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The incidence of *Burkholderia* in epiphytic and endophytic bacterial cenoses in hybrid aspen grown on sandy peat

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Endophytic bacteria are not known from woody plant seedlings. Endophytic and epiphytic bacteria were isolated from leaves, stems and roots of hybrid aspen (*Populus tremula* × *Populus tremuloides*) seedlings. The uncultured 16S rRNA rhizospheric bacterial community was cloned from pristine and polyaromatic hydrocarbon polluted rhizosphere soil. The isolates were subjected to restriction fragment length polymorphism analysis and partial 16S rRNA of selected strains was sequenced for phylogenetic identification. The bacteria could be classified into 16 different genera, showing that epiphytes from plant surfaces were most often Gammaproteobacteria, which composed 47% of the isolates. Endophytes from plant tissue were most frequently Betaproteobacteria (45%). Polyaromatic hydrocarbons caused a shift in the cultured bacterial community in the rhizosphere soil to a Betaproteobacteria dominated one. The root bacterial community showed a strong association of *Burkholderia* bacteria with hybrid aspen. Over 50% of all isolated strains grew on benzoic acid, but only 16% of benzoic acid degraders grew on *m*-toluate.

Introduction

Plants harbour endophytic bacteria that are best known as pathogens, but enthusiasm towards this peculiar group of bacteria has arisen from their beneficial properties (Davison 1988, Compant *et al*. 2005, Lodewyckx *et al*. 2002). Endophytic bacteria are thus defined as those bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plant and do not visibly harm the plant (Hallmann *et al*. 1997). Endophytes are found in internal microenvironments of the shoot and leaves, referred to as the endosphere, and additionally in the microenvironment of the root, defined as the endorhiza (Berg *et al*. 2005). At least 82 genera have been detected from a broad range of plants (Lodewyckx *et al*. 2002), including Ni hyper accumulator plants (Idris *et al*. 2004) and woody plants (Araujo *et al*. 2002, Bent Chanway 2002). Endophytic bacteria may contribute to the well-being of the plant, acting as growth promotors synthesizing phytohormones and enzymes (Lambert Joos 1989) and by fixing

atmospheric nitrogen (Davison 1988). They can potentially protect the plant from pathogenic fungi by their anti-fungal activity (Compant *et al*. 2005, Zachow *et al*. 2008).

An important requirement for successful application of phytotechnology is characterization of plant-associated bacteria, including endophytic and epiphytic strains and assemblage of strain collections. The study of plant-associated bacteria is important not only for understanding the ecological role of such bacteria in their interactions with plants but also for the biotechnological application of these bacteria to areas such as plant growth promotion (Sessitsch *et al*. 2004, Moore *et al*. 2006, Ryan *et al*. 2008). Bacterial endophyte studies have focused on agricultural and horticultural plant species, though endophytes have also been detected in fully grown woody plants, such as citrus, coffee, elm, oak and pine, (Araujo *et al*. 2002, Bent Chanway 2002, Mocali *et al*. 2003, Vega *et al*. 2005). Endophytic and epiphytic bacterial communities are related because of their proximity in plants, where a wound or stomata on the leaf gives an opportunity to penetrate into the plant. Some species might develop epiphytic and endophytic colonization, suggesting that these bacteria could fluctuate between endophytic and epiphytic niche (Kuklinsky-Sobral *et al*. 2005).

Poplar is well suited for use in phytotechnology because it is easy to establish and propagate. The genus *Populus*, poplars, cottonwoods and aspens, contains about 30 species of woody plant, all found in the northern hemisphere and exhibiting the fastest growth rates observed in temperate trees (Taylor 2002). This rapid growth results in high biomass production, and the high transpiration rate and a far-reaching root system are advantageous in remediation. In addition, aspen is amenable to coppicing and short-rotation harvest, as well as *in vitro* propagation and genetic transformation (Confalonieri *et al*. 2003). The poplar genome has also recently been sequenced (Tuskan *et al*. 2004). Hybrid aspen (*Populus tremula* × *Populus tremuloides*) has been proposed for short rotation forests in the boreal zone because of relatively fast growth and modest soil nutrient requirements (Christersson 1996, Asikainen 2007). Studies on the endophytic community composition of *Populus* trees are limited (Moore *et al*. 2006, Ulrich *et al*. 2008), and there are no reports on endophytes in seedlings.

The diversity of bacteria can be estimated by a culture analysis which enables characterization of bacterial strains. Still the great majority of bacteria are not readily culturable and a culture-independent analysis is more convenient by isolation of DNA and subsequent amplification of suitable marker genes which are fingerprinted (Sipilä *et al*. 2008).

To examine and improve the suitability of hybrid aspen for phytotechnology the diversity of bacteria associated with this plant was studied in a greenhouse experiment. Endophytic as well as epiphytic bacteria were simultaneously studied from the different microenvironments of this woody plant to investigate the localization and colonization routes of endophytes. The enophytic degradation capacity of aromatic compounds was also of interest regarding bioremediation. The uncultured community was analyzed from the rhizosphere soil to highlight the main groups of bacteria present in the rhizosphere. We hypothesized that the soil bacterial community will affect the plant associated bacterial flora. When PAH pollution will change the bacterial community structure in the soil, this community structure differentiation will be reflected in the cultured plant associated community.

Material and methods

Cultivation of hybrid aspen

Hybrid aspen (*Populus tremula* × *tremuloides*) seedlings were grown in a greenhouse in pots with 250 g of pristine soil or soil polluted with PAHs containing anthracene, phenanthrene and pyrene. The soil was a mixture of 80% sand (Optiroc, granulometric distribution 0.5–1.2 mm) and 20% of untreated peat (Kekkilä Oyj, Tuusula, Finland). The greenhouse microcosms were illuminated 16 h per day to mimic the local summer day-light length (Osram Fluora and Biolux) and the incubation temperature of the greenhouse was maintained at 18 °C.

After a three-month growth period, the hybrid aspen seedlings were carefully removed from

the pot. Roots with adhering soil were carefully separated from bulk soil by gentle shaking. Root soil was manually sampled to form a composite rhizosphere sample (at least six sub-samples) that was used for analysis of the uncultured rhizosphere soil community. Aspen seedling roots were cut off from the stems and these plant parts were packed into separate plastic bags. Subsequently the plant parts were manually washed in sterile water.

Isolation of hybrid aspen associated bacteria

Both endophytic and epiphytic bacteria were analysed from the different microenvironments of the plant grown in the greenhouse. Epiphytes were isolated from root surface (rhizoplane) and endophytes from within the root (endorhiza). Epiphytes were isolated from the stem surface and endophytes from within the stem (endosphere) and epiphytes from leaf surface (phyllosphere) and endophytes inside the leaf (endosphere). Epiphytic bacteria from stems and leaves were isolated from two seedlings grown in pristine soil (87 pure cultures). Endophytic bacteria were isolated altogether from ten aspen seedlings (135 pure cultures).

For isolation of epiphytic bacteria, stems and leaves were mixed with 50 ml PBS, 1 g sterilized glass beads (0.2 cm in diameter) in a 250 ml conical erlenmeyer flask and shaken at 150 rpm and 28 °C for one hour. A 50 μl aliquot of the suspension was plated on 1/10 TSA plates, and these were incubated at 25 °C. Root associated epiphytes were isolated as follows: roots were washed manually in sterile water, and incubated in 25 ml of sterile phosphate-buffered-saline (PBS) containing 10 g sterile glass beads (0.2 cm in diameter) with constant shaking (150 rpm) for one hour. 100 μl of different dilutions of PBS (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were plated onto 1/10 TSA. After the isolation of epiphytes the same samples were used for isolation of endophytes.

Different protocols for surface sterilization of hybrid aspen were tested using varying concentrations of NaOCl and ethanol and incubation times. The surface sterilization was tested by plating uncut plant parts onto 1/10 TSA and incubation for four days in 25 °C. If growth was not observed, the plant part was considered to have been surface-sterile. The protocol giving the best surface sterilization was used in the experiment, in which leaves were placed into 100 ml infusion flasks, submerged and gently shaken in solution according to the following protocol: 30 s in 75% EtOH, 3 min in 3% NaOCl, rinsing three times in sterile MQ water, 30 s in 75% ethanol and drying ca. 5 min. on a partly open plate. Stems were surface-sterilized using a similar protocol, except that after the ethanol treatment the stems were briefly flamed. Roots were surface-sterilized similarly but with 5% NaOCl. Intact plant parts were also plated to check their surface sterility. The surface sterilized plants were cut into small pieces (0.5 × 0.5 cm) and plated onto 1/10 TSA. Any detected bacterial growth on the plant piece was transferred to 1/10 TSA plates to obtain single colonies.

Aromatic degradation capacities of isolated strains

Aromatic compounds that are taken up by the plant are putative carbon sources for endophytic bacteria (Moore *et al*. 2006). The capacity of endophytic and epiphytic isolates to degrade benzoic acid was tested on minimal medium (Horvath Alexander 1970) containing 0.05% benzoic acid or 0.05% *m*-toluate. Benzoic acid is an aromatic compound that is degraded through the catechol *meta*-pathway (Williams Sayers 1994), and also in some strains via the catechol *ortho*-pathway (Reineke 1998). The strains that grew on benzoic acid were analysed for degradation on *m*-toluate, an aromatic compound that is degraded exclusively through the *meta*-pathway in aerobic metabolism.

The aromatic pollutant can diffuse into the plant if the $\log K_{ow}$ (octanol/water partitioning coefficient) is between 0.5 and 3.5 (Trapp *et al*. 2001). PAHs are known to be poorly soluble in water, due to their high $\log K_{ow}$, through the biodegradation metabolites possess lower $\log K_{ow}$ values and therefore will be more likely to diffuse into the plant tissue where the metabolites can be subjected to bacterial biodegradation

causing a pollution effect to the plant associated community. Benzoic acid and toluate are aromatic compounds formed as intermediates in the biodegradation of PAHs and BTEX compounds (Harayama *et al*. 1987).

DNA isolation and PCR amplification

Total genomic DNA from the hybrid aspen rhizosphere was extracted from 0.25 g composite rhizosphere soil samples (obtained as described) with the PowerSoil™ DNA Isolation Kit (Mo Bio laboratories Inc., USA). Separate colonies from pure cultures were selected and inoculated into 50 μl of sterile water. DNA was isolated using the boiling water method as follows: 10 min incubation in a boiling water bath, 5 min on ice and centrifugation for 5 min at 10 000 g. The extracted DNA was immediately used in a PCR assay. Almost full length 16S rDNA gene was amplified using 27F and 1492R primers (Weisburg *et al*. 1991).

The PCR protocol was: 96 °C 1 min followed by 35 cycles of denaturation at 96 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 1 min 30 s, followed by final elongation at 72 °C for 5 min. Gene Amp thermal cycler (Perkin Elmer, Inc., Wellesley, MA, USA) was used for amplification reactions. The PCR mixture contained 40 pmol of primers, 200 μM dNTP (Finnzymes, Finland), 2 units of DNA polymerase (DyNAzyme™ EXT DNA Polymerase, Finnzymes) and 1 × reaction buffer containing 1.5 mM $MgCl_2$. The PCR products were analyzed by agarose gel electrophoresis using 0.5 μg Lambda *Hind*III digest (Finnzymes) as size standard.

Amplified ribosomal DNA restriction analysis

Amplified ribosomal DNA restriction analysis (ARDRA) was used to classify hybrid aspen associated bacterial isolates from the greenhouse experiment. Almost full length 16S rRNA gene was amplified using 27F and 1492R primers (Weisburg *et al*. 1991). The PCR products were quantified using gel electrophoresis. Restriction reactions were performed in a 20 μl volume comprising 5–10 μl PCR product, 3 units of *Alu*I (Fermentas, GMBH, Germany)/*Msp*I (Promega, Madison, USA) enzyme, 1.5 μl Y+/Tango buffer/Promega buffer B. The resulting mixture was incubated overnight at 37 °C. The digested DNA mixtures were run on a 3% agarose gel (Synergel) and the isolates were divided into haplotypes on the basis of DNA banding pattern of individual strain. Each banding pattern detected on agarose gel constitutes one distinct haplotype on the basis of how the restriction enzyme cuts the PCR product.

Cloning of 16S rRNA from the hybrid aspen rhizosphere

PCR-amplified 16S rRNA genes from PAH polluted and pristine hybrid aspen rhizospheres were cloned to produce environmental clone libraries. Gel purified (Wizard® SV gel and PCR Clean-Up System, Promega) PCR products were ligated to pGEMT vector (pGEMT vector system, Promega) and transformed to competent DH5α cells, prepared by the rubidium chloride method (Hanahan 1983). White positive colonies were randomly selected and transferred into 200 μl liquid media (Luria broth supplemented with 0.1 mg ml^{-1} of ampicillin) and cultured overnight at 37 °C with constant agitation. Diluted (1/10) water stocks were taken from growth media for PCR analysis. The rest of the culture was stored as a glycerol stock at –70 °C (Sambrook *et al*. 1989). The water stocks were used as a template to amplify the 16S rRNA genes using vector-specific RP (5´ TTT CAC ACA GGA AAC AGC TAT GAC 3´) and UP (5´ CGA CGT TGT AAA ACG ACG GCC AGT 3´) primers.

Comparison of DNA libraries and isolated bacterial communities

The Library Compare Tool (Ribosomal data base project) was used to compare the bacterial community structure in pristine and PAH-polluted soil. This test estimates the likelihood that the frequency of membership in a given taxon is the

same for the two libraries using a statistical test first developed for comparing transcript levels in "digital northern" analyses (Audic Claverie 1997).

To visualize similarities of isolated bacterial communities from leaf, stem and root the percentages of haplotypes recovered from the three different plant compartments were converted to a distance matrix, using the Morisita distance algorithm and represented in non-metric multidimensional scaling (MDS) plot.

Sequencing and phylogenetic analysis

After the ARDRA classification, bacterial isolates were selected for sequence analysis according to their fingerprint type. Several isolates were sequenced from major fingerprints. The 16S rRNA gene was amplified from each selected strain and clone using 27F and 1492R primers. The partial 16S rRNA gene was sequenced using D´ primer (Edwards *et al*. 1989) producing a sequence from 27 to 518 (*E. coli* numbering) and analysed with an ABI 3130 genetic analyser with Big Dye ver. 3.1 chemistry (Applied Biosystems). Primer sequences were removed using Trev ver. 1.9 (Rodger Stadens software package for sequence analysis). The sequences were corrected as necessary using Gap4. Sequences were compared with those in the databases using BLAST (NBCI, *see* www.ncbi.nlm.nih.gov/BLAST/). The 16S rRNA gene sequences were aligned using ClustalW (Li 2003) and inspected manually with the Genedoc program ver. 2.6 (Nicholas *et al*. 1997). The phylogenetic tree was constructed with the Treecon program package (Van de Peer De Wachter 1994) using evolutionary distances (Tajima Nei 1984) and the neighbour-joining method (Saitou Nei 1987). 16S rRNA sequences were assigned to a taxonomical hierarchy using Classifier from Ribosomal Data Base (Wang *et al*. 2007).

Results

Endophytic and epiphytic bacteria were isolated (531 strains) from the different parts of aspen plants grown in the greenhouse: root stem and leaf. The main aim was to elucidate the aspen 'bacteriome' i.e. the diversity of plant associated bacteria and to compare this with the uncultured community in the soil to unveil relationships between soil and plant populations.

Classification of bacterial isolates by ARDRA

Both endophytic and epiphytic bacteria from microenvironments of hybrid aspen were simultaneously isolated. A total of 364 bacterial isolates were subjected to an ARDRA analysis resulting in the generation of 24 profiles (haplotypes). The distribution of these haplotypes between the different compartments of the plant (root, stem and leaf) is shown in Fig. 1. Subsequent to classification the endophytic and epiphytic isolates were phylogenetically characterized by selecting isolates from each haplotype for partial sequencing of 16S rRNA. The majority of the 24 haplotypes could be assigned to known bacterial genera by the RDP classifier; leaving six unidentified at the genus level (Table 1).

Isolated community structures (Fig. 1) were subjected to multidimensional scaling, which revealed that both endophytic and epiphytic populations from the leaf were different from those in the root and stem (Fig. 2). In leaves, the dominating epiphytes were determined by sequencing to belong to the genera *Sphingomonas* and *Burkholderia* (Table 1). The stem harbored a community with *Methylobacterium* as the dominant endophyte genus (Fig. 1 and Table 1). Some stem endophytes, such as *Methylobacterium* and *Burkholderia*, were simultaneously cultured from the stem surface, indicating an infection route from stem surface to the interior or *vice versa*.

The most diverse bacterial community was obtained from the root, upon which *Pseudomonas* and *Burkholderia* were dominant (Fig. 1). Other major groups could not be assigned directly to known genera but to the families Enterobacteriaceae and Microbacteriaceae. *Burkholderia* and *Methylobacterium* were most frequently cultured from endorhiza whereas *Pseudomonas*, *Burkholderia*, Enterobacteriaceae and Microbacteriaceae were the most common root epiphytes (Fig. 1). Inspection of cultured bacteria specific

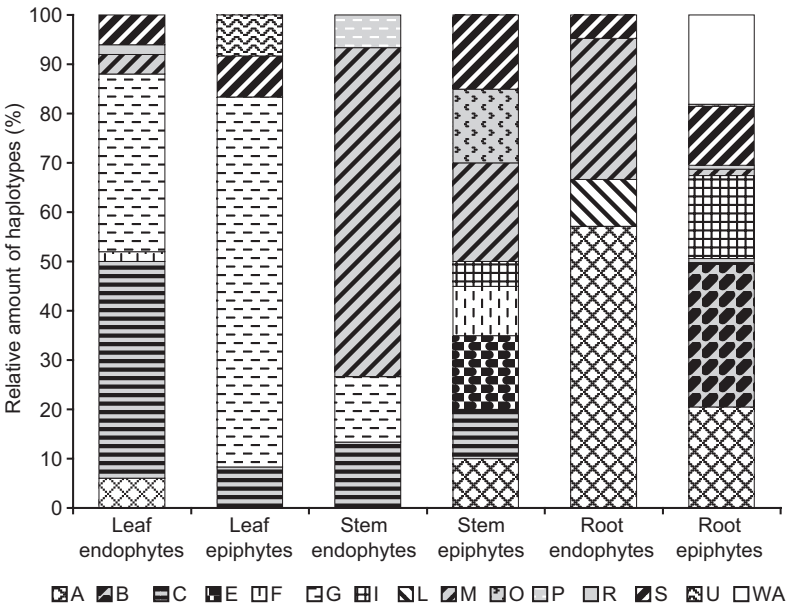

Fig. 1. Distribution of hybrid aspen endophytic and epiphytic bacterial isolates to the different microenvironments (phyllosphere, endosphere of leaf and stem, rhizosphere and endorhiza) with relative abundance of haplotypes. The haplotype WA represents ten bacterial strains unique to the root surface. The haplotypes were defined by ARDRA analysis. Separation of epiphytic from endophytic bacteria was accomplished by means of surface sterilization (*see* Material and methods).

Table 1. RFLP fingerprints (marked with capital letters) representing endophytic and epiphytic bacterial isolates obtained from hybrid aspen roots, stems and leaves.

	Genus	Aspen associated bacterial strains				
					Degraders	
		Root	Stem	Leaf	Benz.	*m*-tol.
ACTINOBACTERIA						
Microbacteriaceae	*Leifsonia* (D)	2	0	0	0	0
	uncl. Microbacteriaceae (S)	31	3	4	3	0
	uncl. Microbacteriaceae (F)	0	2	1	1	1
	uncl. Microbacteriaceae (U)	1	0	1	2	0
Micrococcaceae	*Micrococcus* (AN)	7	0	0	0	2
	Arthrobacter (U)	1	0	1	0	2
PROTEOBACTERIA						
Alphaproteobacteria						
Sphingomonadaceae	*Sphingomonas* (G)	0	2	27	0	0
Methylobacteriaceae	*Methylobacterium* (M)	9	14	2	11	0
Rhizobiaceae	*Agrobacterium* (P)	0	1	0	0	0
	Rhizobium (H)	1	0	0	0	0
	uncl. Rhizobiaceae (AQ)	4	0	0	0	0
Betaproteobacteria						
Burkholderiaceae	*Burkholderia* (A)	63	2	3	49	2
	Burkholderia (C)	3	4	23	30	0
	Burkholderia (AC)	2	0	0	1	0
	Cupriavidus (T/O)	9	0	0	0	6
Comamonadaceae	*Variovorax* (AD/AB)	8	0	0	0	0
Gammaproteobacteria						
Xanthomonadaceae	uncl. Xanthomonadaceae (E)	0	3	0	0	0
	Dyella (AM)	4	0	0	0	0
	Dyella (W)	8	0	0	0	0
Enterobacteriaceae	uncl. Enterobacteriaceae (I)	42	1	0	0	0
Pseudomonadaceae	*Pseudomonas* (R)	2	0	1	3	1
	Pseudomonas (B)	72	0	0	65	38

to hybrid aspen surfaces demonstrated that these epiphytes were most often Gammaproteobacteria (132/281), and constituted 47% of all cultured bacteria from plant surface. The cultured endophytes in their turn were most frequently Betaproteobacteria (39/86), which constituted 45% of the bacteria cultivated from plant tissue.

Aromatic degradation capacities of bacterial isolates

Of 531 tested isolates, 272 grew on benzoic acid. Degraders from the root were distributed into eight haplotypes (Table 1). *Burkholderia* was the main endophytic degrader and *Pseudomonas* the main epiphytic degrader. Inspection of individual isolated strains showed that *Burholderia* isolates were scarce on roots from pristine soil from which only two out of 80 isolates belonged to the genus *Burkholderia*. PAH contamination of the soil increased the occurrence of individual isolates to 34 out of a total of 83 from rhizosphere.

The most common degrader isolated from the stem, *Methylobacterium*, was cultured both as endophyte and epiphyte. The other dominant benzoic acid degrader in the stem was identified as *Burkholderia*. The degraders from the leaf were distributed into five haplotypes and the main epiphyte and endophyte here was *Burkholderia* as well (Table 1).

All benzoic acid degraders were tested for growth on *m*-toluate, a structural analog of toluene, which is degraded via the catechol *meta*-pathway in bacteria. Only 44 of the 272 benzoic degrading isolates grew on *m*-toluate and no degraders were cultured from the stem (Table 1). The *m*-toluate degraders on the leaf were *Burkholderia,* Microbacteriaceae and *Arthrobacter*. On the root, *Pseudomonas* was the most frequently isolated epiphytic degrader, though none of the endophytes grew on *m*-toluate.

Phylogenetic characterization of bacterial isolates and uncultured community

The bacteria cultured from hybrid aspen were placed into ten clusters in the neighbor-joining

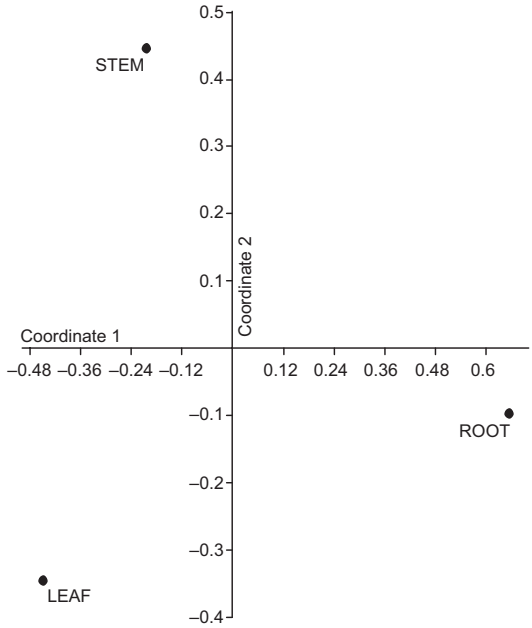

Fig. 2. Non-metric multidimensional scaling (MDS) plot of isolated bacterial communities from leaf, stem and root of hybrid aspen. The percentages of each haplotype in each plant compartment were used to construct the MDS plot.

tree, spanning four bacterial taxa Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Actinobacteria (Fig. 3). *Burkholderia sordidicola* and *Sphingomonas aerolata*/*Sphingomonas aurantiaca* were common in leaves. *Burkholderia sordidicola* strains have been isolated from the white-rot fungi *Phanerochaete sordida* (Lim *et al*. 2003). The most frequently cultured bacterium from leaf endosphere, was identified as *Methylobacterium fujisawaense*. *Methylobacterium* have frequently been associated with terrestrial and aquatic plants, colonizing their roots, leaf surfaces and other parts (Green *et al*. 1988, Madhaiyan *et al*. 2006). In hybrid aspen roots, the three major groups of isolates were identified as *Burkholderia fungorum*, *Pseudomonas koreensis*, *Rahnella aquatilis* respectively (Fig. 3 and Table 1). The partial 16S rRNA gene was sequenced from three isolates of the same haplotype A and all of these were identified as *Burkholderia fungorum* (99% similarity) (Coenye Vandamme 2003, Marx *et al*. 2004). The abundant cultured epiphyte of the I haplotype was assigned to Enterobacte-

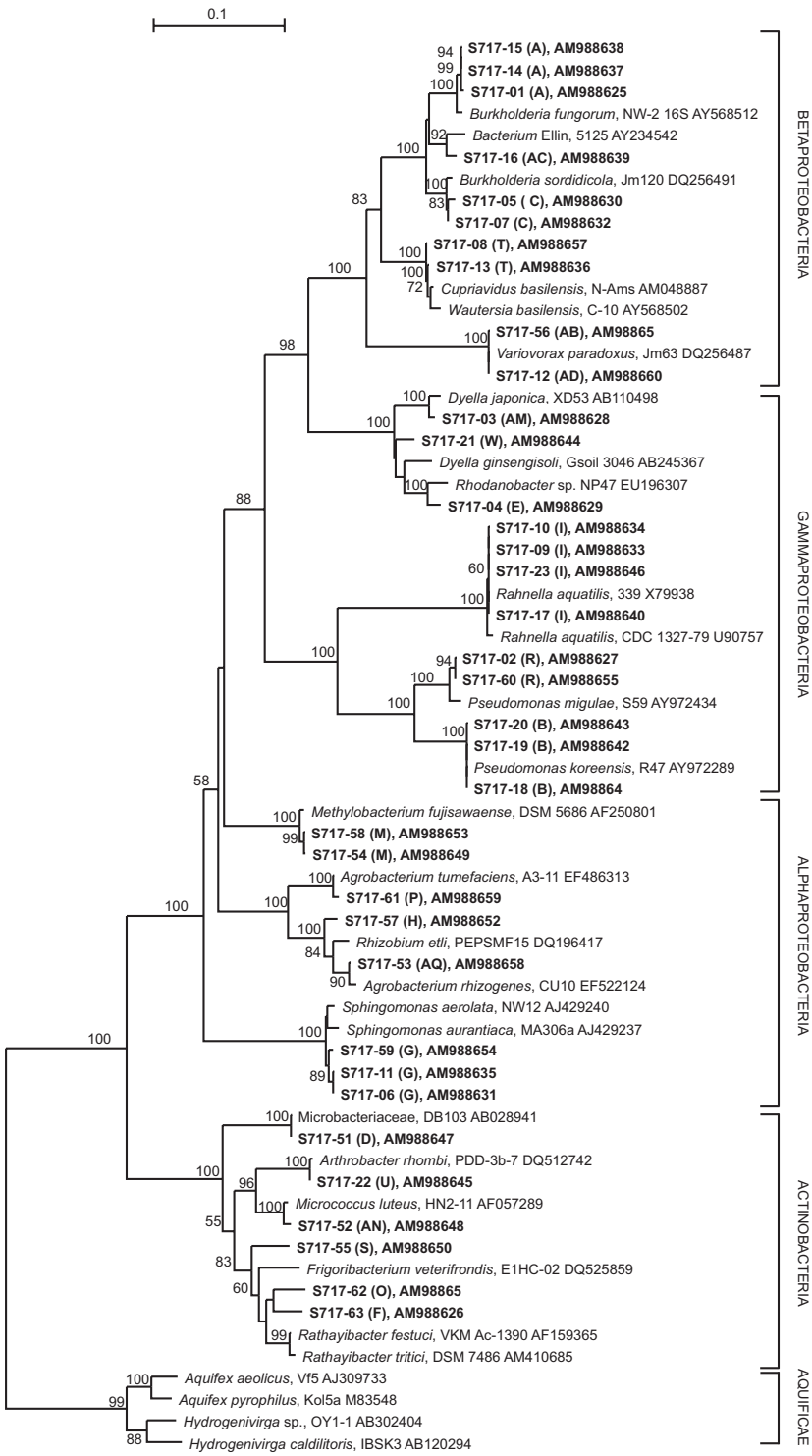

Fig. 3. Phylogenetic dendrogram of bacterial isolates from hybrid aspen and related bacteria. The dendrogram was constructed using partial 16S rRNA gene inferred by the neighbour joining method and Aquificae cluster was used as the root. Isolates are set in boldface with corresponding haplotype. Bootstrap values from a thousand replicates over 50% are shown.

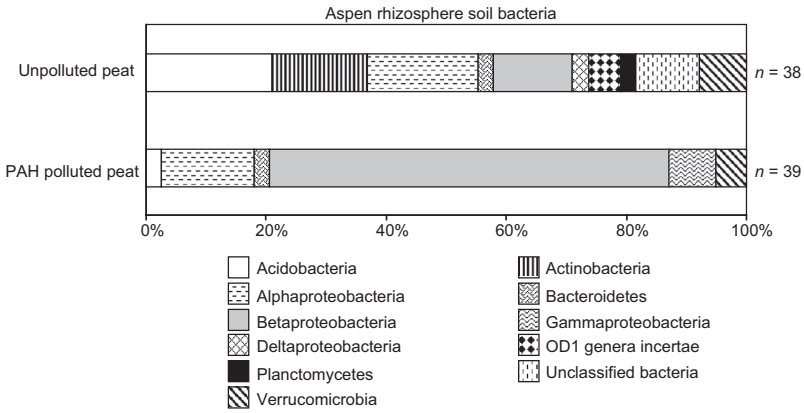

Fig. 4. PCR-amplified 16S rRNA bacterial community structure from the aspen rhizosphere soils. Two clone libraries were analysed: one from pristine soil and one from PAH-polluted soil. The 16S rRNA sequences of 38 and 39 clones respectively were determined and distributed into 11 different taxa by Classifier from Ribosomal Data Base.

riaceae by RDP classifier but in blast search, the best hit was *Rahnella aquatilis* (99%). *Rahnella* are nitrogen fixing enteric Gammaproteobacteria associated with the rhizosphere of wheat and maize (Berge *et al*. 1991).

The uncultured bacteria in the rhizosphere were less easily identified, reflecting the high diversity of the soil micro flora. Acidobacteria and Betaproteobacteria were prevalent phyla in both pristine and polluted rhizosphere. Acidobacteria were identified to the GP1 genus of the family Acidobacteriaceae. Bacteriodetes was represented by the *Sphingobacterium* genus, Planctomycetes by *Isosphaera* and Alphaproteobacteria by *Caulobacter*, *Bradyrhizobium* and *Methylocella*.

Effects of PAHs on bacterial community structure in the rhizosphere

Rhizospheric soil bacteria are the most likely source of endophytes colonizing the endorhiza. A 16S rRNA sequence analysis of the rhizospheral bacterial community showed that the most prevalent groups in pristine rhizosphere soil were Acidobacteria together with Alphaproteobacteria and Actinobacteria (Fig. 4). None of these bacteria were, however, detected from the cultured isolates. The addition of PAHs to the soil changed the uncultured community structure to favor Betaproteobacteria (Fig. 4) and Alphaproteobacteria, demonstrating a strong dominance of Proteobacteria in polluted soil. The Library compare analysis (Ribosomal Database Project:Release 10) of uncultured bacterial communities showed that the Betaproteobacteria class, and more specifically the *Burkholderia* genus, were significantly changed ($P < 0.01$) and at the phylum level, Proteobacteria changed significantly as well ($P < 0.01$).

All Betaproteobacteria in uncultured communities were assigned to *Burkholderia*. Cultured *Burkholderia* from endorhiza and rhizosphere bacteria were also detected in the uncultured Betaproteobacteria community (three and two clones respectively), on the basis of identical 16S rRNA genes, signifying their association with aspen roots. PAH addition caused a clear shift in the diversity of uncultured *Burkholderia* in the rhizosphere of hybrid aspen (Fig. 4). Pristine rhizosphere contained only five (out of 38) *Burkholderia* sequences that clustered in the phylogenetic tree with *Burkholderia sordidicola* and *Burkholderia* sp. In polluted rhizosphere, however, 26 (out of 39) *Burkholderia*-related sequences were retrieved, showing a shift in occurrence of this genus. The diversified population could be divided into 7 phylogenetic clusters. The clusters *B. sordidicola*, *Burkholderia* sp., *B. fungorum*, *B. cepacia*, *Bacterium*, *B. bryophila* and *B. tropica* were solely identified from PAH polluted soil. Additionally *Cupriavidus basiliensis*, of the Burkholderiaceae family, was cultured from hybrid aspen root together with *Variovorax paradoxus* of the Comamonadaceae. Examination of the phylogeny of the cultured Betaproteobacterial community from the different compartments of hybrid aspen showed that they could be placed into five of the nine clusters (Fig. 5).

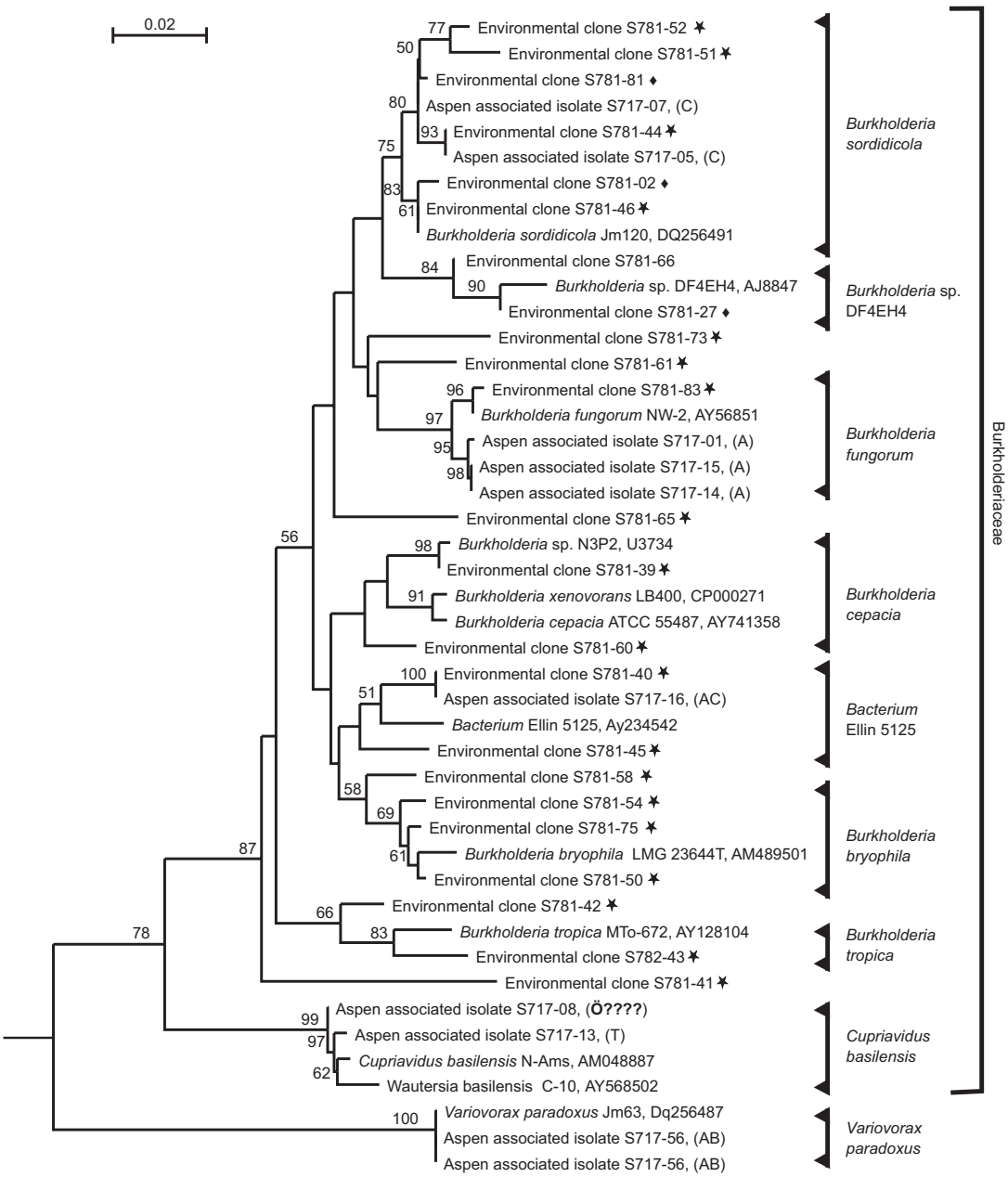

Fig. 5. Neighbour-joining phylogenetic tree presenting relationships of Betaproteobacteria associated with hybrid aspen. Environmental clones and cultured isolates were retrieved from the rhizosphere of hybrid aspen. The stars denote environmental clones from the 900 mg kg^{-1} PAH polluted rhizosphere and circles clones from pristine rhizosphere. The bootstrap values are shown for values with > 50% support in 100 re-sampling. The horizontal scale represents 2% sequence divergence.

The cultured root community was also affected by the addition of PAH mixture. *Burkholderia* were most frequently isolated from hybrid aspen grown in PAH mixture (anthracene, phenanthrene and pyrene) and *Rahnella* was most frequently isolated from hybrid aspen

in pyrene contamined (1200 ppm) soil. Plants grown in pristine soil most frequently harbored *Pseudomonas* and *Cupriavidus* (Table 2).

Discussion

Poplar trees have become increasingly interesting in phytotechnology (Germaine *et al*. 2004, Van Aken *et al*. 2004, Ryan *et al*. 2008, Taghavi *et al*. 2009) and only recently endophytic communities have been described from microenvironments of poplar (Moore *et al*. 2006, Ulrich *et al*. 2008). Our study is the first focusing on hybrid aspen (*Populus tremula* × *tremuloides*) seedlings, in contrast to previous studies of mature trees. Our main hypotheses, that the soil bacterial community will affect the plant associated bacterial flora and that PAH pollution effects will be perceived in cultured plant rhizoplane community, were verified in this study.

Colonisation of aspen

Colonization of plants by microbes occurs mainly through the root, according to the hypothesis of Hallmann, (1997). Other possible entry points are stomata and wounds in the leaf or stem (Lodewyckx *et al*. 2002). The rhizosphere is known to sustain growth of bacteria due to root exudates forming easy available substrate for bacteria (Briones Raskin 2003). We isolated bacteria encompassing 18 haplotypes from hybrid aspen rhizoplane, of which three (*Burkholderia*, *Methylobacterium* and Microbacteriaceae) were found in the endorhiza, suggesting infection via the root (Fig. 1 and Table 1). *Burkholderia* bacteria were cultured most frequently and the dominant endophyte strains were simultaneously detected from the uncultured community, both in pristine and PAH polluted rhizospheric soil, signifying colonisation via the root. The detected *B. fungorum* type bacterium apparently forms viable populations in aspen rhizosphere where they are able to infect endorhiza. Simultaneous study of endophytes and epiphytes interestingly revealed that about half of the endospheric and endorhizal haplotypes were also recovered as epiphytes, supporting the hypothesis that the endophytic bacterial community partly originates from epiphytic bacteria (Hallmann *et al*. 1997, Sturz *et al*. 2000).

PAH effects on soil and plant bacterial diversity

The pristine rhizosphere contained only five *Burkholderia* sequences, whereas polluted rhizosphere contained 26 sequences. The Library compare analysis of the uncultured bacterial

Table 2. Distribution of hybrid aspen rhizoplane isolates. The plants were grown in pristine, and PAH polluted, sandy peat soil with concentrations 150 ppm or 900 ppm of PAH mixture. The pyrene concentration was 1200 ppm. Identification is given as closest relative in the database according to sequence similarity.

Identification	Number of isolates in aspen rhizoplane			
	0 ppm PAH	150 ppm PAH	900 ppm PAH	1200 ppm Pyrene
Cupriavidus sp.	30	2	4	0
Burkholderia sp.	2	34	20	0
Wautersia sp.	1	0	1	0
Pseudomonas sp.	33	6	6	24
Microbacteriaceae	0	2	0	0
Micrococcus sp.	0	4	3	0
Variovorax sp.	0	0	0	0
Agrobacterium sp.	4	0	0	0
Dyella sp.	0	0	12	0
Rhizobium sp.	0	0	1	0
Rahnella sp.	0	0	0	40
Total	70	48	47	64

communities showed that the *Burkholderia* genus changed significantly upon the addition of PAH, although a higher amount of sequences would give more reliable results. The culture independent analysis revealed in any case a clear shift in the bacterial community structure.

The result of *Burkholderia* domination in the PAH-polluted rhizosphere soil was supported by bacterial isolation. Only two *Burkholderia* isolates were retrieved from roots of pristine soil, whereas PAH contamination increased the number of isolates to 34 out of a total of 83 from the rhizosphere (Table 2). This is a clear indication of how soil diversity influences the plant-associated bacterial diversity.

Most benzoic acid degraders from hybrid aspen were either *Burkholderia* or *Pseudomonas*, though also *Methylobacterium* species grew on benzoic acid. The *Burkholderia* endophytes potentially have an important role in the degradation of aromatics within the plant. A structural analogue to toluene, *m*-toluate, was chosen as a substrate that is typically degraded only through the *meta*-pathway. It was used as a growth substrate almost exclusively by *Pseudomonas* isolates which are well known *m*-toluate degraders often harboring the catabolic TOL plasmid (Greated *et al*. 2002). The presence of the TOL plasmid in our *Pseudomonas* isolates is presumably due to observed instability in the toluate-degrading capacity that can be explained by loss of the catabolic plasmid. The relatively low number of detected *m*-toluate degraders may also stem from the instability of the plasmid-encoded *meta*-pathway and easy loss of degradation capacity upon isolation and maintenance of strains.

Endophytic bacterial diversity in two poplar tree varieties growing on a BTEX-contaminated site were studied by Moore *et al*. (2006), who showed that selected endophytes grew on BTEX compounds or were tolerant to BTEX or trichloroethylene, and several isolates were proposed for use in enhanced phytoremediation. The isolation of BTEX degraders from plant tissues supported the idea that endophytes colonise via the roots, since the poplars were growing in BTEX-contaminated soil. The finding that the isolated leaf entophytic community was different from that of the stem and root, was in accordance with our results from hybrid aspen seedlings. We could further conclude that the leaf epiphytic community was also different from the stem and the root epiphyte communities, which had one clearly dominant epiphyte identified as *Sphingomonas aerolata*. This species has previously been isolated from diverse habitats such as air, hydrocarbon-contaminated soil and from 4200-year-old ice from Antarctica (Busse *et al*. 2003). Our discovery that it colonizes the phyllosphere and leaf endosphere strongly suggests an infection route from the air, supported by the fact that they could not be isolated from the hybrid aspen root system.

Burkholderia bacteria associated with hybrid aspen

The most abundant *Burkholderia* species cultured mainly from the root, had the highest similarity to *Burkholderia fungorum*, which is closely related to *B. xenophaga*, the best known PCB degrader which has a very complex genome containing several catabolic pathways (Chain *et al*. 2006). *Burkholderia fungorum* has been isolated from the white-rot fungus, *Phanerochaete chrysosporium*. White rot fungi have been associated with lignin biodegradation in woody plants. It was suggested that there is a symbiotic relationship between the bacteria, *Burkholderia fungorum*, and the fungus, since the bacteria were very efficient degraders of the aromatic compounds derived from the degradation of lignin by white-rot fungi (Seigle-Murandi *et al*. 1996). *Burkholderia* are repeatedly found in contaminated soil and water, and are capable of degrading aromatic compounds, halogenated derivatives and various recalcitrant organic residues (Friedrich *et al*. 2000, Nogales *et al*. 2001). The broad degradation capacities of the *Burkholderia* genus may originally have evolved with their close connection to plants. To form a close relationship with plants, the bacteria have to tolerate and be able to degrade diverse secondary metabolites, including lignin derivates of the host.

The other abundant isolated benzoic-acid degrader from the leaf was similar to *B. sordidicola*, which degrades lignin (Lim *et al*. 2003). The detailed biodegradation capacities of these isolates are worthy of study. *Burkholderia*

have been isolated from *Populus trichocarpa* endorhiza, as well as rhizosphere soil (Moore *et al*. 2006), but surprisingly not in endophyte studies of the aerial parts of hybrid poplar *Populus tremula* × *tremuloides* (Ulrich *et al*. 2008). The lack of *Burkholderia* isolates in their study may be due to the quality of their soil, putatively not containing significant *Burkholderia* populations. As we have shown in this study, the rhizosphere soil has a strong impact on plant colonizing bacterial communities.

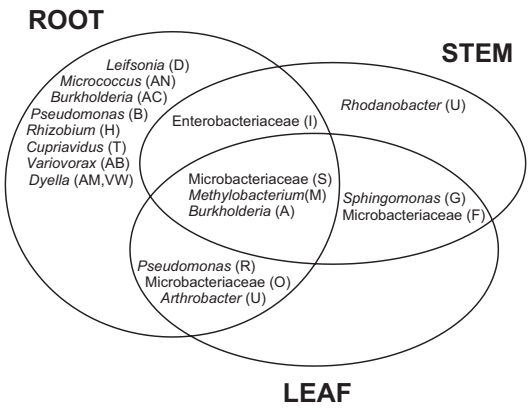

Fig. 6. Distribution of bacterial isolates within different compartments (leaf, stem and root) of hybrid aspen seedlings. Corresponding haplotypes are showed in parenthesis.

Plant compartments

Plant compartments displaying heterogeneity of living conditions are potentially selective for colonisation (Schneider *et al*. 1994, Weber *et al*. 1998). Moore *et al*. (2006) reported strong compartmentalisation when they could not find the same isolates in adjacent plant/soil compartments. Only *Pseudomonas* was found in all compartments, and *Bacillus* and *Arthrobacter* in the roots and stems. *Pseudomonas* was the most abundant genus recovered from the rhizosphere (72 isolates), which was influenced by rhizodeposition that may include organic acids and aromatics, such as phenols (Kamath *et al*. 2004). Their abundance can be explained by the well known ability of *Pseudomonas* to degrade phenols and aromatics. Our results show a less strong compartmentalisation, while several genera were found in multiple locations. *Burkholderia*, *Methylobacterium* and the unidentified Microbacteriaceae were cultured from all parts of the plant. *Arthrobacter*, *Pseudomonas* and another unidentified Microbacteriaceae were found both in the leaf and root. Microbacteriaceae, *Sphingomonas aerolata* and *Sphingomonas aurantica* are closely related to airborne bacteria which tolerate high radiation (Busse *et al*. 2003), and were isolated from stems and leaves. The high incidence of *Sphingomonas* in the phyllosphere of aspen (27 isolates), is explained by the living conditions in this environment, where bacteria have to withstand UV radiation. They have been detected from several plant surfaces (Kim *et al*. 1998) and are often pigmented, which enhances their suitability for conditions on leaf surfaces exposed to radiation (White *et al*. 1996). Unidentified Enterobacteriaceae were recovered from the root as well as from the stem (Fig. 6). The discrepancy of results may partly result from the different growth media used in the studies. Further, and more importantly, the distribution of bacteria in seedlings is likely to be different from that in older trees.

Methylobacterium was a common isolate from within the root (endorhiza) and also the most common endophyte from the stem. These bacteria are evidently well adapted to live in the hybrid aspen endosphere as well as in other plants (Green *et al*. 1988, Madhaiyan *et al*. 2006), though they were a minor group in the soil, since they were not detected from the uncultured community. *Methylobacterium* has been associated with the ability to degrade nitrosubstituted explosives (TNT) (Van Aken *et al*. 2004) and they have been assigned biotechnological potential due to their beneficial properties (Sessitsch *et al*. 2004). Another interesting group of hybrid aspen associated bacteria was the third most frequently isolated root epiphyte assigned to the *Rahnella aquatilis* (haplotype I), but not found as an endophyte in our study. In the sweet flag (*Acorus calamus*) rhizosphere it has been degrading the herbicide atrazine (2-chloro-4-ethylamino-1,3,5 triazine)(Marecik *et al*. 2008). Endobacteria from the ectomycorrhiza, *S. variegatus*, have also been assigned to the *Rahnella* (Izumi *et al*. 2006), though we did not analyse mycorrhizal infection of hybrid aspen roots and

do not know if these isolates, or any other of our root isolates, were endobacteria of fungi.

Conclusions

The holistic approach to the study of bacterial communities in soil simultaneously with both endophytic and epiphytic isolates of hybrid aspen in all compartments of the plant, gave a valuable insight into the ecology of aspen associated bacteria, which can be exploited in the development of the phytotechnology of woody plants.

1. The soil bacterial community affected the plant associated bacterial flora. *Burkholderia* bacteria were common in the soil, as well as in plants grown in PAH-polluted soil.
2. Compartmentalisation was observed, especially in the leaves reflecting the more harsh living conditions, since that community was more different from cultured endophytic and epiphytic communities of stem and root.
3. The impact of pollution on the soil bacterial community was indicated by culture independent bacterial community analysis, in which a shift to a Betaproteobacteria-dominated community was observed.
4. Several bacterial isolates showed potential for use in the development of phytotehcnology of aspen.

Improvement of our understanding of plant associated bacteria is necessary for us to better employ ecosystem services in phytotechnology by facilitating the development of enhanced rhizoremediation and woody plants for energy tree production.

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