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Efficient recovery of environmental DNA for expression cloning by indirect extraction methods

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Abstract

Using direct and cell extraction-based (indirect) isolation methods, DNA was obtained from environmental samples with largely differing characteristics (loam soil, sand soil, sediment, activated sludge, and compost) and evaluated with respect to the comprised bacterial diversity and its suitability for expression cloning in *Escherichia coli*. Indirect DNA extraction methods yielded 10 to 100-fold lower amounts of DNA than direct procedures, but the bacterial diversity of DNA recovered by indirect means was distinctly higher as shown by denaturing gradient gel electrophoresis. Furthermore, much lower amounts of eukaryotic DNA were co-extracted if cell extraction-based methods were used (< 8% of eukaryotic DNA by indirect methods versus 61–93% by direct lysis protocols). Considering the higher purity, i.e. higher cloning efficiency of DNA isolated by indirect methods, similar numbers of clones carrying prokaryotic inserts could be produced by either strategy. Gene banks prepared from directly extracted DNA, however, are expected to contain large portions of clones with eukaryotic inserts, whereas those constructed from indirectly isolated DNA should mainly contain inserts of bacterial origin. As eukaryotic genetic information is generally not expressed in bacterial host organisms but increases the library size, our findings suggest that the use of indirect DNA isolation methods allows the construction of environmental gene banks of superior quality. © 2003 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Expression cloning; Denaturing gradient gel electrophoresis; Environmental gene bank; Cell extraction; Direct DNA isolation

1. Introduction

Recovery, cloning and expression screening of environmental DNA without preceding cultivation is a recent approach to exploit the biocatalytic potential of microbial communities present in environmental samples [1]. It has been of growing interest to both microbial ecologists and to biochemists looking for novel biocatalysts as the genetic information of theoretically all indigenous bacteria can be accessed, including the predominant fraction of microorganisms that is recalcitrant to cultivation. Using this methodology, a number of previously unknown genes, in some cases encoding entire pathways, have been isolated during the last few years [2–8]. Although circumventing the time-consuming microbiological work implicit to cultivation-based screening techniques, the construction of environmental gene banks is still relatively laborious due to size

and complexity of most microbial communities. In view of the large number of 1000–10 000 prokaryotic species that might be present in 1 g of soil [9], large-scale cloning techniques need to be used to cover the collective genomes, which require substantial amounts of high-quality environmental DNA.

Various protocols have been described for DNA recovery from soil and sediment samples, which can be classified as direct and indirect DNA extraction procedures. Direct DNA isolation is based on cell lysis within the sample matrix and subsequent separation of DNA from the matrix and cell debris (pioneered by Ogram et al. [10]), whereas the indirect approach involves the extraction of cells from the environmental material prior to the lytic release of DNA (pioneered by Holben et al. [11]). Cell extraction methods are generally acknowledged to yield DNA of higher molecular mass and greater purity than direct lysis procedures. However, in many cases the amounts of DNA recovered by this strategy are significantly lower, which is why DNA for gene bank construction is commonly isolated from soils by direct lysis protocols that readily yield the required microgram amounts of

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DNA [3–8]. Due to the generally higher DNA yields, direct procedures have also been assumed to access larger fractions of indigenous microbial populations and to recover nucleic acids of larger genetic diversity than indirect methods [12–14]. However, a recent report dealing with the comparison of different direct lysis protocols showed that greater DNA yield does not always equal greater bacterial species richness and that sequence representation is strongly influenced by the extraction method used [15]. Consequently, DNA extracts obtained by indirect methods must not necessarily be less representative for the targeted microbial community than those obtained by direct lysis.

Besides the apparent selectivity of DNA recovery methods for specific groups of bacteria, the amount of co-extracted eukaryotic nucleic acids may also bias the yield–diversity correlation. For construction of expression gene banks in *Escherichia coli* or other bacterial hosts, the eukarya content is a critical parameter of DNA extracts used. As expression in these systems is in most cases limited to prokaryotic genes, eukaryotic DNA will evade analysis and increase the number of clones that need to be prepared and screened. This negative effect is still increased by the generally much larger genome size of eukaryotes (3–140 000 Mb) compared to prokaryotic organisms (0.6–9.5 Mb) [16]. For example, when recovering the complete DNA from an environmental sample comprising only 0.1% of eukaryotic cells, the resulting extract would consist of 91% of eukaryotic nucleic acids, assuming an in average 10 000 times larger genome size of the present eukaryotes. Consequently, an environmental gene bank prepared from such DNA would need to be more than 10-fold larger to cover the same range of prokaryotic genomes than if prepared from eukarya-free nucleic acids. Although previous studies suggested that eukaryotic DNA may be present in DNA extracts prepared by direct lysis [13,17,18], the extent of their recovery by different direct and indirect lysis methods has not been thoroughly investigated.

In this study, we systematically compared the two DNA isolation strategies with respect to their suitability for environmental gene bank construction. Two typical protocols of each category were used for the isolation of DNA from different soils, sediment, compost material, and activated sludge. We evaluated the treatments with respect to yield, molecular mass, and cloning efficiency of the environmental DNA. A major objective was the quantification of co-extracted eukaryotic nucleic acids and the determination of bacterial diversity in the obtained DNA extracts by denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Environmental samples

Single samples of about 500 g were collected of each

environmental material, transferred to the laboratory and stored at -20°C within 2 h after sampling. Marine sludge was collected from the surface of daily-inundated saline mud flats during low tide near Paesens-Moddergat, The Netherlands. Aerobic activated sludge was obtained from a municipal sewage plant (Garmerwolde, The Netherlands) and thermophilic stage compost from an urban composting facility (Groningen, The Netherlands). Sand and loam soils were sampled from the upper 5–15 cm of soil at the lakeshore of the Lauwersmeer (The Netherlands) and a nearby-located agricultural field, respectively. Coarse plant material and stones were removed prior to storage of the samples. Sample pH was measured in a slurry of nine parts distilled water and one part sample material. Moisture contents were determined by drying 10-g aliquots at 100°C for 2 days. To determine the organic matter content, 5–10 g of dried sample material was incubated at 500°C for 3 h and the decrease in weight was measured. The amounts of prokaryotic cells present in the different samples were determined with a direct microscopic count procedure based on DTAF [5-(4,6-dichlorotriazin-2-yl)amino fluorescein] staining of prokaryotic cells [19]. Specimens were examined using an Olympus CK 40 epifluorescence microscope.

2.2. Direct DNA extraction protocols

2.2.1. Soft lysis method

To disrupt microorganisms by solely enzymatic and chemical means a modified protocol of Zhou et al. [20] was used. Triplicate 1-g environmental samples were homogenized by vortexing in 750 μl lysis buffer [100 mM Tris-HCl, 100 mM sodium EDTA, 1.5 M NaCl, 1% hexadecylmethylammonium bromide (CTAB), pH 8] at maximum speed for 5 min in 2-ml screw-cap tubes. 40 μl lysozyme (50 mg ml^{-1}) and 10 μl proteinase K (10 mg ml^{-1}) were added before incubation at 37°C for 30 min. After addition of 200 μl SDS (20%), mixtures were incubated at 65°C for 2 h with vigorous shaking by hand every 30 min. The supernatants were collected by centrifugation at $6000\times g$ for 10 min at room temperature, and the pellets were re-extracted twice by adding 500 μl of lysis buffer, vortexing for a few seconds, and incubating at 65°C for 10 min. Centrifugation was carried out as before. The combined supernatants were extracted with an equal volume of chloroform before precipitating the DNA from the recovered water phase by addition of 0.6 volumes of isopropanol and overnight incubation at 4°C . The precipitates were collected by centrifugation at $16000\times g$, washed with 70% ethanol, and suspended in a total volume of 50 μl TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8).

Lysis efficiencies (LE) were calculated by $\{1 - [\text{cells g}^{-1} (\text{dry wt}) \text{ extracted pellet}] / [\text{cells g}^{-1} (\text{dry wt}) \text{ untreated sample}] \} \times 100$. Moisture contents and cell numbers of the extracted pellets were determined as described for the untreated sample materials.

2.2.2. Harsh lysis method

The effects of mechanical cell disruption were studied by including a bead-milling step in the above protocol. Triplicate 1-g samples were homogenized in lysis buffer and supplied with lysozyme and proteinase K as described above. Additionally, 0.7 g zirconia/silica beads (0.1 mm diameter, BioSpec Products) were added. After incubation at 37°C for 30 min, the mixtures were agitated at top speed on a vortex mixer for 3 min, which corresponds to the vigor of a 1-min treatment in a mini bead beater (BioSpec Products) as judged by the size distributions of recovered DNA fragments (not shown). Subsequent steps were carried out as described for the soft lysis method.

2.3. Indirect DNA extraction protocols

2.3.1. Blending method

A modified repeated blending protocol [11] was used to mechanically release bacterial cells from the sample matrix. Duplicate 50-g environmental samples were dispersed in 100 ml blending buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 0.1% SDS, 1% CTAB, pH 8) and homogenized in a standard blender (Moulinex) for three 1-min intervals, with 1 min breaks in-between to allow cooling. Coarse particles were collected by low-speed centrifugation (1000×g for 10 min at 10°C), resuspended in 100 ml blending buffer, and subjected to another two blending-centrifugation cycles as described above. Supernatants obtained during the three rounds of cell extraction were pooled. Direct microscopic cell counts of the combined supernatants were compared to those of the untreated sample materials to determine cell extraction efficiencies. Supernatants were centrifuged at high speed (10 000×g) for 30 min at 4°C to collect the microbial cell fraction, which was subsequently washed in 150 ml of 0.1% sodium pyrophosphate (4°C). After a second wash in 100 ml Chrombach buffer (0.33 M Tris-HCl, 1 mM EDTA, pH 8), pellets were resuspended in 8 ml lysis buffer, 160 µl lysozyme (50 mg ml⁻¹), and 40 µl proteinase K (10 mg ml⁻¹) solution and incubated at 37°C for 30 min. Lysis was completed chemically by adding 1 ml of 20% SDS and incubation for 2 h at 65°C with rotary shaking (225 rpm). Chloroform extraction and isopropanol DNA precipitation were carried out as described for the direct lysis methods. DNA pellets were dissolved in 250 µl TE buffer.

2.3.2. Cation-exchange method

To disperse cells by chemical means, an adapted protocol from Jacobsen and Rasmussen [21] was used. Duplicate 50-g environmental samples were mixed with 10 g Chelex 100 (Bio-Rad) and 100 ml extraction buffer (0.1% sodium deoxycholate, 2.5% polyethylene glycol 6000) and shaken at room temperature for 1 h at 100 rpm on an orbital shaker. Mixtures were centrifuged at low speed (1000×g) for 15 min at 10°C to remove coarse particles including the cation-exchange resin. Supernatants were

transferred to fresh centrifuge tubes and subjected to high-speed centrifugation (10 000×g) for 30 min at 4°C to harvest microbial cells. Subsequent steps were performed as described for the blending method. DNA pellets were suspended in 100 µl TE.

2.4. Total DNA yield and molecular mass

Crude DNA extracts were analyzed on 0.4% (w/v) agarose gels that were post-run stained with ethidium bromide. Gel photographs were scanned and analyzed with the NIH image software [22]. Genomic DNA was quantified using a calibration curve that was prepared for each image with the five smallest fragments of the Smart Ladder molecular weight marker (Eurogentec) being present in known amounts (20–100 ng per band).

2.5. DNA purification

For molecular analysis and cloning experiments, replicate crude DNA extracts were pooled and purified by preparative gel electrophoresis on 3% (w/v) agarose gels. Genomic DNA was extracted from gel with the QIAEX II gel extraction kit (Qiagen). Care was taken to extract all DNA bands visible on the gel to ensure also the recovery of plasmid borne DNA. Recovery was routinely > 80%.

2.6. Quantitative dot blot hybridization

Four digoxigenin-labeled oligonucleotide probes (Eurosequence, The Netherlands) were used to trace the origin of environmental nucleic acids (Table 1). Purified DNA was denatured at 95°C for 10 min, and 1–1.5 µg aliquots were spotted in triplicate on positively charged nylon membranes (Roche). Nucleic acids were cross-linked to the air-dried membranes by baking at 120°C for 30 min. Membranes were prehybridized for 2 h at the probe-specific hybridization temperature T_h in a buffer consisting of 5× SSC (SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7), 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroyl sarcosine, and 1% Blocking Reagent (Roche). Hybridization was carried out overnight at T_h in 15 ml of the same buffer supplemented with 200 pmol of the probe. Membranes were washed twice with 2× SSC, 0.1% SDS for 5 min at room temperature and twice with 0.5× SSC, 0.1% SDS for 15 min at T_h . Chemoluminescent detection of the hybridized probe by anti-DIG-alkaline phosphatase Fab fragments and CSPD (disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate] and subsequent removal of bound probe was carried out according to the instructions of the manufacturer (Roche). Membranes were successively hybridized to all four probes as described above. X-ray films were digitally photographed and analyzed using the NIH image software [22]. Bacterial, archeal, and eukaryotic response factors of the four probes were determined with

Table 1
Small subunit rRNA gene probes and PCR primers used in this study

Probe/primer	Specificity	Sequence (5' → 3')	Target site ^a	T _h ^b [°C]	Reference
UNIV1390	Universal	GAC GGG CGG TGT GTA CAA	1390–1407	45	[23]
EUB341	Bacteria	CCT ACG GGA GGC AGC AG	341–357	45	[24]
ARC915	Archea	GTG CTC CCC CGC CAA TTC CT	915–934	53	[25]
EUC502	Eukarya	ACC AGA CTT GCC CTC C	502–517	39	[26]
U968	Bacteria	AAC GCG AAG AAC CTT AC	968–984	n.a. ^c	[27]
L1401	Bacteria	CGG TGT GTA CAA GAC CC	1385–1401	n.a. ^c	[27]

^a*E. coli* numbering.

^bT_h, probe-specific hybridization temperature.

^cn.a., not applicable.

two-fold dilution series of genomic DNA (6.4 µg to 100 ng) of *Bacillus megaterium*, *Pyrococcus furiosus*, and *Hansenula polymorpha*.

2.7. PCR

Primers U968 and L1401 (Table 1) were used to amplify a 402-bp section of bacterial 16S rRNA genes, including the highly variable V6 region [27]. A GC clamp (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3') was attached to the 5'-end of primer U968 to confer melting stability to the PCR products during DGGE. Specific amplification of the target sequences was routinely achieved by using 1–10 ng of template DNA in a total volume of 80 µl PCR reaction mixture [300 µg ml⁻¹ BSA, 1.25 nmol ml⁻¹ of each primer, 200 µM of each dNTP, 1× PCR buffer, and 25 U ml⁻¹ of *Taq* polymerase (Roche)]. After an initial denaturation step of 3 min at 94°C, PCR temperature cycles of 1 min of denaturation at 94°C, 1 min of annealing, and 1 min of primer extension at 72°C were performed. During 10 initial touchdown cycles, the annealing temperature was lowered from 56 to 47°C in steps of 1°C per cycle. Subsequently, 25 cycles were done at 46°C followed by a final extension step of 10 min at 72°C. PCR products were purified on 0.8% (w/v) agarose gels using the QIAquick gel extraction kit (Qiagen).

2.8. DGGE analysis

DGGE was essentially performed as described previously [24]. A device manufactured by Ingeny International BV (The Netherlands) was used to separate PCR products on 9% polyacrylamide gels containing a 30–70% gradient of urea and formamide [100% solution: 40% (v/v) formamide, 7 M urea]. Samples containing 100–150 ng of PCR products were loaded per gel slot and run for 16 h at a constant voltage of 110 V and a temperature of 60°C. Gels were silver stained [28] and digitally photographed. Pixel density profiles were obtained by using the ImageQuaNT software version 4.1b (Molecular Dynamics) that allowed the determination of exact position, number (richness *S*) and intensity (relative abundance *p_j*) of individual bands.

Shannon–Weaver diversity indices were calculated by $H' = -\sum p_j \log_2 p_j$ [29].

2.9. Sequencing of DGGE bands

Small amounts of acrylamide containing the DNA fragments of interest were scraped from gel by use of a sterile needle that was subsequently immersed in 50 µl of PCR reaction mixture without BSA. PCR products were prepared as described above and cloned into pCR4-TOPO according to the instructions of the manufacturer (Invitrogen). Inserts of at least three clones per DGGE band were sequenced at the Medical Biology Department of the University of Groningen to test for unspecific PCR amplification products (background) and whether different 16S rRNA gene segments were present. Sequences were compared to the 16S rDNA database of the Ribosomal Database Project RDP-II [30] by using the Sequence Match service [31], and to GenBank entries by using BLAST software [32].

2.10. Cloning

The purity of DNA extracts was estimated in terms of blunt-end cloning efficiencies (transformants per µg of environmental DNA fragments) in the *EcoRV* site of the high-copy plasmid vector pZero-2 (Invitrogen). Environmental DNA fragments of 4–6 kb were prepared by mechanical shearing of DNA using a nebulizer (Invitrogen), and subsequent blunting with Klenow and T4 polymerase according to the instructions of the manufacturer (Roche). Ligation using a 1:10 vector:insert ratio and transformation to *E. coli* TOP10 cells [Δ (ara-leu)7697] by electroporation was done as described in the manual provided with pZero-2, using a Gene Pulser apparatus (Bio-Rad). Transformants were spread on Luria–Bertani (LB) agar medium [15 g l⁻¹ agar (Difco)], containing 10 mg l⁻¹ kanamycin for the selection of transformants carrying a pZero-2-derived recombinant plasmid. After incubation at 37°C for 1 day, colony forming units were enumerated. At least 20 transformants were separately grown overnight in 5 ml LB medium supplied with 10 mg l⁻¹ kanamycin. Plasmid DNA was isolated from these cultures using the High

Pure Plasmid Isolation kit (Roche), and checked for insert size by running *Bam*HI/*Xho*I (Roche) digested samples on a 0.8% agarose gel and comparing bands to the Smart Ladder molecular mass standard (Eurogentec). Average insert sizes were found to be 5.2 kb in all gene banks, with background levels of self-ligated vector molecules of $\leq 5\%$.

2.11. Gene bank screening

Transformants obtained from loam soil DNA (blending method) were washed from LB agar plates with minimal medium [per liter: 5.3 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.4 g KH_2PO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 1 ml vitamin solution [33], and 5 ml of a trace element solution; the trace element solution contained per liter 780 mg $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 200 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg H_3BO_3 , 10 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 4 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 3 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, and 2 mg $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$]. Aliquots from this amplified gene bank were spread on a number of different agar media containing either selective or indicator agents, and incubated at 30°C for 3 days before scoring the positive clones. All media contained kanamycin at a concentration of 10 mg l^{-1} . Amidase-expressing clones were selected on minimal medium plates [15 g l^{-1} MP agarose (Roche)] supplied with 0.2% (w/v) glucose and 10 mg l^{-1} phenylacetyl-L-leucine as a sole source of leucine [34]. Selection for β -glucosidase encoding transformants was carried out on minimal agar medium (15 g l^{-1} agar) plus 5 mg ml^{-1} leucine and 2 mM β -D-glucovanillin as the only source of carbon. Clones with β -lactamase activity were selected on LB agar plates, containing 50 mg l^{-1} of ampicillin. The presence of amylase activity was indicated by a bright orange halo when flooding replicate Bacto Starch agar plates with Bacto Stabilized Gram Iodine (Difco) after 3 days of growth. Lipase activity was tested on Spirit Blue agar supplied with Lipase Reagent (Difco) with a clear halo indicating positive transformants. Due to the use of an amplified gene bank, active clones obtained in each screen needed to be analyzed by enzymatic restriction to determine the number of unique transformants exhibiting the respective enzyme activity.

3. Results and discussion

3.1. Isolation of DNA from environmental samples

In the last 15 years, a vast number of protocols for DNA extraction from environmental samples have been published and even commercial soil DNA extraction kits have become available (e.g. from Bio101, La Jolla, CA, USA [35]). These kits as well as most of the other published methods have improved the original direct DNA

extraction procedure of Ogram et al. [10] mainly in terms of DNA yield and ease of use by varying the lysis conditions in many ways. However, the basic concept of cell lysis by enzymatic (lysozyme) and/or hot detergent (SDS) treatment is still the core of many recent DNA extraction methods [7,20,36]. Besides, various protocols make use of mechanical forces created by, e.g., bead beating, freeze-thawing or grinding to disrupt more rigid cell structures. We therefore chose a classical soft lysis protocol as well as a harsh lysis procedure including the widely used bead beating [35–38] to represent the direct approach of DNA extraction (Fig. 1). Compared to direct methods, indirect DNA extraction methods are less diverse, all relying on the release of microorganisms from the sample matrix by either mechanical or chemical means (or a combination of both [39]) and their subsequent collection by differential or density gradient centrifugation. Blending or rotating pestle homogenization of loam soil have been described to be most efficient in mechanical soil dispersion [40]. While the addition of Chelex 100 did not improve cell yields in these cases, cation-exchange resins have proven to be useful in soil dispersion on their own [41]. To reveal possible differences between the two dispersion strategies, a standard blending protocol and a method solely based on the use of Chelex 100 were selected (Fig. 1). In both cases, bacterial cells were recovered by differential centrifugation, which allows higher DNA yields than the collection of cells in a density (e.g. Nycodenz) gradient although the purity of DNA extracts may be lower [40].

All four protocols were used to recover DNA from five different environmental samples (Table 2). The amounts of DNA isolated by direct lysis were 10- to 100-fold higher than for protocols based on cell extraction (Table 3). Activated sludge and loamy sand allowed highest recoveries with all protocols, corresponding to their high indigenous cell numbers. Although purely enzymatic and chemical lysis methods are regarded as not sufficiently vigorous to

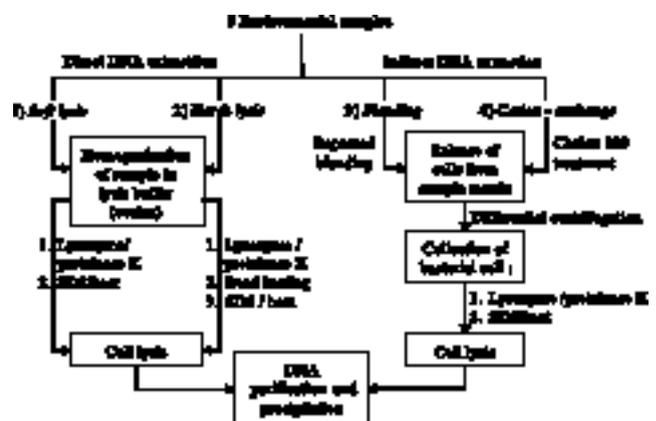


Fig. 1. The four DNA recovery protocols used in this study. To emphasize the effects of the different methods, all protocols comprised the same basic procedure for cell lysis (lysozyme, proteinase K, and hot SDS treatment) followed by chloroform extraction and isopropanol precipitation of the released DNA.

Table 2
Characteristics of the environmental samples used in this study

Sample	pH	Moisture content [%]	Organic matter content [%] ^a	Cells g ⁻¹ (wet weight) of sample ^b
Marine sludge	8.0	52	10.8	(4.79 ± 0.43) × 10 ⁸
Activated sludge	6.4	94	n.d. ^c	(14.90 ± 0.20) × 10 ⁸
Compost	7.9	54	n.d. ^c	(6.18 ± 0.53) × 10 ⁸
Sand soil	8.5	8	1.3	(2.43 ± 0.11) × 10 ⁸
Loam soil	8.2	21	2.4	(11.10 ± 0.08) × 10 ⁸

^aThe percentage of organic matter content refers to dry sample material.

^bProkaryotic cell numbers (mean count ± standard deviation, *n* = 3) were determined by a DTAF-based direct microscopic cell count procedure [19].

^cNot determined (n.d.) as the organic matter content of dried sample material is obviously close to 100%.

lyse a large number of microorganisms [37,38], soft direct lysis outperformed the harsh treatment by recovering up to two times more DNA from most environmental samples. Whereas Jacobsen and Rasmussen [21] found about equal DNA yields from a seeded soil by both indirect methods, our results show two- to eight-fold higher DNA recoveries with blending. This is in agreement with the higher cell extraction efficiencies reached by this treatment for all environmental samples studied.

3.2. Co-extraction of eukaryotic DNA

The portions of bacterial, archeal, and eukaryotic nucleic acids in the recovered DNA extracts were determined by consecutive hybridization of environmental DNA with three domain-specific oligonucleotide probes (Table 1). To calculate absolute amounts of DNA from hybridization signals, response factors (hybridization signal/ng of DNA) were determined for each probe by using known amounts of genomic control DNA (*B. megaterium* representing bacteria, *P. furiosus* for archaea, and *H. polymorpha* for eukarya). Hybridization assays with these control DNAs confirmed the high specificity of the probes since no cross-hybridization was detected. As an additional control, a fourth probe that binds to all three types of DNA was used. The responses obtained with this universal probe (Table 1) were in good agreement with the re-

sponses that were expected on basis of the composition of DNA extracts which was found with the domain-specific probes.

DNA quantification of complex communities by hybridization with probes that target small subunit rDNA suffers from the fact that the number of probe targets, i.e. the number of rRNA operons, varies between organisms. While archeal cells typically contain one or two *rrn* copies, bacteria have been found to comprise about four copies in average (Ribosomal RNA Operon Copy number Database, <http://rrndb.cme.msu.edu> [42]). Regarding the copy numbers of the control organisms *P. furiosus* (one copy) and *B. megaterium* (seven copies), quantification of prokaryotic organisms should maximally deviate by a factor of 2. Concerning eukaryotes, four-fold variation in rDNA copy number has been found for yeasts [43], but a comprehensive study of copy number in higher eukaryotes is still lacking. The assumption that targeted species on average have the same *rrn* copy numbers as the control organisms should therefore be regarded as an approximation, especially in the case of eukaryotes. However, total amounts of DNA determined by hybridization experiments agreed reasonably well with those found by gel electrophoresis, which excludes a major quantification bias.

Depending on the sample material used, direct DNA extracts contained 61–93% of eukaryotic nucleic acids (Fig. 2), which may be due to the partial lysis of indige-

Table 3
Lysis and cell extraction efficiencies of indigenous prokaryotic organisms and total DNA yields

Sample	Direct DNA extraction				Indirect DNA extraction			
	soft lysis		harsh lysis		blending		cation-exchange	
	LE ^a [%]	μg g ⁻¹ sample ^c	LE ^a [%]	μg g ⁻¹ sample ^c	CEE ^b [%]	μg g ⁻¹ sample ^c	CEE ^b [%]	μg g ⁻¹ sample ^c
Marine sludge	91	10.9 ± 0.9	92	8.2 ± 0.1	24	0.7 ± 0.0	7	0.1 ± 0.0
Activated sludge	98	71.4 ± 2.3	99	107.8 ± 17.0	68	5.0 ± 0.0	19	1.3 ± 0.1
Compost	86	15.3 ± 1.5	89	7.0 ± 1.0	39	1.2 ± 0.1	19	0.5 ± 0.0
Sand soil	96	11.5 ± 1.8	99	9.6 ± 0.7	79	0.8 ± 0.0	21	0.1 ± 0.0
Loam soil	97	42.6 ± 4.6	99	36.5 ± 3.2	55	3.3 ± 0.3	14	0.8 ± 0.1

^aLE, lysis efficiency of indigenous prokaryotic organisms (mean value, *n* = 3) reached by direct DNA extraction methods. Coefficients of variation were below 7% for all data.

^bCEE, cell extraction efficiency (mean value, *n* = 2) reached by indirect DNA extraction methods. LE for the extracted cells were larger than 99% as determined by microscopical means. Coefficients of variation were below 7% for all data.

^cTotal DNA yields (mean value ± standard deviation, *n* = 3 for direct lysis protocols, *n* = 2 for indirect methods). Values are related to g (wet weight) of environmental sample material.

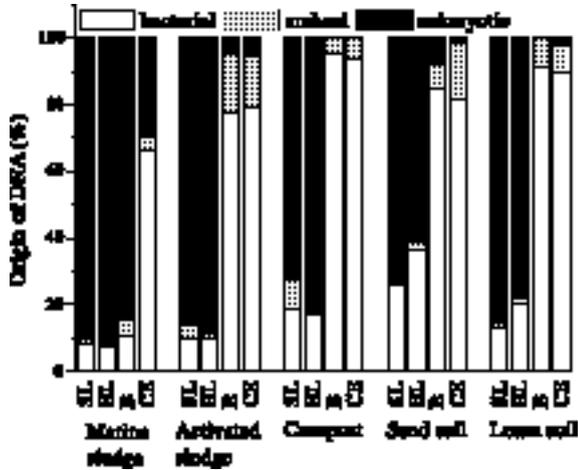


Fig. 2. Origin of DNA prepared by soft lysis (SL), harsh lysis (HL), blending (B), and cation-exchange (CE) protocols. White columns correspond to bacterial DNA, gray columns to archaeal DNA, and black columns to eukaryotic DNA.

nous eukaryotic organisms such as fungi, algae, and protozoa, or it may be caused by lysis of residual plant material. This is not surprising, as conditions similar to those of the harsh lysis protocol have been described for the lysis of yeasts, fungi, and plant and animal tissues [35,44–46]. Plant DNA can be even accessed with simple hot SDS treatment after initial disruption of the tissue structure by freezing and grinding [47]. Nevertheless, the observed large extent of co-extraction of eukaryotic DNA was not expected and shows that even though they yield a lot of DNA, direct lysis methods should be avoided when gene banks are prepared in bacterial hosts strains. In contrast, DNA obtained by cell extraction was primarily derived from bacterial cells (>92%) due to the separation from eukarya by differential centrifugation, which makes it suitable for expression cloning. Only from marine sludge, large amounts of eukaryotic nucleic acids were also obtained after cell extraction, which may be due to the high content of easily detachable microalgae in this environment.

3.3. Bacterial diversity

PCR-DGGE is a widely used technique for profiling microbial communities in a variety of ecosystems (e.g. [48,49]). A drawback of the method, however, is its rather high detection limit that only allows revelation of target genomes accounting for more than 0.1% [50] or even 1% [51] of the total population. Despite this rather low sensitivity, PCR-DGGE analysis can provide valuable information about the redundancy that is to be expected in an environmental gene bank of limited size by revealing the number and frequency of the most abundant species in DNA extracts. Obviously, for the construction of gene banks with maximal biotransformation diversity, a high complexity of the source DNA is of advantage. Typical

DGGE profiles of environmental DNA obtained by soft lysis (A) and blending (B) are shown in Fig. 3. When analyzing by DNA sequencing eight intense bands of activated sludge DNA prepared by blending (Fig. 3), only one band (no. 6) contained more than one dominant 16S rDNA sequence (Table 4). Sequences fell into at least six different genera, including Gram-positive and Gram-negative species, indicating that a broad spectrum of bacteria was accessed by the blending protocol.

The complexity of DGGE profiles can be described by the number of bands present (richness S) and the Shannon–Weaver diversity index H' , which incorporates both the number of bands as well as their intensities (relative abundance p_i) [29]. While counting of bands is only limited by the resolution of the gel and the minimal amount of DNA required for detection, determination of relative band abundance might be compromised by biased PCR amplification. As a measure to prevent PCR bias, we performed reactions with dilution series of template, ensuring that the overall amplification process was still in its exponential phase (not shown). Other potential sources of bias could not be excluded however. Varying G/C contents of target DNA, primer degeneracies, or even the absence of primer binding sites in yet unknown organisms have been suspected to cause differential amplification [52]. To minimize these effects, we chose a primer pair (U968-GC and L1401) that has been shown to equally amplify an approximately 400-bp 16S rDNA segment from a broad range of bacterial taxa and uncultured soil bacteria [27]. Integrated amplification efficiencies were found to be similar for all DNA extracts used, which shows that compounds interfering with PCR amplification had been equally removed from all samples after purification on agarose gels.

Although it is commonly assumed that higher DNA yield equates to larger diversity, DNA extracts prepared

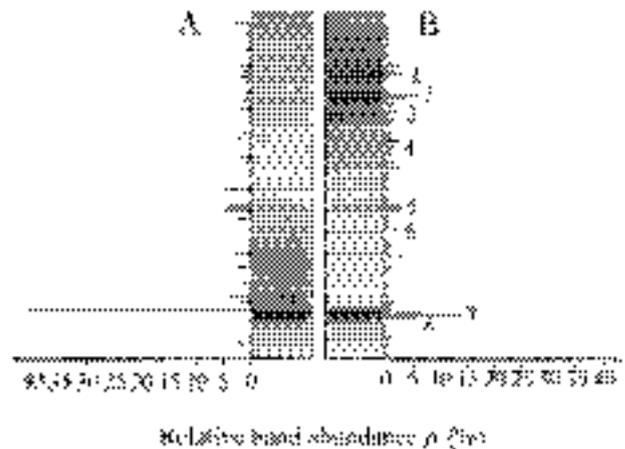


Fig. 3. DGGE profiles of DNA extracted from activated sludge by (A) soft lysis and (B) blending. Pixel density plots of the digitized images were used to determine the exact position, number (richness S) and relative abundance (p_i) of bands. Ribosomal RNA gene fragments present in the labeled bands (numbers 1–8) were sequenced and assigned to their closest database relatives (Table 6). The DGGE profiles are 47% identical as calculated by $100 \times (2 \times \text{common bands}) / (\text{total bands})$.

Table 4
Phylogenetic origin of 16S rRNA gene segments PCR amplified from activated sludge DNA

DGGE band ^a	Closest RDP/GenBank relative		Similarity [%]
	Phylogenetic group	Genus and species	
1	Gram-positive bacteria	<i>Butyrivibrio fibrisolvens</i> , U77339	95
2	β-Proteobacteria	<i>Acidovorax</i> 7078, AF078767	99
3	β-Proteobacteria	<i>Hydrogenophilus thermoluteolus</i> , AB009829	99
4	β-Proteobacteria	<i>Pseudomonas saccharophila</i> , AF368755	99
5	Gram-positive bacteria	unidentified bacterium from activated sludge, Z94008	97
6	Gram-positive bacteria	<i>Staphylococcus epidermidis</i> ^{b,c} , AY030342	99
7	Gram-positive bacteria	<i>Lactosphaera pasteurii</i> , L76599	99
8	Gram-positive bacteria	<i>Staphylococcus epidermidis</i> ^c , AY030342	99

^aDGGE band numbers refer to numbering of bands in Fig. 3B.

^bBand no. 6 contained at least two non-identical sequences (99% similarity) both aligning to the same RDP/GenBank entries.

^cThe 16S rDNA segments found in DGGE bands 6 and 8 were not identical (99% similarity).

by indirect methods were in most cases more diverse than those obtained by direct lysis regarding both diversity indices (Table 5). This seems surprising, because significantly lower proportions of indigenous microorganisms were accessed by cell extraction procedures (see CEE versus LE, Table 3), which is thought to be the result of selective recovery of easily detachable cells. Nevertheless, very different types of bacteria were recovered, as shown for activated sludge (Table 4). The higher diversity of DNA found with indirect methods may be explained by the presence of abundant bacterial species that cannot be efficiently recovered by cell extraction due to shielding by the sample matrix, but that are readily accessed by direct lysis agents. Due to the predominance of these species, DNA extracts prepared by direct methods should be of lower complexity than those obtained by indirect procedures. Furthermore, the initial composition of free DNA released by direct lysis may be altered. Effects like selective and irreversible adsorption of DNA to the sample matrix, chemical DNA degradation, or shearing of less stable DNA molecules due to relatively vigorous extraction conditions can in principle lead to the loss of genetic information especially of Gram-negative species. At present, it remains difficult to judge which approach provides the better analysis of bacterial communities in terms of quantitative composition. For the construction of gene banks, however, this is not a major issue as this technique aims at qualitative results, i.e. revealing the presence of certain

genes in a bacterial community. To maximize the chance of cloning all genes present in a microbial community, richness S and Shannon–Weaver index H' describing a DNA extract should both be as large as possible.

Besides ribotype richness and diversity, also the composition of DGGE profiles was strongly affected by the DNA recovery method used (Fig. 3). When comparing the similarity of DGGE profiles obtained by the two basic approaches, differences including up to 80% of the detected bands were observed. Even within the same category of protocols DGGE profiles varied by at least 20%, emphasizing the impact of the DNA recovery method chosen on the outcome of any ecological experiment.

3.4. Suitability of DNA for cloning

To date, most gene banks described in the literature have been constructed in cosmid, fosmid, lambda or plasmid expression vectors using relatively small insert sizes [2,3,6,53]. In terms of molecular mass, DNA extracted by soft lysis was suitable for cloning in these systems, making insert sizes of up to 30 kb possible (Fig. 4). By omitting the separation of soil particles before chloroform extraction as well as the re-extraction of soil pellets, Rondon et al. succeeded in the construction of a bacterial artificial chromosome library with an average insert size of even 44.5 kb [7]. When we included bead beating in the procedure, DNA was strongly sheared, which may com-

Table 5
Diversity indices based on bacterial 16S rRNA gene segments amplified from environmental DNA

Sample	Direct DNA extraction				Indirect DNA extraction			
	soft lysis		harsh lysis		blending		cation-exchange	
	H'^a	S^b	H'^a	S^b	H'^a	S^b	H'^a	S^b
Marine sludge	3.1	16	3.0	11	4.1	25	4.01	21
Activated sludge	3.7	30	4.0	31	5.0	47	4.3	37
Compost	4.5	47	4.3	35	4.3	32	4.6	29
Sand soil	4.5	28	4.2	29	4.4	42	4.2	31
Loam soil	4.7	32	4.6	31	5.2	44	5.1	43

^aShannon–Weaver indices H' were calculated by $H' = -\sum p_j \log_2 p_j$, where p_j is the relative abundance of the j^{th} band in a DGGE profile.

^bRichness S corresponds to the total number of distinct bands in a DGGE profile.

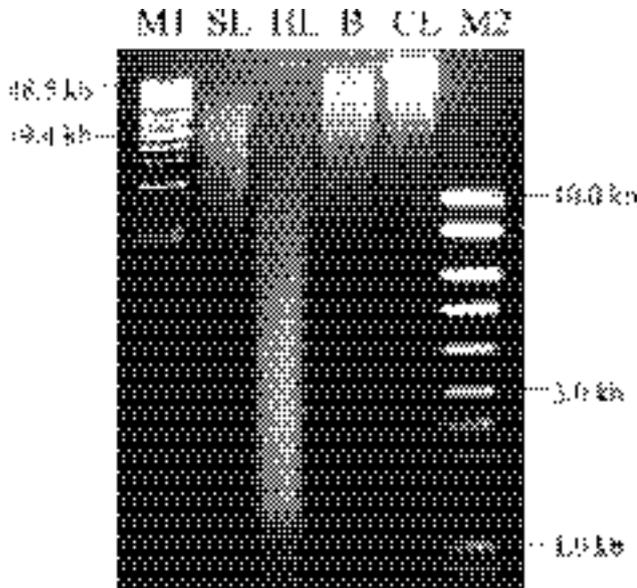


Fig. 4. Molecular mass of DNA fragments isolated by soft lysis (SL), harsh lysis (HL), blending (B), and cation-exchange (CE) protocols from loam soil. Fragment size distributions obtained by the different methods are representative for all environmental samples studied. Lane M1: High Molecular Weight DNA (Invitrogen). Lane M2: Smart Ladder molecular weight marker (Eurogentec).

promise the cloning of gene-sized fragments (Fig. 4). Harsh lysis conditions comparable to those described in the present study should therefore be avoided if DNA is used for expression cloning. Cell extraction methods routinely recovered DNA of high molecular mass (> 50 kb), allowing the preparation of large insert gene banks.

To compare the purity of DNA extracts obtained by both DNA extraction strategies, DNA recovered from three environmental samples by soft lysis and blending, respectively, was used for blunt-end cloning in pZero-2, a high-copy plasmid vector. Inserts were prepared by mechanical shearing, which avoids selective, methylation pattern-dependent fragmentation of DNA, and the possible loss of whole genomes for cloning as encountered when cutting DNA by enzymatic digestion. Between two- and three-fold higher cloning efficiencies, i.e. transformants per μg of insert DNA, were obtained with nucleic acids isolated by the blending method as compared to the soft lysis protocol (Table 6). When cells were extracted with Chelex 100, even higher efficiencies were reached, as we found with activated sludge (data not shown). Due to its low

total DNA yield however, we recommend the use of this method only when purity is of paramount concern or levels of indigenous eukaryotic microorganisms are extremely high as, e.g., in marine sludge. Worst results were found with DNA recovered from compost, probably due to co-extracted polyphenolic substances like humic acids that are known to interfere with various enzymatic reactions and decrease transformation efficiencies [44].

Besides with activated sludge, high cloning efficiencies were reached with DNA extracted from loam soil using the blending protocol. To estimate the functional diversity present in the loam soil gene bank, we conducted initial screens for five different enzymatic activities on agar media. When screening 80 000 clones that comprised about 200 Mb of total insert DNA, as determined by enzyme restriction analysis, two clones expressing amidase activity, two clones degrading β -D-glucovanillin, four clones with β -lactamase activity, and one clone with amylase activity were found. Lipase activity was not revealed. To assure plasmid-encoded activity, recombinant plasmids were isolated from the identified positive clones and retransformed to *E. coli* TOP10 cells. Although the gene bank was not sufficiently large to cover the complete genomes of the at least 44 abundant species present in the sample (Table 5), clones expressing four from the five enzymatic activities tested could be isolated. This shows that an environmental gene bank of high quality can be prepared from DNA extracted by the blending protocol.

Although the indirect DNA extraction protocols recovered smaller amounts of total DNA than the direct methods, similar numbers of clones carrying prokaryotic DNA should be obtained by either method due to the higher selectivity of the indirect approach towards prokaryotic DNA and higher cloning efficiencies. The apparent drawback of the indirect approach, the low DNA yields, may be overcome by adjusting the experimental conditions to the specific environmental material used. A study of Duarte et al. [54] suggests, for instance, that DNA yields close to the theoretical maximum can be obtained from different soils with a cell extraction method based on shaking with gravel in pyrophosphate buffer. Although the four protocols described in this paper have not been optimized for the largely differing environmental samples studied, a clear trend towards higher bacterial diversity, lower eukaryotic DNA content, and superior purity has been observed for DNA isolated by indirect means. Our results therefore indicate that the use of indirect DNA

Table 6
Cloning efficiencies of environmental DNA

Sample	Soft lysis [10^3 transformants μg^{-1} DNA] ^a	Blending [10^3 transformants μg^{-1} DNA] ^a
Activated sludge	52.9 ± 5.5	107.8 ± 13.9
Compost	3.2 ± 1.0	9.6 ± 0.7
Loam soil	17.7 ± 2.8	44.4 ± 18.3

^aCloning efficiencies (mean value \pm standard deviation, $n = 3$) are given as number of transformants obtained per μg of environmental DNA fragments used for ligation in pZero-2.

isolation strategies generally result in environmental gene banks of improved quality.

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