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Bacterial rhizosphere populations of black poplar and herbal plants to be used for phytoremediation of diesel fuel

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Abstract

Phytoremediation—an environmentally sound method to clean up moderate and superficial site contamination—is based on the stimulation of rhizosphere microorganisms by plant roots. Tolerance of plants to the contamination is a basic criterion for successful phytoremediation. Degradation may be accelerated by inoculation with appropriate microorganisms that are able to break down pollutants and to compete with indigenous microorganisms.

An outdoor pot experiment, in which 16 black poplar clones and four herbal plant varieties were cultivated in soil contaminated with diesel fuel, was performed in order to identify plants with high tolerance to the pollution. The effect of plant variety and diesel contamination on rhizosphere bacterial communities was tested using a 16S rRNA-based cultivation-independent approach to characterize the rhizoflora. To obtain potential inoculant strains, diesel-degrading microbes were isolated from the rhizosphere and analyzed for their ability to utilize *n*-alkanes as carbon sources, the presence of genes encoding alkane hydroxylase (*alkB*) and their abundance in the rhizosphere.

All plant varieties were strongly affected by the diesel contamination, however, black poplar clones exhibited higher sensitivities than herbal plants. One black poplar clone (Brandaris) showed significantly higher tolerance to diesel fuel contamination, whereas the growth of the remaining clones were severely inhibited. Analysis of rhizosphere communities indicated that the diesel contamination had a more pronounced effect than the plant variety. Fourteen diesel degrading strains were isolated from the rhizosphere and identified by 16S rRNA gene sequencing. Taking into account the presence of *alk* B genes, the ability to utilize alkanes as well as the abundance in the rhizosphere, three promising plant/microbe combinations were identified for future phytoremediation applications.

Keywords: Diesel; Phytoremediation; 16S rRNA gene; Terminal restriction fragment length polymorphism; *alk* B

1. Introduction

Phytoremediation has been suggested as a feasible alternative to clean up moderately polluted soils compared to expensive excavation techniques combined with on/off-site treatment or deposition (Frick et al., 1999). Field trials have shown that remediation of petroleum contaminated sites can be enhanced by cultivation of plants (Qiu et al., 1997; Carman et al., 1998; Flathman and Lanza, 1998; Banks et al., 1999). To date, a great variety of grass species, legumes (Aprill and Sims, 1990; Flathman and Lanza, 1998; Pradhan et al., 1998; Banks et al., 1999; Kulakow et al., 2000) and fast growing trees with high transpiration rates such as poplar (Jordahl et al., 1997), alder or willow (Carman et al., 1998; Hübner and Tischer, 1999) have been applied for phytoremediation. These plants provide large surface areas for root–soil contact due to their expansive root systems. Roots provide ideal attachment sites for microbes and a food supply of exudates consisting of amino acids and organic acids, sugars, enzymes, and complex carbohydrates (Shim et al., 2000). Several studies demonstrated the stimulating effect of the rhizosphere on hydrocarbon degrading bacteria. Jordahl et al. (1997) reported that populations of benzene-, toluene- and xylene degrading microbes were five times more abundant in the rhizosphere of poplar trees than in the surrounding soil. Degraders of hexadecane, benzoic acid, pyrene and phenanthrene proved to concentrate in the rhizosphere of alfalfa and alpine bluegrass as compared to the bulk soil (Nichols et al., 1997). Likewise, Radwan et al. (1998) found that roots of several desert plants were densely

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associated with hydrocarbon degraders. A broad phylogenetic range of bacteria including species/strains of *Achromobacter*, *Acidovorax*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus,* *Sphingomonas* and *Xanthomonas* have been identified to participate in the breakdown of hydrocarbons (Bossert and Bartha, 1984; Radwan et al., 1998; Riser-Roberts, 1998; Fritsche and Hofrichter, 1999).

Diesel fuel—a common contaminant—has a high content of alkanes between C_8 and C_{26} (Adam and Duncan, 1999). It is a pollutant comparatively easy to degrade, as alkanes between C_{10} and C_{25} are in general most suitable to microbial attack (Atlas and Bartha, 1993). Nevertheless, compared to other medium distillate fuels, it has the highest content of environmentally persistent PAHs and total aromatics (Wang et al., 1990).

Sotsky et al. (1994) reported that most culturable soil bacteria (gram-positives and gram-negatives) occurring at hydrocarbon polluted environments contain genes encoding oxygenases. These enzymes, including alkane hydroxylase (encoded by *alk*B), the prototype of non haem, iron integral membrane monooxygenases, are essential for monoterminal oxidation—the main pathway of alkane degradation. This reaction proceeds aerobically via the formation of the corresponding alcohol, aldehyde and fatty acid. Analysis of these genes seems to be an appropriate method to predict the potential function of microbes.

The aims of the present study were to investigate the effect of diesel fuel on plant growth and on the rhizosphere microflora in order to identify suitable plant/microbe combinations to be used for phytoremediation of diesel fuel. Sixteen black poplar (*Populus nigra*) clones and four herbal plant variants were tested for their tolerance of diesel fuel. Rhizosphere microbial population structures of best performing plants were further analysed. As only a minority of naturally occurring bacteria are culturable (Amann et al., 1995), a cultivation-independent approach was used in order to identify dominant rhizosphere community members. In parallel, hydrocarbon-degrading strains were isolated and characterized regarding their phylogeny, the presence of *alk*B genes as well as their abundance in the rhizopshere.

2. Materials and methods

2.1. Experimental setup and plant development

Diesel fuel contaminated substrate was prepared by spiking agricultural soil (pH 7.4, 27 g sand kg^{-1}, 621 g silt kg^{-1}, 352 g clay kg^{-1}) with 5, 10 and 25 g diesel fuel kg^{-1} fresh soil. Pots (diameter: 20 cm, height: 22.3 cm) were filled with either 9.1 kg (equivalent to 7.5 kg dry matter) of spiked or uncontaminated control soil. Spiking was done by thoroughly mixing the appropriate amount of diesel fuel (47, 95 and 240 ml, respectively) with fresh soil designated for one pot. Pots were subsequently placed outdoors, and to avoid leakage a plastic film was placed underneath containers. Pots containing diesel-spiked soil and uncontaminated soil were arranged in a completely randomized block design. In April two rootless cuttings of each of the 16 black poplar clones (Brandaris, L51, L55, L69, E1, E11, T5, T10, T39, T97, T170, T171, T179, T182, T188, T191) were planted and later thinned to one plant. During dry periods plants were manually watered, but water supply was kept to a minimum in order to prevent leaching. Except one commercial clone (*Populus nigra* cv. Brandaris) that was bought from a nursery (Vermeeringsuin Zeewolde, NL), clones were collected from native Austrian populations. Clones came from Lobau (L), Eferding (E) and from a clone collection of the Austrian Federal Forest Research Centre (T). In addition to black poplar the following herbal plants were sown into the same pots: 2 g annual ryegrass (*Lolium multiflorum* cv. Lolita), 2 g alfalfa (*Medicago sativa* cv. Europ) and a combination of both species (1 g each). Furthermore, ryegrass (2 g) was cultivated and supplemented with compost (9.7 g dry matter kg^{-1} soil, according to 10 ton compost dry matter ha^{-1}). Three replicates of each plant treatment and contamintion level were prepared.

Plants were harvested in October after one growth period, biomass and length of roots and shoots were determined. Significant differences between means were calculated using a commercial statistics software (STATISTICA 6.0, Statsoft, Inc, Tulsa, USA).

Rhizosphere soils from E11, L55, T179, Brandaris, compost + ryegrass and alfalfa + ryegrass grown in soil spiked with 10 g diesel oil kg^{-1} and in control soil were sampled for subsequent characterization of microbial shifts caused by the contaminant and for the isolation of diesel degrading strains. These plant variants were chosen because of greater biomass production and lower injury level in the presence of the contaminant.

2.2. DNA isolation

DNA was isolated from rhizosphere samples (0.3 g of lyophilized soil) according to a protocol described by Miskin et al. (1999), in which DNA was extracted by polyethyleneglycol. Humic acids were bound to potassium acetate. Finally, DNA was precipitated with ethanol and resuspended in TE (10 mM Tris–HCl, pH 8). For further purification, spin-columns were prepared containing sepharose CL-6B (Pharmacia) and polyvinylpyrrolidone (20 mg ml^{-1} CL-6B). In most cases, passage through two columns was needed to remove all PCR-inhibiting substances.

2.3. Terminal restriction fragment length polymorphism (T-RFLP) analysis

The eubacterial primers 8f (Weisburg et al., 1991)

labeled at the 5′ end with 6-carboxyfluorescein (6-Fam, MWG) and 1406r (Suzuki and Giovannoni, 1996) were used to amplify 16S rRNA genes. PCR reactions were carried out with a PTC-100™ thermocycler (MJ Research, Inc.) applying an initial denaturation step of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 1 min annealing at 54 °C and 2 min extension at 72 °C. PCR reactions (50 μl) contained 1 × reaction buffer (Gibco, BRL), 200 μM each dATP, dCTP, dGTP and dTTP, 0.15 μM of each primer, 3 mM $MgCl_2$, 2.5 U Taq DNA polymerase (Gibco, BRL) and 20 ng template DNA. Three independent PCR reactions were performed on each sample, which were subsequently pooled. T-RFLP analysis using AluI (Gibco, BRL) as restriction enzyme was performed and representative sample profiles were determined as described by Sessitsch et al. (2001). Cluster analysis was performed by STATISTICA software (Complete Linkage, 1-Pearsons r).

2.4. Enrichment and isolation of hydrocarbon degraders

Fresh soil (10 g) and sterile sand (5 g) were added to 50 ml of a 0.23% NaCl solution containing 10 μl SDS (20%). Soil suspensions were shaken for 40 min at 180 rpm and 50 μl of the supernatant was transferred to 5 ml minimal medium (Alef, 1994) containing 10 μl filter-sterilized diesel ml^{-1}. After incubation for 10 days with constant shaking at 28 °C, aliquots (100 μl) of 10-fold dilutions were spread onto plates containing the same medium solidified with 15 g l^{-1} agar noble (Difco, USA). After 10 days incubation at 28 °C 16 colonies were randomly picked resulting in 324 isolates.

2.5. Characterization of isolates by PCR-RFLP and sequence analysis of 16S rRNA genes

Isolates were characterized by 16S rRNA analysis in order to distinguish different phylotypes. 16S rRNA genes were amplified as described above using the primers 8f and 1406r and digested individually with the enzymes *AluI* and *Hae*III. Isolates with identical restriction profiles were grouped and a representative isolate was chosen for further analysis. For taxonomic identification 16S rRNA genes were partially sequenced. Partial 16S rRNA genes were amplified by PCR from genomic DNA applying primers 8f and 1406r under the same conditions as described above. PCR products were purified using the NucleoTraPCR kit (Macheroy-Nagel) according to the manufacturer's instructions and used as template in sequencing reactions using the primer 518r (Liu et al., 1997). DNA sequencing was performed using an ABI 373A automated DNA sequencer (PE Applied Biosystems Inc., Faster City, CA, USA) and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin–Elmer). Sequences were subjected to a BLAST analysis (Altschul et al., 1990) with the National Center for Biotechnology Information database and were compared with sequences available in the ribosomal database project (RDP) (Maidak et al., 1997).

2.6. Alkane degradation and PCR detection of alkane hydroxylases

Hydrocarbon-degrading potential was determined with liquid cultures containing 'Bath' minimal medium (Bodrossy et al., 1999) and 5 μl l^{-1} of either n-octane, *n*-dodecane, *n*-hexadecane or *n*-octadecane (Merck, Darmstadt) as the sole carbon source. Control variants were prepared using the same minimal medium containing glucose and sucrose for carbon provision. Cultures were incubated for 6 days and constant shaking at 28 °C.

The presence of *alk* B genes was determined by PCR as described previously (Smits et al., 1999) using the degenerate primer pairs TS2S/deg1RE and TS2Smod2/deg1RE2. PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen) and ligated into the pGEM-T plasmid (Promega). *Escherichia coli* DH5α competent cells were transformed with ligation products, and insert sequences were amplified by PCR using the primers M13for and M13rev under the conditions described above. Purified PCR products were used as template in sequencing reactions applying M13for as sequencing primer. Sequences were subjected to a BLAST analysis (Altschul et al., 1990) with the National Center for Biotechnology Information database.

2.7. Nucleotide sequence accession numbers

The sequences obtained in this study have been deposited in GenBank under accession numbers AF451840–AF451853 (partial eubacterial 16S rDNA sequences) and AF451153–AF451155 (*alk B* PCR products).

3. Results

3.1. Plant development

Black poplar clones did not survive on substrate spiked with 25 g diesel kg^{-1} soil, with the exception of Brandaris, E11 and T97, which were severely stunted, thus this contamination level is not considered in the further discussion of poplar development. At lower contamination levels, Brandaris and L55 performed best. Brandaris and L55 showed highest shoot and root dry weights at all contamination levels (Table 1). Brandaris had a shoot dry weight of 13.01 g on control soil, 4.02 g on 5 g diesel kg^{-1} soil and 3.78 g on 10 g diesel kg^{-1} soil, whereas L55 produced 12.08 g shoot dry weight on control soil, 2.12 g on 5 g diesel kg^{-1} soil and 1.13 g on 10 g diesel kg^{-1} soil. Similar observations were made with data on root biomass (data not shown). Although Brandaris had the highest biomass production in uncontaminated soil, shoot and root dry weights

Table 1
Shoot biomass of 5 month old poplar clones and herbal plants grown on control and diesel contaminated soil

Shoot biomass (g)				
	Control	5 g kg^{-1}	10 g kg^{-1}	25 g kg^{-1}
Poplar clone				
Brandaris[a]	13.01 ± 3.54[b]	4.02 ± 1.8*[c]	3.78 ± 0.61*	
L55	12.08 ± 3.71	2.12 ± 0.95**	1.13 ± 0.87**	
E11	10.18 ± 3.07	0.69 ± 0.62**	0.33 ± 0.15**	
T179	1.31 ± 0.53	1.81 ± 0.73	0.23 ± 0.13*	
L51	5.64 ± 1.5	0.44 ± 0.38***	0.31 ± 0.16***	
L69	10.56 ± 0.64	0.84 ± 0.61***	0.53 ± 0.01***[a]	
E1	1.76 ± 0.49	NG[d]	0.53 ± 0.37	
T188	8.71 ± 0.85	1.55 ± 0.4***	NG	
T182	7.96 ± 1.2	1.31 ± 0.49*	NG	
T171	NG	0.30	NG	
T170	4.34 ± 2.9	1.97 ± 0.13	0.24 ± 0.11	
T97	NG	1.94 ± 1.03	0.21 ± 0.25	
T39	2.51 ± 1.98	1.06 ± 0.21	0.26	
T10	0.81	0.94 ± 0.7	0.67 ± 0.2	
T9	4.11 ± 1.27	0.31 ± 0.28	0.6	
Herbal plant				
Comp + ryegrass	5.40 ± 22.49	3.28 ± 0.33	2.52 ± 0.09*	0.83 ± 0.46**
Alfalfa + ryegrass	6.11 ± 1.26	8.48 ± 0.62*	4.01 ± 11.3*	0.64 ± 0.09***
Ryegrass	4.72 ± 1.1	2.97 ± 0.75*	2.11 ± 0.75**	0.37 ± 0.21***
Alfalfa	5.41 ± 0.87	9.38 ± 2.2	4.23 ± 2.99	NG

[a] Variants that have been chosen for microbial investigations are underscored.
[b] Mean value ± SD.
[c] Asterisks indicate significant differences between control and diesel contaminated soil calculated by the LSD test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
[d] NG, no growth observed.

were not significantly different ($p < 0.05$) from most clones (L55, E11, L69, L51, E1, T188, T182, T39, T9). Brandaris showed a reduction of its biomass due to diesel contamination (on 5 g diesel kg^{-1} 69% (shoot) and 74% (root) and on 10 g diesel kg^{-1} 71% (root) and 83% (root), respectively), whereas other clones were significantly stunted by the lowest contamination level (L55, L69, E11 and T188) or were even killed by 10 g diesel kg^{-1} soil (T182 and T188). Nevertheless, all clones showed vital growth in uncontaminated soil. After cultivation in soil spiked with 10 g diesel kg^{-1} soil Brandaris produced significantly more shoot and root biomass than all other clones.

After 2 months of growth, biomass of all grass/legume variants was significantly reduced ($p < 0.01$) due to the presence of diesel, even at the lowest contamination level. However, after three further months biomass production was only slightly inhibited (Table 1). Interestingly, alfalfa as well as alfalfa combined with ryegrass produced more biomass when cultivated on 5 g diesel kg^{-1} soil compared to the control soil.

Diesel fuel content after harvest ranged from 162 to 973 mg kg^{-1} in soil spiked with 5 g diesel kg^{-1}, from 1850 to 4583 mg kg^{-1} in soil spiked with 10 g diesel kg^{-1} and from 9600 to 13330 mg kg^{-1} in soil spiked 25 g diesel kg^{-1}.

3.2. Rhizosphere microbial communities

According to the physiological development and injury level of the plants, six variants (Brandaris, L55, T179, E11, alfalfa + ryegrass and compost + ryegrass) were chosen for microbiological analysis. Rhizosphere bacterial communities found in soil spiked with 10 g diesel fuel kg^{-1} soil were compared to those present in control soil. A total of 31 terminal restriction fragments (T-RFs) was detected by T-RFLP analysis. The total number of T-RFs found in representative sample profiles was 22 (Table 2), as some T-RFs were only present in individual profiles and/or disappeared due to the normalization procedure. The number of T-RFs in control soils ranged from 6 (T179) to 11 (compost + ryegrass), whereas spiked soils showed 3 (L55) to 8 (alfalfa + ryegrass) (Table 2). In general, contamination with diesel fuel caused a decreased number of dominant species. However, the combination of alfalfa and ryegrass contained comparable numbers of T-RFs in both treatments.

Control soils and soils spiked with diesel showed different microbial population structures. Some bacteria disappeared or decreased their abundance, whereas others were enriched in the presence of the pollutant. Fragments that were found in most control soils and were inhibited by the contaminant included sizes of 226, 246 and 250 bp. A T-RF of 243 bp disappeared in all diesel containing rhizospheres, except that of Brandaris and T179.In association with the poplar clone T179 the 243 bp fragment showed increased abundance as compared to the control treatment. A T-RF of 208 bp was detected in the rhizospheres of all

Table 2
Comparison of representative T-RFLP profiles of bacterial communities in control (C) and diesel contaminated (DF) soils and rhizospheres

T-RF (bp ± 1)	61	**69**[a]	72	**112**	**122**	**155**	179	**205**	208	216	218
Variant											
C-unplanted			□				□		□		
DF-unplanted			□			■			□		
C-L55			□				□	□	□		
DF-L55						□		□			
C-E11	□								□		□
DF-E11						□		□	□		
C-Brandaris									□	□	
DF-Brandaris						□			□		
C-T179									□		
DF-T179									□		
C-alfalfa + ryegrass	□								□		□
DF-alfalfa + ryegrass		□		□	□	□			□		
C-comp + ryegrass			□			□			□		
DF-comp + ryegrass					□	■			□		

T-RF (bp ± 11)	**224**	226	**233**	240	243	246	248	250	**275**	**282**	**310**
Variant											
C-unplanted		□	□		□	□	□	□			
DF-unplanted	□		■						□		
C-L55			■	□	□	□		□			
DF-L55			■								
C-E11		□	■		□	□		□			
DF-E11			■						□		
C-Brandaris		□	□	□	□		□	□			
DF-Brandaris			■	□	□			□	□		
C-T179		□	□		□	□		□			
DF-T179			■		□	□		□			
C-alfalfa + ryegrass		□	□		□	□		□			
DF-alfalfa + ryegrass			□						□	□	
C-comp + ryegrass	□		□	□	□		□	□	□	□	
DF-comp + ryegrass			■						□	□	□

□ 50–250 fluorescence units; ■ >250 fluorescence units; others denote < 50 fluorescence units.

[a] TR-Fs which showed increasing fluorescence units in the DF variant compared to the corresponding control or occurred exclusively in the polluted variant are indicated in bold letters and are underscored.

plants with similar intensities. Several fragments showed highly increased abundances in soils spiked with diesel such as 69, 112, 122, 155, 205, 224, 233, 243, 275, 282 and 310 bp (Table 2).

Some T-RFs were specifically found in the rhizospheres of particular plants, however, most specific fragments were exclusively present in control soils. Several plant-specific T-RFs induced by diesel fuel were detected in the rhizospheres of alfalfa and ryegrass including fragments of 69, 112, 122, 282 and 310 bp.

A cluster analysis demonstrated the effect of diesel fuel on the soil and rhizosphere microflora (Fig. 1). Two clusters were found comprising microbial populations of control and diesel treatments. The only exception was T179 grown in the presence of diesel fuel that clustered with control treatments.

3.3. Diesel degrading isolates

In parallel to a cultivation-independent community analysis, 14 representative isolates were obtained that grew on diesel as sole carbon source and showed distinct 16S rDNA RFLP profiles. The bacteria obtained as well as their closest relatives found in the NCBI database are shown in Table 3. Most isolates belonged to the β- and γ-Proteobacteria, whereas one isolate (dO) showed 98% similarity to *Clavibacter michiganensis* and could be classified as low G + C gram-positive. The population profiles of bacteria culturable on diesel as sole carbon source were highly different in various treatments (Fig. 2). The most widespread phylotype dA (*Pseudomonas putida*) occurred in unplanted soil as well as in the rhizospheres of all plants except poplar clone L55. Furthermore, the phylotypes dC, dD and dO were only found in control soils, whereas the phylotypes dP, dR, dS, dT and dU were exclusively detected in diesel polluted soils. Six isolates (dC, dP, dQ, dR, dT, dU) possessed theoretical T-RFs corresponding to T-RFs of community fingerprints (155 and 233 bp), which showed increased abundances in most diesel containing soils (Table 3).

Five strains (dM, dN, dR, dS and dU) did not utilize any

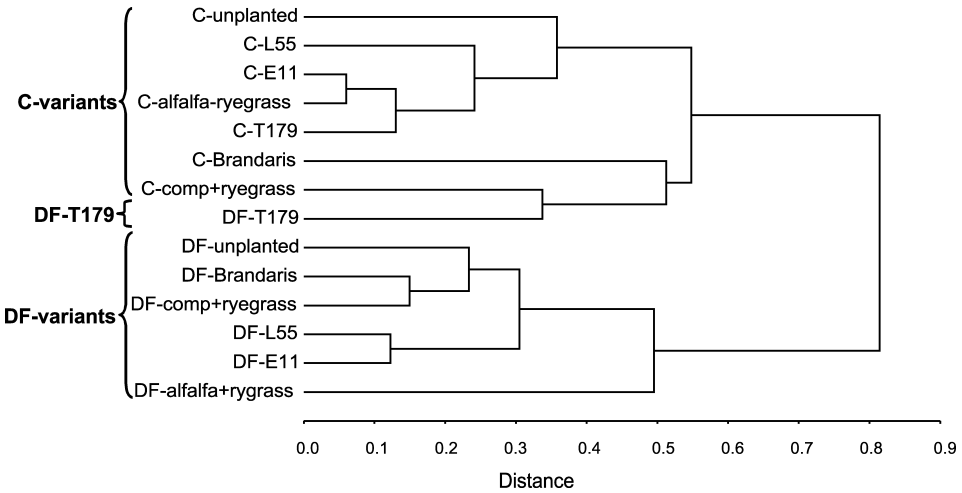

Fig. 1. Cluster analysis of rhizosphere communities of plants growing on soil spiked with 10 g diesel fuel kg^{-1} soil (DF-plant) and the uncontaminated control soil (C-plant) based on normalized T-RFLP data.

of the tested alkanes as sole carbon source (Table 3), although they were able to grow on diesel. Some strains such as isolate dA, dG and dO could utilize all alkanes in the range of C_8 to C_{18} with strain dO showing the highest growth rate, whereas others (dC, dD, dK, dP, dQ, dT) were only able to grow on a limited number of alkanes. Most alkane metabolizing strains showed a PCR product of 550 bp using degenerate *alk B* PCR primers (Table 3). No

Table 3
Diesel degrading isolates obtained from rhizosphere soils, their theoretical T-RF sizes, alkane utilization and *alk* B amplification

Isolate	Closest relative[a] (NCBI database)[b]	Percent similarity	Theor. T-RF size (bp)[c]	Corresp. T-RF in soil profiles (bp)	Utilization of alkanes[d]				*alk* B PCR product [e]
					C_8	C_{12}	C_{16}	C_{18}	
A	*Pseudomonas putida* str. ATCC 17514 (AF094741)	99	72	72	+	+	+	+	+[g]
C[f]	*Achromobacter xylosoxidans sbsp. xylosoxidans Cm*4 (AF302097)	**99**	**156**	**155**	−	+	+	+	−
D	*Pseudomonas putida* (AF291048)	99	72	72	−	−	−	+	+[g]
G	*Xanthomonas melonis* (Y10756)	99	240	240	+	+	+	+	+
K	*S. maltophilia str.c* 20 (AJ293469)	98	73	72	−	+	−	−	+
M	*Xanthomonas melonis* (Y10756)	99	241	240	−	−	−	−	−
N	*Zoogloea* sp.GOBB3-C110 (AF321021)	99	71	72	−	−	−	−	−
O	*Clavibacter michiganensis* str. *JCM* 3345 (D45059)	98	73	72	+	+	+	+	+[g]
P	*Acinetobacter* sp.(U37348)	**99**	**232**	**233**	−	−	−	+	−
Q	*Acinetobacter xylosoxidans* (AF302096)	**99**	**235**	**233**	+	−	+	+	+
R	*Pseudomonas fluorescens* F113 (AF375844)	**99**	**234**	**233**	−	−	−	−	−
S	*Variovorx paradoxus* str. Bm 2 (AF288737)	100	150		−	−	−	−	−
T	*Alcaligenes sp*. isolate 151 (AJ0022802)	**99**	**156**	**155**	−	+	+	+	+
U	*Alcaligenes* sp. isolate 151 (AJ002802)	**98**	**156**	**155**	−	−	−	−	−

[a] The closest relative was determined by BLAST analysis and is based on app. 500 bp of the 16S rRNA gene sequence.
[b] Accession numbers of closest database matches are given in parentheses.
[c] Theoretical T-RFs were calculated using the 16S rRNA sequences and *Alu* I.
[d] Abbreviations: C_8, *n*-octane, C_{12}, *n*-dodecane, C_{16}, *n*-hexadecane, C_{18}, *n*-octadecane.
[e] PCR products of the appropriate length of app. 550 bp were obtained applying the primers TS2S and deg1RE.
[f] Bold letters indicate isolates, which show theoretical T-RFs corresponding with T-RFs in community fingerprints that were specifically induced by diesel.
[g] PCR products which showed high sequence homologies to either alkane-1-monooxygenase or putative xylene monooxygenase genes deposited in the NCBI database.

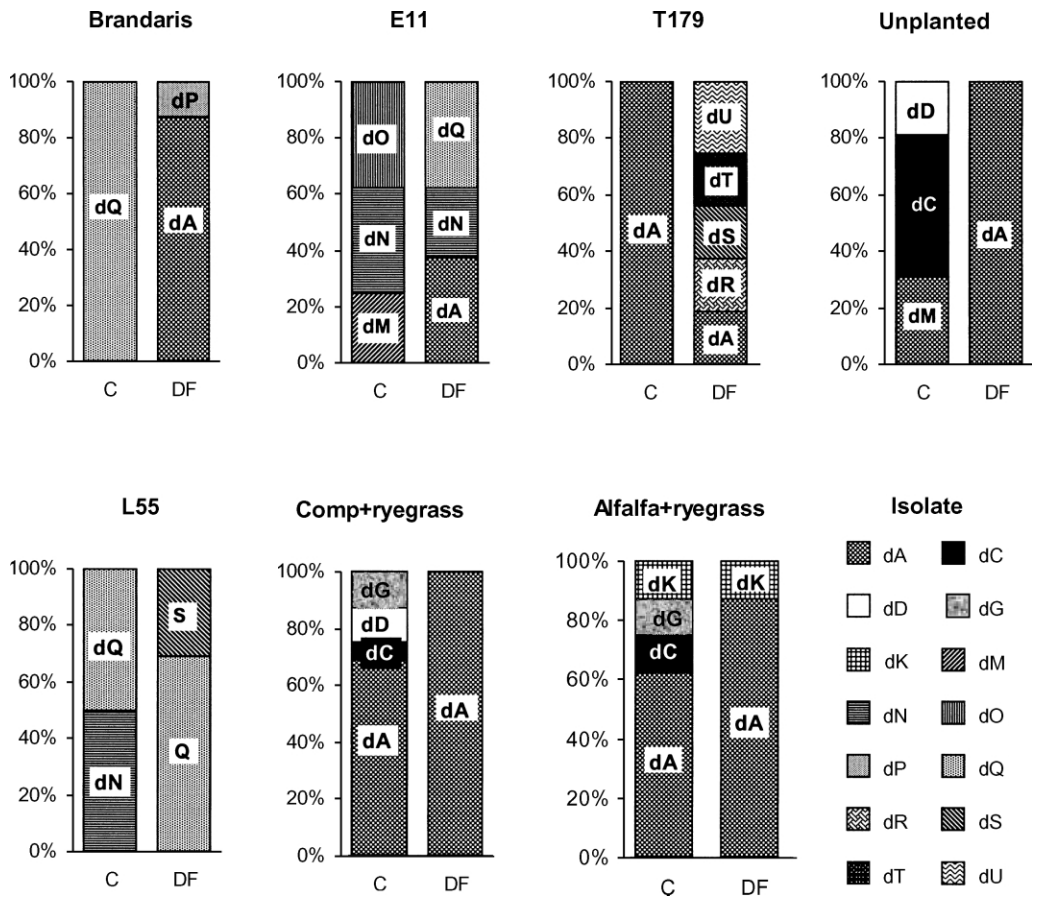

Fig. 2. Distribution of diesel degrading isolates found by RFLP analysis of 16S rRNA genes of isolated strains in control (C) and diesel contaminated (DF) soils.

PCR products were obtained with strains dC, dP and dQ, although utilization of alkanes was observed. Sequence analysis revealed that PCR products of dA, dD and dO showed protein sequence identities of 95–98% to either alkane-1-monooxygenase or putative xylene monooxygenase and DNA sequence identities of 88–99%. The remaining sequences did not show any homology to NCBI database entries.

4. Discussion

Various plants, together with their associated microflora, have been reported to degrade petroleum hydrocarbons from contaminated soils (Carman et al., 1998; Flathman and lanza, 1998; Pradhan et al., 1998; Banks et al., 1999). Grasses have high phytoremediation potential due to their excessive, fibrous root systems and their competitive advantage to become established under unfavourable conditions (Aprill and Sims, 1990; Qiu et al., 1997; Banks et al., 1999; Kuiper et al., 2001). In addition, legumes are of high interest because of their ability to fix atmospheric nitrogen and a variety of legumes have been found to grow naturally on oil-contaminated sites (Gudin and Syratt, 1975). Poplar is an interesting plant for phytoremediation because of its rapid growth (3–5 m a^{-1}), the possibility to proliferate it from cuttings as well as to regrow plants from stumps after harvest and a high transpiration rate (up to 180 l d^{-1} per plant) (Shim et al., 2000). As poplar trees are phreatophytes their roots can withdraw water from depths up to 3 m (Schnoor et al., 1995).

Varying degrees of growth reduction have been observed for plants commonly used for phytoremediation of hydrocarbon contaminated soils (Kulakow et al., 2000). Our study confirmed these findings and furthermore showed that not only the plant species but even different clones of black poplar were differently affected by mineral oil hydrocarbons. However, the application of compost as a fertilizer had a beneficial effect, particularly at early plant growth stages, which may be explained by the addition of nutrients and microorganisms that participated in the breakdown of pollutants.

Kulakow et al. (2000) reported a 77% reduction of ryegrass biomass after 30 days growth on soil contaminated with 25 g petroleum hydrocarbons kg^{-1}. In our experiment ryegrass was much more stunted under comparable conditions (96% reduction in biomass). Even 5 g diesel kg^{-1} soil led to a biomass reduction of 82%.

Although herbal plant variants were less affected (biomass reduction of 85–92%) than poplar clones (reduction 95–100%), 25 g diesel kg^{-1} soil seems to be the critical contamination level for cultivation of these plants. This may be due to the use of freshly spiked soil, which was reported to be more toxic to plants and microorganisms than aged contaminations (Bäk and Krömer, 1997), where pollutants are in general adsorbed to a higher degree on soil particles and therefore less available for degradation.

Diesel had a strong effect on rhizosphere microbial communities. The plant species and also the plant clone affected the rhizosphere microflora, which is in accordance to Grayston et al. (1998) and Smalla et al. (2001), however, to a lower extent than the pollutant. Similar findings were also reported by Siciliano et al. (2001), who found that the kind and amount of a certain contaminant had a greater impact on the occurrence of catabolic rhizosphere bacteria as well as endophytes than the plant species. Nevertheless, we could detect clone-specific differences. In general, the rhizospheres of the four poplar clones analysed were colonized by different microbial populations, which had a high degree of similarity when exposed to diesel fuel. Interestingly, poplar clone T179 showed comparably bad growth on diesel and possessed rhizosphere communities similar to those found in non-contaminated rhizospheres. Possibly, the associated microflora of this variety was not involved in diesel degradation and did therefore not reduce the stress exposed onto the plant. Diesel pollution caused a decreased number of highly dominant bacterial species in the rhizosphere. Clone T179 with its poor growth performance on diesel showed the lowest number of induced bacterial species, whereas the alfalfa and ryegrass mixture possessed a particularly high number of microbes specifically detected in the presence of the contaminant. Various bacteria were not able to persist in the presence of the pollutant, and perhaps were outcompeted by the newly formed populations. Alternatively, this may be a consequence of the toxicity of *n*-alkanes, iso-alkanes, cycloalkanes and aromatic hydrocarbons with carbon numbers below 10, due to their penetration into cell membranes (Hornick et al., 1983; Atlas and Bartha, 1993; Bäk and Krömer, 1997).

During phytoremediation, both, plants and microorganisms, take part in the degradation process, either independently or through synergistic effects. Microorganisms possess a huge metabolic potential and have furthermore the ability to adapt to new environmental conditions (Bollag et al., 1994). Inoculation of contaminated sites with degrading microbes is a promising strategy to improve bioremediation processes. However, frequently inoculant strains are rapidly outcompeted by the natural microflora (Cunningham et al., 1996). The identification and application of microbial strains adapted to a particular environment may overcome this limitation. We were therefore interested in identifying bacteria with the ability to degrade *n*-alkanes as well as to compete well in the rhizosphere with the indigenous microflora. Therefore, strains were isolated from the rhizosphere and characterized regarding their diesel-degrading potential and abundance in the rhizosphere. The majority of isolates were able to degrade alkanes. Isolates that did not show growth on alkanes (dM, dN, dR, dS and dU) were possibly involved in the degradation of other diesel components such as aromatics and/or PAHs. Amplification of *alk*B genes corresponded well with alkane utilization. The degenerate primers developed by Smits et al. (1999) could be employed to detect *alk*B genes in phylogenetically different genera. However, several sequences that were amplified by these primers did not show homologies to known genes indicating that further improvement of primers is necessary. Isolates were identified by 16S rRNA gene sequencing. Sequences showed 98–100% similarity to 16S rRNA genes deposited in the NCBI database and some of the closely related organisms were also obtained from sites polluted with organochemicals. *Stenotrophomonas maltophilia* strain c20 that showed highest homology to isolate dK was found in a PAH contaminated site in Melbourne/Australia. As dK showed weak utilization of only dodecane and no *alk* B PCR product was obtained, this strain may be involved in the degradation of the aromatic rather than of the alkane fraction of diesel fuel. Similarly, isolate dP, an *Acinetobacter* sp., did not grow well on *n*-alkanes and its closest relative was isolated from a soil contaminated with PAHs (Müller et al., 1997). Three isolates (dC, dQ and dU) proved to be members of the genus *Achromobacter*, however, they showed different patterns of alkane utilization. Strains of the genus *Achromobacter* have been found previously in soils contaminated with hydrocarbons in dense association with the crop plants *Vicia faba* and *Lupinus albus* (Radwan et al., 1998). Furthermore, the remaining isolates belong to species that have already been described in connection with hazardous organic pollutants (Unz and Farrah, 1972; Hanson et al., 1997; Rooney-Varga et al., 1999; Cavalca et al., 2000; Belimov et al., 2001; Kuiper et al., 2001; Seffernick et al., 2000).

Population analysis demonstrated that several isolates had theoretical T-RFs that were specifically induced by the presence of diesel such as isolates dC, dT and dU (155 bp) and isolates dP, dQ and dR (233 bp). Our results showed that all of these isolates, except strains dR and dU, are able to degrade *n*-alkanes. Although phylotypes of the isolates dT and dU occurred in the rhizosphere of T179 grown in contaminated soil, they were not a member of the dominant rhizosphere population. The phylotype of isolate dC could only be isolated from control rhizosphere soils, although its corresponding T-RF was found in most treatments. Bacteria possessing a theoretical T-RF of 155 bp may belong to different genera or species or alternatively, cells of isolate dC may have entered a viable but non-culturable status in the presence of diesel. The phylotype of isolate dQ was found in various poplar rhizospheres, both in soils with and without diesel fuel, whereas strain dP was exclusively

isolated from the rhizosphere of poplar clone Brandaris cultivated in the presence of diesel. Isolate dQ was able to grow on several *n*-alkanes, possessed an *alk*B gene, and had a theoretical T-RF that showed high abundance in diesel contaminated soils. The fact that it could be isolated from rhizospheres of various poplar clones in high numbers further indicates that it possibly was a vital component of the *n*-alkane degrading microbial community. Therefore, isolate dQ, belonging to *Achromobacter xylosoxidans*, may be a promising inoculant strain for the poplar clones E11 and L55 and eventually Brandaris. Isolate dP may in turn be used for the inoculation of the cultivar Brandaris.

In conclusion, appropriate poplar clones were selected based on biomass production in the presence of diesel fuel. We found that many culturable bacteria utilizing diesel fuel under laboratory conditions may not necessarily contribute to hydrocarbon remediation in situ probably because of their insufficient competitiveness. In this paper a strategy was chosen to obtain potential bioremediating strains that are also frequently associated with a given plant. This approach increases the likelihood of the selected inoculant strain to establish well in the rhizosphere. For future phytoremediation applications the poplar clone Brandaris in combination with isolate dP and clone L55 with isolate dQ were identified as promising plant–microbe combinations. Their performance will be further evaluated in the greenhouse using different soil substrates as well as in field experiments.

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