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Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and Birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment

Sohail Yousaf[a,1], Verania Andria[a,1], Thomas G. Reichenauer[b], Kornelia Smalla[c], Angela Sessitsch[a,*]

[a] *Bioresources Unit, AIT Austrian Institute of Technology GmbH, A-2444 Seibersdorf, Austria*
[b] *Unit of Environmental Resources & Technologies, AIT Austrian Institute of Technology GmbH, A-2444 Seibersdorf, Austria*
[c] *Julius Kühn-Institut - Federal Research Centre for Cultivated Plants (JKI), Institute for Epidemiology and Pathogen Diagnostics, D-38104 Braunschweig, Germany*

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ABSTRACT

Twenty-six different plant species were analyzed regarding their performance in soil contaminated with petroleum oil. Two well-performing species, Italian ryegrass (*Lolium multiflorum* var. Taurus), Birdsfoot trefoil (*Lotus corniculatus* var. Leo) and the combination of these two plants were selected to study the ecology of plant-associated, culturable alkane-degrading bacteria. Hydrocarbon degrading bacteria were isolated from the rhizosphere, root interior and shoot interior and subjected to the analysis of 16S rRNA gene, the 16S and 23S rRNA intergenic spacer region and alkane hydroxylase genes. Furthermore, we investigated whether alkane hydroxylase genes are plasmid located. Higher numbers of culturable, alkane-degrading bacteria were associated with Italian ryegrass, which were also characterized by a higher diversity, particularly in the plant interior. Only half of the isolated bacteria hosted known alkane hydroxylase genes (*alkB* and cytochrome P153-like). Degradation genes were found both on plasmids as well as in the chromosome. In regard to application of plants for rhizodegradation, where support of numerous degrading bacteria is essential for efficient break-down of pollutants, Italian ryegrass seems to be more appropriate than Birdsfoot trefoil.

1. Introduction

Phytoremediation employs plants and their associated microorganisms for the clean-up of contaminated sites. Plants and microorganisms interact to absorb, degrade, or remove toxic pollutants from contaminated soil, ground- and surface-water [1]. In respect to phytoremediation of organic contaminants, which are slowly transferred from the soil to the plant, rhizodegradation (i.e. degradation by microorganisms in the rhizosphere of plants) is the major mechanism of detoxification [2]. Degrading bacteria are supported through the release of root exudates. Several plant species were reported to tolerate petroleum hydrocarbons. Among others, prairie grasses and legumes have been shown to be suitable for phytoremediation of petroleum hydrocarbon contaminants [3,4]. Grasses have a fibrous root system which creates a high root surface area and which may penetrate to a soil depth of up to 3 m [5], whereas legumes have the advantage to form a symbiosis with nitrogen-fixing bacteria [6] and thus can potentially improve the N:C-ratio in a soil contaminated with hydrocarbons.

Apart from mineralizing organic contaminants, bacteria can also reduce the phytotoxicity of contaminants to a level where plants can grow under unfavourable soil conditions [7,8]. The ability of bacteria to degrade aliphatic compounds found in petroleum is primarily conferred by enzymes such as the alkane monooxygenase encoded by *alkB*. Bacterial oxidation of n-alkanes is a very common phenomenon in oil contaminated soil and water as alkanes constitute about 20–50% of crude oil. Although very common among various bacterial domains [9], only the *Pseudomonas putida* strain GPo1 alkane hydroxylase system (*alk* gene cluster) has been studied in detail with respect to enzymology and genetics [10,11]. In strain GPo1 the *alkB* gene is located on a broad-host range IncP-2 plasmid [12,13], whereas many other strains contain homologues of the GPO1 *alkB* gene on the chromosome [14]. Quite divergent *alkB* homologues to GPO1 were found mainly in Gram-positives [10]. Alkane-degrading yeasts and fungi mainly possess enzymes related to cytochrome P450 class II (CYP52) alkane hydroxylase [15]. Recent studies showed that a range of bacteria also contain CYP153 genes encoding cytochrome P450 alkane hydroxylase [16]. The *alkB* and CYP153 hydroxylase genes allow bacteria to grow in media containing long chain length alkanes [9].

* Corresponding author. Tel.: +43 050 5503509; fax: +43 050 5503666.
E-mail address: angela.sessitsch@ait.ac.at (A. Sessitsch).
[1] Both authors contributed equally to this paper.

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Table 1
List of plant species tested in a two-month pot experiment.

Common name	Acr.	Scientific name	Biomass (mean (g) ± SD)		
			Control	Without compost	With compost
Italian ryegrass	IT	*Lolium multiflorum* var. Taurus	4.62 ± 0.42[a,*]	1.51 ± 0.18[b]	1.66 ± 0.09[b]
Birdsfoot trefoil	BT	*Lotus corniculatus* var. Leo	1.76 ± 0.54[a]	0.66 ± 0.10[b]	1.61 ± 0.04[a]
Mix Italian ryegrass and Birdsfoot trefoil	IT+BT	Mix *Lolium multiflorum* var. Taurus and *Lotus corniculatus* var.Leo	3.19 ± 0.23[a]	1.01 ± 0.14[c]	1.71 ± 0.19[b]
Perennial ryegrass, English ryegrass	EG	*Lolium perenne* var. Prana	1.81 ± 0.49[a]	0.40 ± 0.06[b]	0.23 ± 0.16[b]
Birdsfoot trefoil	HR	*Lotus corniculatus* var. Rocco	1.24 ± 0.15[a]	0.64 ± 0.11[b]	1.19 ± 0.13[a]
Alfalfa	LH	*Medicago sativa* var. Harpe	1.77 ± 0.37[a]	0.94 ± 0.12[b]	2.07 ± 0.33[a]
Red clover	RG	*Trifolium pratense* var. Gumpensteiner	2.02 ± 0.48[a]	0.10 ± 0.09[b]	0.03 ± 0.16[b]
Red fescue	RR	*Festuca rubra* var. Reverent	2.92 ± 0.74[a]	0.40 ± 0.15[b]	0.31 ± 0.02[b]
White clover	WH	*Trifolium repens* var. Gr. Huta	1.96 ± 0.51[a]	0.32 ± 0.08[b]	0.47 ± 0.15[b]
Sainfoin	EI	*Onobrychis viciifolia*	0.75 ± 0.33[a]	0.10 ± 0.09[b]	0.38 ± 019[ab]
Kidney-vetch	WU/1	*Anthyllis vulneraria*	1.40 ± 0.1[a]	0.12 ± 0.16[b]	1.04 ± 0.25[c]
Spelt	ZS	*Triticum spelta* var. Album	1.75 ± 0.44[a]	0.08 ± 0.10[b]	0.40 ± 0.12[b]
Emmer	SW	*Triticum dicoccon*	0.79 ± 0.34[a]	0.24 ± 0.08[b]	0.56 ± 0.25[ab]
Perennial ryegrass, English ryegrass	EP	*Lolium perenne* var. Prana	3.59 ± 0.45[a]	0.17 ± 0.07[b]	
Hybrid ryegrass	BG	*Lolium boucheanum* var. Gumpensteiner	3.05 ± 0.29[a]	0.05 ± 0.02[b]	
Black medic	GV	*Medicago lupulina* var. Virgo	1.71 ± 0.12[a]	0.02 ± 0.01[b]	
Orchardgrass	KT	*Dactylis glomerata* var. Tandem	1.80 ± 0.07[a]	0.04 ± 0.01[b]	
Timothy	TT	*Phleum pratense* var. Tiller	2.59 ± 0.47[a]	0.10 ± 0.08[b]	
Kentucky bluegrass	WC	*Poa pratensis* var. Compact	1.28 ± 0.62[a]	0.03 ± 0.04[b]	
Kentucky bluegrass	WO	*Poa pratensis* var. Oxford	1.38 ± 0.37[a]	0.01 ± 0.01[b]	
Kidney bean	BW/1	*Phaseolus vulgaris* var. vulgaris	3.13 ± 0.01[a]	1,392 ± 0.0[b]	
Grasspea	PM	*Lathyrus sativus* var. Colorolus Merkur	1.03 ± 0.72	Not growing	
Cereal rye	JO	*Secale multicaule*	0.61 ± 0.54	Not growing	
Falseflax	LE	*Camelina sativa*	0.98 ± 0.67	Not growing	
Kidney bean	BA/1	*Phaseolus vulgaris* var. vulgaris	1.70 ± 0.01	Not growing	
Livid amaranth	RM	*Amaranthus lividus*	1.44 ± 0.56	Not growing	

Control pots contained agricultural soil whereas treatment pots contained crude-oil contaminated soil with and without 10% (v/v) compost amendment. Italian ryegrass, Birdsfoot trefoil and a mixture of IT and BT amended with 10% compost (v/v) were selected for the isolation of hydrocarbon-degrading bacteria. SD, standard deviation.
*The same letter following mean ± SD shows that the mean value is not significantly different with a level of confidence 95% (Least Significant Difference test, $P < 0.05$).

Several studies showed that different plant species host distinct microbial populations in their rhizo- and endospheres, which is most likely due to different root exudates and substances produced by the plant [17–19]. Furthermore, different compartments of the plant harbor different bacterial assemblages [20,21]. In regard to phytoremediation of petroleum oil-contaminated soil, less information is available on degrading bacteria colonizing different plant species and their degradation genes. Therefore, the aim of this study was to determine whether two different plant species, both growing well in petroleum oil-contaminated soil, are colonized by distinct oil degrading bacteria, which might influence the phytoremediation efficiency. Based on their capacity to grow in diesel-contaminated soil, we selected two plant species, Italian ryegrass and Birdsfoot trefoil, and analyzed the diversity of culturable, alkane-degrading bacteria and their alkane hydroxylase (*alk*) genes in the rhizo- and endosphere of these plants.

2. Materials and methods

2.1. Plant screening

The petroleum oil-contaminated soil (17.2 g total hydrocarbon kg^{-1} soil as determined by Fourier-Transformation-IR-spectroscopy) was sampled from a landfill used for deposition of crude oil-contaminated soil from oil pumping sites in Zistersdorf, Lower Austria. The soil was taken randomly from 10 sampling points at the landfill each from 0 to 25 cm soil depth. Subsequently, all soil samples were mixed, homogenized and sieved by a 2 mm sieve. The soil was filled into pots (10 cm × 10 cm × 11 cm) and 200 mg seeds of each plant were sown in triplicates (Table 1). In the first screening experiment (without compost amendment) 26 plant species were tested. Plants were cultivated in contaminated soil as well as in uncontaminated, agricultural soil mixed with 40% sand. In the second screening experiment (with 10% (v/v) compost amendment), the 12 best performing plant species (i.e. plants producing highest biomass) as well as a mixture of Italian ryegrass (IT) and Birdsfoot trefoil (BT) were tested. The mixture was prepared by sowing 100 mg seeds of IT and BT in the same pot. Plants were grown for 3 months until being harvested. The tolerance of each plant species towards petroleum was measured based on comparing above-ground biomass with that produced in the control soil. The biomass was harvested, heat-dried and weighed. The Least Significant Difference statistical test ($P < 0.05$) was applied to compare mean values of results obtained from various treatments.

2.2. Isolation of alkane-degrading bacteria

Alkane-degrading bacteria were isolated from the rhizosphere, shoot interior and root interior of IT, BT and the mixture (IT + BT) of these plants. The plants were pulled and shaken to dislodge the bulk soil attached to the roots, and the shoots were cut (2 cm above soil). The rhizosphere soil was obtained by putting the roots with its attached soil into a 50 ml Erlenmeyer flask with 3 ml of 0.9% (w/v) NaCl and shaking at 180 rpm for 30 min. Subsequently, agitated roots and cut shoots were washed for 2 min in sterile distilled water before surface sterilization with 70% ethanol for 5 min (shoots) or 10 min (roots), followed by a 1 min wash in 1% NaOCl amended with 0.01% Tween 20 solution, and then a final rinse in sterile distilled water (3 times, 1 min each time). The surface sterility was checked on 10% Tryptic Soy Agar (TSA) medium. The plate was incubated at 30 °C for 48 h, no growth was observed. The isolation of bacteria from IT + BT was conducted by collecting and processing shoots from IT and BT separately. Roots were collected together as roots could not be separated.

2.2.1. Isolation with enrichment

One millilitre rhizosphere soil slurry or 1 g of surface sterilized and macerated shoots and roots were transferred to Falcon

tubes containing 10 ml liquid minimal basal medium (MBM) [22] amended with 1% (v/v) filter-sterilized diesel and incubated for 5 days at 30 °C with agitation at 180 rpm. For the isolation of rhizosphere, shoot and root bacteria, aliquots (100 μL) of serial dilutions up to 10^{-3} were spread onto 10% TSA and MBM amended with 1% (v/v) filter-sterilized diesel. Both media were amended with cycloheximide (100 mg L^{-1}) to avoid fungal growth and 15 g agar (Difco, USA). Isolation media were incubated at 30 °C for 2 days (TSA medium) and for 14 days (MBM). Bacterial colonies on each plate were selected according to their distinguishable colony morphologies. Three to five colonies of similar colony type were picked, grown on solid MBM amended with 2% (v/v) filter-sterilized diesel followed by incubation at 30 °C for 14 days.

2.2.2. Isolation without enrichment

Surface sterilized roots and shoots (1 g) were homogenized individually with 2 mL 0.9% (w/v) NaCl solution. Aliquots (100 μL) of this suspension as well as 10-fold dilutions of the rhizosphere slurry were spread onto 10% TSA and MBM containing 1% diesel.

2.3. Identification of alkane-degrading isolates

All hydrocarbon-degrading isolates were subjected to restriction fragment length polymorphism (RFLP) analysis of the 16S–23S rRNA intergenic spacer (IGS) region to distinguish different strains. Genomic DNA was extracted from liquid cultures by using the Microbial DNA Isolation Kit (Mo Bio Laboratories, USA). The IGS region was amplified by PCR using the primers pHr (5′-TGCGGCTGGATCACCTCCT-3′) and P23SR01 (5′-GGCTGCTTCTAAGCCAAC-3′) [23] as described by Rasche et al. [24]. Digestion of 10 μL of IGS PCR products was performed with 5 U of endonuclease HhaI (Invitrogen) at 37 °C for 4 h. The resulting DNA fragments were analyzed by gel electrophoresis in 2.5% (w/v) agarose gels. The IGS characterization distinguished 81 bacterial strains which were further analyzed. A representative isolate of each IGS type was identified by partial 16S rRNA gene sequencing. 16S rRNA genes were amplified by applying PCR primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) [25] and 1520rev (5′-AAGGAGGTGATCCAGCCGGA-3′) [26] as described by Rasche et al. [24]. PCR amplicons were purified with Sephadex™ G-50 (Amersham) and used as template for sequence analysis. Partial sequencing of 16S rRNA genes was performed by applying the BigDye V3.1 Terminator Kit (Applied Biosystem, Warrington, UK) and the reverse primer 518rev (5′-ATTACCGCGGCTGCTGG-3′) [27], resulting in sequences of approximately 500 bp length. Sequences were subjected to BLASTN analysis with NCBI database (accession numbers FJ013273–FJ013353).

2.4. Growth on different n-alkanes and polycyclic aromatic hydrocarbons

Strains were tested for their ability to utilize alkanes as sole carbon source by growing them on plates containing solid MBM amended with either 2% (v/v or w/v) of diesel and n-alkanes (C_6, C_8, C_{10}, C_{12} and C_{16}). Plates were prepared as described by Daane et al. [28]. For control, strains were grown on 10% TSA containing 0.2% (w/v) glucose. The hydrocarbon-containing plates were incubated for 7 days at 30 °C and the control was incubated for 2 days. All hydrocarbons used were at least 98% pure (Sigma–Aldrich).

2.5. Detection, sequencing and localization of alkane hydroxylase (alk) genes

The presence of known (*alk*) genes was tested by applying three sets of published PCR primers for the detection of *alk* genes: (1) *P. putida alkB* genes derived primers: Pp alkB-for (5′-TGGCCGGCTACTCCGATGATCGGAATCTGG-3′) and Pp alkB-rev (5′-CGCGTGGTGATCCGAGTGCCGCTGAAGGTG-3′) resulting in a 870 bp fragment [29]; (2) primers based on *P. oleovorans* GPO1 *alkB* and *Acinetobacter sp.*ADP1 *alkM*: TS2S (5′-AAYAGAGCTCAYGARYTRGGTCAYAAG-3′) and deg1RE (5′-GTGGAATTCGCRTGRTGRTCIGARTG-3′) resulting in a 557 bp fragment [30]; and (3) primers detecting cytochrome P153 *alk* genes: P450fw1 (5′-GTSGGCGGCAACGACACSAC-3′) and P450rv3 (5′-GCASCGGTGGATGCCGAAGCCRAA-3′) resulting in a 339 bp fragment [15]. The *alk* PCR analysis was performed as described by Smits et al. [15], Whyte et al. [29] and van Beilen et al. [30]. Amplicons obtained from PCR of *alk* genes were sequenced in two directions by using either the forward or reverse primer. The sequencing procedure and BLASTN analysis were conducted as for partial 16S rRNA genes described above. Alignments of resultant sequences with related sequences at the NCBI database were done with the Multalin alignment (http://bioinfo.genotoul.fr/multalin/multalin.html). The TREECON software [31] was used to calculate distance matrices by the Jukes and Cantor algorithm and to generate phylogenetic trees using nearest-neighbor criteria.

All strains, in which known *alk* genes were detected (37 strains), were further tested to determine whether the *alk* genes are localized on a plasmid or on the chromosome. Due to the lack of knowledge regarding the presence of plasmids and their size in the different strains isolated, several plasmid isolation methods were applied as described by Crosa et al. [32], Birnboim and Doly [33], Olsen [34] (and modified for Gram-positive strains by addition of 5 mg mL^{-1} of lysozyme at the lysis step), [35] and by using the Plasmid Midi Kit (Qiagen). Isolated plasmid DNA was checked by electrophoresis on 0.5% (w/v) or 2% (w/v) agarose gels. Contamination of chromosomal DNA in isolated plasmid DNA was examined by conducting 16S rRNA PCR analysis. Contamination of chromosomal DNA was removed by applying Plasmid Safe ATP-dependent DNase (Epicentre Biotechnologies), which efficiently digested chromosomal DNA. Furthermore, the localization of *alk* genes was tested by performing *alk* PCR as described above using pure plasmid DNA as template. Reference strains used as positive controls in plasmid isolation were *Pseudomonas putida* PaW701 (DSMZ 3938, ATCC 12633) containing a 87 kb plasmid, *Bacillus subtilis* subsp. *subtilis* BD170 (DSMZ 10, ATCC 6051) carrying a 7 kb plasmid and *Escherichia coli* V517 carrying 9 different plasmids in range of 2.7–54 kb (obtained from L. Phillips, University of Saskatchewan, Canada).

2.6. PCR-based screening for IncP-1 and IncP-9 plasmids

Isolated plasmid DNA was analyzed by PCR with primers targeting the backbone of IncP-1 [36] and IncP-9 plasmids [37]. PCR amplicons obtained by IncP-1 specific primers (*trfA2*) were confirmed by Southern blot hybridization with a mixed probe generated from IncP-1α (RP4) and IncP-1β (R751).

2.7. Plasmid curing

Plasmid curing was performed by using acridine orange as curing agent with incubation at sub-lethal temperature and daily sub-culturing. The method was a modification of Trevors [38] and Mesas et al. [39]. Ten percent of a dense plasmid-harboring bacterial culture in Luria Bertani (LB) broth was inoculated into LB agar medium supplemented with 200 μg mL^{-1} acridine orange, and grown at 40 °C. The culture was sub-cultured every day into fresh LB agar medium containing 200 μg mL^{-1} acridine orange. On the 5th and 10th day, the sub-culture was diluted and spread onto LB plates as well as on MBM containing 1% filter-sterilized diesel. Single colonies from LB plates were randomly picked and grown

overnight in LB medium. The presence of *alk* genes was checked by colony PCR with *alk*-targeting PCR primers. The native strain was used as positive control and the IGS PCR-RFLP was conducted to assure the chromosomal identity of the cured strain with its parental strain. Cured strains were also compared with parental strains by plasmid detection according to Eckhardt [40].

2.8. Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences determined in this study were deposited in the GenBank database with the accession numbers FJ013273–FJ013353. The nucleotide sequences of partial *alk* genes determined in this study were deposited as well with the accession numbers FJ014891–FJ014897 for CYP153 genes, FJ014898–FJ014906 for Pp-*alkB* genes and FJ014916–FJ014920 for TS2S-*alkB* genes.

3. Results

3.1. Screening of petroleum oil-tolerant plant species

Twenty-six different legume and grass species and varieties were screened for growth on soil contaminated with crude petroleum oil (Table 1). Most plant species showed significant growth reduction, particularly when grown without compost amendment, and few plants (grasspea (*Lathyrus sativus*), cereal rye (*Secale multicaule*), false flax (*Carnelina sativa*), kidney bean (*Phaseolus vulgaris*) and livid amaranth (*Amaranthus lividus*)) were not able to grow at all. Biomass production of some plant species (Birdsfoot trefoil (*Lotus corniculatus*), alfalfa (*Medicago sativa*)) was not affected by the contamination when grown with compost. Italian ryegrass (IT, *Lolium multiflorum* var. Taurus), Birdsfoot trefoil (BT, *L. corniculatus* var. Leo) and the mixture of these two plant species were selected for analysis of plant-associated microbial communities as they demonstrated similar growth in polluted soil compared to that in unpolluted soil and better growth than other plant species in polluted soil.

3.2. Diversity of alkane degrading isolates

Generally, enrichment for 5 days in MBM containing diesel resulted in less culturable bacterial isolates. More strains were isolated by using TSA medium containing diesel oil without enrichment. However, strains, in which *alk* genes were detected, were primarily isolated by using MBM containing diesel oil without enrichment. Isolation of plant-associated bacteria from IT without enrichment resulted in 2.9×10^2 CFU g^{-1} fresh shoot, 4.4×10^2 CFU g^{-1} fresh root and in 10^6 CFU ml^{-1} rhizosphere soil slurry. BT hosted 1.2×10^2 CFU g^{-1} fresh shoot, 3.3×10^2 CFU g^{-1} fresh root and 6.3×10^4 CFU ml^{-1} rhizosphere soil slurry. From IT + BT 87 CFU g^{-1} were isolated from IT fresh shoot and 1.2×10^2 CFU g^{-1} from BT fresh shoot, whereas 3.1×10^2 CFU g^{-1} were isolated from fresh roots and 2.2×10^4 CFU ml^{-1} rhizosphere soil slurry. A total 266 colonies were obtained, which after being tested for their growth capability on minimal basal medium with 2% (v/v) diesel as sole carbon source, decreased to 164 alkane-degrading isolates. The rhizosphere yielded 18 isolates from IT, 9 from BT and 18 from IT + BT. RFLP analysis of the 16S–23S IGS region resulted in 81 different IGS types. From each IGS type one representative isolate was chosen for further analysis (Tables 2–4). Only one strain, MixRH13 (*Pseudomonas anguiliseptica*) (Supplementary information (SI) Table 1), was isolated from the rhizosphere of all three treatments (IT, BT and IT + BT). Only few strains, which were found in IT or BT were found in the IT + BT treatment.

The majority of alkane-degrading isolates was obtained from IT. Most strains isolated from IT, particularly the endophytes, belonged to the *Gammaproteobacteria* comprising mostly *Pseudomonas* and *Pantoea* species (Table 2, SI Table 1, Fig. 1). Besides some *Alpha-* and *Betaproteobacteria* and a *Bacteroidetes* strain, several high G + C (*Rhodococcus, Microbacterium, Arthrobacter*, *Dietzia, Cellulosimicrobium* and *Streptomyces*) and low G + C Gram-positives (*Bacillus, Paenibacillus*) were isolated from the IT rhizosphere. Most strains showed 97–100% similarity to known 16S rRNA genes, whereas several endophyte strains (ITSI32, ITSI67, ITRI66, ITRI59, and ITSI21) were only distantly related to known bacteria (93–96% similarity) and probably represent novel genera within the *Bacteroidetes* group as well as within the *Beta-* and *Gammaproteobacteria* (Table 2, SI Table 1). Only few Gram-positive strains were isolated from the root or shoot interior of IT plants (Fig. 1).

Compared to IT, fewer strains were obtained from BT. Isolates belonged mostly to the *Beta-* (*Achromobacter*, *Alcaligenes*) and *Gammaproteobacteria* (*Pseudomonas*, *Pantoea*) as well as to the low G + C Gram-positives (*Bacillus*, *Paenibacillus*) (Table 3, SI Table 1, Fig. 1). In addition, one *Flavobacterium* strain and one high G + C Gram-positive strain were obtained. Most strains showed 97–100% similarity to known bacteria, only three strains (BTSI16, BTSI34, and BTRH72) had lower similarity values (91–96%).

3.3. Growth on hydrocarbons and diversity of alkane hydroxylase genes

The growth capability test performed with all (81) degrading isolates on different n-alkanes (C_6, C_8, C_{10}, C_{12} and C_{16}) demonstrated that most of the isolates, which could grow, utilized all tested n-alkanes. Exceptions were ITRI24 (*Pseudomonas* sp.), which was only able to grow on short n-alkanes C_6 and C_8.

Although all isolates were able to grow on n-alkanes, only less than the half of all isolates carried detectable *alk* genes. Mostly strains isolated from the IT rhizosphere contained *alk* genes, as revealed by PCR (Fig. 1, Tables 2 and 3). Also, besides hosting a large amount and variety of alkane degraders, in strains isolated from IT various *alk* gene types were detected. The prevailing detectable *alk* gene type was highly homologous to *alkB* encoding alkane-monooxygenase commonly found in *Pseudomonas* strains (detected by using PpalkB and TS2S-deg1RE PCR primer sets). The identical partial *alkB* DNA sequence and closely related homologues were found within and across genera of *Alpha-*, *Beta-* and *Gammaproteobacteria*, and high and low G + C Gram-positives (Figs. 2 and 3). The degenerated TS2S-deg1RE primer was designed to amplify *alkB* genes from Gram-positive and Gram-negative strains [30], but we experienced that the primer only amplified *alkB* genes from Gram-negative strains (Fig. 3). Some cytochrome P153 (CYP153)-like *alk* genes were detected in *Alpha-* and *Gammaproteobacteria* and in low G + C Gram-positive strains isolated from IT and BT. Only distantly related DNA sequences of known CYP153 genes were found among the isolates (Fig. 4).

3.4. Plasmid analysis

Furthermore, we tested whether the *alk* genes detected by PCR were located on a plasmid or on the chromosome. Both plasmid as well as chromosome-located genes were found (Tables 2–4). Even identical *alk* gene fragments were located on a plasmid as well as on the chromosome. No correlation could be detected between the location of the *alk* gene and the phylogeny of the strain or the host plant. PCR analysis of Gram-negative isolates with primers targeting IncP-1 or IncP-9 plasmids revealed that only ITRH1 (*Ochrobactrum*), MixRI74 and MixRI75 (both *Pseudomonas*) and ITSI67 (*Alcaligenes*) carried plasmids belonging to the incompatibility group IncP-1. IncP-9 specific regions were not identified.

To further confirm that *alk* genes are indeed located on plasmids, plasmid curing was performed with few strains that had identi-

Table 2
Bacterial strains isolated from IT in which alkane hydroxylase encoding genes (*alk* genes) were detected by PCR. Rhizosphere strains are termed ITRH, root endophytes ITRI and shoot endophytes ITSI.

IGS type	16S rRNA gene similarity (NCBI accession number /%)	Phylogenetic group	PCR primers for *alk* genes detection	*alk* seq. type	Tentative location of detected *alk* genes	Isolation medium
Rhizosphere						
Proteobacteria						
ITRH1	*Ochrobactrum anthropi* (AB120120/99)	*Alphaproteobacteria*	PpalkB[a]	Ia	Chromosome	Basal
ITRH31	*Pseudoxanthomonas sp.* E16 (AY488509/99)	*Gammaroteobacteria*	CYP450[b]	IIa	Plasmid	Basal
ITRH16	*Pseudomonas* sp. MACL12A (EF198249/99)	*Gammaproteobacteria*	TS2S-degRe1[c]	IIIa	Plasmid	10% TSA
ITRH17	*Pseudomonas* sp. MACL12A (EF198249/99)	*Gammaproteobacteria*	TS2S-degRe1	IIIa	Chromosome	10% TSA
ITRH25	*Pseudomonas alcaligenes* (AY297790/99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal
ITRH76	*Pseudomonas cuatrocienegasensis* (EU791282/100)	*Gammaproteobacteria*	PpalkB	Ib	Chromosome	Basal
ITRI22	*Pseudomonas* sp. BWO (EU006700/99)	*Gammaproteobacteria*	PpalkB	Ie	Chromosome	10% TSA
Gram positives						
ITRH39	*Bacillus* sp. G2DM-19 (DQ416802/99)	Low GC, G+	CYP450	IIb	Chromosome	10% TSA
ITRH43	*Rhodococcus rhodochrous* (AB183422/99)	High GC, G+	PpalkB	Ia	Plasmid	Basal
ITRH47	*Microbacterium lacus* (AB286030/99)	High GC, G+	PpalkB	Ia	Chromosome	Basal
ITRH49	*Arthrobacter oxydans* (EU086791/99)	High GC, G+	PpalkB	Ia	Plasmid	Basal
ITRH56	*Dietzia* sp. BBDP47 (DQ337507/99)	High GC, G+	PpalkB	Ia	Plasmid	Basal
ITRH48	*Arthrobacter tecti* (AJ639829/99)	High GC, G+	PpalkB	Ic	Chromosome	Basal
ITRH51	*Streptomyces sp.* CNQ-023 (EU214930/99)	High GC, G+	PpalkB	Ic	Plasmid	Basal
Root interior						
Proteobacteria						
ITRI4	*Sphingopyxis macrogoltabida* (AB372255/100)	*Alphaproteobacteria*	CYP450	IIc	Plasmid	Basal
ITRI24	*Pseudomonas boreopolis* (AJ864722/100)	*Gammaproteobacteria*	CYP450	IId	Plasmid	Basal
ITRH16	*Pseudomonas* sp. MACL12A (EF198249/99)	*Gammaproteobacteria*	TS2S-degRe1	IIIa	Plasmid	10% TSA
ITRI19	*Pseudomonas* sp. SMCC B0205 (AF500277/100)	*Gammaproteobacteria*	TS2S-degRe1	IIIb	Plasmid	10% TSA
ITRI22	*Pseudomonas* sp. BWO (EU006700/ 99)	*Gammaproteobacteria*	PpalkB	Id	Chromosome	10% TSA
ITRI53	*Pseudomonas anguiliseptica* (AM902193/99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal
ITRI73	*Pseudomonas* sp. 01WB03.3-1 (FM161461/100)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal
Shoot interior						
Proteobacteria						
ITSI70	*Achromobacter xylosoxidans* (DQ659433/99)	*Betaproteobacteria*	CYP450	IIe	Chromosome	10% TSA Enriched
ITSI67	*Alcaligenes xylosoxidans* (FJ883978/94)	*Betaproteobacteria*	TS2S-degRe1	IIId	Plasmid	Basal
ITRH16	*Pseudomonas* sp. MACL12A (EF198249/99)	*Gammaproteobacteria*	TS2S-degRe1	IIIa	Plasmid	10% TSA
ITRI19	*Pseudomonas* sp. SMCC B0205 (AF500277/100)	*Gammaproteobacteria*	TS2S-degRe1	IIIb	Plasmid	10% TSA
ITRI22	*Pseudomonas* sp. BWO (EU006700/99)	*Gammaproteobacteria*	PpalkB	IIId	Chromosome	10% TSA
ITSI32	*Enterobacter* sp. CTSP29 (EU855207/91)	*Gammaproteobacteria*	TS2S-degRe1	IIIc	Plasmid	Basal

[a] Alkane hydroxylase genes were detected by using PCR primers derived from published *Pseudomonas putida* GPO1 *alkB* genes sequence, commonly known as *Pseudomonas oleovorans* ATCC 29347 [11].
[b] Alkane hydroxylase genes were detected by using PCR degenerate primer CYP153 for detecting cytochrome P450 alkane hydroxylase [29].
[c] Alkane hydroxylase genes were detected by using degenerate PCR primers detecting alkane hydroxylase genes in Gram-negative and Gram-positive strains [15].

cal *alkB* gene fragments (ITRH43, ITRI53 and MixRI75). Although the *alkB* genes were no longer detected after plasmid curing, the capacity of cured strains to grow on n-alkanes did not vanish completely, however, the range of degraded alkanes changed (Table 5). This indicates the presence of more than one *alk* gene, which all together were responsible for the observed degradation capacity. The cured strain of ITRH43 (*Rhodococcus*) lost its ability to grow on naphthalene and pyrene as well as on medium chain alkanes ($C_{10,}$ C_{12}, and C_{16}) but still could grow on short chain alkanes (C_6 and C_8). In contrast, the cured strain of ITRI53 (*Pseudomonas*) was no longer able to grow on short alkanes but was able to grow on medium chain alkanes (C_{12} and C_{16}) (Table 5).

4. Discussion

Different plant species are not equally suited for phytoremediation applications, in which they preferably should produce substantial biomass and support the appropriate, degrading microorganisms. Frequently, plants are fertilized with compost to optimize nutrient availability under unfavorable conditions. The plant screening performed in this study revealed that different plant species respond differently to petroleum oil as well as to compost amendment. Italian ryegrass, Birdsfoot trefoil and alfalfa were the best performers in regard to tolerance towards petroleum oil contamination, however, they responded differently to compost amendment suggesting that even under unfavorable conditions the

Table 3
Bacterial strains isolated from BT in which alkane hydroxylase encoding genes (*alk* genes) were detected by PCR. Rhizosphere strains are termed BTRH, root endophytes BTRI and shoot endophytes BTSI.

IGS type	16S rRNA gene similarity (NCBI accession number /%)	Phylogenetic group	PCR primers for *alk* genes detection	*alk* seq. type	Tentative location of detected *alk* genes	Isolation medium
Rhizosphere						
Proteobacteria						
BTRH5	*Achromobacter xylosoxidans* (GQ359326/99)	*Betaproteobacteria*	PpalkB[a]	Ie	Plasmid	Basal enriched
ITRI19	*Pseudomonas* sp. SMCC B0205 (AF500277/100)	*Gammaproteobacteria*	TS2S-degRe1[b]	IIIb	Plasmid	10% TSA
BTRH79	*Pantoea agglomerans* (AY335552/100)	*Gammaproteobacteria*	CYP450[c]	IIf	Plasmid	10% TSA
BTRH11	*Pantoea sp.* iCTE592 (DQ122350/99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal enriched
Gram positives						
BTRH40	*Bacillus macroides* (X70312/99)	Low GC, G+	PpalkB	If	Plasmid	10% TSA
Shoot interior						
Proteobacteria						
ITRI19	*Pseudomonas* sp. SMCC B0205 (AF500277/100)	*Gammaproteobacteria*	TS2S-degRe1	IIIb	Plasmid	10% TSA
Gram positives						
BTSI33	*Bacillus licheniformis* (EU445292/99)	Low GC, G+	CYP450	IIg	Plasmid	Basal enriched

[a] Alkane hydroxylase genes were detected by using PCR primers derived from published *Pseudomonas putida* GPO1 *alkB* genes sequence, commonly known as *Pseudomonas oleovorans* ATCC 29347 [29].
[b] Alkane hydroxylase genes were detected by using degenerate PCR primers detecting alkane hydroxylase genes in Gram-negative and Gram-positive strains [30].
[c] Alkane hydroxylase genes were detected by using PCR degenerate primer CYP153 for detecting cytochrome P450 alkane hydroxylase [16].

Table 4
Bacterial strains isolated from IT+BT in which alkane hydroxylase encoding genes (*alk* genes) were detected by PCR. Rhizosphere strains are termed MixRH and root endophytes MixRI.

IGS Type	16S rRNA gene similarity (NCBI accession number /%)	Phylogenetic Group	PCR primers for *alk* genes detection	*alk* seq. type	Tentative location of detected *alk* genes	Isolation medium
Rhizosphere						
MixRH30	*Enterobacteriaceae bacterium* 58 (AY579163/99)	*Gammaproteobacteria*	PpalkB[a]	If	Plasmid	10% TSA
Root interior						
MixRI74	*Pseudomonas* sp. BWDY-40 (DQ200853/99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal
MixRI75	*Pseudomonas* sp. BWDY-40 (DQ200853/99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal

[a] Alkane hydroxylase genes were detected by using PCR primers derived from published *Pseudomonas putida* GPO1 *alkB* genes sequence, commonly known as *Pseudomonas oleovorans* ATCC 29347 [29].

need of fertilization depends (in addition to the nutrient status of the soil) on the plant species.

The investigated plant species, IT and BT, not only did respond differently to petroleum contamination and compost treatment, but also showed highly different degrading isolates from the rhizosphere and plant endosphere. Italian ryegrass, which tolerated petroleum oil also without compost amendment, but produced less biomass than control plants, hosted higher numbers and a higher variety of culturable, degrading bacteria. Similarly, Siciliano et al. [17] reported that the enrichment of hydrocarbon-degrading bacteria by plants depends, in addition to the type and amount of contaminant, on the plant species. Root exudates and other plant metabolites determine to a great extent which microorganisms colonize the rhizo- or endosphere [41,42]. We can only speculate on the kind of metabolites produced by Italian ryegrass, but they allowed a better interaction with bacteria degrading alkanes. Grasses have been reported to contain alkanes *in planta* [43], which might be associated with the enrichment of alkane-degrading bacteria.

Overall, we observed that only few strains were found in both plants investigated indicating that distinct plant species acted as reservoirs of different degrading populations. Interestingly, in the treatment in which Italian ryegrass and Birdsfoot trefoil were grown together, less strains were isolated, and only three strains were detected carrying *alk* genes. Lower numbers and diversities of degrading isolates in the mixed treatment may be due to com-

Table 5
Hydrocarbon utilization performance of the isolates after plasmid curing, grown on minimal basal medium containing 2% hydrocarbon (Glu, glucose (as control), Die, diesel, Naph, naphthalene, and Pyr, pyrene). The isolates were detected carrying *alkB* genes on plasmid.

IGS	16S rRNA gene homology (NCBI accession number/%)	Plasmid curing	Glu (0.2%)	Die	Naph	Pyr	n-C_6	n-C_8	n-C_{10}	n-C_{12}	n-C_{16}
ITRH43	*Rhodococcus rhodochrous* (AB183422/99)	Before	+	+	+	+	+	+	+	+	+
		After	+	–	–	–	+	+	–	–	–
ITRI53	*Pseudomonas anguiliseptica* (AM902193/99)	Before	+	+	–	–	+	+	+	+	+
		After	+	+	–	–	–	–	–	+	+
MIXRI75	*Pseudomonas sp.* BWDY-40 (DQ200853/99)	Before	+	+	–	–	+	+	+	–	–
		After	+	–	–	–	+	–	–	–	–

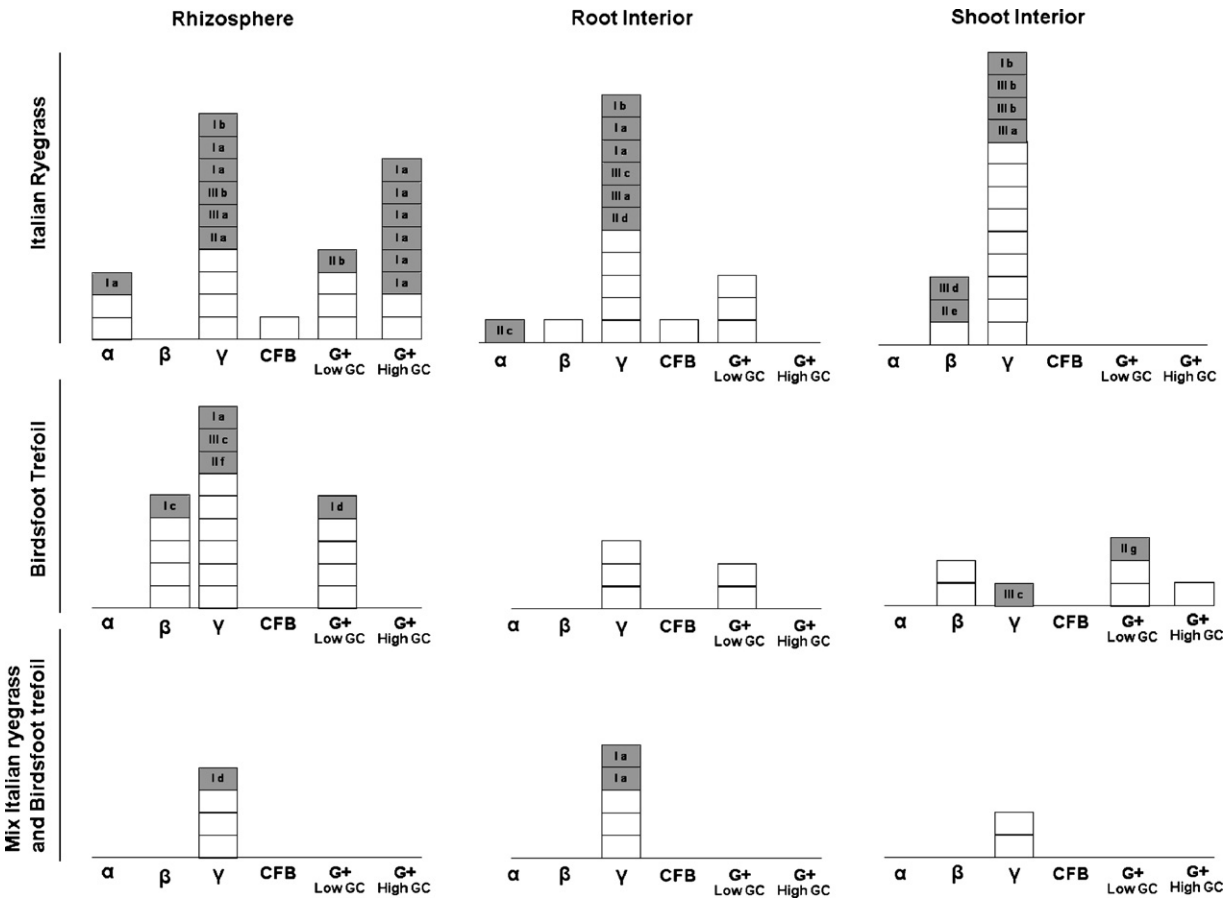

Fig. 1. Distribution of alkane-degrading isolates in relation to the host plant, site of isolation, detectable alkane hydroxylase (*alk* genes) and diversity of *alk* gene homologues. Each box represents one bacterial strain. The empty box means no *alk* genes were detected, whereas shadowed boxes indicate that *alk* genes were detected. The initial (I, II, and III) shows the type of *alk* genes detected using: I (PpalkB primer set), II (CYP450 primer set), and III (TS2S-deg1RE primer set). The same letter following the initial means identical nucleotide sequences of *alk* genes.

petition between plants for space and nutrients, which might have impacted the production of root exudates [44,45].

A high number of different endophyte strains belonging to diverse phylogenetic groups were isolated from Italian ryegrass but not from Birdsfoot trefoil. Production of alkanes by grasses or endophytic fungi as reported by Marseille et al. [43] or uptake of alkanes from soil might explain the comparably high abundance and diversity of alkane degraders within the plant. Mostly, endophytes were different from rhizosphere strains and also roots and shoots hosted distinct culturable, degrading bacterial isolates. Only few strains were found in the rhizosphere, root and shoot interior of selected plants, such as *Pseudomonas* strains ITRH16 and ITRI19. It has been reported that most endophytes originate from the rhizosphere [41], however, the plant apoplast provides different growth conditions and therefore different strains efficiently colonize the plant interior. Endophytes were dominated by *Gammaproteobacteria* and many strains belonged to *Pseudomonas*, a genus, which has been frequently reported to be involved in the degradation of aliphatic hydrocarbons [46].

Known alkane hydroxylases genes were only detected in approximately 50% of the strains, although both enzyme types known for alkane degradation, namely *alkB* and CYP153 genes, were analyzed by using several published PCR primer pairs. This indicates that either only distantly related genes of the same enzyme classes or new enzyme classes might be responsible for the alkane degradation observed. Furthermore, plasmid curing indicated that several strains contained alkane degradation genes in addition to the genes detected by the PCR detection methods used in this study. According to van Beilen and Funhoff [9] the occurrence of multiple alkane hydroxylases with overlapping substrate range is a common phenomenon among alkane degraders. Several degrading strains, particularly endophytes, in which alkane hydroxylase genes could not be identified, showed very low 16S rRNA gene similarity to known species indicating that new species or genera were isolated. Unknown taxa might also host novel alkane degradation genes.

The PpalkB primers are based on the sequence of the *alkB* gene of *Pseudomonas* GPo1 [47], however, our study demonstrated a much wider phylogenetic distribution than previously known. Identical or highly similar sequences of *alkB* genes of that type were found in *Alpha*-, *Beta*- and *Gammaproteobacteria* as well as in low and high G+C Gram-positives. This suggests that these genes might have spread by horizontal gene transfer, which is supported by the finding that many alkane hydroxylase genes were located on a plasmid. Frequently, related genes have been detected on plasmids [14] or within mobile elements in the chromosome [15]. The high number of strains belonging to distinct phyla hosting identical *alkB* gene fragments together with the fact that the genes identified in our study were highly related to those found in *Pseudomonas* strains obtained from various other environments [11,28,47] indicate that this type of *alkB* gene has been prone to horizontal gene transfer. Interestingly, the most abundant *alkB* type (type Ia) was located in some strains on the chromosome (or possibly on a linear plasmid), whereas two-thirds of the strains contained this gene on a (circular) plasmid. However, the broad host range plasmids IncP-1 or IncP-9 were only rarely found. In contrast to the PpalkB-type *alkB* genes,

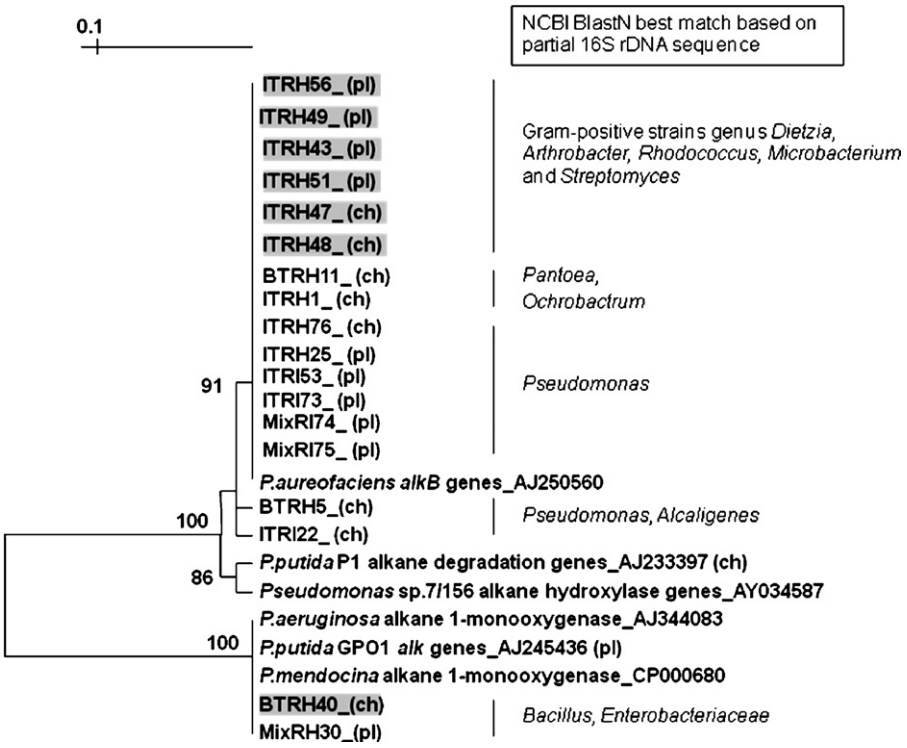

Fig. 2. Neighbor-joining phylogenetic tree of *alkB* genes detected with the PpalkB primer pair and closely related *alkB* gene sequences deposited in the NCBI database. The genus assignment based on 16S rRNA gene analysis is shown on the right side. Gram-positive strains are highlighted in grey. Written in brackets, 'pl' means tentative location of *alkB* genes is on plasmid, while 'ch' indicates a chromosomal location.

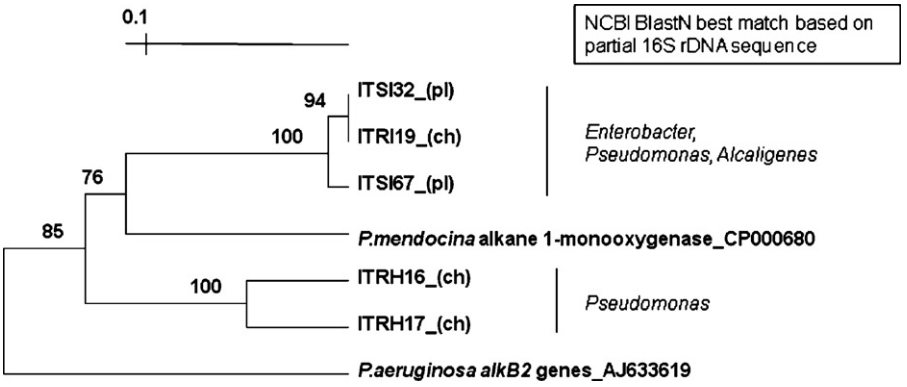

Fig. 3. Neighbour-joining phylogenetic tree of *alkB* genes detected with the TS2S-deg1Re primer pair and closely related *alkB* gene sequences deposited in the NCBI database. The genus assignment based on 16S rRNA gene analysis is shown on the right side. Written in brackets, 'pl' means tentative location of *alkB* genes is on plasmid, while 'ch' indicates a chromosomal location.

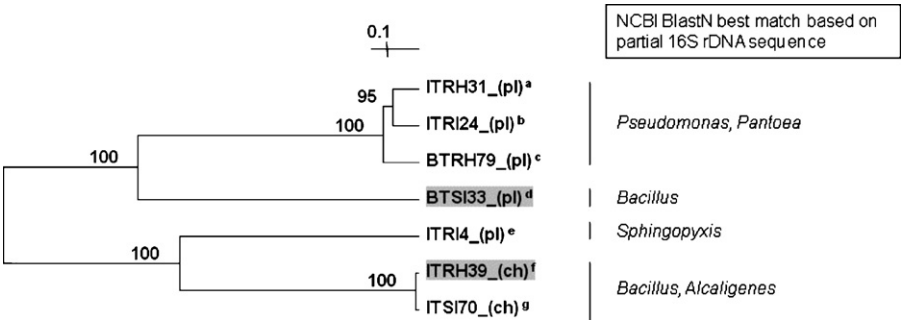

Fig. 4. Neighbour-joining phylogenetic tree of cytochrome P153 alkane hydroxylase genes obtained in this study. The genus assignment based on 16S rRNA gene analysis is shown on the right side. Gram-positive strains are highlighted in grey. Written in brackets, 'pl' means tentative location of *alkB* genes is on plasmid, while 'ch' indicates a chromosomal location.

those detected with the TS2S-deg1Re primer pair were only found in *Proteobacteria* and were only distantly related to other *alkB* genes deposited in the NCBI database as well as to each other. Only one strain (ITSI67) hosted an IncP-1 plasmid combined with a plasmid location of *alkB*. In regard to the occurrence of alkane degradation genes, CYP153-like genes were detected in association with both plants, whereas *alkB* genes amplified with the PpalkB primer pair was mostly found in the rhizosphere and endosphere of Italian ryegrass.

Horizontal gene transfer has been reported to support the biodegradation potential [48,49] by accelerating the development of an efficiently degrading microbial community upon contamination. Efficient horizontal gene transfer of catabolic genes might affect the speed and efficiency of degradation as well as the range of substrates degraded. The fact that most strains containing identical *alkB* gene fragments were isolated from the Italian ryegrass rhizosphere suggests that horizontal gene transfer may have contributed to the comparably high richness of degrading strains. The rhizosphere has been described as a hot spot of horizontal gene transfer [50–52]. It has been reported that the pea rhizosphere is approximately 10 times more conducive to plasmid transfer than barley rhizosphere [53,54], which has been mostly attributed to the higher root exudation in pea leading to higher donor cell concentrations on roots [54]. Similarly, Italian ryegrass may show different root exudation characteristics as compared to Birdsfoot trefoil, which promoted the growth of donor cells. Furthermore, potential exudation of aliphatic hydrocarbons in form of wax [55] and specific co-metabolites for degradation of aliphatic hydrocarbons might have enhanced the selective pressure for degraders [56] or horizontal gene transfer [57]. Alternatively, Italian ryegrass may have been the better host for microbial species such as *Pseudomonas* being highly competent for DNA transfer [58] or microbial strains being highly susceptible to the acquisition of plasmids [59]. The finding of identical *alkB* gene fragments in plasmids and chromosomes indicates that not only plasmid transfer occurred but also transfer of other mobile elements.

In this study we only addressed culturable alkane degraders. Culturable bacteria often represent only a minority of the total microbial community, however, it can be assumed that the recovery rate is higher by using selective conditions as well as by combining isolation procedures. Nevertheless, we are aware that we might have missed degraders that do not easily form colonies on solid nutrient media. On the other hand, our study revealed that by targeting alkane hydroxylase genes directly, the diversity of degraders is not fully addressed, as many strains contain novel or only distantly related degradation genes. Furthermore, by applying a cultivation approach we could observe that very distinct strains contain identical or highly similar alkane hydroxylase genes and thereby obtain information on horizontal gene transfer in different plant environments. Particularly in association with Italian ryegrass we detected a high diversity of alkane degrading bacteria and further analysis will reveal whether, the detected alkane hydroxylase genes are all involved in the degradation of hydrocarbons and to which extent endophytes contribute to the break-down of alkanes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2010.08.067.

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