polycyclic aromatic hydrocarbon degradation. *Biochemical and Biophysical Research Communications* 327:656-662.
Da Silva MLB Kamath R & Alvarez PJJ (2006) Effect of simulated rhizodeposition on the relative abundance of polynuclear aromatic hydrocarbon catabolic genes in a contaminated soil. *Environmental Toxicology and Chemistry* 25:.
Daane L Harjono I Zylstra G & Haggblom M (2001) Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. *Applied and Environmental Microbiology* 67:2683-2691.
Daubner SC Gadda G Valley MP & Fitzpatrick PF (2002) Cloning of nitroalkane oxidase from *Fusarium oxysporum* identifies a new member of the acyl-CoA dehydrogenase superfamily. *Proceedings of the National Academy of Sciences* 99:2702.
de Cárcer DA Martín M Karlson U & Rivilla R (2007) Changes in bacterial populations and in biphenyl dioxygenase gene diversity in a polychlorinated biphenyl-polluted soil after introduction of willow trees for rhizoremediation. *Applied and Environmental Microbiology* 73:6224-6232.
Demaneche S Meyer C Micoud J Louwagie M Willison JC & Jouanneau Y (2004) Identification and functional analysis of two aromatic-ring-hydroxylating dioxygenases from a *Sphingomonas* strain that degrades various polycyclic aromatic hydrocarbons. *Applied and Environmental Microbiology* 70:6714-6725.
Denome S Oldfield C Nash L & Young K (1994) Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8. *Journal of Bacteriology* 176:6707-6716.
Denome S Stanley D Olson E & Young K (1993) Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. *Journal of Bacteriology* 175:6890-6901.
Di Gregorio S Zocca C Sidler S Toffanin A Lizzari D & Vallini G (2004) Identification of two new sets of genes for dibenzothiophene transformation in *Burkholderia* sp. DBT1. *Biodegradation* 15:111-123.
Dillon SC & Bateman A (2005) The Hotdog fold: wrapping up a superfamily of thioesterases and dehydratases. *BMC Bioinformatics* 5:109-123.
Donnelly P Hegde R & Fletcher J (1994) Growth of PCB-degrading bacteria on compounds from photosynthetic plants. *Chemosphere (United Kingdom)* 28: 981-988.
Doolittle WF (1999) Phylogenetic Classification and the Universal Tree. *Science* 284:2124 2128.
Eaton R (1994) Organization and evolution of naphthalene catabolic pathways: sequence of the DNA encoding 2-hydroxychromene-2-carboxylate isomerase and trans-o-hydroxybenzylidenepyruvate hydratase-aldolase from the NAH7 plasmid. *Journal of Bacteriology* 176:7757-7762.
Eaton R & Chapman P (1992) Bacterial metabolism of naphthalene: construction and use of recombinant bacteria to study ring cleavage of 1, 2-dihydroxynaphthalene and subsequent reactions. *Journal of Bacteriology* 174:7542-7554.
Eckert K A & Kunkel T A (1991) DNA polymerase fidelity and the polymerase chain reaction. *Genome Research* 1:17-24.
Eichorst SA Breznak JA & Schmidt TM (2007) Isolation and characterization of soil bacteria that define *Terriglobus* gen. nov., in the Phylum Acidobacteria. *Applied and Environmental Microbiology* 73:2708-2717.
Eltis LD & Bolin JT (1996) Evolutionary relationships among extradiol dioxygenases. *Journal of Bacteriology* 178:5930-5937.
Farrelly V Rainey F & Stackebrandt E (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* 61:2798-2801.

Feng X Ou L & Ogram A (1997) Plasmid-mediated mineralization of carbofuran by *Sphingomonas* sp. strain CF06. *Applied and Environmental Microbiology* 63:1332-1337.
Ferrero M Llobet-Brossa E Lalucat J Garcia-Valdes E Rossello-Mora R & Bosch R (2002) Coexistence of two distinct copies of naphthalene degradation genes in *Pseudomonas* strains isolated from the western mediterranean region. *Applied and Environmental Microbiology* 68:957-962.
Foster R (1986) The ultrastructure of the rhizoplane and rhizosphere. *Annual Reviews in Phytopathology* 24:211-234.
Fredrickson J Brockman F Workman D Li S & Stevens T (1991) Isolation and characterization of a subsurface bacterium capable of growth on toluene, naphthalene, and other aromatic compounds. *Applied and Environmental Microbiology* 57:796-803.
Fredrickson JK Balkwill DL Drake GR Romine MF Ringelberg DB & White DC (1995) Aromatic-degrading *Sphingomonas* isolates from the deep subsurface. *Applied and Environmental Microbiology* 61:1917-1922.
Friedmann S Alber BE & Fuchs G (2006) Properties of succinyl-coenzyme A: D-citramalate coenzyme A transferase and its role in the autotrophic 3-hydroxypropionate cycle of *Chloroflexus aurantiacus*. *Journal of Bacteriology* 188:6460-6468.
Fuenmayor SL Wild M Boyes AL & Williams PA (1998) A Gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. Strain U2. *Journal of Bacteriology* 180:2522-2530.
Fuhrman JA Schwalbach MS & Stingl U (2008) Proteorhodopsins: an array of physiological roles? *Nature Reviews Microbiology* 6:488.
Fuhrman J McCallum K & Davis A (1993) Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. *Applied and Environmental Microbiology* 59:1294-1302.
Fuhrman JA McCallum K & Davis AA (1992) Novel major archaebacterial group from marine plankton. *Nature* 356:148-149.
Furukawa K Simon J & Chakrabarty A (1983) Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. *Journal of Bacteriology* 154:1356-1362.
Gaillard M Vallaeys T Vorhölter FJ Minoia M Werlen C Sentchilo V Pühler A & van der Meer JR (2006) The clc element of *Pseudomonas* sp. strain B13, a genomic island with various catabolic properties. *Journal of Bacteriology* 188:1999-2013.
Galand PE Saarnio S Fritze H & Yrjälä K (2002) Depth related diversity of methanogen Archaea in Finnish oligotrophic fen. *FEMS Microbiology Ecology* 42:441-449.
Gerlt JA & Babbitt PC (2001) Divergent evolution of enzymatic functions: Mechanistically diverse superfamilies and functionally distinct suprafamilies. *Annual Review of Biochemistry* 70:209-246.
Ghisla S & Thorpe C (2004) Acyl-CoA dehydrogenases. *European Journal of Biochemistry* 271:494-508.
Gibson DT & Parales RE (2000) Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Current opinion in biotechnology* 11:236-243.
Gomes NCM Borges LR Paranhos R Pinto FN Krögerrecklenfort E Mendonça-Hagler LCS & Smalla K (2007) Diversity of ndo genes in mangrove sediments exposed to different sources of polycyclic aromatic hydrocarbon pollution. *Applied and Environmental Microbiology* 73:7392-7399.
Gomes N Heuer H Schönfeld J Costa R Mendonca-Hagler L & Smalla K (2001) Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. *Plant and Soil* 232:167-180.
Green R & Noller HF (1997) Ribosomes and translation. *Annual Review of Biochemistry* 66:679-716.
Habe H Ashikawa Y Saiki Y Yoshida T Nojiri H & Omori T (2002) *Sphingomona*s sp. strain KA1, carrying a carbazole dioxygenase gene homologue, degrades chlorinated dibenzo-p-dioxins in soil. *FEMS Microbiology Letters* 211:43-49.
Handelsman J Rondon MR Brady SF Clardy J & Goodman RM (1998) Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chemistry & biology* 5:245-249.
Harayama S Kok M & Neidle E (1992) Functional and evolutionary relationships among diverse oxygenases. *Annual Reviews in Microbiology* 46:565-601.
Harayama S & Rekik M (1989) Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. *Journal of Biological Chemistry* 264:15328-15333.
Harley, J.L. and Smith, S.E. (1983) *Mycorrhizal symbiosis.* : Academic Press London.
Heider J (2001) A new family of CoA-transferases. *FEBS letters* 509:345-349.

Heiss G Stolz A Kuhm AE Muller C Klein J Altenbuchner J & Knackmuss HJ (1995) Characterization of a 2,3-dihydroxybiphenyl dioxygenase from the naphthalenesulfonate-degrading bacterium strain BN6. *Journal of Bacteriology* 177:5865-5871.

Hendrickx B Dejonghe W Faber F Boenne W Bastiaens L Verstraete W Top EM & Springael D (2006) PCR-DGGE method to assess the diversity of BTEX mono-oxygenase genes at contaminated sites. *FEMS Microbiology Ecology* 55:262-273.

Hendrickx B Dejonghe W Boenne W Brennerova M Cernik M Lederer T Bucheli-Witschel M Bastiaens L Verstraete W Top EM Diels L & Springael D (2005) Dynamics of an oligotrophic bacterial aquifer community during contact with a groundwater plume contaminated with benzene, toluene, ethylbenzene, and xylenes: an in situ mesocosm study. *Applied and Environmental Microbiology* 71:3815-3825.

Hernandez B Koh SC Chial M & Focht D (1997) Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphenyls in soil. *Biodegradation* 8:153-158.

Heuer H Krsek M Baker P Smalla K & Wellington E (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* 63:3233-3241.

Hofer B Backhaus S & Timmis K (1994) The biphenyl/polychlorinated biphenyl-degradation locus (bph) of *Pseudomonas* sp. LB 400 encodes four additional metabolic enzymes. *Gene(Amsterdam)* 144:9-16.

Holben WE Jansson JK Chelm BK & Tiedje JM (1988) DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Applied and Environmental Microbiology* 54:703-711.

Holmes B Owen R Evans A Malnick H & Willcox W (1977) *Pseudomonas paucimobilis*, a New species isolated from human clinical specimens, the hospital environment, and other sources. *International Journal of Systematic and Evolutionary Microbiology* 27:133-146.

Hu X Mamoto R Fujioka Y Tani A Kimbara K & Kawai F (2008) The pva operon is located on the megaplasmid of *Sphingopyxis* sp. strain 113P3 and is constitutively expressed, although expression is enhanced by PVA. *Applied Microbiology and Biotechnology* 78:685-693.

Huber JA Morrison HG Huse SM Neal PR Sogin ML & Mark Welch DB (2009) Effect of PCR amplicon size on assessments of clone library microbial diversity and community structure. *Environmental Microbiology* 11:1292-1302.

Hugenholtz P Goebel BM & Pace NR (1998) Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *Journal of Bacteriology* 180:4765-4774.

Hugenholtz P (2002) Exploring prokaryotic diversity in the genomic era. *Genome biology* 3:3.1-3.8

Hurt RA Qiu X Wu L Roh Y Palumbo AV Tiedje JM & Zhou J (2001) Simultaneous Recovery of RNA and DNA from Soils and Sediments. *Applied and Environmental Microbiology* 67:4495-4503.

Janssen PH Yates PS Grinton BE Taylor PM & Sait M (2002) Improved culturability of soil bacteria and solation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Applied and Environmental Microbiology* 68:2391-2396.

Jeon CO Park M Ro HS Park W & Madsen EL (2006) The naphthalene catabolic (nag) genes of *Polaromonas naphthalenivorans* CJ2: evolutionary implications for two gene clusters and novel regulatory control. *Applied and Environmental Microbiology* 72:1086-1095.

Jouanneau Y Micoud J & Meyer C (2007) Purification and characterization of a three-component salicylate 1-hydroxylase from *Sphingomonas* sp. Strain CHY-1. *Applied and Environmental Microbiology* 73:7515-7521.

Jouanneau Y & Meyer C (2006) Purification and characterization of an arene cis-dihydrodiol dehydrogenase endowed with broad substrate specificity toward polycyclic aromatic hydrocarbon dihydrodiols. *Applied and Environmental Microbiology* 72:4726-4734.

Julkunen-Tiitto R Rousi M Bryant J Sorsa S Keinänen M & Sikanen H (1996) Chemical diversity of several Betulaceae species: comparison of phenolics and terpenoids in northern birch stems. *Trees-Structure and Function* 11:16-22.

Junca H & Pieper DH (2004) Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. *Environmental Microbiology* 6:95-110.

Junca H & Pieper DH (2003) Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria. *Journal of Microbiological Methods* 55:697-708.

Jurgens G Lindstrom K & Saano A (1997) Novel group within the kingdom Crenarchaeota from boreal forest soil. *Applied and Environmental Microbiology* 63:803-805.

Kasai Y Shindo K Harayama S & Misawa N (2003) Molecular characterization and substrate preference of a polycyclic aromatic hydrocarbon dioxygenase from *Cycloclasticus* sp. strain A5. *Applied and Environmental Microbiology* 69:6688-6697.
Kasuga I Nakajima F & Furumai H (2007) Diversity of catechol 2, 3-dioxygenase genes of bacteria responding to dissolved organic matter derived from different sources in a eutrophic lake. *FEMS Microbiology Ecology* 61:449-458.
Keck A Conradt D Mahler A Stolz A Mattes R & Klein J (2006) Identification and functional analysis of the genes for naphthalenesulfonate catabolism by *Sphingomonas xenophaga* BN6. *Microbiology* 152:1929-1940.
Khan A Kuek C Chaudhry T Khoo C & Hayes W (2000) Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. *Chemosphere* 41:197-207.
Kilpi S (1980) Degradation of some phenoxy acid herbicides by mixed cultures of bacteria isolated from soil treated with 2-(2-methyl-4-chloro) phenoxypropionic acid. *Microbial Ecology* 6:261-270.
Kilpi S Backström V & Korhola M (1983) Degradation of catechol, methylcatechols and chlorocatechols by *Pseudomonas* sp. HV3. *FEMS microbiology letters* 18:1-5.
Kilpi S Backström V & Korhola M (1980) Degradation of 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2, 4-dichlorophenoxyacetic acid (2, 4-D), benzoic acid and salicylic acid by *Pseudomonas* sp. HV3. *FEMS microbiology letters* 8:177-182.
Kilpi S Himberg K Yrjälä K & Backström V (1988) The degradation of biphenyl and chlorobiphenyls by mixed bacterial cultures. *FEMS Microbiology Letters* 53:19-26.
Kim E & Zylstra G (1999) Functional analysis of genes involved in biphenyl, naphthalene, phenanthrene, and *m*-xylene degradation by *Sphingomonas yanoikuyae* B1. *Journal of Industrial Microbiology and Biotechnology* 23:294-302.
Kim E & Zylstra G (1995) Molecular and biochemical characterization of two meta-cleavage dioxygenases involved in biphenyl and m-xylene degradation by *Beijerinckia* sp. strain B1. *Journal of Bacteriology* 177:3095-3103.
Kim S Shin H Park Y Youngsoo K Min K & Kim Y (1999) The 2, 3-dihydroxybiphenyl 1, 2-dioxygenase gene (phnQ) of *Pseudomonas* sp. DJ77: nucleotide sequence, enzyme assay, and comparison with isofunctional dioxygenases. *Journal of Biochemistry and Molecular Biology* 32:399-404.
Kleinsteuber S Riis V Fetzer I Harms H & Muller S (2006) Population dynamics within a microbial consortium during growth on diesel fuel in saline environments. *Applied and Environmental Microbiology* 72:3531-3542.
Kleppe K Ohtsuka E Kleppe R Molineux I & Khorana HG (1971) Studies on polynucleotides. XCVI. Repair replications of short synthetic DNA's as catalyzed by DNA polymerases. *Journal of Molecular Biology* 56:341-361.
Kohler HP (1999) *Sphingomonas herbicidovorans* MH: a versatile phenoxyalkanoic acid herbicide degrader. *Journal of Industrial Microbiology & Biotechnology* 23:336-340.
Korkama-Rajala T Müller MM & Pennanen T (2008) Decomposition and fungi of needle litter from slow- and fast-growing Norway spruce (*Picea abies*) clones. *Microbial Ecology* 56:76-89.
Kozdroj J & van Elsas JD (2001) Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. *Journal of Microbiological Methods* 43:197-212.
Kuhm AE Stolz A & Knackmuss HJ (1991) Metabolism of naphthalene by the biphenyl-degrading bacterium *Pseudomonas paucimobilis* Q1. *Biodegradation* 2:115-120.
Kuiper I Lagendijk EL Bloemberg GV & Lugtenberg BJJ (2004) Rhizoremediation: A beneficial plant-microbe interaction. *Molecular Plant-Microbe Interactions* 17:6-15.
Kuntze K Shinoda Y Moutakki H McInerney MJ Vogt C Richnow HH & Boll M (2008) 6-Oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolases from obligately anaerobic bacteria: characterization and identification of its gene as a functional marker for aromatic compounds degrading anaerobes. *Environmental Microbiology* 10:1547-1556.
Leander M Vallaeys T & Fulthorpe R (1998) Amplification of putative chlorocatechol dioxygenase gene fragments from and proteobacteria. *Canadian Journal of Microbiology* 44:482-486.
Lee D Zo Y & Kim S (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. *Applied and Environmental Microbiology* 62:3112-3120.
Leys NM Ryngaert A Bastiaens L Verstraete W Top EM & Springael D (2004) Occurrence and phylogenetic diversity of Sphingomonas strains in soils contaminated with polycyclic aromatic hydrocarbons. *Applied and Environmental Microbiology* 70:1944-1955.

Li W Shi J Wang X Han Y Tong W Ma L Liu B & Cai B (2004) Complete nucleotide sequence and organization of the naphthalene catabolic plasmid pND6-1 from *Pseudomonas* sp. strain ND6. *Gene* 336:231-240.

Liesack W & Stackebrandt E (1992) Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *Journal of Bacteriology* 174:5072-5078.

Liles MR Williamson LL Rodbumrer J Torsvik V Goodman RM & Handelsman J (2008) Recovery, purification, and cloning of high-molecular-weight DNA from soil microorganisms. *Applied and Environmental Microbiology* 74:3302-3305.

Lim JS Jung MK Kim MS Ahn JH & Ka JO (2004) Genetic and phenotypic diversity of (R/S)-mecoprop [2-(2-methyl-4-chlorophenoxy)propionic acid]-degrading bacteria isolated from soils. *Journal of Microbiology* 42:87-93.

Little AEF Robinson CJ Peterson SB Raffa KF & Handelsman J (2008) Rules of Engagement: Interspecies Interactions that Regulate Microbial Communities. *Annual Review of Microbiology* 62:375-401

Lloyd-Jones G Laurie AD Hunter DWF & Fraser R (1999) Analysis of catabolic genes for naphthalene and phenanthrene degradation in contaminated New Zealand soils. *FEMS Microbiology Ecology* 29:69-79.

Lu J Nomura N Nakajima-Kambe T & Nakahara T (2000) Cloning and expression of genes encoding *meta*-cleavage enzymes from 4, 6-dimethyldibenzothiophene-degrading *Sphingomonas* strain TZS-7. *BBA-Gene Structure and Expression* 1492:493-498.

Mackova, M., Dowling, D.N. and Macek, T. (2006) *Phytoremediation Rhizoremediation: Theoratical Background.* : Springer Verlag. Germany

Maeda K Nojiri H Shintani M Yoshida T Habe H & Omori T (2003) Complete nucleotide sequence of carbazole/dioxin-degrading plasmid pCAR1 in *Pseudomonas resinovorans* strain CA10 indicates its mosaicity and the presence of large catabolic transposon Tn4676. *Journal of Molecular Biology* 326:21-33.

Maruyama T Park HD Ozawa K Tanaka Y Sumino T Hamana K Hiraishi A & Kato K (2006) *Sphingosinicella microcystinivorans* gen. nov., sp. nov., a microcystin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology* 56:85-89.

Masai E Sugiyama K Iwashita N Shimizu S Hauschild JE Hatta T Kimbara K Yano K & Fukuda M (1997) The bphDEF *meta*-cleavage pathway genes involved in biphenyl/polychlorinated biphenyl degradation are located on a linear plasmid and separated from the initial bphACB genes in *Rhodococcus* sp. strain RHA1. *Gene* 187:141-149.

McDonald IR & Murrell JC (1997) The particulate methane monooxygenase gene pmoA and its use as a functional gene probe for methanotrophs. *FEMS Microbiology Letters* 156:205-210.

McLeod MP Warren RL Hsiao WWL Araki N Myhre M Fernandes C Miyazawa D Wong W Lillquist AL & Wang D (2006) The complete genome of Rhodococcus sp. RHA1 provides insights into a catabolic powerhouse. *Proceedings of the National Academy of Sciences* 103:15582.

McMahon B & Mayhew SG (2007) Identification and properties of an inducible phenylacyl-CoA dehydrogenase in *Pseudomonas putida* KT2440. *FEMS microbiology letters* 273:50-57.

Mesarch MB Nakatsu CH & Nies L (2000) Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. *Applied and Environmental Microbiology* 66:678-683.

Meyer S Moser R Neef A Stahl U & Kaempfer P (1999) Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. *Microbiology*1731-1741.

Miller RW Donahue RL and Miller JU (1990) *Soils: An Introduction to Soils and Plant Growth.* : Prentice Hall Englewood Cliffs, NJ (USA).

Mühling M Woolven-Allen J Murrell JC & Joint I (2008) Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *The ISME Journal* 2:379-392.

Muyzer G De Waal E & Uitterlinden A (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59:695-700.

Nagata Y Kamakura M Endo R Miyazaki R Ohtsubo Y & Tsuda M (2006) Distribution of γ-hexachlorocyclohexane-degrading genes on three replicons in *Sphingobium japonicum* UT26. *FEMS Microbiology Letters* 256:112-118.

Nair DR Burken JG Licht LA & Schnoor JL (1993) Mineralization and uptake of triazine pesticide in soil-plant systems. *Journal of Environmental Engineering* 119:842-854.

Ni Chadhain SM Norman RS Pesce KV Kukor JJ & Zylstra GJ (2006) Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon biodegradation. *Applied and Environmental Microbiology* 72:4078-4087.
O'Donnell AG Young IM Rushton SP Shirley MD & Crawford JW (2007) Visualization, modelling and prediction in soil microbiology. *Nature Reviews Microbiology* 5:689-699.
Ogram A Sayler GS & Barkay T (1987) The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods* 7:57-66.
Ogram A Duan YP Trabue S Feng X Castro H & Ou LT (2000) Carbofuran degradation mediated by three related plasmid systems. *FEMS Microbiology Ecology* 32:197-203.
Palo RT (1984) Distribution of birch (*Betula* spp.), willow (*Salix* spp.), and poplar (*Populus* spp.) secondary metabolites and their potential role as chemical defense against herbivores. *Journal of Chemical Ecology* 10:499-520.
Park SJ & Lee SY (2003) Identification and characterization of a new enoyl coenzyme A hydratase involved in biosynthesis of medium-chain-length polyhydroxyalkanoates in recombinant *Escherichia coli*. *Journal of Bacteriology* 185:5391-5397.
Park Y So J & Koh SC (1999) Induction by carvone of the polychlorinated biphenyl (PCB)-degradative pathway in *Alcaligenes eutrophus* H850 and its molecular monitoring. *Journal of microbiology and biotechnology* 9:804-810.
Peng RH Xiong AS Xue Y Fu XY Gao F Zhao W Tian YS & Yao QH (2008) Microbial biodegradation of polyaromatic hydrocarbons. *FEMS Microbiology Reviews* 32:927-955.
Pinyakong O Habe H Yoshida T Nojiri H & Omori T (2003a) Identification of three novel salicylate 1-hydroxylases involved in the phenanthrene degradation of *Sphingobium* sp. strain P2. *Biochemical and Biophysical Research Communications* 301:350-357.
Pinyakong O Habe H & Omori T (2003b) The unique aromatic catabolic genes in Sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs). *Journal of General Applied Microbiology* 49:1-1-19.
Prosser JI Rangel-Castro JI & Killham K (2006) Studying plant–microbe interactions using stable isotope technologies. *Current Opinion in Biotechnology* 17:98-102.
Rappe MS & Giovannoni SJ (2003) The uncultured microbial majority. *Annual Review of Microbiology* 57:369-394.
Reineke W (1998) Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. *Annual Review of Microbiology* 52:287-331.
Reysenbach A Giver L Wickham G & Pace N (1992) Differential amplification of rRNA genes by polymerase chain reaction. *Applied and Environmental Microbiology* 58:3417-3418.
Romine MF Stillwell LC Wong K Thurston SJ Sisk EC Sensen C Gaasterland T Fredrickson JK & Saffer JD (1999) Complete Sequence of a 184-Kilobase Catabolic Plasmid from *Sphingomonas aromati*civorans F199. *Journal of Bacteriology* 181:1585-1602.
Rossello-Mora R & Amann R (2001) The species concept for prokaryotes. *FEMS Microbiology Reviews* 25:39-67.
Rotthauwe J Witzel K & Liesack W (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology* 63:4704-4712.
Rovira AD (1969) Plant root exudates. *The Botanical Review* 35:35-57.
Sakai M Masai E Asami H Sugiyama K Kimbara K & Fukuda M (2002) Diversity of 2, 3-dihydroxybiphenyl dioxygenase genes in a strong PCB degrader, *Rhodococcus* sp. strain RHA1. *Journal of Bioscience and Bioengineering* 93:421-427.
Salminen JM Tuomi PM Suortti AM & Jørgensen KS (2004) Potential for aerobic and anaerobic biodegradation of petroleum hydrocarbons in boreal subsurface. *Biodegradation* 15:29-29-39.
Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989*) Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press,New York
Schnoor JL Light LA McCutcheon SC Wolfe NL & Carreia LH (1995) Phytoremediation of organic and nutrient contaminants. *Environmental Science & Technology* 29:318-323.
Seeger M Timmis K & Hofer B (1995) Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the bph locus of *Pseudomonas* sp. strain LB400. *Applied and Environmental Microbiology* 61:2654-2658.
Sei K Asano K Tateishi N Mori K Ike M & Fujita M (1999) Design of PCR primers and gene probes for the general detection of bacterial populations capable of degrading aromatic compounds via catechol cleavage pathways. J*ournal of Bioscience and Bioengineering* 88:542-550.

Shintani M Urata M Inoue K Eto K Habe H Omori T Yamane H & Nojiri H (2007) The *Sphingomonas* Plasmid pCAR3 Is Involved in Complete Mineralization of Carbazole. *Journal of Bacteriology* 189:2007-2020.
Singer AC Crowley DE & Thompson IP (2003) Secondary plant metabolites in phytoremediation and biotransformation. *Trends in Biotechnology* 21:123-130.
Sipos R Szekely AJ Palatinszky M Revesz S Marialigeti K & Nikolausz M (2007) Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. *FEMS Microbiology Ecology* 60:341-350.
Soltis D Soltis P Morgan D Swensen S Mullin B Dowd J & Martin P (1995) Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. *Proceedings of the National Academy of Sciences* 92:2647-2651.
Song B & Ward BB (2005) Genetic diversity of benzoyl coenzyme A reductase genes detected in denitrifying isolates and estuarine sediment communities. *Applied and Environmental Microbiology* 71:2036-2045.
Song F Zhuang Z Finci L Dunaway-Mariano D Kniewel R Buglino JA Solorzano V Wu J & Lima CD (2006) Structure, function, and mechanism of the phenylacetate pathway hot dog-fold thioesterase PaaI. *Journal of Biological Chemistry* 281:11028.
Sota M Yano H Ono A Miyazaki R Ishii H Genka H Top EM & Tsuda M (2006) Genomic and functional analysis of the incP-9 naphthalene catabolic plasmid NAH7 and its transposon Tn 4655 suggests catabolic gene spread by a tyrosine recombinase. *Journal of Bacteriology* 188:4057-4067.
Sota M Kawasaki H & Tsuda M (2003) Structure of haloacetate-catabolic IncP-1 beta plasmid pUO1 and genetic mobility of its residing haloacetate-catabolic transposon. *Journal of Bacteriology* 185:6741-6745.
Sprent JI (2007) Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. *New Phytologist* 174:11-25.
Stackebrandt E Liesack W & Goebel B (1993) Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *The FASEB Journal* 7:232-236.
Stolz A (2009) Molecular characteristics of xenobiotic-degrading sphingomonads. *Applied Microbiology and Biotechnology* 81:793-811.
Story S Parker S Hayasaka S Riley M & Kline E (2001) Convergent and divergent points in catabolic pathways involved in utilization of fluoranthene, naphthalene, anthracene, and phenanthrene by *Sphingomonas paucimobilis* var. EPA505. *Journal of Industrial Microbiology and Biotechnology* 26:369-382.
Stott MB Crowe MA Mountain BW Smirnova AV Hou S Alam M & Dunfield PF (2008) Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. *Environmental Microbiology*. 10:2030-2041
Suenaga H Ohnuki T & Miyazaki K (2007) Functional screening of a metagenomic library for genes involved in microbial degradation of aromatic compounds. *Environmental Microbiology* 9:2289-2297.
Susarla S Medina VF & McCutcheon SC (2002) Phytoremediation: An ecological solution to organic chemical contamination. *Ecological Engineering* 18:647-658.
Suzuki M & Giovannoni S (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* 62:625-630.
Takeuchi M Hamana K & Hiraishi A (2001) Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. *International Journal of Systematic and Evolutionary Microbiology* 51:1405-1417.
Takeuchi M Sawada H Oyaizu H & Yokota A (1994) Phylogenetic evidence for *Sphingomonas* and *Rhizomonas* as nonphotosynthetic members of the alpha-4 subclass of the Proteobacteria. *International Journal of Systematic Bacteriology* 44:308-314.
Takizawa N Iida T Sawada T Yamauchi K Wang YW Fukuda M & Kiyohara H (1999) Nucleotide sequences and characterization of genes encoding naphthalene upper pathway of *Pseudomonas aeruginosa* PaK1 and *Pseudomonas putida* OUS82. *Journal of bioscience and bioengineering* 87:721-731.
Tani A Charoenpanich J Mori T Takeichi M Kimbara K & Kawai F (2007) Structure and conservation of a polyethylene glycol-degradative operon in sphingomonads. *Microbiology* 153:338.
Taylor PM Medd JM Schoenborn L Hodgson B & Janssen PH (2002) Detection of known and novel genes encoding aromatic ring-hydroxylating dioxygenases in soils and in aromatic hydrocarbon-degrading bacteria. *FEMS Microbiology Letters* 216:61-66.

Tervahauta AI Fortelius C Tuomainen M Åkerman ML Rantalainen K Sipilä T Lehesranta SJ Koistinen KM Kärenlampi S & Yrjälä K (2009) Effect of birch (*Betula* spp.) and associated rhizoidal bacteria on the degradation of soil polyaromatic hydrocarbons, PAH-induced changes in birch proteome and bacterial community. *Environmental Pollution* 157:341-346.
Thompson JR Marcelino LA & Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by reconditioning PCR. *Nucleic Acids Research* 30:2083-2088.
Thorpe C & Kim J (1995) Structure and mechanism of action of the acyl-CoA dehydrogenases. *The FASEB Journal* 9:718-725.
Tiirola MA Wang H Paulin L & Kulomaa MS (2002) Evidence for natural horizontal transfer of the pcpB gene in the evolution of polychlorophenol-degrading sphingomonads. *Applied and Environmental Microbiology* 68:4495-4501.
Torsvik V & Øvreås L (2002) Microbial diversity and function in soil: from genes to ecosystems. *Current Opinion in Microbiology* 5:240-245.
Torsvik V Øvreas L & Thingstad TF (2002) Prokaryotic Diversity-Magnitude, Dynamics, and Controlling Factors. *Science* 296:1064-1066.
Torsvik VL (1980) Isolation of bacterial DNA from soil. *Soil Biology & Biochemistry* 12:15-21.
Toussaint A Merlin C Monchy S Benotmane MA Leplae R Mergeay M & Springael D (2003) The biphenyl- and 4-chlorobiphenyl-catabolic transposon Tn 4371, a member of a new family of genomic islands related to IncP and Ti plasmids. *Applied and Environmental Microbiology* 69:4837-4845.
Tringe SG von Mering C Kobayashi A Salamov AA Chen K Chang HW Podar M Short JM Mathur EJ & Detter JC (2005) Comparative Metagenomics of Microbial Communities. *Science* 308:554-557.
Tsuda M & Iino T (1990) Naphthalene degrading genes on plasmid NAH7 are on a defective transposon. *Molecular Genetics and Genomics* 223:33-39.
Ueda T Suga Y & MatsuguchI T (1995) Molecular phylogenetic analysis of a soil microbial community in a soybean field. *European Journal of Soil Science* 46:415-421.
Vaillancourt FH Bolin JT & Eltis LD (2006) The ins and outs of ring-cleaving dioxygenases. *Critical Reviews in Biochemistry and Molecular biology* 41:241-267.
Vallaeys T Fulthorpe RR Wright AM & Soulas G (1996) The metabolic pathway of 2, 4-dichlorophenoxyacetic acid degradation involves different families of tfdA and tfdB genes according to PCR-RFLP analysis. *FEMS Microbiology Ecology* 20:163-172.
van der Meer J. (1997) Evolution of novel metabolic pathways for the degradation of chloroaromatic compounds*. Antonie van Leeuwenhoek 71:*159-178.
Vedler E (2008) *Microbial Megaplasmids*. Springer verlag, Berlin
Venter JC Remington K Heidelberg JF Halpern AL Rusch D Eisen JA Wu D Paulsen I Nelson KE & Nelson W (2004) Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science* 304:66-74.
Whipps J & Lynch J (1986) The influence of the rhizosphere on crop productivity. *Advances in Microbial Ecology* 9:187-244.
White-Stevens RH & Kamin H (1972) Studies of a flavoprotein, salicylate hydroxylase I. Preparation, properties, and the uncoupling of oxygen reduction from hydroxylation. *Journal of Biological Chemistry* 247:2358-2370.
Widada J Nojiri H Kasuga K Yoshida T Habe H & Omori T (2002a) Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. *Applied Microbiology and Biotechnology* 58:202-209.
Widada J Nojiri H & Omori T (2002b) Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacteria and their catabolic genes in bioremediation. *Applied Microbiology and Biotechnology* 60:45-59.
Wikström P Wiklund A Andersson AC & Forsman M (1996) DNA recovery and PCR quantification of catechol 2, 3-dioxygenase genes from different soil types. *Journal of Biotechnology* 52:107-120.
Williams PA & Sayers JR (1994) The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. *Biodegradation* 5:195-217.
Willison JC (2004) Isolation and characterization of a novel sphingomonad capable of growth with chrysene as sole carbon and energy source. *FEMS microbiology letters* 241:143-150.
Witzig R Junca H Hecht H & Pieper DH (2006) Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Applied and Environmental Microbiology* 72:3504-3514.
Woese C (1987) Bacterial evolution. *Microbiology and Molecular Biology Reviews* 51:221-271.
Yabuuchi E Kosako Y Fujiwara N Naka T Matsunaga I Ogura H & Kobayashi K (2002) Emendation of the genus *Sphingomonas* Yabuuchi et al. 1990 and junior objective synonymy of the species of three

genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, in conjunction with *Blastomonas ursincola*. *International Journal of Systematic and Evolutionary Microbiology* 52:1485-1496.
Yabuuchi E Yano I Oyaizu H Hashimoto Y Ezaki T & Yamamoto H (1990) Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. *Microbiology and immunology* 34:99-119.
Yano H Garruto CE Sota M Ohtsubo Y Nagata Y Zylstra GJ Williams PA & Tsuda M (2007) Complete sequence determination combined with analysis of transposition/site-specific recombination events to explain genetic organization of IncP-7 TOL plasmid pWW53 and related mobile genetic elements. *Journal of Molecular Biology* 369:11-26.
Yeates C Holmes AJ & Gillings MR (2000) Novel forms of ring-hydroxylating dioxygenases are widespread in pristine and contaminated soils. *Environmental Microbiology* 2:644-653.
Yen KM & Serdar CM (1988) Genetics of naphthalene catabolism in pseudomonads. *Critical reviews in microbiology* 15:247-268.
Yrjälä K Suomalainen S Suhonen EL Kilpi S Paulin L. & Romantschuk M (1998) Characterization and reclassification of an aromatic- and chloroaromatic-degrading *Pseudomonas* sp., strain HV3, as *Sphingomonas* sp. HV3. *International Journal of Systematic Bacteriology* 48:1057-1062.
Yrjälä K Paulin L Kilpi S & Romantschuk M (1994) Cloning of cmpE, a plasmid-borne catechol 2,3-dioxygenase-encoding gene from the aromatic- and chloroaromatic-degrading *Pseudomonas* sp. HV3. *Gene* 138:119-121.
Yrjälä K Paulin L & Romantschuk M (1997) Novel organization of catechol *meta*-pathway genes in Sphingomonas sp. HV3 pSKY4 plasmid. *FEMS microbiology letters* 154:403-408.
Zhang YX Denoya CD Skinner DD Fedechko RW McArthur HA Morgenstern MR Davies RA Lobo S Reynolds KA & Hutchinson CR (1999) Genes encoding acyl-CoA dehydrogenase (AcdH) homologues from *Streptomyces coelicolor* and *Streptomyces avermitilis* provide insights into the metabolism of small branched-chain fatty acids and macrolide antibiotic production. *Microbiology* 145:2323-2334.
Zhou J Bruns MA & Tiedje JM (1996) DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* 62:316-322.
Zhuang Z Gartemann KH Eichenlaub R & Dunaway-Mariano D (2003) Characterization of the 4-hydroxybenzoyl-coenzyme A thioesterase from *Arthrobacter* sp. strain SU. *Applied and Environmental Microbiology* 69:2707-2711.
Zylstra G & Kim E (1997) Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. *Journal of Industrial Microbiology and Biotechnology* 19:408-414.