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A new method for the detection of alkane-monooxygenase homologous genes (*alkB*) in soils based on PCR-hybridization

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Abstract

An improved method was developed that allowed the specific detection of the gene *alkB* (coding for the rubredoxin dependent alkane monooxygenase) from bacteria without any obvious strain specific discrimination using a combination of PCR and hybridization. This approach enabled a fast culture-independent monitoring of environmental samples for the occurrence of *alkB*, and an estimation of the gene copy number and the genetic diversity. Both parameters provide useful informations for an assessment of the intrinsic biodegradation potential that is present at a site.

The method was applied to soil samples from different uncontaminated sites. *alkB* was highly abundant and redundant in all soils tested. Potential biodegradation of *n*-alkanes was also demonstrated for these soils with substrate utilization assays. Cell numbers of hydrocarbon degraders estimated as MPN varied from 10^3 to $10^6\,g^{-1}$ soil (dry weight) for the different soils. Gene copy numbers estimated with MPN-PCR ranged within $1–40 * 10^4\,ng^{-1}$ soil DNA. Analysis of the diversity of the *alkB* sequences obtained from a grassland and an agricultural soil indicated that the alkane degrading microbial populations occurring at these sites were rather diverse. Compared on protein level, three major clusters were distinguishable for both soils that showed highest similarities to AlkB from the Gram-positives *Nocardioides* and *Mycobacterium*, and the Gram-negative *Alcanivorax*. The majority of the cloned AlkB sequences were homologous to proteins from the Gram-positive bacteria. However, significant differences from published sequences were observed; homologies varied from 50% to 90% (identity of amino acids).

Keywords: *alkB*; Alkane monooxygenase; Hydrocarbon biodegradation; PCR-hybridization; MPN-PCR

1. Introduction

Aliphatic *n*-alkanes are a group of hydrocarbons that are present in crude and refined oils. They were formed by reduction of organic material during the geochemical formation of oil. However, *n*-alkanes are also produced by plants and microorganisms and form part of the biomass (Bird and Lynch, 1974; Taylor and Calvin, 1987; Kunst and Samuels, 2003). Geogenic and biogenic alkane fractions differ in the complexity of the mixture and the mean chain length found (Hellmann, 1991). Introduced in greater amounts into the environment, e.g. by oil spills, aliphatic alkanes may become environmental pollutants. The bioavailability and toxicity of *n*-alkanes depends on their chain length (Gill and Ratledge, 1972). Only short chain *n*-alkanes are directly toxic, acting as solvents for cellular fats and membranes (Sikkema et al., 1995). However, long chain *n*-alkanes can contribute to the formation of oil films and slicks (Leahy and Colwell, 1990). These are hazardous to the

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macro- and microflora of the contaminated site blocking the exchange of water, water soluble nutrients and gases.

A wide range of non-related bacteria and fungi can use *n*-alkanes as sole carbon and energy source (Rehm and Reiff, 1981; Watkinson and Morgan, 1990; van Beilen et al., 2003). Bacterial degradation of *n*-alkanes is possible under aerobic and anaerobic conditions (Widdel and Rabus, 2001). However, activation of the otherwise chemically inert aliphates with molecular oxygen allows much faster degradation rates. Although various mechanisms of aerobic activation have been described (Rehm and Reiff, 1981; Maeng et al., 1997; van Beilen et al., 2003), only the terminal oxidation pathway that involves an alkane monooxygenase as key enzyme has been investigated in greater detail so far. Three types of alkane monooxygenases are known. A rubredoxin dependent [2FeO] protein has been described for bacteria (van Beilen et al., 1994; Shanklin and Whittle, 2003). This enzyme encoded by the gene *alkB* (van Beilen et al., 1994; termed *alkM* in *Acinetobacter* sp., Ratajczak et al., 1998) is found in a variety of non-related bacteria (Smits et al., 1999; van Beilen et al., 2002). A second alkane hydroxylase that is alternatively found in bacteria belongs to the CYP153 family of the cytochrome P450 monooxygenases (Asperger et al., 1981; Maier et al., 2001). A cytochrome P450, the NADH dependent CYP52, encoded by the gene *CYP52*, is also found in fungi (Müller et al., 1991; Yadav et al., 2003). The alkane-inducible P450s of the fungi form a distinct family in the P450 superfamily (Sanglar and Loper, 1989). Alternative pathways of biodegradation that involve different mechanisms of *n*-alkane activation were identified in bacteria by metabolite analysis, but biochemical and molecular biological data are very limited (Rehm and Reiff, 1981; Maeng et al., 1997).

Hybridization probes derived from various organisms have been used for the detection of *alkB* in microbial communities after isolation of the bacteria (Sotzsky et al., 1994; Whyte et al., 1995; Vomberg and Klinner, 2000). Investigations with PCR-based methods were also mostly performed with cultured isolates (Smits et al., 1999; Vomberg and Klinner, 2000; van Beilen et al., 2002). The high sequence divergence found in *alkB* of bacteria from different taxonomic groups (Smits et al., 1999; van Beilen et al., 2003) caused a group-specificity in the detection of alkane degraders with the PCR and hybridization methods described in the literature. Therefore, comprehensive analysis of environmental samples required the use of multiple primer and probe sets targeting the respective subgroups (Luz et al., 2004; Heiss-Blanquet et al., 2005).

Here, an improved PCR based method is described that in combination with hybridization allowed a specific and sensitive detection of the *alkB* gene in environmental samples without obvious discrimination of any of the known bacterial groups possessing this gene.

2. Material and methods

2.1. Microbiological methods

2.1.1. Reference strains and media

Strains used as references were *Acinetobacter* sp. ADP1 (DSMZ 586), *Pseudomonas putida* GPo1 (ATCC 29347), *Bacillus subtilis* strain Marburg (DSMZ 10) and *Escherichia coli* TG1. Strains were obtained from W. Hillen (Germany), B. Witholt (Switzerland), W. Zimmer, (Germany) and DSMZ (Germany). They were grown in NB medium (8 g l^{-1} nutrient broth No. 4, Fluka, Buchs, Switzerland).

HC medium contained per 1:2 g NH_4NO_3, 4 g KH_2PO_4, 6 g $Na_2HPO_4 \cdot 2H_2O$, 200 mg $MgSO_4$, 50 mg $CaCl_2 \cdot 2H_2O$, 136.3 μg $ZnCl_2$, 3.2 mg $FeCl_3$, 2.0 mg $MnCl_2 \cdot 4H_2O$, 170.5 μg $CuCl_2 \cdot 2H_2O$, 475.9 μg $CoCl_2 \cdot 6H_2O$, 61.8 μg HBO_3, 3.9 μg $NaMoO_4 \cdot 2H_2O$. The pH of the medium was adjusted to 7.0 with KOH before autoclaving. The appropriate C-source was autoclaved or filter sterilized separately before addition to the medium.

2.1.2. Isolation of soil bacteria

One gram of soil was resuspended in 10 ml 10 mM K-phosphate-buffer pH 7.0, vortexed vigourously and shaken for 1 h to prepare a cell suspension. The sample was allowed to settle for 15 min. Further dilutions were made from the supernatant.

Serial dilutions of the cell suspension were plated on NB medium supplemented with 250 μg ml^{-1} cycloheximide. Plates were incubated for 5 d at 30 °C. Colonies were replicated on fresh NB plates for further cultivation.

2.1.3. MPN counts of oil degrading soil microorganisms

MPN counts were performed in microtiter plates with 8 parallels. Serial dilutions of the cell suspensions (see Section 2.1.2) were prepared with HC medium (Section 2.1.1) directly in the microtiter plates, leaving the last row as sterile control. To a total volume of 270 μl medium 25 μl *n*-hexadecane or heavy mineral oil (density 0.84 g ml^{-1}, Sigma, Deisenhofen) were added after inoculation. Plates were incubated for 6 weeks at 30 °C and growth judged from turbidity.

2.1.4. Substrate utilization tests for soil microbial communities

Cell suspensions were prepared from the soils as described above (Section 2.1.2). Cell numbers were determined with epifluorescence microscopy after DAPI staining (Porter and Feig, 1980) and the cell suspensions than diluted into HC medium (Section 2.1.1) to a final cell titer of $10^7 * ml^{-1}$ cells. Aliquots of 1 ml of these dilutions were transferred to 15 ml culture tubes with screw caps and complemented with one of the following carbon sources: 1% (*w*/*v*) of pyruvate, malate, glucose or sucrose, 2% (*w*/*v*) of tridecane, hexadecane, eicosane, tricosane, tetracosane or tetracontane, or 5% (*w*/*v*) of hexane or decane. Vials were closed airtight and those vials containing short alkanes ($<C_{16}$) additionally sealed with PTFE film at the screw rim. All samples were incubated for 6 weeks at 30 °C. Growth was judged from turbidity or film formation at the water/hydrocarbon interphase.

2.2. Molecular biological methods

2.2.1. Preparation of genomic and environmental DNA

For preparation of genomic DNA from pure cultures cells were grown over night and harvested by centrifugation. Cells were resuspended in 320 µl 1 × TE (10 mM Tris pH 7.5, 1 mM EDTA) and incubated for 1 h. After addition of 80 µl 10% SDS samples were incubated at 65 °C for 2 h. The final DNA extraction was done with the Plant Mini Kit (Qiagen, Hilden, Germany) starting with the addition of buffer AP2 according to the manufacturer's protocol.

Environmental DNA from soil was prepared with the QBio Kit for Soil (MP Biomedicals, Heidelberg, Germany) according to the instructions given with the kit.

DNA of cell lysates was used for colony-PCR. Approximately 1 µl cell mass was sucked directly from agar plate, resuspended into 100 µl water and lysed for 15 min at 105 °C. One microliter of this lysate was used as a template in PCR.

Suitability of all DNA preparations for PCR was checked by 16S rDNA amplification with the primers 27f and 1492r (Lane, 1991). Annealing temperature was 52 °C. All other PCR conditions were the same as described for the amplification of *alkB* (see Section 2.2.2).

2.2.2. Detection of alkane monooxygenase gene alkB

For primer and probe design, all full length protein sequences of the rubredoxin dependent alkane monooxygenase AlkB available in GenBank were aligned with CLUSTALW using the identity matrix for pairwise and the gonnet matrix for multiple alignment (Thompson et al., 1994). Conserved regions were identified and degenerate oligonucleotide primers designed from the consensus regions by back translation into DNA. The specificity of these oligonucleotide primers was checked in silico by comparison to the AlkB and AlkB related protein sequences found in the public databases.

PCR primers were tested with reference strains of known geno- or phenotype. The following primers proved to be successful: alkB-1f 5′-AAYACNGCNCAY GARCTNGGNCAYAA (coding for the peptide NTA HELGHK′) and alkB-1r 5′-GCRTGRTGRTCNG ARTGNCGYTG (coding for QRHSDHHA′). The PCR mix contained in a final volume of 50 µl: 5 µl buffer (provided with Taq polymerase), 1.5 µl $MgCl_2$ (50 mM), 5 µl dNTP-mix (2.5 mM each nucleotide), 5 µl each primer (10 µM), 10 ng purified DNA of cultured strains or 5 ng soil DNA and 2.5 U Taq-DNA polymerase (Invitrogen, Karlsruhe, Germany). Cycling was performed with initial denaturation for 5 min at 90 °C, 35 cycles with 30 s 90 °C, 30 s 55 °C, 60 s 72 °C, and final elongation for 5 min at 72 °C. PCR products were separated in 1.5% agarose gels. Gels were blotted on positively charged nylon membrane (Amersham Bioscience, Braunschweig, Germany) by capillary transfer with 0.4 M NaOH for hybridization. Hybridization was performed with the oligonucleotide alkB-5f 5′-AAY-TAYCTNGARCAYTAYGGNCT (coding for NYLE-HYGL′). The oligonucleotide was labeled with Dig-*dd*UTP using the 3′-end labeling kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Five hundred picomoles of oligonucleotide were used per 50 ml hybridization buffer containing 5 × SSC (20 × SSC: 3 M NaCl, 0.3 M Na_3-citrate), 0.1% Na-lauroylsulfate, 0.02% Na-*N*-lauroylsarcosine, 0.5% blocking reagent. Hybridization was performed at 42 °C over night and was followed by two washes with 2 × SSC/0.1% SDS for 5 min at room temperature, and two washes with 0.5 × SSC/0.1% SDS at 42 °C. The Colour Detection Kit (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions to detect hybrids.

2.2.3. Cloning and sequencing of alkB amplicons

PCR fragments obtained for the reference strains were directly cloned into the pST-Blue vector and transformed into *E. coli* NovaBlue competent cells following the provider's manual (Novagen, Schwalbach, Germany).

PCR fragments obtained for the soil samples were purified before cloning. Bands of the expected size were cut from the gel and extracted with the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). DNA was

eluted in a final volume of 10 µl. Ends of the PCR products were filled with Klenow enzyme. The reaction mix contained in a final volume of 20 µl: the total purified DNA, 1 mM dTTP, 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 2 U Klenow enzyme. Samples were incubated for 15 min at 37 °C and stopped by addition of 2 µl 0.2 M EDTA pH 8.0. Samples were cleaned again with the MinElute Reaction Purification Kit (Qiagen, Hilden, Germany). Half of the purified DNA (5 µl, ~25 ng DNA) were used for cloning into the pCR-Blunt vector and transformation into *E. coli* TOP10 competent cells following the protocols of the manufacturer (Invitrogen, Karlsruhe, Germany).

Plasmids from transformants were prepared using the method of Birnboim and Doly (1979) and checked for the presence of an insert of the expected size. A fast initial screening of the transformants was performed by colony hybridization according to the method of Grunstein and Hogness (1975). The oligonucleotide alkB-5f was used under the hybridization conditions described above (Section 2.2.2).

Plasmids used for sequencing were further purified with the Plasmid Mini Kit (Qiagen, Hilden, Germany). Sequencing was performed with the BigDye Terminator Kit 3.1 on an ABI 3730 48-capillary sequencer (both Applied Biosystems, Foster City, CA, USA). Both strands were read from double stranded plasmid with the opposing vector primers T7 and M13reverse. Sequences were compared to Genbank with BLASTN or BLASTX (Altschul et al., 1997). Derived protein sequences were aligned to the existing AlkB alignment (Section 2.2.2) of the full length sequences with CLUSTALWProf (Thompson et al., 1994). Trees were calculated with PHYLIP (Felsenstein, 1989).

Sequences are available under the GenBank accession numbers DQ288026–DQ288068 (clones of the grassland soil), DQ287991–DQ288004 (isolates of the grassland soil), and DQ288005–DQ288025 (clones of the barley field soil).

2.3. Soil sampling

Soil was sampled from different field sites in the area of Röhrmoos, Bavaria, Germany, in June–July, 2004. Samples from the grassland were taken after the first mowing. Maize was in the early stage of development, barley and rape fruiting. Samples were taken from the upper 5 cm of the soil. All soils were silty loams. Those of the spruce forest and the dung hill contained to a major part organic covering material. No history of anthropogenic hydrocarbon contamination was known for any of the sites. Two samples (appr. 1 m distance) were taken from each site and analysed separately.

3. Results and discussion

3.1. Detection of alkB by PCR and hybridization

Specific detection of *alkB* was achieved combining PCR amplification with oligonucleotide hybridization (Fig. 1). The PCR primers target essentially the same regions as the primers described by Smits et al. (1999). However, the reverse primer was shifted 6 bases, and both primers used here were more degenerate. The expected band of 550 bp was amplified from DNA of the reference strains *Acinetobacter* sp. ADP1 and *P. putida* GPo1 containing *alkM* or *alkB*, respectively (Fig. 2A).

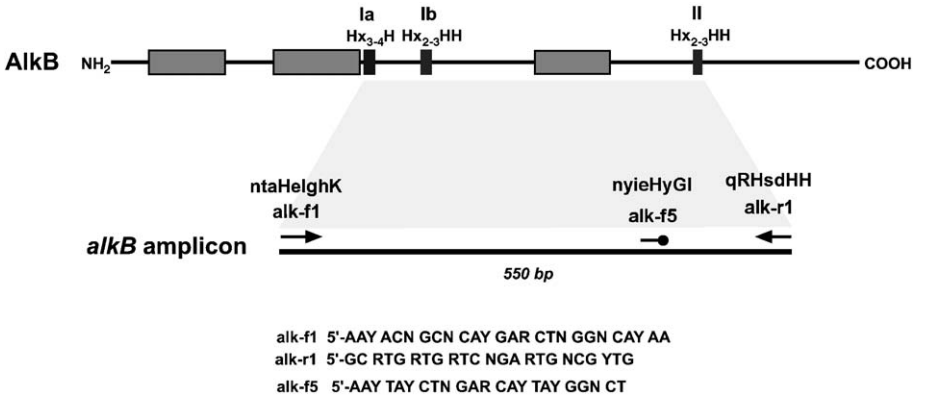

Fig. 1. Primer and probe design for *alkB*. The amplified gene fragment is shown in relation to the structure of the protein. Light grey boxes indicate the transmembrane helices, dark grey boxes the histidine clusters involved in Fe binding at the active centre of the AlkB protein. Consensus sequences of the protein stretches used for primer and probe design are given above their location on the *alkB* PCR amplicon. Only amino acids in capitals are conserved in all AlkB proteins compared. Primer and probe DNA-sequences are given underneath.

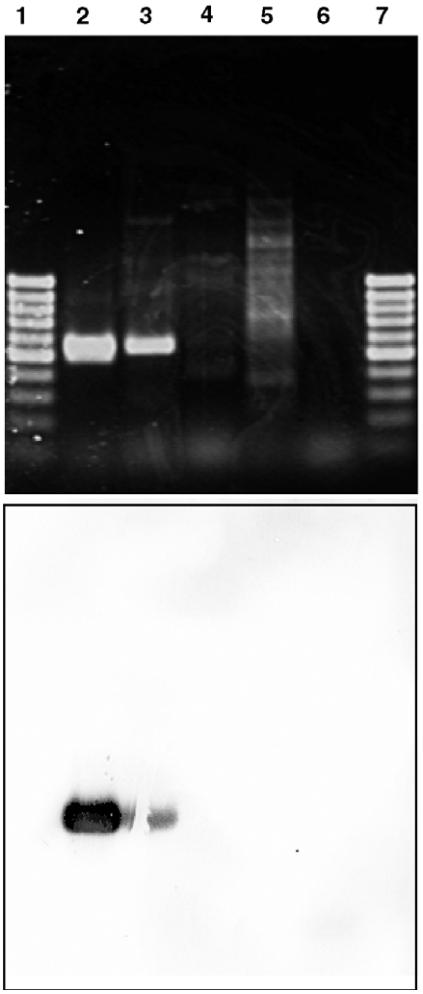

Fig. 2. Detection of *alkB* in reference organisms. A. PCR, B. hybridization. Lane 2: *Acinetobacter* sp. ADP1, 3: *Pseudomonas putida* GPo1, 4: *Bacillus subtilis* strain Marburg, 5: *Escherichia coli* TG1, 6: no DNA. Lanes 1+7: 100 bp ladder.

Sequences of these PCR products differed only in the primer sequences from those published. Unspecific PCR products of various sizes were obtained with the non-degrading strains *Bacillus subtilis* strain Marburg and *Escherichia coli* TG1. Hybridization with an oligonucleotide probe binding to *alkB* within the amplicon allowed to distinguish specific and non-specific products (Fig. 1). Hybridization signals were obtained only for the expected products of the two alkane degraders (Fig. 2B).

The detection limit of the combined PCR/hybridization assay was estimated with chromosomal DNA of the reference strains *Acinetobacter* sp. ADP1 and *P. putida* GPo1 to be 0.1 ng DNA. Assuming a mean bacterial genome size of 3.6 Mb (Fogel et al., 1999) this detection limit corresponded to $2.6 * 10^4$ gene copies.

3.1.1. Evaluation of the alkB detection method with soil isolates

The PCR/hybridization method was used to screen soil isolates for the occurrence of *alkB* by colony-PCR. Isolates were obtained under non-selective conditions from a grassland soil. Ninety-six randomly picked isolates were screened for each of the two independent samples. A single PCR product of the expected size was obtained for 8% and 10% of the isolates. None or unspecific products were observed for all other isolates. Again, only PCR products of the expected size hybridized with the internal probe. Sequencing of these PCR products after cloning confirmed their homology to *alkB*. Derived protein sequences showed highest homologies to AlkB from *Acinetobacter calcoaceticus* 69-V (AJ009582 partial gene, Smits et al., 1999; 95–98% identity, 6 isolates), *Pseudomonas fluorescens* DSM 50106 (AF090329, Khalameyzer et al., 1999; 96% identity, 4 isolates) and *Rhodococcus* Sp. 1BN (AJ401611 partial gene, Andreoni et al., 2000; 84–88% identity, 3 isolates) (also compare Fig. 4). Only for one isolate, W34, DNA sequences from two independently obtained clones (alkW34-1, alkW34-2) showed significant differences indicating that this isolate had two genes of *alkB* with homologies to *Rhodococcus* Sp. 1BN (AJ401611; 86% identity) and *Nocardia farcinica* IFM 10152 (AP006618; 84% identity), respectively. For all other isolates, sequences obtained from separate clones varied aside from single base exchanges only in the primer sequences. Multiple genes of *alkB* with significant sequence divergence have been described for strains of *Rhodococcus*, *Nocardia*, *Acinetobacter*, and *Pseudomonas* (Whyte et al., 2002; Tani et al., 2001; van Beilen et al., 2002; Marin et al., 2003). The encoded AlkB isoenzymes can vary in their specificity towards *n*-alkanes of different chain lengths and are differentially regulated by substrate availability, accordingly. However, also differential expression in dependence of the growth state was observed (Marin et al., 2003).

3.1.2. Evaluation of the alkB detection method with a clone library from soil

The PCR for *alkB* was also applied to total environmental DNA isolated directly from the same grassland soil. PCR products of the expected size of 550 bp were obtained, but additional products having a wide range of sizes were also observed (Fig. 3, lanes 8+9). Hybridization labeled only the band of the expected size. This band was purified from the gel and a gene library was constructed. In an initial random screening 58 clones carrying inserts of the appropriate size (±50 bp were allowed) were sequenced. Sequence analysis showed that *alkB*

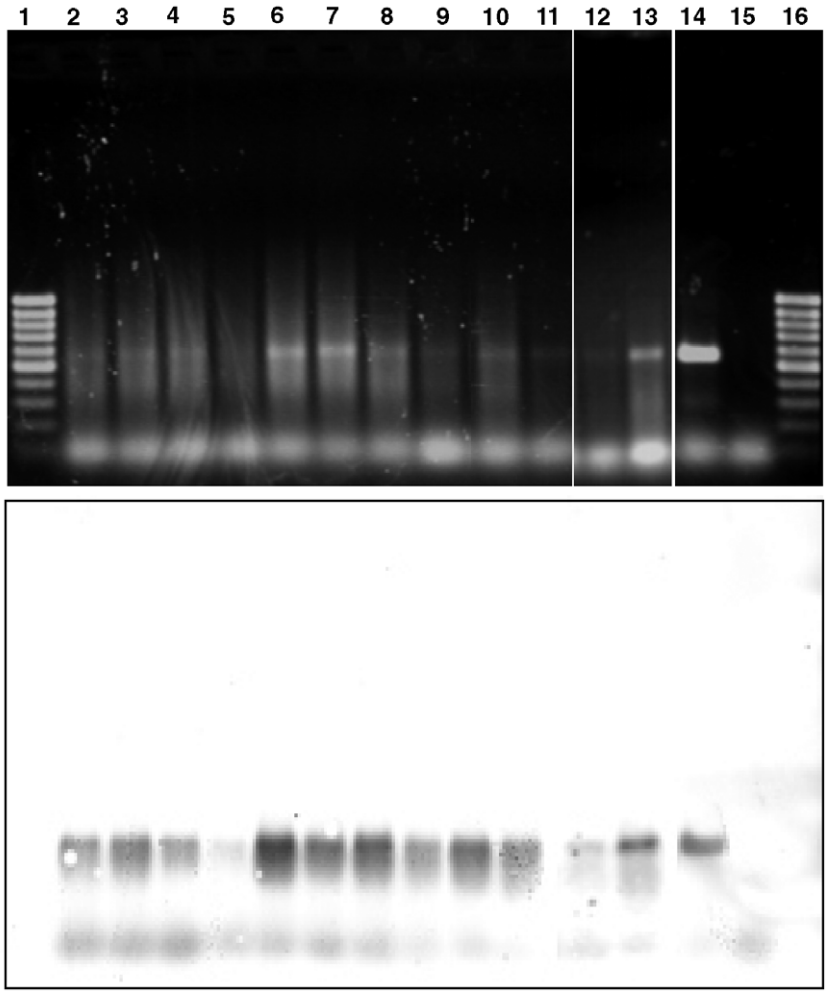

Fig. 3. Detection of *alkB* in soils. A. PCR, B. hybridization. Lane 2+3: barley field, lane 4+5: maize field, lane 6+7: rape field, lane 8+9: grassland, lane 10+11: spruce forest, lane 12+13: dung hill, lane 14: *Pseudomonas putida* GPo1, lane 15: no DNA; lanes 1+16: 100 bp ladder.

homologous gene fragments were cloned. However, an appreciable number of clones carried *alkB* non-related gene fragments. Hybridization of the cloned amplicons with the oligonucleotide probe, again, allowed a differentiation: Only those inserts having significant sequence homology (>50% identity of the amino acid sequence) to other alkane monooxygenases gave hybridization signals. The perfect correlation of sequencing and hybridization results confirmed that the combined PCR/hybridization approach allowed a specific detection of *alkB* and demonstrated that it is also applicable to environmental DNA.

The primary sequences of most of the cloned *alkB* genes obtained from the environmental gene library showed a high divergence from published sequences (Figs. 4 and 5). Homologies to published sequences were in most cases only detectable on protein level but not on DNA level. Publicly available *alkB* sequences also show a high diversity, as became apparent from an alignment of all full-length DNA-sequences retrieved from the databases (data not shown). Accordingly, published trees that are calculated based on the alignment of partial DNA-sequences showed deep branching for the sequences from non-related bacteria (van Beilen et al., 2003; Heiss-Blanquet et al., 2005). These data corresponded to the observation that cross hybridization of *alkB* from different organisms under stringent conditions is limited to close relatives (Whyte et al., 1995; Smits et al., 1999; Vomberg and Klinner, 2000). The oligonucleotide probe developed and used in this study allowed to overcome this limitation. *alkB* homologous sequences of all Gram-negative and Gram-positive bacteria were detected alike for the isolates and in the environmental clone library. Therefore, it could be concluded that the combined PCR/hybridization

Fig. 4. Diversity of AlkB in a grassland soil. Amino acid sequences derived for the cloned PCR products from isolates (boxed dark grey) and environmental DNA (boxed light grey) of the grassland soil were added to an alignment of published AlkB sequences (see Material and methods for alignment parameters). Sequences from the database are labeled with the name of the strain and the gene (for multiple genes) and the GenBank protein identification number. Note: partial sequences from the database are not included in this tree.

method developed here allowed the detection of *alkB* without an obvious specificity for any bacterial group.

The formation of additional unspecific PCR products made the cloning of *alkB* sequences rather inefficient when working with environmental DNA. This problem did not occur when DNA of pure cultures or isolates was used as a template. Here, the occurrence of an *alkB* fragment suppressed the formation of unspecific

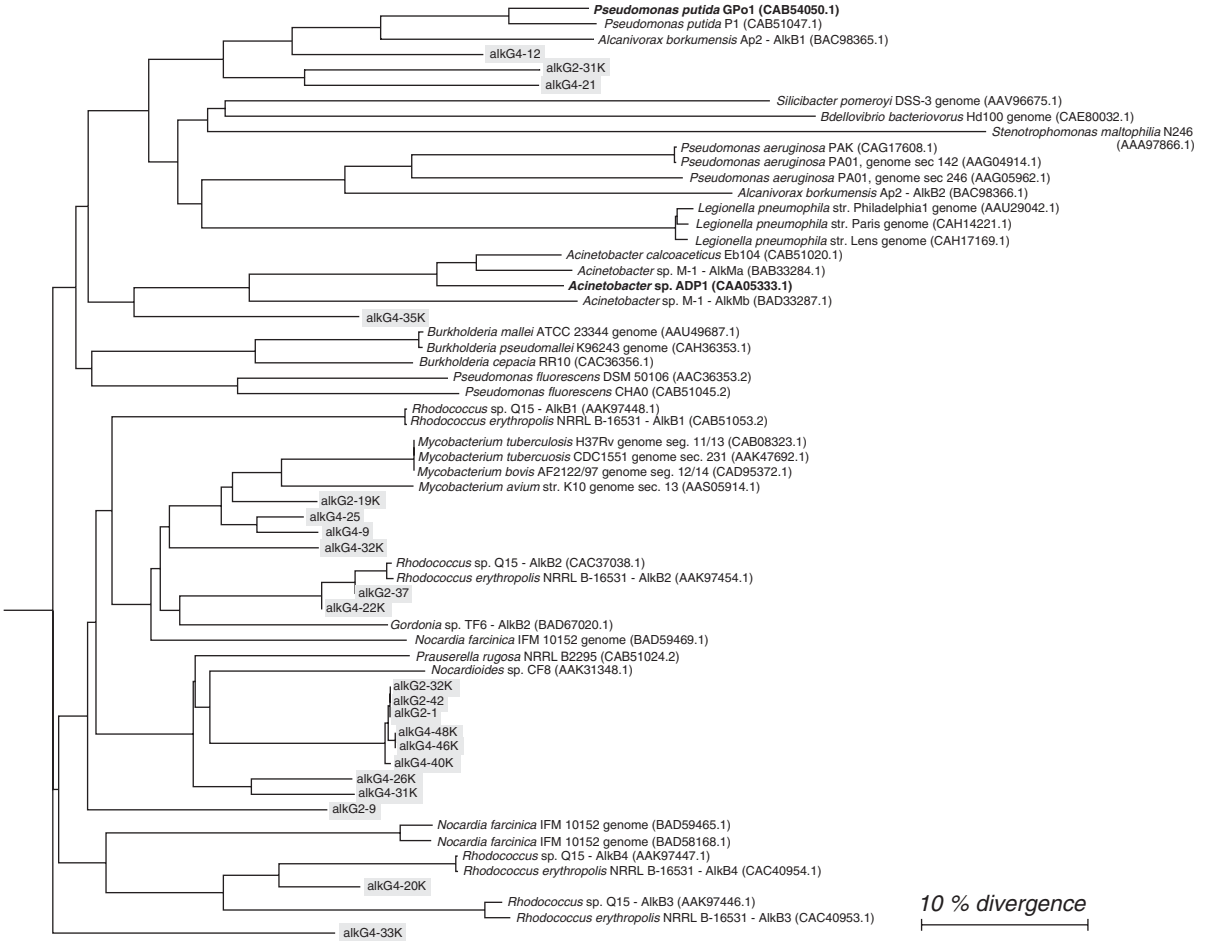

Fig. 5. Diversity of AlkB in soil from a barley field. For details see Fig. 4.

products. Efforts to optimize PCR conditions for the application to environmental DNA were not successful but resulted in a loss of all products. However, the oligonucleotide probe could be used as a convenient screening tool to identify *alkB* sequences in the clone libraries by dot blot or colony hybridization, allowing the screening of a high number of clones.

3.2. Occurrence of alkB and alkane degrading microorganisms in soils

The described PCR/hybridization method was applied to screen uncontaminated soils for the occurrence of *alkB* and the results were compared to those obtained with alternative microbiological methods. The soils investigated were from a small area covered with different types of vegetation. *alkB* homologues were detectable for all samples (Fig. 3) indicating an *n*-alkane biodegradation potential for all sites tested. This was confirmed with substrate utilization assays performed for the same samples. Growth of soil microorganisms with *n*-alkanes that were provided as sole carbon sources was observed in all cases (Table 1). However, short (C_6) and long (C_{40}) hydrocarbons were not as good substrates as those with medium length (C_{13}–C_{23}). This is supposedly due to the higher toxicity of the first and the reduced bioavailability of the latter (Gill and Ratledge, 1972). Compared to the carboxylic acids and sugars used as controls, growth on all hydrocarbon substrates was significantly slower.

Gene copy numbers estimated with MPN-PCR/hybridization were in the range of $0.9–38 * 10^4\,ng^{-1}$ soil DNA for the soils tested (Table 2). Cell numbers determined with classical MPN counts for alkane degrading bacteria ranged from 10^3 to $10^6\,g^{-1}$ soil (dry weight) for the same samples (Table 2). No significant differences in numbers were observed when pure hexadecane or complex mineral oil was used as a substrate. Both estimates indicated that alkane degraders were present in all soils investigated with similar population densities and in appreciable numbers. MPN-PCR

Table 1
Substrate utilization assay

C-source	Sample					
	Barley	Maize	Rape	Meadow	Spruce	Dung
C_6	−/+	+/+	+/+	+/+	−/+	−/−
C_{10}	−/−	+/+	+/+	+/+	+/+	+/+
C_{13}	−/−	+/+	+/+	+/+	+/+	+/+
C_{16}	−/−	+/+	+/+	+/+	+/+	+/+
C_{20}	+/+	+/+	+/+	+/+	+/+	+/+
C_{23}	+/+	+/+	+/+	+/+	+/+	+/+
C_{24}	+/+	+/+	+/+	+/+	+/+	+/+
C_{40}	+/+	+/+	+/+	+/+	+/+	−/−
Pyruvate	+/+	+/+	+/+	+/+	+/+	+/+
Malate	+/+	+/+	+/+	+/+	+/+	+/+
Glucose	+/+	+/+	+/+	+/+	+/+	+/+
Sucrose	+/+	+/+	+/+	+/+	+/+	+/+

indicated a slightly higher abundance of alkane degraders in the un-cultivated soils. This was not confirmed by the MPN counts of cell numbers (Table 2). However, statistical more sound data will be required for a final analysis. A strict correspondence between the two approaches cannot be expected: genetic potential and enzyme activity are not directly linked. In addition, the occurrence of degradation pathways that do not use AlkB is possible and likely, and microorganisms using these pathways will also be detected in the MPN counts.

3.3. Diversity of alkane degraders in soils

Detailed analysis of the *alkB* sequences was performed to estimate the diversity of alkane degrading bacteria in a grassland soil and an agricultural soil covered with barley. Data are summarized in the trees in Figs. 4 and 5.

A total of 42 sequences were obtained from clone libraries prepared from the grassland soil DNA (34 for sample W1 and 8 for the parallel sample W2) (Fig. 4). Only two sequences occurred twice with no base exchange. Two more sequences occurred 2 and 3 times with a maximum of three exchanges (primer sequences neglected). Overall, sequence homologies ranged from 57% to 90% (identity of the amino acids) to published sequences, only one having 100% identity (amino acids) to the published sequences derived from *Rhodococcus alkB1* (AF388181, AJ009586, Whyte et al., 2002). The majority of the AlkB sequences were most similar to those from the Gram-positive bacteria *Rhodococcus* (*alkB1* and *alkB2*), *Nocardioides* and *Mycobacterium*. Here, two major clusters were observed: one mostly related to *Nocardioides* sp. CF8 (AF350429, Hamamura et al., 2001), the other to the putative *Mycobacterium* AlkB proteins (AE000516.2, AE017239.1, BX842582, BX248345.1). Sequences in the latter cluster showed even higher homologies to the partial sequence of an unidentified Gram-positive bacterium HXN600 (AJ300338, van Beilen et al., 2002). A third major cluster contained sequences related to AlkB from Gram-negative bacteria with highest homologies to the *Alcanivorax borkumensis* Ap2 *alkB2* gene (AJ295164, van Beilen et al., 2004). AlkB populations derived from the culture independent screening differed significantly from those found in the culture dependent screening. With the latter, sequences homologue to those of the Gram-negative bacteria *A. calcoaceticus* or *P. fluorescens* were predominant (also discussed above in Section 3.1.1). These sequences could not be detected in the corresponding clone library from soil DNA.

For the agricultural soil covered with barley, a total of 21 sequences (7 for sample G2 and 14 for the parallel sample G4) were obtained. Again a significant diversity of the cloned sequences was observed (Fig. 5). Only 2 sequences with base exchanges of less than 1% were found to be repeated 2 or 3 times. Derived AlkB sequences showed 50% to 90% (identity of amino acids) to published sequences, most of them again had highest homologies to AlkB from Gram-positive bacteria. The same three major clusters with closest relation to *Nocardioides* sp. CF8, *Mycobacterium* sp. and *A. borkumensis*, respectively, described above for the grassland soil samples were also observed for the soil samples from the barley field.

The populations of *alkB* determined for the two different sites implied a similar pattern of microbial diversity. Unfortunately, the low number of positive clones obtained in this study did not allow an estimation of the true diversity of alkane degraders present in these soils which might have been much higher. A further improvement of the cloning procedure may help to get a more comprehensive overview on the microbial diversity. Though the homologies of the environmental *alkB* sequences do not allow a taxonomic assignment, it is not unlikely that they belonged to bacteria of the genera

Table 2
Enumeration of alkane degrading bacteria by MPN counts and MPN-PCR

Sample	Cell number [g^{-1} soil]		Gene copy number
	Hexadecan	Mineral oil	*alkB* [ng^{-1} DNA]
Barley	$1.7*10^4/6.5*10^3$	$8.0*10^4/5.6*10^3$	$6.2*10^4/2.3*10^4$
Maize	$2.5*10^4/1.1*10^4$	$5.3*10^4/8.2*10^4$	$2.3*10^4/2.3*10^4$
Rape	$4.4*10^5/2.1*10^5$	$1.0*10^5/2.1*10^4$	$1.0*10^4/6.0*10^4$
Grassland	$1.1*10^4/7.8*10^3$	$3.3*10^4/3.2*10^4$	$2.4*10^5/3.8*10^5$
Spruce	$1.6*10^4/2.2*10^4$	$7.6*10^3/1.5*104$	$1.6*10^5/5.5*10^5$
Dung	$9.5*10^5/1.6*10^6$	$2.4*10^6/2.2*106$	$-/1.0*10^4$

Rhodococcus, *Nocardioides, Mycobacterium* or close relatives. Actinomycetes are known to have aerobic biodegradation capabilities for a wide range of organic substrates (Warhurst and Fewson, 1994; Lofgren et al., 1995) including *n*-alkanes (Bouchez-Naitali et al., 1999; Vomberg and Klinner, 2000; van Beilen et al., 2003). A similar pattern, also showing the occurrence of *alkB* in uncontaminated soils and the dominance of *Rhodococcus* homologous sequences in these populations has been observed before (Whyte et al., 1995; Luz et al., 2004). None of the *alkB* sequences identified in this study had a significant homology to *alkB* from *P. putida* GPo1. In this strain all genes necessary for alkane degradation are located on the non-conjugative OCT plasmid (Chakrabarty et al., 1973). Interestingly, the occurrence of *P. putida*-like genes seemed to be increased in contaminated habitats (Sotzsky et al., 1994; Whyte et al., 1995; Luz et al., 2004, Heiss-Blanquet et al., 2005). The abundance and diversity of *alkB* found in the uncontaminated soils indicated that the potential for alkane degradation and the use of alkanes as substrates is a widespread trait in many soil microorganisms. This may be the basis for a fast adaptation of the indigenous microbial communities and than biodegradation of spilled oils.

4. Conclusion

A method was developed that combined PCR and hybridization and thereby allowed the specific detection of the alkane monooxygenase gene *alkB* from non-related bacteria in environmental samples. Its applicability was tested with uncontaminated soils. Molecular data were in accordance with physiological experiments for detection and enumeration of alkane degrading bacteria. Results showed that the capability for alkane degradation is a common trait in soil microbial communities. The method can be a very useful tool for the fast estimation of the biodegradation potential at polluted sites. The microbial diversity of alkane degraders estimated from sequence polymorphisms of *alkB* seems to be vast, even in uncontaminated soils. This indicated that the function of *alkB* in environmental microbial communities is not restricted to alkane degradation in the case of oil spills.

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