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Application of polymerase chain reaction-denaturing gradient gel electrophoresis for comparison of direct and indirect extraction methods of soil DNA used for microbial community fingerprinting

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Abstract We used polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to compare bacterial community patterns obtained with target DNA extracted from a soil by direct and indirect methods. For this purpose, two direct extraction methods, i.e. cell lysis by bead beating and cell disruption by grinding in liquid N, and two indirect methods, i.e. cell extraction followed by DNA extraction, and combined RNA/DNA extraction from the bacterial cell fraction, were performed. Crude extracts were purified and amplified using universal bacterial primers. PCR products were then analysed by DGGE, and similarity between the profiles obtained was determined by unweighted pair group with mathematical averages clustering. The results showed clear profiles that presumably represented the dominant bacterial fractions in the samples. The profiles generated by all four methods were similar, indicating that the methods were of approximately equal efficiency in the extraction of target DNA representative of the soil bacterial community. However, the patterns of clustering also indicated that different populations of bacteria could be detected in the same soil using different soil DNA extraction methods. The application of two dilution levels of DNA in PCR-DGGE showed that the most stable profile of the soil bacterial community could be generated by the direct methods. The indirect methods gave clustered profiles at both dilution levels. It is likely that these methods extracted DNA from a major, easily desorbed, bacterial fraction, consisting of low-density populations. PCR-DGGE was found to be a suitable technique with which to assess differences in methods for DNA extraction from soil, which can be further used for the determination of microbial community diversity at the molecular level.

Key words Deoxyribonucleic acid extraction · Community structure · Denaturing gradient gel electrophoresis fingerprint · Polymerase chain reaction · Temperature gradient gel electrophoresis

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Introduction

Soil is a complex and heterogeneous habitat where a vast diversity of microorganisms exist. However, until recently, this diversity has been underestimated because the majority of studies were based on methods for culturing organisms, which can characterise only a small fraction of all bacteria living in soil due to the fact that a large proportion of soil bacteria is not culturable (Torsvik et al. 1996). Selectivity of media and the lack of knowledge of the real conditions under which most bacteria grow in soil, difficulties with soil sampling and detection of bacteria because of small cell size and bacterial viability are critical limitations when trying to gain an understanding of bacterial diversity and community structure in soil (Trevors 1998).

Recently, molecular techniques based on total community DNA extracted from soil have been widely applied for assessing the structural diversity of microorganisms (Head et al. 1998; Muyzer 1998; Muyzer and Smalla 1998). These include reassociation analysis of DNA (Clegg et al. 1998; Ovreas and Torsvik 1998), community DNA hybridisation (Griffiths et al. 1999), percentage G plus C profiling (Clegg et al. 1998), restriction digestion and sequence comparison (Yap et al. 1996), single-strand-conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR) products of 16S rDNA (Lee et al. 1996; Schwieger and Tebbe 1998), restriction fragment length polymorphism (RFLP) and terminal RFLP (T-RFLP) of 16S rDNA (Liu et al. 1997; Dunbar et al. 1999), amplified ribosomal DNA restriction analysis (ARDRA) of 16S rRNA

genes (Smit et al. 1997), repeated extragenic palindromic sequence PCR (Ovreas and Torsvik 1998), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) of 16S rDNA (Heuer and Smalla 1997, 1999; Duarte et al. 1998; Felske et al. 1998; Kowalchuk et al. 1998; Smit et al. 1999).

The major limitations of these techniques are quantity and quality, and consequently, the representativity of the DNA extracted for the total microbial community. Two intrinsically different approaches are used for DNA extraction from soil, i.e. direct in situ lysis followed by extraction and a cell extraction/cell lysis approach (Trevors and van Elsas 1995; van Elsas et al. 1998). Critical features of direct soil DNA extraction are the efficient lysis of microbial cells in soil, the separation of the microbial DNA from extracellular DNA, and the contamination of extracted DNA by humic acids, proteins, polysaccharides, metals and other inhibitors of PCR (Liesack et al. 1997). A range of lysis treatments has been used in the different protocols, including enzymatic (lysozyme, proteinase K) treatment, sodium dodecyl sulphate (SDS), microwaves, ultrasonication and bead beating, as well as different combinations of these treatments (Trevors and van Elsas 1995).

The second approach to soil DNA extraction, called the indirect method, can be advantageous in the assessment of the numbers (diversity) of specific DNA sequences (targets) present inside bacterial cells. However, a major obstacle in most protocols is the efficient extraction of bacterial fractions from soil (van Elsas et al. 1998). A rapid protocol was developed recently, which combined the efficiency of sodium pyrophosphate in resolving soil aggregates with the efficient cell lysis and DNA extraction (purification) provided by the direct method (van Elsas et al. 1997).

DGGE and TGGE of PCR-amplified 16S rDNA fragments seem to be very useful for comparing the efficiency and reproducibility of different DNA extraction protocols (Heuer and Smalla 1997; Liesack et al. 1997). Therefore, the aim of this study was to compare the community fingerprints obtained by PCR-DGGE based on target DNA extracted from a soil by direct and indirect methods. For this purpose, two direct methods, i.e. cell lysis by bead beating (van Elsas et al. 1997) and cell disruption by grinding in liquid N (Volossiouk et al. 1995), and two indirect methods, i.e. cell extraction and lysis according to van Elsas et al. (1997) and combined RNA/DNA extraction from the bacterial cell fraction (Duarte et al. 1998), were performed.

Materials and methods

Soil

Soil samples were collected from the surface layer (0–10 cm) of Flevo silt loam soil contained in a microplot near the IPO-DLO in Wageningen. The soil was described by Richaume et al. (1992). Briefly, it was a silt loam with about 2% organic matter and pH 7.2. The soil samples were sieved (2-mm mesh) and stored moist at 4 °C. Prior to analysis, the soil samples were acclimatised for a few (5–7) days at room temperature.

DNA extraction and analysis

Two direct and two indirect methods were used for DNA extraction from bacterial communities. The first direct method used for DNA extraction from soil was the method proposed by van Elsas et al. (1997), based on the original protocol of Ogram et al. (1987). Briefly, 2 g soil in 3 ml phosphate buffer (120 mM Na_2HPO_4/NaH_2PO_4, pH 8) with 3 g of 0.1-mm-diameter glass beads was processed in a bead beater (Brown, Germany), followed by SDS treatment, cold phenol extraction and sequential CsCl and KAc precipitation steps. Final purification was performed using a Wizard spin column (Wizard DNA cleanup system, Promega, USA). The second direct method used in this study was based on the method applied by Volossiouk et al. (1995) and originally developed for fungal DNA. Briefly, 2 g soil was ground with liquid N by using a mortar and pestle for about 5 min until a fine powder remained. The powdered soil was suspended in milk powder solution followed by centrifugation, treatment with SDS extraction buffer, phenol (pH 8.0) extraction, CsCl precipitation and final purification using Wizard spin columns.

For DNA extraction from bacterial cells, the indirect method of van Elsas et al. (1997) was used. Briefly, bacterial cells were separated from soil particles (2 g) by blending in 0.1% sodium pyrophosphate (NaPP) buffer followed by differential centrifugation (pelleting of soil particles for 3 min at 121 *g*, harvesting of bacterial fraction for 20 min at 20,200 *g*). The bacterial fraction was lysed in phosphate buffer by bead beating followed by SDS treatment, cold phenol extraction, first purification with hexadecyltrimethyl ammonium bromide/NaCl solution and final purification with glassmilk (GeneClean II kit; La Jolla, USA). The second indirect method for DNA extraction from bacterial cells was adapted from the protocol proposed for rRNA extraction from soil (Duarte et al. 1998). In this protocol, the bacterial fraction was dispersed from soil (4 g) by blending in 0.1% NaPP buffer followed by differential centrifugation (pelleting of soil particles for 3 min at 121 *g*, centrifugation of the bacterial fraction for 20 min at 21,000 *g*). The bacterial pellet was resuspended in phosphate buffer (pH 5.8), and, after addition of glass beads (0.1 mm diameter), SDS and acid phenol (pH 5.0), lysed by bead beating (twofold, separated by incubation at 60 °C). The slurry was then sequentially extracted with acid phenol and a mixture of acid phenol:chloroform:isoamyl alcohol (25:24:1), pH 5. Nucleic acids were precipitated with 3 M NaAc (pH 5.5) and isopropanol. DNA was purified by precipitation with CsCl followed by purification using the Wizard spin column clean-up system. Absorbency measurements at A_{260} and A_{280} to calculate the A_{260}/A_{280} purity ratio of DNA samples (Crecchio and Stotzky 1998) were determined with a GeneQuant RNA/DNA calculator (Pharmacia, Sweden) using a small-volume quartz cuvette. DNA quality (size) and quantity was checked by electrophoresis in 0.8% (w/v) horizontal agarose gel run in 0.5% Tris-borate-ethylenediaminetetraacetate (TBE, pH 8.3) TBE buffer and stained with 0.9 μg ml^{-1} ethidium bromide (Sambrook et al. 1989). A molecular size marker (1-kb ladder) was used as the reference.

A 1-μl volume (roughly 5–10 ng in undiluted form) of each DNA preparation was amplified by PCR with a Peltier thermal cycler PTC 200 (MJ Research, USA). The PCR mixture used contained 0.2 μM of each primer, 200 μM of each deoxynucleotide (dNTP) dNTP, 5 μl of 10× Stoffel buffer (Perkin-Elmer, USA), 5 U of Ampli*Taq* Stoffel fragment (Perkin-Elmer, USA), 3.75 mM $MgCl_2$, 0.5 μl of 1% (v/v) formamide, 0.25 μg T4 gene 32 protein (Boehringer, Mannheim, Germany) and sterile Milli-Q water, to a final volume of 50 μl. The primers for PCR were specific for conserved bacterial 16S rDNA sequences (Heuer and Smalla 1997). PCR with primers R1401 (5′ GCG TGT GTA CAA GAC CC-3′) and F968GC (5′ GC clamp-AAC GCG AAG AAC CTT AC-3′) amplified a bacterial 16S rDNA fragment

from positions 968 to 1401 (*Escherichia coli* numbering). The GC-rich sequence attached to the 5′ end of primer F968GC prevented complete melting of the PCR products during subsequent separation on a denaturing gradient during DGGE (Muyzer et al. 1993). PCR amplification was performed for 40 cycles in a touchdown scheme (Duarte et al. 1998) as follows: after initial denaturation of 4 min at 94 °C, each cycle consisted of denaturation at 94 °C for 1 min, primer annealing at the annealing temperature (T_A) for 1 min, and primer extension at 72 °C for 1 min. In the first ten cycles, T_A decreased by 2 °C every second cycle from 65 °C in the first cycle to 57 °C in the tenth. In the last 30 cycles, T_A was 55 °C. Cycling was followed by final primer extension at 72 °C for 10 min. PCR products were visualised by electrophoresis in 1.2% (w/v) agarose gels after ethidium bromide (0.9 μg ml^{-1}) staining (Sambrook et al. 1989). Strong bands of the expected size (450 bp) were subjected to DGGE analysis.

DGGE (Muyzer et al. 1993; Heuer and Smalla 1997) was performed with an Ingeny phor U-2 system (Leiden, The Netherlands). Samples of 20 μl of PCR product were loaded onto 6% (w/v) polyacrylamide gels in 0.5 strength Tris-acetate-ethylenediaminetetraacetate (TAE, pH 8.0) TAE buffer. The polyacrylamide gels were prepared with a denaturing gradient ranging from 45% at the top of the gel to 65% at the bottom (where 80% denaturant contained 5.6 M urea and 32% formamide). The electrophoresis was run for 16 h at 60 °C and 100 V. After the runs, gels were removed from the set-up and stained for 60 min with SYBR green I nucleic acid gel stain (Molecular Probes, The Netherlands). The stained gels were immediately photographed on a UV transillumination table with a CCD camera and scanned (Biozym, The Netherlands). Digital images of the gels showed banding patterns that were analysed by the Molecular Analyst Fingerprinting software (BioRad, The Netherlands). To obtain clustering trends among the soil samples analysed, cluster analysis using the unweighted pair group with mathematical averages (UPGMA) was performed using the 1/0 clustering method of the NT-SYS program (Exeter Software, New York).

Results

Different methods were used for the extraction of bacterial DNA from soil. These can be divided into two categories, i.e. direct and indirect methods. The direct methods (i.e. bead beating or cold disruption lysis of cells) extracted the highest amounts of crude DNA from the soil (data not shown). After purification and gel electrophoresis of the pure extracts, bands of high molecular weight (10–40 kb) DNA were observed for all methods, with the highest band intensities being recorded for the bead beating-based methods. This observation was supported by estimation of DNA concentrations in the gel (comparison to standard), as well as assessment of the A_{260} values. The direct bead beating method yielded 53.0 ± 19.9 μg DNA g^{-1} soil, the direct cold disruption method 16.3 ± 2.5 μg DNA g^{-1} soil, the indirect bead beating method (van Elsas et al. 1997) 25.1 ± 8.3 μg DNA g^{-1} soil, and the indirect RNA/DNA method 8.3 ± 5.9 μg DNA g^{-1} soil. Although the highest yield of DNA was obtained with the direct bead beating method, the highest purity of DNA (A_{260}/A_{280} 1.62) was obtained by the indirect method (van Elsas et al. 1997). By comparison, the A_{260}/A_{280} ratio was 1.52 in the case of DNA extracted by the direct methods. All DNA extracts obtained after Wizard purification were sufficiently pure for PCR amplification, and no traces of brown colour (i.e. humic contaminants) were observed. Electrophoresis of PCR products in all cases revealed bands of approximately 450 bp.

Samples (around 5 ng per reaction) of the undiluted soil DNA extracted by different methods showed little variation in banding patterns when analysed by PCR-DGGE. In all patterns, 17–26 bands of various intensities were detected per sample, with about 12 bands shared among all samples (Fig. 1). UPGMA cluster analysis (dice coefficient of similarity) of the profiles revealed that all profiles were about 82% similar, with distinct separation of the profile obtained with the indirect RNA/DNA method (as one cluster) from those obtained with the other methods, which composed the second group. In this latter group, clustering of the profiles obtained with the direct and indirect bead beating methods was noticed, with separation of the profile obtained with the direct cold disruption method (Fig. 2). Replicates of the same DNA extraction method showed the highest similarity. A strong band around 55% denaturant was found in both replicates extracted with the indirect RNA/DNA method. In the profiles generated with DNA from the direct methods, bands that occurred at low denaturant concentrations, presumably of low GC content, were more numerous than in the profiles of the indirect methods (Fig. 1).

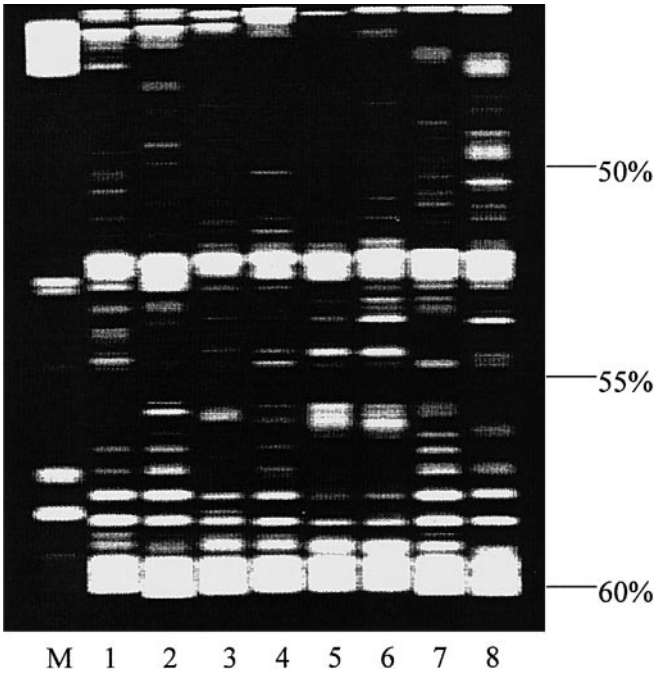

Fig. 1 Denaturing gradient gel electrophoresis (DGGE) patterns of 16S rDNA fragments from soil after image analysis by Molecular Analyst Fingerprinting (MAF-2) software. DNA was extracted by two direct and two indirect methods. *Percentages* indicate the percentage of denaturant at each position. *M* 1-kb DNA ladder marker (Gibco BRL), *1* bead beating (direct method) soil sample (A), *2* bead beating (direct method) soil sample (B), *3* DNA extraction (indirect method) A, *4* DNA extraction (indirect method) B, *5* RNA/DNA extraction (indirect method) A, *6* RNA/DNA extraction (indirect method) B, *7* cold disruption (direct method) A, *8* cold disruption (direct method) B

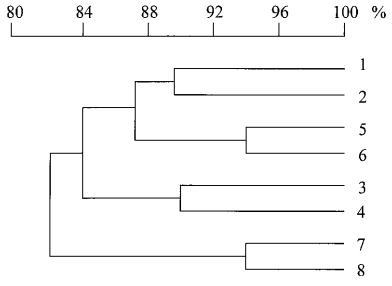

Fig. 2 Genetic similarity of microbial-community profiles obtained with polymerase chain reaction (PCR)-DGGE from the target DNA extracted by four methods from soil. *Numbers* correspond to the same methods described for Fig. 1

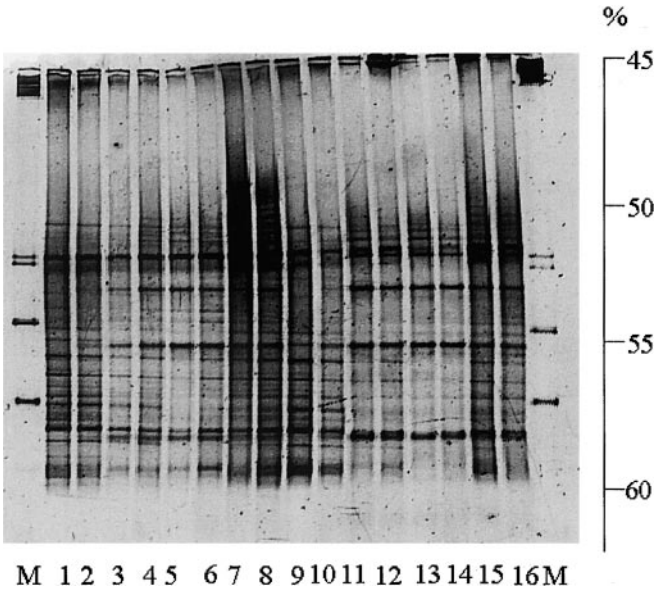

Fig. 3 DGGE patterns of 16S rDNA fragments from soil after dilution of the target DNA. DNA was extracted by two direct and two indirect methods from the soil. *Percentages* indicate the percentage of denaturant at each position. *Successive lanes* (two per method) in *each group* represent bead beating (direct method), cell extraction followed by DNA extraction (indirect method; van Elsas et al. 1997), RNA/DNA extraction (indirect method), and cold disruption (direct method), respectively. *M* 1-kb DNA ladder marker (Gibco BRL), *lanes 1–8* 1:10 dilutions of target soil DNA, *lanes 9–16* 1:50 dilutions of target soil DNA

Dilution of target DNA generated significant variation in banding patterns when analysed by PCR-DGGE. In the profiles, 17–28 bands were visible for the different samples (i.e. extraction method, and dilution), with only a limited number of bands being shared among all samples. However, about 12 bands in the same position occurred in the tracks that represented target DNA diluted 1:10, while only five bands were common at dilution 1:50 (Fig. 3). The highest number of bands (i.e. 26 ± 2) was found in the tracks representing target DNA obtained with the cold disruption method and diluted 1:10. Clustering of the profiles showed that replicates invariably were closely related, at >90% similarity. Moreover, two broad clusters, corresponding to the extraction method and soil DNA dilution applied, were distinguished, at a similarity level of 51%. In contrast to the profiles obtained with both direct methods, in which the dilutions clustered at about 80%, a higher similarity (i.e. 85%) was found between the profiles of the indirect extractions at the same dilution than between the same method at different dilution (Fig. 4). The profiles of the direct cold disruption method clustered closer to those of the indirect extractions than those of the direct bead beating method. The highest numbers of, and generally stronger, bands were found in the profiles of bacterial community DNA extracted by the direct methods. In addition, a substantial number of bands, comprising a large part of the profiles, were present at the same denaturant concentrations when comparing both dilutions of the bacterial DNA extracts (Fig. 3).

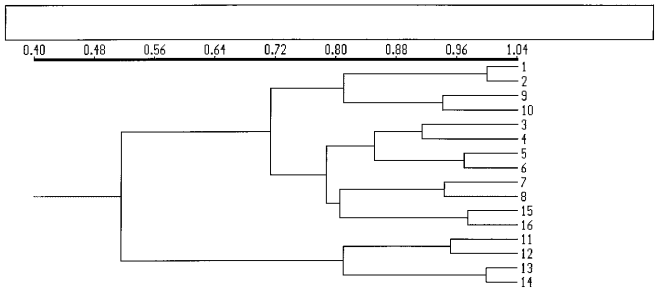

Fig. 4 Genetic similarity of microbial-community profiles obtained with PCR-DGGE from the diluted target DNA extracted by four methods from soil. *1–8* soil DNA dilutions of 1:10; *9–16* soil DNA dilutions of 1:50; *1, 2, 9, 10* bead beating (direct method); *3, 4, 11, 12* DNA extraction (indirect method; van Elsas et al. 1997); *5, 6, 13, 14* RNA/DNA extraction (indirect method); *7, 8, 15, 16* cold disruption (direct method)

Discussion

The first step in soil microbial community characterisation by molecular methods is the extraction of nucleic acids from soil (Bej and Mahbubani 1996). Then, the extracted DNA or RNA has to be pure enough for PCR amplification and subsequent cloning, sequencing, restriction analyses (e.g. RFLP, ARDRA, T-RFLP), DGGE/TGGE or SSCP profiling, and hybridisation. The two intrinsically different approaches (i.e. direct and indirect) for DNA extraction from soil used in this study generated yields of DNA comparable to those reported in other papers (Saano et al. 1995; Smalla and van Elsas 1995; Zhou et al. 1996; Clegg et al. 1997; van Elsas et al. 1997; Yeates et al. 1997; Duarte et al. 1998; Gelsomino et al. 1999). A major, critical step in direct soil DNA extraction is the efficient lysis of the microbial cells in soil. The bead beating method used for cell lysis in soil gave a higher DNA yield than that generated by cell disruption with liquid N followed by mortar

and pestle grinding. However, this difference was not statistically significant. Van Elsas et al. (1997) found up to 35 μg DNA g^{-1} dry soil in different soil types using the same bead beating procedure. They also applied various steps for purification of the crude DNA depending on the soil type and organic matter levels. In this study, the complete procedure, including a Wizard spin column clean-up step, was performed. In this way, relatively pure DNA ready for PCR amplification was obtained. By comparison, another procedure for the direct extraction of DNA from soil by high-salt/SDS/heat treatment recovered 2.5–26.9 μg DNA g^{-1} soil, with A_{260}/A_{280} ratios between 1.17 and 1.23 according to the purification treatment (Zhou et al. 1996). An improved yield and higher purity of DNA after bead beating in comparison with hot-SDS extraction, three freeze-thaw cycles or a combination of treatments were also found by Cullen and Hirsch (1998). It is likely that the bead beating method is quite effective in establishing bacterial cell lysis, whereas the brown humic compounds extracted together with DNA from soil can be readily removed in subsequent purification steps. Also, in another study, cold disruption of cells with liquid N in soil and suspension of the soil particles in the presence of skimmed milk enhanced lysis of cells and protected crude DNA from severe humic contamination, respectively (Volossiouk et al. 1995).

An indirect soil DNA extraction method, which combined sodium pyrophosphate solubilisation of cells and soil aggregates with efficient cell lysis by bead beating followed by DNA extraction and purification, was recently developed (van Elsas et al. 1997). As expected, the DNA yields obtained with this method were somewhat reduced compared to those obtained by the direct bead beating extraction method, suggesting the DNA obtained with the former might have been more representative of the bacterial communities in soil. The second indirect (RNA/DNA) extraction method used in this study was even less efficient with regard to the amount of DNA obtained. This might have been due to the acid phenol treatment, which tends to be favourable for RNA rather than DNA extraction (Duarte et al. 1998).

Several authors have reported that the efficiency of PCR amplification of target soil DNA depends on the relationship between the level of target DNA and that of contaminants in the crude extract, which in turn is affected by the efficacy of the purification procedure (Saano et al. 1995; Bej and Mahbubami 1996; Yeates et al. 1997; Cullen and Hirsch 1998). Positive PCR amplification of soil-extracted DNA is a good indicator of purity of the sample, as *Taq* polymerase can be inhibited by quite low amounts (e.g. <1 μg ml^{-1}) of humic acids (Tsai and Olson 1992; Tebbe and Vahjen 1993). All four methods used to obtain DNA from the soil resulted in successful amplification of 16S rRNA genes. Thus, the community DNA yielded by all methods was pure enough for further DGGE analysis. Also, physical treatment by bead beating to lyse the microbial cells did not result in heavily sheared template DNA as reported by others (Dijkmans et al. 1993; Zhou et al. 1996; Clegg et al. 1997; Porteus et al. 1997).

The results obtained with PCR-DGGE performed on soil DNA extracted by different methods showed clear profiles that likely represented the dominant bacterial fractions in the samples. Taking into account the scarcely detectable specific genome numbers, the total microscopic cell count and estimated losses of cells and DNA, Gelsomino et al. (1999) suggested that these fractions represent at least 0.5–1.5% of the total microscopically detectable bacterial community. They also suggested that the bacterial PCR-DGGE system will generally detect a limited number of dominant, ubiquitous and ecologically recalcitrant bacterial types in a given soil. Grossly speaking, the profiles generated by all four extraction methods were largely similar (i.e. revealed 82% similarity), indicating that the methods were of similar efficiency in the extraction of the target DNA underlying these consistent bands in the DGGE patterns. Also, Duineveld et al. (1998) recognised 82% similarity between profiles as an indicator of the lack of significant difference in community structure. However, the differences that were apparent above this level (i.e. $>18\%$ difference) indicated that varying populations of bacteria were detected in the same soil by the different methods of soil DNA extraction. Heuer and Smalla (1997) applied TGGE analysis of 16S rDNA fragments amplified from DNA extracted and purified with different protocols. They reported that fingerprints generated with DNA extracted after harsh lysis of cells were different from those obtained after a soft lysis procedure. Also, Liesack et al. (1997) reported that environmental DNA extracted from the same soil sample using different lysis protocols (e.g. bead beating homogenisation alone versus a combination of methods) produced different 16S rDNA profiles when analysed by DGGE. The aforementioned results indicate that the relative composition of DNA extracted from the same soil sample can be strongly influenced by the lysis procedure applied. In this respect, it should be noted that due to the very nature of the PCR-DGGE approach, comparisons between DGGE profiles based on clustering are obviously of relative value. First, bands below detection, even though they might represent substantial bacterial populations, will not be scored. Secondly, the conditions in the PCR reaction that determine the relative rate of amplification of each band can be slightly different between different extracts, resulting, after 30–45 cycles, in widely divergent profiles. Thirdly, the primer-product versus product-product competitive effect that is often observed in later stages of the PCR might be different in the different PCR reaction mixes due to different amplification efficiencies and target DNA amounts. Nevertheless, the relative similarities observed by us between the profiles obtained by four methods seemed to indicate that, for the most part, very similar bacterial populations were being extracted and amplified.

The dilution of template DNA extracted by the various methods resulted in divergent fingerprints generated by PCR-DGGE. Various bacterial populations could be detected depending on the dilution level of the total target soil DNA. Presumably, populations that were less dominant might have become apparent by PCR-DGGE when the dominant groups were diluted out. The application of two dilution levels allowed the finding that the most stable profile of the soil bacterial community was generated by the direct methods. Thus, direct lysis of bacterial cells by bead beating or grinding in liquid N generated the highest numbers of different 16S rDNA fragments which were most stable upon dilution of the template DNA. By contrast, the profiles obtained with the indirect methods clustered together in different groups, and were separated by dilution level. It is likely that these methods both preferentially yielded DNA from a major, easily desorbed, bacterial fraction, which consisted of low density populations. Also, Gelsomino et al. (1999) reported the separate clustering of DGGE profiles obtained with direct versus indirect extraction methods. They indicated that this difference was based entirely on the presence or absence of weak bands, suggesting that less numerous bacterial types might have been differentially extracted.

The differences observed between the profiles obtained should be explained very carefully, as they are the result of competitive PCR followed by DGGE separation of the amplicons. It is often assumed that the number and intensity of the bands observed reflects the relative dominance of the targets (i.e. bacteria). However, several problems can distort this interpretation, and these include problems inherent in the extraction methods. First, each soil DNA extraction method yields different levels of inhibitors, which may affect the nature of the competition in the PCR reaction and, finally, the patterns. Secondly, albeit apparently infrequent, each single band can theoretically have different origins, i.e. relate to different underlying organisms (different sequences with similar melting behaviour). Thirdly, one organism might produce more than one band in the DGGE profiles due to the presence of several copies of the *rrN* operon with slightly differing sequences (Muyzer and Smalla 1998). Fourth, some of the bands in the profiles might be, in reality, chimeric forms produced during PCR. Moreover, if certain targets amplify more readily than others, the distribution of band intensities is distorted. Finally, the dilution effect may shift the balance to a quite different competitive PCR with a different outcome due to, for example, different primer-product versus product-product annealing competition (Gelsomino et al. 1999).

In conclusion, the aim of this study was to assess to what extent the procedure used for soil DNA extraction would affect the profiles representing the composition of the bacterial community, i.e. which method would yield the most representative pattern? Presumably, the answer is equivocal, if only because of the virtual impossibility of extracting and lysing all bacterial cells present in a given soil. Moreover, problems of soil heterogeneity at the microscale level play a role. The methods selected should, therefore, be used bearing in mind the soil type and the aim of the study, acknowledging that, much like plating of soil bacteria, a magical method which allows the visualisation of the total bacterial diversity is impossible to achieve. However, the results of the PCR-DGGE analysis carried out here, especially those obtained with the diluted target DNA, allowed us to arrange the methods for DNA extraction from soil with respect to the decreasing diversity (reflected in numbers of bands) of bacterial populations found, as follows: direct cold disruption ≥ direct bead beating > indirect cell extraction/lysis method > indirect RNA/DNA extraction. PCR-DGGE was found to be a suitable technique with which to find the best method for DNA extraction from soil, which could be used further for the determination of microbial community diversity at the molecular level.

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References

Bej AK, Mahbubani MH (1996) Current development and applications of nucleic acid technology in the environmental sciences. In: Dangler CA (ed) Nucleic acid analysis. Principles and bioapplications. Wiley-Liss, New York, pp 231–273

Clegg CD, Ritz K, Griffiths BS (1997) Direct extraction of microbial community DNA from humified upland soils. Lett Appl Microbiol 25:30–33

Clegg CD, Ritz K, Griffiths BS (1998) Broad-scale analysis of soil microbial community DNA from upland grasslands. Antonie van Leeuwenhoek 73:9–14

Crecchio C, Stotzky G (1998) Binding of DNA on humic acids: effect on transformation of *Bacillus subtilis* and resistance to DNase. Soil Biol Biochem 30:1061–1067

Cullen DW, Hirsch PR (1998) Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biol Biochem 30:983–993

Dijkmans R, Jagers A, Kreps S, Collard J-M., Mergeay M (1993) Rapid method for purification of soil DNA for hybridization and PCR analysis. Microb Releases 2:29–34

Duarte GF, Rosado AS, Seldin L, Keijzer-Wolters AC, Elsas JD van (1998) Extraction of ribosomal RNA and genomic DNA from soil for studying the diversity of the indigenous bacterial community. J Microbiol Methods 32:21–29

Duineveld BM, Rosado AS, Elsas JD van, Veen JA van (1998) Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum via denaturing gel electrophoresis and substrate utilization patterns. Appl Environ Microbiol 64:4950–4957

Dunbar J, Takala S, Barns SM, Davis JA, Kuske CR (1999) Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl Environ Microbiol 65:1662–1669

Elsas JD van, Mantynen V, Wolters A (1997) Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCP-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. Biol Fertil Soils 24:188–195

Elsas JD van, Duarte GF, Rosado AS, Smalla K (1998) Microbiological and molecular methods for monitoring microbial inoculants and their effects in the environment. J Microbiol Methods 32:133–154

Felske A, Wolterink A, Lis R van, Akkermans ADL (1998) Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils. Appl Environ Microbiol 64:871–879

Gelsomino A, Keijzer-Wolters AC, Cacco G, Elsas JD van (1999) Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. J Microbiol Methods 38:1–15

Griffiths BS, Ritz K, Ebblewhite N, Dobson G (1999) Soil microbial community structure: effects of substrate loading rates. Soil Biol Biochem 31:145–153

Head IM, Saunders JR, Pickup RW (1998) Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microb Ecol 35:1–21

Heuer H, Smalla K (1997) Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In: Elsas JD van, Trevors JT, Wellington EMH (eds) Modern soil microbiology. Dekker, New York, pp 353–373

Heuer H, Smalla K (1999) Bacterial phyllosphere communities of *Solanum tuberosum* L. and T4-lysozyme-producing transgenic variants. FEMS Microbiol Ecol 28:357–371

Kowalchuk GA, Bodelier PLE, Heiling GHJ, Stephen JR, Laanbroek HJ (1998) Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol Ecol 27:339–350

Lee D-H, Zo Y-G, Kim S-J (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl Environ Microbiol 62:3112–3120

Liesack W, Janssen PH, Rainey FA, Ward-Rainey NL, Stackebrand E (1997) Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques. In: Elsas JD van, Trevors JT, Wellington EMH (eds) Modern soil microbiology. Dekker, New York, pp 375–433

Liu W-T, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

Muyzer G (1998) Structure, function and dynamics of microbial communities: the molecular biological approach. In: Carvalho GR (ed) Advances in molecular ecology. IOS, Amsterdam, pp 87–117

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73:127–141

Muyzer G, De Wall EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Ogram A, Sayler GS, Barkay TJ (1987) DNA extraction and purification from sediments. J Microbiol Methods 7:57–66

Ovreas L, Torsvik V (1998) Microbial diversity and community structure in two different agricultural soil communities. Microb Ecol 36:303–315

Porteous LA, Seidler RJ, Watrud LS (1997) An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. Mol Ecol 6:787–791

Richaume A, Smit E, Faurie G, Elsas JD van (1992) Influence of soil type on transfer of plasmid RP4p from *Pseudomonas fluorescens* to introduced recipient and to indigenous bacteria. FEMS Microbiol Ecol 101:281–292

Saano A, Tas E, Pippola S, Lindstrom K, Elsas JD van (1995) Extraction and analysis of microbial DNA from soil. In: Trevors JT, Elsas JD van (eds) Nucleic acids in the environment: methods and applications. Springer Verlag, Heidelberg, pp 49–67

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning – a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Schwieger F, Tebbe CC (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl Environ Microbiol 64:4870–4876

Smalla K, Elsas JD van (1995) Application of the PCR for detection of antibiotic resistance genes in environmental samples. In: Trevors JT, Elsas JD van (eds) Nucleic acids in the environment: methods and applications. Springer Verlag, Heidelberg, pp 241–256

Smit E, Leeflang P, Wernars K (1997) Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. FEMS Microbiol Ecol 23:249–261

Smit E, Leeflang P, Glandorf B, Elsas JD van, Wernars K (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. App Environ Microbiol 65:2614–2621

Tebbe CC, Vahjen W (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl Environ Microbiol 59:2657–2665

Torsvik V, Sorheim R, Goksoyr J (1996) Total bacterial diversity in soil and sediment communities – a review. J Ind Microbiol 17:170–178

Trevors JT (1998) Bacterial biodiversity in soil with an emphasis on chemically-contaminated soils. Water Air Soil Pollut 101:45–67

Trevors JT, Elsas JD van (1995) Nucleic acids in the environment: methods and applications. Springer Verlag, Heidelberg

Tsai Y-L, Olson BH (1992) Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl Environ Microbiol 58:2292–2295

Volossiouk T, Robb EJ, Nazar RN (1995) Direct DNA extraction for PCR-mediated assays of soil organisms. Appl Environ Microbiol 61:3972–3976

Yap WH, Li X, Soong TW, Davies JE (1996) Genetic diversity of soil microorganisms assessed by analysis of hsp70 (*dna*K) sequences. J Ind Microbiol 17:179–184

Yeates C, Gillings MR, Davidson AD, Altavilla N, Veal DA (1997) PCR amplification of crude microbial DNA extracted from soil. Lett Appl Microbiol 25:303–307

Zhou J, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. Appl Environ Microbiol 62:316–322