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ORIGINAL ARTICLE

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

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Genes encoding key enzymes of catabolic pathways can be targeted by DNA fingerprinting to explore genetic degradation potential in pristine and polluted soils. We performed a greenhouse microcosm experiment to elucidate structural and functional bacterial diversity in polyaromatic hydrocarbon (PAH)-polluted soil and to test the suitability of birch (*Betula pendula*) for remediation. Degradation of PAHs was analysed by high-performance liquid chromatography, DNA isolated from soil amplified and fingerprinted by restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP). Bacterial 16S rRNA T-RFLP fingerprinting revealed a high structural bacterial diversity in soil where PAH amendment altered the general community structure as well as the rhizosphere community. Birch augmented extradiol dioxygenase diversity in rhizosphere showing a rhizosphere effect, and further pyrene was more efficiently degraded in planted pots. Degraders of aromatic compounds upon PAH amendment were shown by the changed extradiol ring-cleavage community structure in soil. The RFLP analysis grouped extradiol dioxygenase marker genes into 17 distinct operational taxonomic units displaying novel phylogenetic clusters of ring-cleavage dioxygenases representing putative catabolic pathways, and the peptide sequences contained conserved amino-acid signatures of extradiol dioxygenases. A branch of major environmental TS cluster was identified as being related to *Parvibaculum lavantivorans* ring-cleavage dioxygenase. The described structural and functional diversity demonstrated a complex interplay of bacteria in PAH pollution. The findings improve our understanding of rhizoremediation and unveil the extent of uncharacterized enzymes and may benefit bioremediation research by facilitating the development of molecular tools to detect and monitor populations involved in degradative processes.
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Introduction

The relatively recent introduction of toxic chemicals to soils, sediments and aquifers has disturbed natural catabolic processes, and the spread of pollutants constitutes a threat to ecosystem functioning and human health (Susarla *et al.*, 2002). Polyaromatic hydrocarbons (PAHs), constituents of petroleum hydrocarbons and wood preservatives like creosote, are toxic to organisms in the ecosystems because of their lipophilic character and harmful due to their acute toxicity, mutagenicity or carcinogenicity (Fewson, 1988; Collins *et al.*, 1998). Organic pollutants are subjected to microbial biodegradation exemplified by monitored natural attenuation (Dojka *et al.*, 1998; Stapleton *et al.*, 1998; Gieg *et al.*, 1999), and corresponding pathways have been depicted from bacterial strains (Cerniglia, 1992; Habe and Omori, 2003). Present knowledge about catabolic pathways is restricted to culturable

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strains constituting a great minority of the bacteria inhabiting soils and sediments. Uncharacterized degradation pathways most likely exist and in the study of little explored pathways in nature, incisive experimental strategies are required for finding novel reactions (Galvao *et al.*, 2005; Suenaga *et al.*, 2007).

The structural diversity of bacteria in soil (Torsvik and Oevreas, 2002) has been assessed by 16S rRNA marker gene analysis, most often not connected to a specific function in the ecosystem. Within functional marker gene studies, an activity like aromatic ring hydroxylation or ring cleavage in biodegradation is, however, connected to the retrieved community. Knowledge of this catabolic potential for example, biodegradation genes in soils, is valuable in the development of efficient bioremediation strategies (Whyte *et al.*, 1999; Baldwin *et al.*, 2003; Dionisi *et al.*, 2004). Degradation genes are potential biomarkers for the detection of toxic hydrocarbons and for the evaluation of toxicological risks intrinsic to the presence of these pollutants (Power *et al.*, 1998).

Extradiol dioxygenase genes encoding aromatic ring-cleaving dioxygenases have been used as functional markers for catabolic bacterial communities (Erb and Wagner-Doebler, 1993; Daly *et al.*, 1997; Sei *et al.*, 1999; Mesarch *et al.*, 2000; Junca and Pieper, 2004). Functional primers used in these studies suffer, however, from relatively narrow specificity. Upper and lower extradiol dioxygenases group into separate phylogenetic clusters (Eltis and Bolin, 1996). The lower-pathway catechol dioxygenases form subfamilies I.2.A, I.2.B, I.2.C, I.2.D and I.2.E. The upper-pathway families I.1 and I.3–I.5 are not closely related to the lower-pathway enzymes. Subfamilies I.3.A, I.3.B, I.3.C, I.3.D and I.3.E are diverse displaying little similarity. We developed an extradiol dioxygenase marker gene assay (group I.3.E) to target bacteria with capacity to cleave the persistent aromatic ring that frequently is part of environmental pollutants like PAHs and petroleum hydrocarbons (Sipilä *et al.*, 2006). The target subfamily I.3.E encloses enzymes for ring cleavage of biphenyl, naphthalene and evidently also for polyaromatic compounds such as dibenzothiophene and phenanthrene, containing three aromatic rings (Denome *et al.*, 1993; Pinyakong *et al.*, 2003). These enzymes have been isolated and described from the proteobacterial genera of *Sphingomonas*, *Pseudomonas*, *Ralstonia* and *Burkholderia*, and from high GC genus of *Rhodococcus*.

In bioremediation of soil, biodegradation can be enhanced by making conditions favourable for the catabolic process. Plants in phytoremediation of hydrocarbon contaminants have been proposed to improve degradation conditions in soil (Kuiper *et al.*, 2004), and some improvement in biodegradation has been observed in plant microcosms/microecosystems (Miya and Firestone, 2000). Plant-associated microbes, especially in the root zone, potentially play a central role in rhizoremediation (Parrish *et al.*, 2004; White *et al.*, 2006) because plants mediate a rhizosphere effect illustrated by plant-specific microbial communities (Smalla *et al.*, 2001; Costa *et al.*, 2006). Generally, the rhizosphere of plants harbours a higher diversity of bacteria than the surrounding bulk soil due to root exudates and oxygen that stimulate bacteria (Briones *et al.*, 2003). Most interest in phytoremediation with woody plants has been devoted to *Populus* (Spriggs *et al.*, 2005; Widdowson *et al.*, 2005; Zalesny *et al.*, 2005) and *Salix* (Vervaeke *et al.*, 2003) with the aim to combat heavy metal pollution. Not much attention has been devoted to elucidate applicability of woody plants for remediation of organic pollution (Tesar *et al.*, 2002; Mueller and Shann, 2006; Palmroth *et al.*, 2006). One requisite for successful rhizoremediation of PAH is the presence and diversity of PAH biodegradation genes in the rhizosphere, which has not previously been assessed using cultivation-independent methods.

We hypothesized that birch amendment will diversify aerobic aromatic ring-cleavage bacterial populations in rhizosphere-associated soil. Newly designed broad-specificity primers targeting I.3.E group extradiol dioxygenases and the 16S rRNA marker gene were, in our study, used to elucidate functional and structural diversity, respectively, in rhizoremediation of PAHs to demonstrate how genes encoding biodegradation enzymes can be used as marker genes in soil with high bacterial diversity to show pertinent communities in the biodegradation process.

Materials and methods

Greenhouse experiment and sampling

A pot microcosm experiment was set up to study biodegradation of PAHs in soil where heavy metal-tolerant birch (*Betula pendula*) clone, Wales W008, was tested for suitability in rhizoremediation. Birch seedlings (4–6 cm) were planted into individual pots containing 500 g of soil after manually removing nursery soil from the plant roots. Microcosms without plants, both PAH-polluted and -unpolluted, were included. All treatments were done in triplicate.

The soil was a mixture of 50% sand (Optiroc, granulometric distribution 0.5–1.2 mm) and 50% of untreated peat (Kekkilä Oyj, Tuusula, Finland). Soil was contaminated with a PAH mixture of anthracene, phenanthrene, fluoranthene and pyrene in acetone. Microcosms were treated with two concentrations, 50 or 300 mg kg^{-1}, of each polyaromatic compound making a final concentration of 200 and 1200 mg kg^{-1}, respectively, of PAHs. The acetone was evaporated from the soil in a fume chamber for 72 h. Microcosms were illuminated 16 h per day imitating Nordic summer day light with light of colour 77 and 965 (Osram Fluora and Biolux), and

the incubation temperature in greenhouse was kept at 18 °C.

Two replicate microcosm from treatments pristine nursery soil (Bulk-0), PAH-polluted soil 1200 mg kg^{-1} (Bulk-1200), pristine rhizosphere (Rhiz-0) and polluted rhizosphere 1200 mg kg^{-1} (Rhiz-1200) were subjected to bacterial community analysis. Composite soil samples (six subsamples), 20 g, were taken from bulk soil microcosms at the end of the 3-month incubation. From rhizosphere-associated soil, composite soil samples were taken as follows: birch seedlings were lifted up from the pot leaving soil stuck on the roots; subsequently, they were physically shaken by hand, and soil falling off from the roots was sampled; six subsamples were taken and pooled together to a composite Rhiz sample for community DNA analysis.

For chemical analysis, the whole batch of extant soil (480 g) from the pots, after removal of plant, was analysed for PAH compounds by HPLC (high-performance liquid chromatography) (planted-soil sample). The 'bulk soil' sample was all the soil from a pot without a plant. The soil was extracted with 1500 ml of 50% acetonitrile–50% water solution in a shaker for 120 min, and 10 ml of the upper phase was filtrated with Whatman PVDF micro filter (0.45 μm) for HPLC analysis.

DNA isolation and PCR amplification

Total DNA for analysis of extradiol dioxygenase gene was extracted from 0.25 g soil from greenhouse microcosm experiment with the PowerSoil DNA Isolation Kit (Mo Bio laboratories Inc., Carlsbad, CA, USA). The bead-beating step was performed using Cell Homogenizer MSK (B Braun Biotech International GmbH, Melsungen, Germany), by 3 × 40 s beating and 20 s pause on ice between the bead beatings. No further purification of the DNA was needed. The quality of the total community DNA was assessed using agarose gel electrophoresis.

Recently published primers targeted to the I.3.E subfamily of extradiol ring-cleavage dioxygenases were selected for analysis of aromatic ring-cleavage diversity in microcosms (Sipilä *et al*., 2006). The deduced amino-acid fragment peptide (154 amino acids) corresponds to the almost complete N-terminal part of the intact enzyme, and included the His145, crucial for iron binding in the catalytic centre (Sato *et al*., 2002). The BP-F sequence is 5′-TCTAYCTVCGNATGGAYHDBTGGCA-3′ and BP-R 5′-TGVTSNCGNBCRTTGCARTGCATGAA-3′.

Extradiol dioxygenase genes were amplified from two replicate microcosm experiments using PCR protocol as described (Sipilä *et al*., 2006). The general 16S rRNA bacterial marker gene was amplified from two replicate microcosms using the primer pair 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (ACGGCTACCTTGTTACGACTT) (Weisburg *et al*., 1991). The 27F primer was labelled with 5́-carboxyfluorescein. The PCRs contained 40 pmol of primers, 200 μM dNTP, 2 U of DNA polymerase (Biotools; B&M Labs, Madrid, Spain), 1 × reaction buffer containing 200 μM MgCl2 and 0.03 mg bovine serum albumin. The PCR protocol was 60 °C 5 min followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 90 s, followed by final elongation at 72 °C for 5 min.

T-RFLP analysis of bacterial communities

Amplified 16S rRNA genes were fingerprinted by terminal restriction fragment length polymorphism (T-RFLP). T-RFLP analysis is a highly reproducible and robust technique that yields high-quality fingerprints consisting fragments of precise sizes (Osborn *et al*., 2000). Several four-cutter restriction enzymes were tested and *Hae*III was selected giving best size distribution of terminal restriction fragments (T-RFs). PCR products were diluted 1/50, and 2 μl of diluted PCR product was digested using 3 U of *Hae*III restriction enzyme (Promega Corporation, Madison, WI, USA) using restriction buffer C as recommended by the manufacturer. Digestion was performed overnight at 37 °C to ensure complete restriction in 20 μl volume. The *Hae*III restriction enzyme produces blunt restriction sites minimizing the potential effect of polymerase-end filling on the terminal fragment diversity (Hartmann *et al*., 2007). Digested fragments were ethanol-precipitated and solubilized in formamide (15 μl). Gene Scan 500 standard (Applied Biosystem, Foster City, CA, USA) was used in capillary electrophoresis (ABI 310 DNA sequencer; Applied Biosystem).

The fragment size, peak area and height were measured in fluorescence units (Gene Scan software; Applied Biosystem). Fragments 50–500 bp were analysed. Three T-RFLP electropherograms from same community DNA were compared to identify true peaks from artefacts. The proportional percentage of each peak area was calculated from the total amount of fluorescence. Peak shoulders were manually checked and the intensities were summed up when appropriate. Peaks with intensity higher than 1% of total fluorescence were accepted for analysis. Accepted peaks of samples were aligned allowing ± 1 bp difference in size. 16S rRNA genes from PAH-polluted and pristine bulk soil were cloned to produce environmental libraries for identification of T-RFLP peaks. The 16S rRNA of clones was subjected to T-RFLP analysis along with community PCR products. After identification of T-RF sizes, selected clones were submitted to sequence analysis. The selected clones were amplified with PCR using vector-specific RP (5′-TTTCA CACAGGAAACAGCTATGAC-3′) and UP (5′-CGA CGTTGTAAAACGACGGCCAGT-3′) primers. PCR products were sequenced using 27F primer with an ABI 3130 genetic analyser with Big Dye v.3.1 chemistry (Applied Biosystem). The 16S rRNA gene

sequences were submitted to GeneBank with accession numbers AM981263–AM981270.

Cloning and RFLP
PCR-amplified aromatic ring-cleavage gene fragments from four different treatments from two replicate microcosms were cloned to produce eight environmental DNA libraries. Cloning and subsequent restriction fragment length polymorphism (RFLP) analysis were performed according to Sipilä *et al.* (2006). Gel-purified PCR products (Wizard SV gel and PCR Clean-Up System; Promega) were ligated to pGEMT vector (pGEMT vector system; Promega) and transformed to competent DH5α cells prepared by the rubidium chloride method (Hanahan, 1983). On average, 51 clones were picked for RFLP analysis from each library. Clones were divided into operational taxonomic units (OTUs) on the basis of DNA-banding pattern of individual clones. Each banding pattern found on agarose gel constitutes one distinct OTU according to how the restriction enzyme cuts the PCR product. The OTUs were later confirmed by sequence analysis.

Statistical analysis
Statistical analysis was done using the Past v. 1.73c program (Hammer *et al.*, 2001). Shannon diversity indices $H' = -\sum_{i=1}^{S}(n_i/N)\ln(n_i/N)$ (Shannon and Weaver, 1963) and Simpson diversity indices $(1-D) = -\sum_{i=1}^{S}(n_i/N)^2$ (Simpson, 1949) take into consideration both species richness and species dominance. In both equations, *ni* is the number of clones assigned to OTU *i* in a library, *N* is the total number of clones analysed from the library and *S* is the total number of OTUs. Shannon (H') and Simpson (1–D) diversity indices increase with OTU richness and evenness but the highest possible value for Simpson diversity index is 1.

Nonparametric analysis of similarity (ANOSIM) (Clarke and Warwick, 1994) was used to test the significance of the differences between communities as suggested (Rees *et al.*, 2005). The ANOSIM tests the hypothesis that the average rank similarity between the objects within a group is the same as the rank similarity between objects between groups. ANOSIM produces *R* statistics that can range from −1 to 1. Objects that are more dissimilar between groups than within groups will be indicated by *R* statistic greater than 0. An *R*-value 0 indicates that the null hypothesis is true. Exploratory tool similarity percentage analysis (SIMPER) was used to study the similarity between complex T-RFLP profiles.

The 16S rRNA T-RFLP profiles and RFLP extradiol dioxygenase libraries from different treatments were analysed by principal component analysis (PCA) to find important components correlated with the treatments. The variance–covariance matrix was used to find the eigen values and eigen vectors. Shapiro–Wilk test of normality was used to assess the normal distribution in PAH degradation data. Student's test (*t*-test) was used to compare degradation of PAHs in planted and bulk soil pots.

Phylogenetic analysis of extradiol dioxygenase peptides
Clones representing each OTU were chosen for sequence analysis. Several clones were amplified from dominant OTUs to assess the sequence similarity within the OTU. The selected clones were amplified with routine PCR using vector-specific RP (5′-TTTCACACAGGAAACAGCTATGAC-3′) and UP (5′-CGACGTTGTAAAACGACGGCCAGT-3′) primers. PCR products were sequenced using T7 primer with an ABI 3130 genetic analyser with Big Dye v.3.1 chemistry (Applied Biosystem). Sequences were compared with those in the databases using BLAST (NBCI, URL http://www.ncbi.nlm.nih.gov/BLAST/). The amino-acid sequences were aligned using ClustalW (Li, 2003) and inspected manually with the Genedoc program v. 2.6 (Nicholas *et al.*, 1997). The phylogenetic tree was constructed with the Treecon program package (Van de Peer and De Wachter, 1994) using evolutionary distances (Tajima and Nei, 1984) and the neighbour-joining method (Saitou and Nei, 1987).

To identify and compare signature amino-acid sequences of amplified extradiol dioxygenases, a peptide sequence alignment was compared to the three-dimensional structure of BphC enzyme from *Pseudomonas* KKS102 (Sato *et al.*, 2002) belonging to the group I.3.A (Eltis and Bolin, 1996). A simultaneous comparison of the alignment with the three-dimensional structure was made using the Cn3D v. 4.1 program (http://130.14.29.110/Structure/CN3D/cn3d.shtml). Extradiol dioxygenase gene sequences were deposited in the EMBL database with the accession numbers AM418471–AM418514.

HPLC apparatus and chromatographic conditions
Samples were analysed according to the modified EPA 500/500.1 method, using Hewlett-Packard HPLC (Waldbronn Analytical Division) with 1100 quaternary pumps, an auto sampler and a UV detector, linked to HP ChemStation data-handling system. The reversed-phase separation was performed in a C_{18}–RP column (Bondapack C_{18}–RP, 250×4.6 mm, 5 μm particles). The PAH compounds were separated with gradient elution using water (A) and acetonitrile (B). The elution system was as follows: 0–2.5 min, 40–60% of A in B; 2.5–12 min, 60–90% of A in B; and 12–20 min, 100% of B. Injection volume was 10 μl and the flow rate 1.5 ml min^{-1}. The PAH compounds were detected at 270 nm and identified according to their retention times and UV spectra.

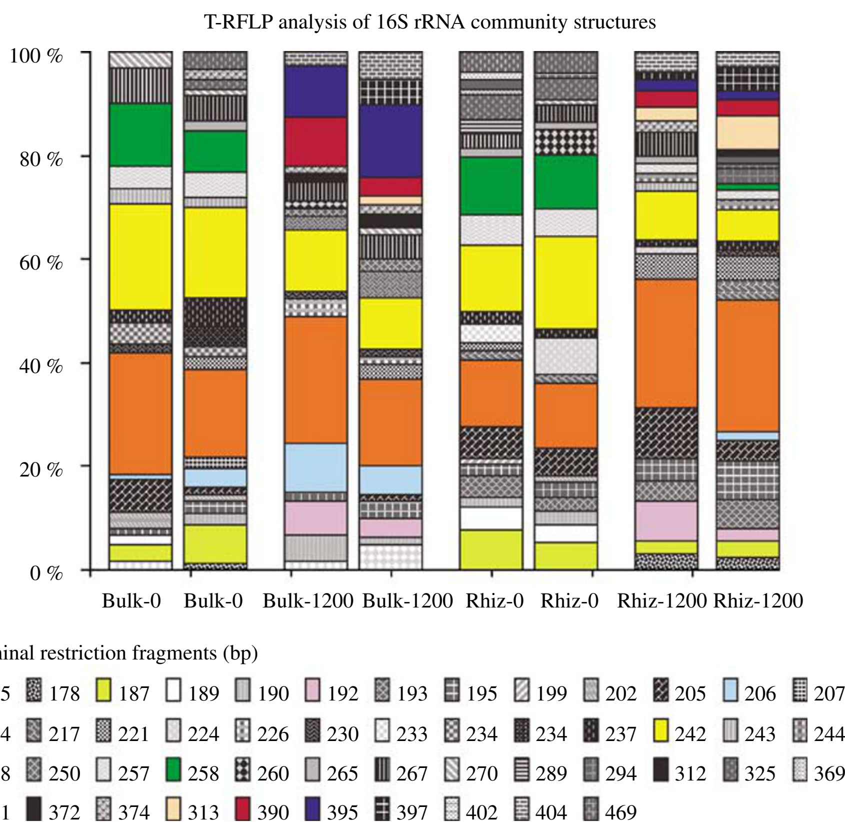

Figure 1 Relative abundances of 16S rRNA T-RFs from microcosms by T-RFLP analysis after 3-month experiment. T-RF size is given in base pairs, and the relative abundance of T-RFs is given as percentage of total peak area. The T-RFs contributing most to the treatment-specific differences (SIMPER) are marked with colours. 'Rhiz-0' is rhizosphere-associated soil without PAH amendment. 'Bulk-0' is bulk soil without PAH amendment. '1200' denotes addition of 1200 mg kg^{-1} PAH. PAH, polyaromatic hydrocarbon; T-RF, terminal restriction fragment.

High-performance liquid chromatography analysis of each sample was repeated four times with same PAH extract, and the mean value constituted the result from one separate pot. Recoveries for the four studied PAH compounds were measured by contaminating soil samples with pure standard solutions. Standard concentration levels of 200, 800 and 1200 mg l^{-1} were spiked into the soil, mixed for 30 min in a roller, and stored for 14 days before extraction and HPLC analysis. Each concentration level was studied in five parallel tests. The average recovery percentages for anthracene, phenanthrene, fluoranthene and pyrene from the soil matrix were 83.9%, 70.7%, 80.6% and 73.8% respectively (with corresponding standard deviations of the recoveries 10.6%, 1.6%, 1.6% and 0.9% respectively). This part of the PAHs is considered as the available fraction used in calculations of biodegradation percentage.

Results

Soil microcosms were designed to study the effect of PAHs and birch (*Betula pendula*) on structural and functional diversity in rhizoremediation. The microcosm setup (see Materials and methods) entailed treatments analysed by DNA fingerprinting, which are as follows: pristine nursery soil (Bulk-0), PAH-polluted soil 1200 mg kg^{-1} (Bulk-1200), pristine rhizosphere (Rhiz-0) and polluted rhizosphere 1200 mg kg^{-1} (Rhiz-1200). The birch grew substantially in height during the 3-month incubation. In microcosms with high (1200 mg kg^{-1}) PAH concentration, phytotoxicity symptoms were shown using Hsp70 immunoassay, proteomic profiling of birch roots and growth measurements (data not shown).

T-RFLP analysis of 16S rRNA communities

The 16S rRNA marker gene was fingerprinted with T-RFLP to analyse treatment-specific structural changes in bacterial communities in soil. Totally, 49 individual T-RFs were accepted for analysis. After the 3-month experiment, all microcosm treatments showed different bacterial communities (Figure 1), and the replicates were more similar to each other than to those in any other treatments (ANOSIM, $R = 0.95$). 16S rRNA communities in Bulk-0 and Bulk-1200 soil were disparate (overall dissimilarity, 57.4 SIMPER), and most contributing T-RFs were 395, 258, 242, 390, 206 and 187 bp. Pollution affected the rhizosphere-associated soils as well (overall dissimilarity, 56.4), and the

Table 1 Identification of terminal restriction fragments (T-RFs) of 16S rRNA community profiles. The occurrence of T-RFs in different treatments (Bulk-0, Bulk-1200, Rhiz-0 and Rhiz-1200) is shown as the relative means of peak areas in T-RFLP profiles. Identification of T-RFs was based on T-RFLP analysis of 16S rRNA gene from clones and subsequent sequencing of corresponding partial 16S rRNA gene

T-RF length (bp)[a]	*Clone id*	*Relative mean of T-RF area in community samples*				*Identification of the sequence*
		Bulk-0	*Bulk-1200*	*Rhiz-0*	*Rhiz-1200*	
165	09-654B03	0.8	3.0	0.0	0.0	Acetobacteraceae
189	39-654C11	1.0	0.0	3.6	0.0	Rhizobiales
195	26-654E08	1.6	2.3	2.3	5.2	Alphaproteobacteria
214	45-654A12	16.8	30.2	12.3	22.8	Burkholderia
226	01-654E01	0.0	2.2	0.0	0.0	Acetobacteraceae
233	31-654C09	0.0	0.0	1.7	0.0	Acetobacteraceae
242	73-656C05	19.2	10.5	10.7	6.7	Genus Gp1 (Acidobacteriaceae)
270	60-656A03	2.0	0.6	0.5	0.0	Genus Gp1 (Acidobacteriaceae)

Abbreviation: T-RFLP, terminal restriction fragment length polymorphism.
[a]Size estimation by Gene Scan software.

contributing T-RFs were 214, 258, 242, 192, 313 and 187. Interestingly, part of the contributing T-RFs (214 and 313) were not the same as detected in Bulk-0 and Bulk-1200 microcosm, suggesting incongruent development of bacterial community in the rhizosphere. Not surprisingly, bacterial communities of Bulk-1200 and Rhiz-0 were highly contrasting (overall dissimilarity, 66.8) with contributing T-RFs, 395, 258, 214, 206, 187, 390 and 257 bp (Figure 1). Bulk-0 and Rhiz-0 soil showed lower, 36.2 dissimilarity, and an emergent rhizosphere effect was detected in a comparison of the polluted samples (Rhiz-1200 and Bulk-1200) (dissimilarity, 48.5).

The dominant T-RFs, 214 and 242, were identified to contain 16S rRNA gene sequences similar to *Burkholderia* genus and Gp1 (Acidobacteriaceae), respectively (Table 1). The partial 16S rRNA sequence (T-RFs, 214) was 99% similar to *Burkholderia glathei* isolate Hg 11, known to degrade naphthalene (Wilson *et al*., 2003). The relative peak area of T-RF 214 increased upon PAH addition in birch rhizosphere-associated soil. The relative abundance of Acidobacteria (T-RFs, 242) seemed to be lower in polluted soil.

Treatment-specific changes in community structure were revealed by PCA. Component 1 explains the PAH-pollution effect (49.5% of the variance) and component 2, evidently, the rhizosphere effect (19.1% of the variance) best detected in PAH-amended soils. The unpolluted rhizosphere and bulk soil were quite much a like (Figure 2a).

Extradiol dioxygenase community structures

Restriction groups of functional genes estimated by PCR-RFLP were explicitly defined as OTUs (Figure 3). The fingerprints detected in agarose gel were in good agreement with *in silico* digestion although the resolution of agarose gel is low for small fragments <50 bp. In total, 407 clones from eight environmental DNA libraries were fingerprinted, on average 51 clones per library (Table 2). The amplified extradiol dioxygenase marker genes grouped in 17 OTUs (Figure 4) from two replicate microcosms of four different treatments and richness curves showed representative sampling of each clone library (Figure 5). Marker gene community structures displayed in Figure 4 were subjected to PCA. Replicate microcosms placed together indicating good reliability of analysis. Treatment-specific differences were demonstrated by PCA (Figure 2b) and ANOSIM analysis ($R=0.91$, $P<0.05$). Component 1 separated Bulk-1200 soil from all other treatments. Component 2 separated the amended soils, Bulk-1200, Rhiz-0 and Rhiz-1200 from Bulk-0.

Bulk-0 samples grouped together and PAH addition changed their ring-cleavage community structure. In Bulk-0 soil, eight OTUs were detected with unique OTU 69 as the dominating one (Figure 4). Addition of PAH, 1200 mg kg^{-1}, resulted in a population with seven OTUs, where OTU 72 was clearly the most dominating one and seems to be important in PAH degradation. The effect of PAH was also reflected in Shannon diversity index (H') decreasing from 1.3 to 0.93 and Simpson index ($1-D$) as well from 0.65 to 0.45 (Table 2). Rhiz-0 and Rhiz-1200 soils displayed a more diverse ring-cleavage community than in the bulk soil microcosms (Table 2, Figure 4) with 13 OTUs, almost twice as many found in bulk soil. OTU 48 was the most characteristic one, and OTUs 40, 49, 52, 60 and 70 were specific for Rhiz soil. After 3-month incubation, Rhiz-0 and Rhiz-1200 soils displayed only minor differences in aromatic ring-cleavage communities (Figure 4).

Phylogenetic analysis of dioxygenases

The extradiol dioxygenase peptide sequences retrieved from this study grouped into seven distinct clusters (Figure 6). These clusters represent putative independent genetic pathways describing catabolic

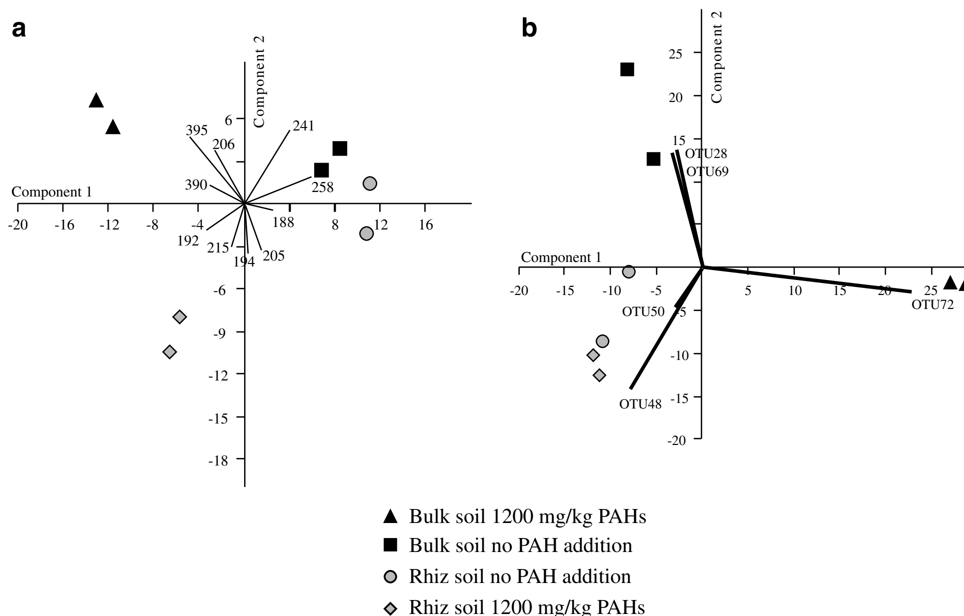

Figure 2 (**a**) Principal component analysis of 16S rRNA gene communities derived from T-RFLP fingerprinting (selected T-RF are shown). Component 1 represents 49.5% and component 2, 19.1% of the variance. (**b**) Biplot presentation of principal component analysis of extradiol dioxygenase gene libraries (selected OTU are shown) from bulk soil, bulk soil amended with 1200 mg kg^{-1} PAH (Bulk-1200), rhizosphere-associated soil and rhizosphere-associated soil amended with 1200 mg kg^{-1} PAH (Rhiz-1200). Component 1 represents 58.1% and component 2, 29.0% of the variance. OTUs, operational taxonomic units; PAH, polyaromatic hydrocarbon; T-RF, terminal restriction fragment.

*Hha*I RFLP	Clone	OTU	*In silico Hha*I fragments	Cluster in phylogenetic tree (Figure 6)
	163_A02	28	166,163,115,16	WZ
	163_B02	28	166,163,115,16	WZ
	168_F09	40	280,187,8	CE
	168_G08	40	280,187,8	CE
	167_B05	42	177,162,79,52	KY
	167_B09	42	177,162,79,52	KY
	408_ B04	48	229,160,79	KY/HR
	409_C02	48	229,160,79	KY/HR
	168_D09	49	270,111,84,10	TS
	409_B02	49	270,111,84,10	TS
	408_G02	50	108, 104, 95, 75,54,30,6,2	TS
	409_G01	50	108, 104, 95, 75,54,30,6,2	TS
	409_C01	52	387, 82	
	167_D04	54	197,102,101,57	TS
	167_G04	56	213,176,84	TS
	167_G08	56	213,176,84	TS
	164_B06	57	159,81,79,51,47,41,6,2	BPHC
	164_G06	57	159,81,79,51,47,41,6,2	BPHC
	167_D06	59	213,121,84,57	TS
	168_F04	60	270,111,92,10	TS
	168_G04	62		
	168_E09	63	no sites	KY
	408_B03	63	no sites	KY
	163_A01	69	115,110,79,75,41,36,9	CE
	163_E02	69	115,110,79,75,41,36,9	CE
	408_D04	70	159,111,111,84,10	TS
	409_D02	70	159,111,111,84,10	TS
	164_C06	72	335,134	THNC
	164_H06	72	335,134	THNC

Figure 3 *Hha*I RFLP fingerprints from microcosms representing OTUs (operational taxonomic units) and their phylogenetic affiliation. The name of OTUs, the sizes of *Hha*I fragments and name of phylogenetic cluster are shown. Fingerprints from gel electrophoresis were normalized using Gelcompar II version 4.6 (Applied Mach). OTUs, operational taxonomic units.

Table 2 Description of eight extradiol dioxygenase libraries from four treatments. 'Bulk-0' represents composite soil samples from microcosms without birch and PAH amendment; 'Rhiz-0' represents rhizosphere-associated soil without PAH amendment; '1200' denotes addition of 1200 mg kg^{-1} PAH

Sample	*Treatment*	*Clones*	*OTUs*	*Shannon index* (H′)	*Simpson index* (1−D)
Bulk-0	—	46	4	1.1	0.6
Bulk-0	—	42	7	1.5	0.7
Bulk-1200	1200 mg kg^{-1} PAH mixture[a]	57	6	1.2	0.6
Bulk-1200	1200 mg kg^{-1} PAH mixture[a]	43	4	0.6	0.3
Rhiz-0	W008 birch clone	54	12	2.3	0.9
Rhiz-0	W008 birch clone	53	9	1.7	0.8
Rhiz-1200	W008 birch clone and 1200 mg kg^{-1} PAH mixture[a]	59	10	1.9	0.8
Rhiz-1200	W008 birch clone and 1200 mg kg^{-1} PAH mixture[a]	53	10	1.6	0.7

Abbreviations: OTUs, operational taxonomic units; PAH, polyaromatic hydrocarbon.
[a]PAH mixture; anthracene, phenanthrene, fluoranthene and pyrene, 300 mg kg^{-1} of each.

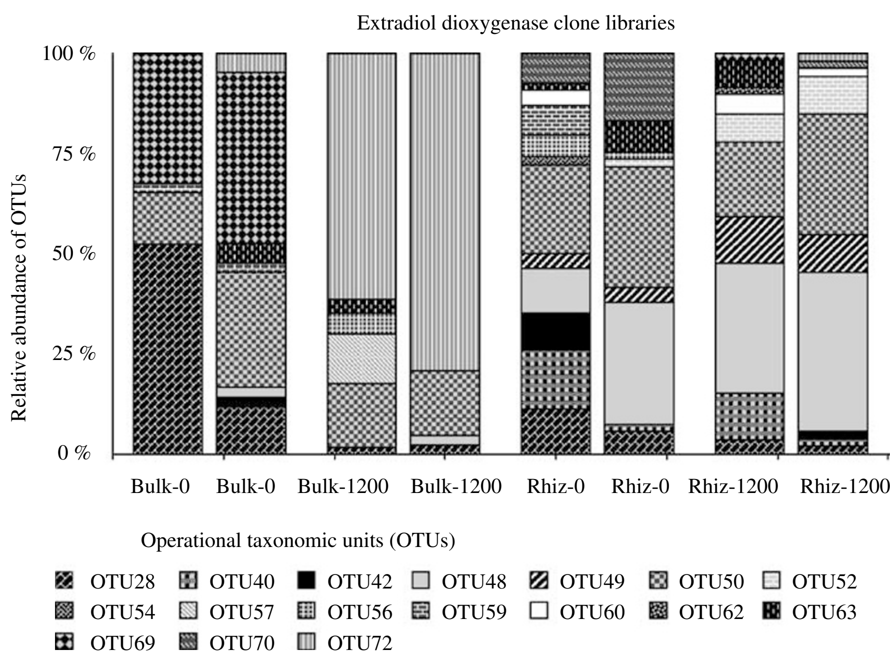

Figure 4 Relative abundance of operational taxonomic units (OTUs) of extradiol dioxygenase gene libraries deduced by RFLP analysis. The *Hha*I RFLP patterns reflect changes in extradiol dioxygenase community structure in biodegradation. 'Rhiz-0' is rhizosphere-associated soil without PAH amendment and 'Bulk-0' is soil without birch and PAH amendment. '1200' denotes addition of 1200 mg kg^{-1} PAH. Two replicates from different pots were included in the analysis (for details, see Materials and methods). PAH, polyaromatic hydrocarbon.

diversity, pathways that may have similar biochemical function, but different gene organization shaped by evolution. Phylogenetic analysis of deduced dioxygenase peptides of OTU sequences showed that the great majority of them were of novel kind. The most abundant TS cluster contained seven OTUs counting for 34% of all clones analysed and representing an ecologically important cluster of extradiol dioxygenases. The large amount of different peptide sequences within the TS cluster reflects either functional redundancy or functional diversification of dioxygenases or both. TS cluster dioxygenases were dominant in polluted sites with long history of petroleum hydrocarbon contamination from southern Finland (Sipilä *et al.*, 2006). A branch of this cluster was closely related to extradiol dioxygenase from recently sequenced genome of *Parvibaculum lavantivorans* DS-1. The strain was isolated from activated sludge as an alkylbenzensulphonate surfactant degrader (Schleheck *et al.*, 2004), but its ring-cleavage dioxygenase has not been characterized. The TS cluster affiliated to α-proteobacteria (Rhizobiales) is an example of an uncharacterized group of putative environmentally important aromatic degraders.

The OTU 57 was closely related to the sequence of *Sphingomonas aromaticivorans* BphC enzyme. The *bphC* gene was found in pNL1 plasmid with

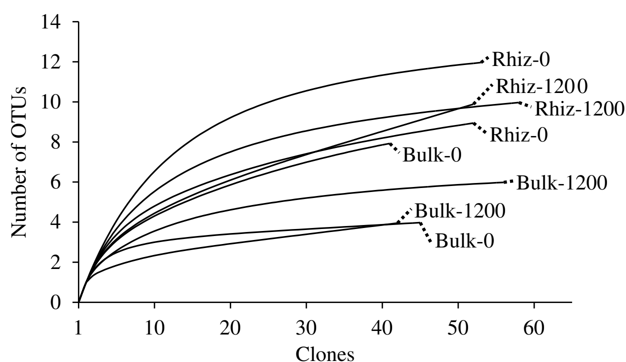

Figure 5 OTU richness curves of extradiol dioxygenase gene libraries. The curves were obtained by rarefaction calculations of OTU abundance data and they represent libraries from the different soil microcosms. 'Rhiz-0' is rhizosphere-associated soil without PAH amendment. 'Bulk-0' is bulk soil without PAH amendment. '1200' denotes addition of 1200 mg kg^{-1} PAH. Two replicates were included in the analysis. OTUs, operational taxonomic units; PAH, polyaromatic hydrocarbon.

complex aromatic degradation pathway for naphthalene, phenanthrene, biphenyl, xylene derivates, toluene and cresol (Romine *et al.*, 1999). The dominant OTU 72 in Bulk-1200 soil (69 clones in both replicates) was placed in the ThnC cluster named according to the *thnC* gene from *Sphingopyxis macrogoltabida* TFA strain. The ThnC enzyme catalyses the 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).

Four clusters of extradiol dioxygenase peptides (XY, KY, HR and CE) found in our study were only distantly related to the extradiol dioxygenases from known pathways (Figure 6), and bacterial hosts are evidently not known.

The identity of novel ring-cleavage peptides was confirmed by alignment construction with known upper-pathway dioxygenases to detect conserved regions and amino-acid signatures. The peptide alignment (78 environmental sequences) was mapped to the three-dimensional structure of the BphC enzyme from *Pseudomonas* sp. KKS102 (Sato *et al.*, 2002) to detect where the conserved amino acids are situated in the structure (data not shown). In the alignment of peptides, we identified two conserved regions, one β-sheet structure located between two domains that can be structurally important or involved in interactions between the domains and another region that is a loop on the surface of the enzyme connecting the N- and C-terminal domain. Nineteen conserved amino-acid residues were found in the alignment of deduced amino-acid sequences of the novel environmental peptides. They contained the His145 signature amino acid that is crucial for the binding of iron in the catalytic centre of the enzyme. The position 147 in the alignment, which is close to the oxygen-binding cavity, contained hydrophobic amino acids like Val, Ala, Met and Ile.

PAH degradation in soil microcosms

Overall biodegradation was measured from whole batch of extant soil in microcosms. Almost all extractable (estimated from average PAH recoveries from soil matrix) anthracene and fluoranthene was dissipated from microcosms, but residuals of these PAHs were detected in microcosms amended with 1200 mg kg^{-1} PAH (90–98%) (Figure 7a). Anthracene degradation was enhanced by the planting of birch, but the fluoranthene degradation was more complete in the bulk soil pots. Pyrene degradation was greater in pots with plant (52%) than in bulk soil pots (27%), revealing plant effect in the biodegradation of PAHs ($P<0.01$). The degradation of phenanthrene in planted pots was 29% and in bulk soil without birch, 34%. In pots with low, 200 mg kg^{-1} PAH concentration, only partial degradation of phenanthrene and pyrene was detected. In the bulk soil microcosms, both phenanthrene and pyrene were 95% degraded but in the planted pots, lower degradation rates were detected (58% and 61% respectively) (Figure 7b).

Discussion

Treatment-specific changes of bacterial communities both at functional (extradiol dioxygenases) and at structural (16S rRNA) level revealed the complex interplay of bacteria in rhizoremediation, which has not explicitly been shown before. Birch amendment diversified aerobic aromatic ring-cleavage dioxygenases in rhizosphere-associated soil according to our hypothesis. In rhizosphere, the detected diversity of novel ring-cleavage dioxygenases highlighted a broad-spectrum degradation capacity of environmental contaminants and natural aromatics. Enhanced degradation of pyrene was detected in planted microcosms, revealing a positive rhizoremediation effect.

There is an increasing interest in how pollutants affect the overall bacterial communities, both on functional and on structural levels (Ni Chadhain *et al.*, 2006; Muckian *et al.*, 2007). Fingerprinting of functional genes for key enzymes in catabolic pathways offers a powerful tool for assessing biodegradation potential in the environment. Previous studies on assessing the genetic potential for PAH degradation have been hampered by the relatively narrow scope of the PCR primers that are mainly targeted to the lower-pathway 1.2.A group extradiol dioxygenases, which catalyses ring cleavage of the catechols (Meyer *et al.*, 1999; Widada *et al.*, 2002; Junca and Pieper, 2004), a common intermediate of aromatic degradation. The targeted upper-pathway I.E.3 dioxygenases in our study are functionally different because the substrate is a double-hydroxylated aromatic compound including polyaromatic, substituted polyaromatic and biphenyl structures, which demand different substrate specificities for assorted structures (Eltis and Bolin, 1996;

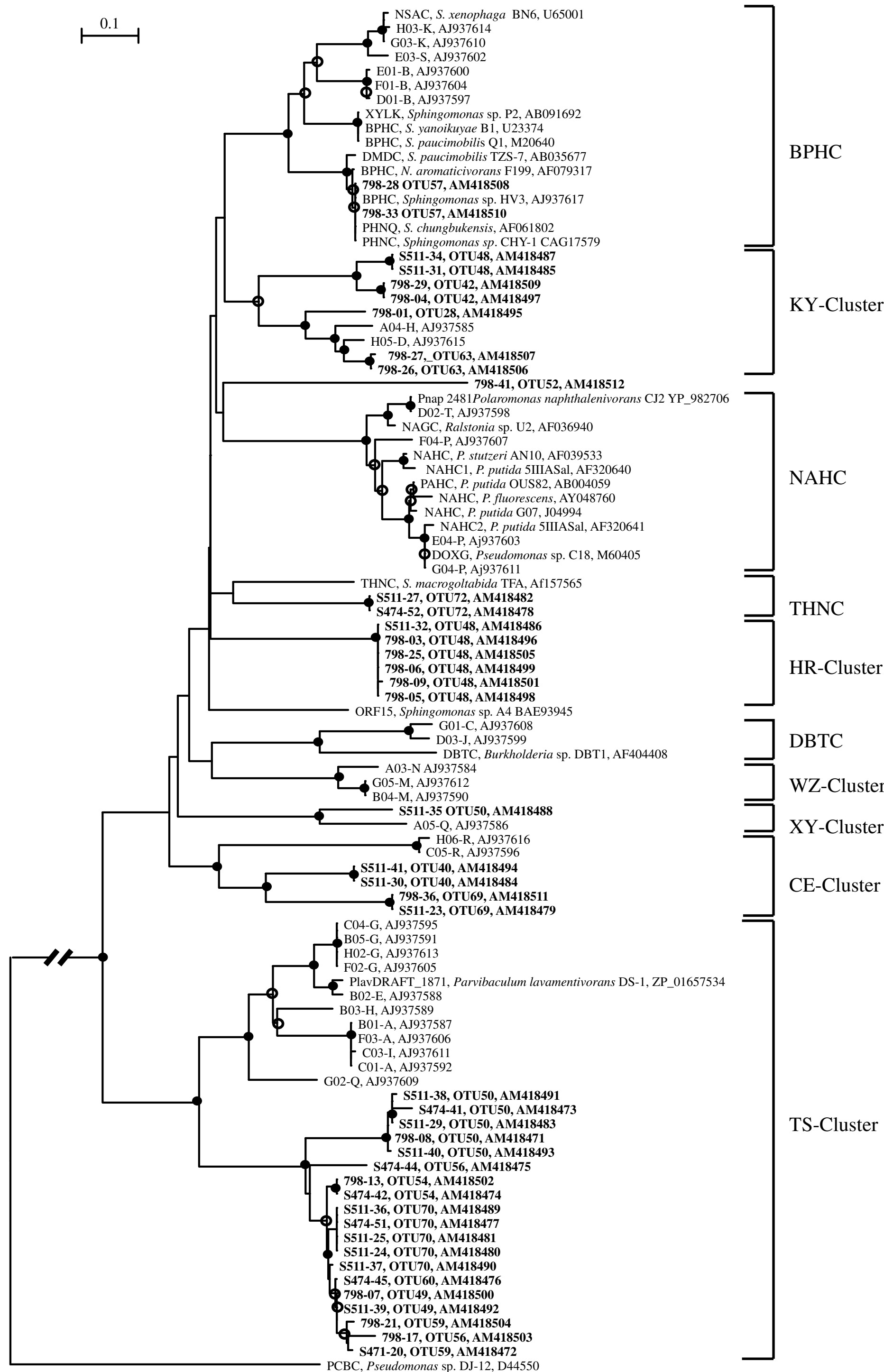

Figure 6 Neighbour-joining dendrogram of upper-pathway extradiol dioxygenase deduced peptides from soil microcosms, pristine and polluted, together with relevant reference sequences. Bootstrap values (50–80%) are marked with open circles, and values exceeding 80% are marked with filled circles. Sequences retrieved from the study (marked in bold) were placed in seven separate clusters. The generated phylogenetic tree was rooted with the PcbC from *Pseudomonas* sp. DJ-12 belonging to subfamily I.3.D according to Eltis and Bolin (1996).

Vaillancourt *et al.*, 2006). This is, to the best of our knowledge, the first study in which upper-pathway dioxygenase gene has successfully been used to note a rich functional gene diversity putatively residing from α-, β- and γ-proteobacteria and Gram positives involved in biodegradation and rhizoremediation. Recently, the influence of individual PAHs on ring-hydroxylating dioxygenase gene communities in soil enrichments and a soil sample was successfully assessed (Ni Chadhain *et al.*, 2006). The interesting

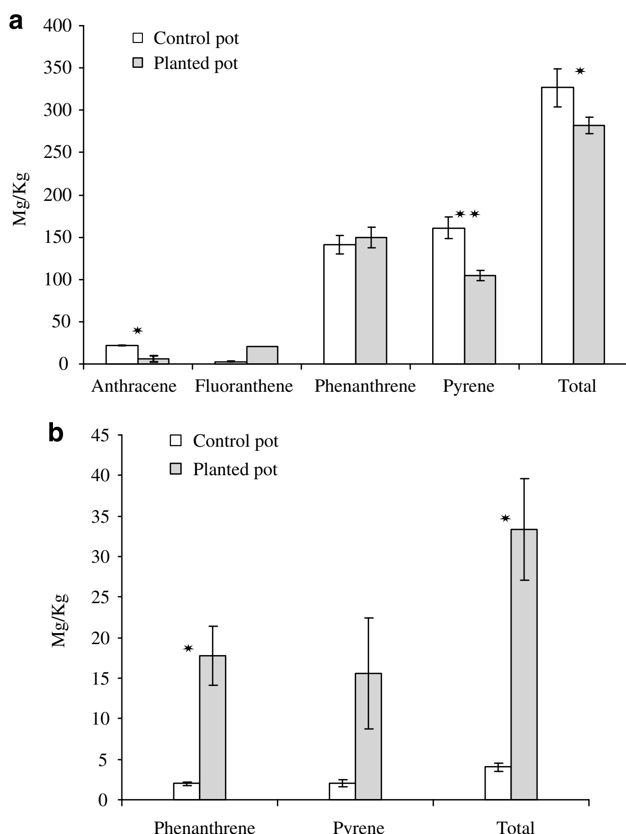

Figure 7 Extracted PAHs (mg kg^{-1}) from microcosms after 3-month incubation analysed by HPLC. (**a**) Microcosms amended with 300 mg kg^{-1} of anthracene, phenanthrene, fluoranthene and pyrene. Fluoranthene was successfully analysed only from one replicate microcosm. (**b**) Microcosms amended with 50 mg kg^{-1} of anthracene, phenanthrene, fluoranthene and pyrene. Residual fluoranthene and anthracene were not detected after 3-month experiment. Student's *t*-test was applied to assess statistical differences (**$P<0.01$; *$P<0.05$) between planted microcosm and bulk soil microcosm. HPLC, high-performance liquid chromatography; PAH, polyaromatic hydrocarbon.

study demonstrated changes in ring-hydroxylating dioxygenase gene communities only in liquid enrichments that could not be related to soil treatments. The benefit of our microcosm experiment is that we could relate both bacterial ring cleavage and 16S rRNA gene communities directly to different treatments in PAH bioremediation in soil. The effect of PAH amendment was evident for general bacterial and extradiol dioxygenase communities in bulk soil. The change in extradiol dioxygenase community in bulk soil demonstrated nicely how the I.E.3 group dioxygenases responded to PAH pollution. In the rhizosphere, pollution changed the 16S rRNA bacterial community structure but not the targeted functional ring-cleavage community. The targeted I.E.3 group extradiol dioxygenases could be involved in the turn over of natural aromatic compounds derived from decaying roots, root exudates and even PAHs in peat (Dreyer *et al.*, 2005), and were therefore not largely affected by PAH addition.

In the microcosms planted with birch, we observed that the aromatic ring-cleavage populations were more diverse and different from those in bulk soil displaying a rhizosphere effect. The birch trees are known to produce phenols as secondary metabolites (Bradley and Fyles, 1995; Priha *et al.*, 1998/1999). The phenols might end up in the rhizosphere from decaying roots or as root exudates and sustain the detected diverse ring-cleavage community including endophytic bacteria in the root. Very recently, it has been shown that *Pseudomonas* type aromatic degrading genes are upregulated in corn rhizosphere indicating that corn root exudates contain aromatic compounds (Matilla *et al.*, 2007). Simultaneous assay of 16S rRNA and ring-cleavage marker genes illustrated contrasting rhizosphere effects at different bacterial community levels. A rhizosphere effect in birch root-associated soil was detected by changed 16S rRNA communities in PAH-amended microcosms. This was in agreement with results of Corgie *et al.* (2004), where the most evident rhizosphere effect in phenanthrene-amended sandy soil was detected closest to roots. In this study, the bacterial diversity was, however, very low probably due to sterile quartz sand and artificial bacterial inoculum. Our microcosms were, in contrary, hosting a diverse bacterial community with complex T-RFLP patterns. In high microbial diversity (Costa *et al.*, 2006) of pristine soil planted with strawberry and oil seed rape, a structural rhizosphere effect was shown.

Biodegradation of PAHs was evident in all microcosms. The high bacterial diversity in bulk soil with changing communities due to PAH amendment explains the modest planting effect where the nursery soil was already rich in microbes. A positive planting effect in degradation of individual PAHs could be detected at 300 mg kg^{-1} PAH amendment. On the other hand, at the lower PAH-pollution level 50 mg kg^{-1}, the planting of birch did not enhance PAH degradation. The planting effect seems to be dependent on PAH concentration. At the high PAH concentration, phytotoxicity symptoms were observed. The pollution stress might affect the root exudate composition or even result in some form of plant–microbe interaction. Stress has been shown to affect root exudates of poplar (*Populus tremula* L) (Qin *et al.*, 2007) and wheatgrass (*Agropyroncristatum*) (Henry *et al.*, 2007). Bacterial products, such as lumichrome, are known to stimulate root respiration and thereby increase the availability of root exudates for bacteria (Phillips *et al.*, 1999). Poplar (*Populus trichocarpa*) has been shown to sense *Pseudomonas aeruginosa* PAO1, a pathogen in rhizosphere, and changes were detected in the transcriptome of both species (Attila *et al.*, 2008). Different levels of root exudates at high and low PAH levels could explain the concentration dependence of birch-planting effect.

Several novel extradiol ring-cleavage dioxygenase clusters were detected in the microcosms indicating existence of putative novel pathways. Very recently, two new putative upper-pathway extradiol dioxygenase groups were proposed I.3.M and I.3.N in a metagenomic study of activated sludge in wastewater treatment plant (Suenaga *et al.*, 2007). Upper ring-cleavage marker genes encode enzymes for degradation of aromatic hydrocarbons with different structures, and their different types reside from putative degradation pathways. For example, detection of *bphC Sphingobium* gene is indication of a *Sphingomonas* type of complex aromatic pathway and that of *nahC* gene is indication of the well-studied *Pseudomonas* type of aromatic pathway (Yen and Serdar, 1988; Kim *et al.*, 1996). In many instances, the ring-cleavage dioxygenase is an important determinant of the specificity of the pathway (Vaillancourt *et al.*, 2006). The relatively broad-specificity BP primers (Sipilä *et al.*, 2006) were proven to be useful for evaluating catabolic potential in soil microcosms. With these primers, it was possible to assess not only gene diversity but also putative pathway diversity. The identification of one branch of the novel TS cluster, to be similar to extradiol dioxygenase from *P. lavantivorans* DS-1 (Rhizobiales), illustrated the value of our approach to detect a broad range of ring-cleavage genes in the environment as a first step in identification of novel pathways. Aromatic ring-cleavage properties of Rhizobia are poorly known, and aromatic-degradation capacities and specificity of the enzyme of the DS-1 strain are not known. The abundance of the TS cluster in several polluted sites (Sipilä *et al.*, 2006) and in the microcosms of this study suggests that TS cluster-hosting bacteria are important degraders of aromatics in nature. These results show that new environmentally important pathways and bacteria exist to be isolated and characterized. If these pathways have escaped detection because of hosts being difficult to culture, they could be detected by metagenomic approaches (Handelsman, 2005; Suenaga *et al.*, 2007).

Connecting community structure with functions is one ultimate goal in microbial ecology (Leigh *et al.*, 2007). Effects of pollution, or even more effect of plants, on the often very complex microbial communities in soil environments are challenging to depict. In our study, we demonstrated how treatments like PAH addition or planting of birch caused different responses at specific bacterial community levels. We showed that these processes, fundamental in bioremediation, can be monitored by combined analysis of marker genes encoding catalytic enzymes, 16S rRNA genes and biodegradation of pollutants. Novel aromatic ring-cleavage dioxygenases in both polluted and pristine soil underpin the fact that we are unfortunately only in the beginning of grasping the overwhelming diversity of bacteria involved in biodegradation in soil.

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