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Bacterial Community DNA Extracted from Soils Polluted with Heavy Metals

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Abstract

This paper describes the application of two direct and one indirect methods for the extraction of microbial community DNA from soils polluted with heavy metals. DNA was extracted directly from soil using a gentle method based on soil incubation at 37°C with proteinase K and SDS, or the method was modified by the addition of a bead beating step. The indirect approach was based on the RNA/DNA extraction method. The level of soil contamination did not affect the yields of DNA extracted and PCR amplification of the target DNA. The results indicate that the DNA obtained by the applied protocols was sufficiently pure for further molecular analyses.

Keywords: DNA extraction, PCR amplification, heavy metals, polluted soil

Introduction

The application of molecular biology techniques in microbial ecology allows for a more realistic assessment of microorganisms and their interactions in various environments [1]. Soil is an example of a heterogeneous habitat where traditional techniques used for microflora characterization have been improved by methods based on DNA/RNA analysis. However, the use of DNA-based techniques has one major limitation; DNA of sufficient quality and quantity is required. To isolate microbial DNA from soil, two approaches are usually applied, direct extraction of DNA from cells within the soil, and extraction of DNA from microorganisms separated from soil particles by fractionation techniques. Critical steps in direct soil DNA extraction are the efficiency of lysis of microbial cells in soil, the separation of cell DNA from extracellular DNA, contamination of extracted DNA by humic acids, proteins, polysaccharides, metals and other inhibitors of PCR [2]. A range of lysis treatments has been used in the different protocols, including enzymatic (lysozyme, proteinase K) treatment, SDS, microwave, ultrasonication and bead beating, as well as different combinations of these treatments [1]. For indirect approach of DNA extraction, a major obstacle in most protocols is the efficient extraction of bacterial fractions from soil [3]. Direct extraction generally generates greater yields of DNA from soil than microbial fractionation, and is regarded to be more representative of the soil microbial community [4]. However, the purity of DNA obtained by the indirect methods is usually greater than that from direct extraction [5]. Direct lysis *in situ* results in the co-extraction of humic impurities that can inhibit subsequent molecular reactions as PCR amplification and restriction enzyme digestion [6, 7]. In addition, when DNA is extracted from a contaminated soil, heavy metals may be significant inhibitors of the DNA amplification by *Tag* polymerase [7].

The objective of this study was to determine whether soil contamination with heavy metals has any effect on the quantity of microbial community DNA extracted directly from the soil or obtained from a bacterial fraction previously separated from the soil aggregates. The quality of the DNA recovered from soils containing different

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concentrations of heavy metals was compared with that from uncontaminated soil by the assessment of PCR amplification of the target DNA.

Materials and Methods

Soil

Soil samples were collected from the surface (0-10 cm) at four sites (i.e. PS1, PS2, PS19 and PS22) of Piekary Slaskie in Silesia, a highly industrialized region of Poland. Two soils with lower contents of heavy metals were silt loam PS1 (37% sand, 54% silt, 11% clay, 3.4% organic C, pH_{KCl} 6.2, 160 mg Pb kg^{-1}, 4.4 mg Cd kg^{-1}, 330 mg Zn kg^{-1}) and sandy loam PS2 (59% sand, 34% silt, 7% clay, 2.7% organic C, pH_{KCl} 5.6, 141 mg Pb kg^{-1}, 3.1 mg Cd kg^{-1}, 190 mg Zn kg^{-1}). Higher contaminated were silt loam PS19 (37% sand, 54% silt, 10% clay, 4.6% organic C, pH_{KCl} 6.3, 1830 mg Pb kg^{-1}, 23.3 mg Cd kg^{-1}, 2390 mg Zn kg^{-1}) and sandy loam PS22 (71% sand, 24% silt, 5% clay, 4.0% organic C, pH_{KCl} 6.4, 730 mg Pb kg^{-1}, 24.0 mg Cd kg^{-1}, 1215 mg Zn kg^{-1}). Concentrations of heavy metals were determined by atomic absorption spectrophotometry after soil extraction with aqua regia (cone. HNO_3/conc. HC1, 1:3, v/v). For comparison, an uncontaminated Flevo soil was sampled from a microplot near the IPO-DLO in Wageningen. The soil was described by Richaume et al. [8]. Briefly, it was a silt loam with 4.1% organic matter and pH 7.5. All soil samples were sieved (2 mm-mesh) and stored moist at 4°C. Prior to analysis, the soil samples were acclimatized for 5-7 days at room temperature.

DNA Extraction

Two direct and one 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 Saano and Lindstrom [9]. Briefly, 1-g soil sample was mixed with phosphate buffer (pH 8.0), 1% (w/v) SDS and proteinase K, and incubated at 37°C with occasional shaking. The suspension was treated with 5 M NaCl followed by incubation with hexadecyltrimethyl ammonium bromide (CTAB)/NaCl mixture at 65°C and extraction with chloroform. DNA was purified by precipitation with CsCl followed by final purification on a Wizard spin column (Promega, USA). The same method, but modified by soil bead beating in phosphate buffer [10] before the incubation step with proteinase K, was used as the second direct method for DNA extraction from soil. As the indirect method of DNA extraction from bacterial cells, the method adapted from the protocol proposed for rRNA extraction from soil was used [11]. Briefly, the bacterial fraction was dispersed from soil (4 g) by blending in 0.1% tetra-sodium diphosphate (NaPP) buffer followed by differential centrifugation. The bacterial pellet was resuspended in phosphate buffer (pH 5.8), and after addition of glassbeads (0.1 mm), 20% (w/v) SDS and acid phenol (pH 5.0), the pellet was lysed by bead beating (2-fold, separated by incubation at 60°C). The slurry was then sequentially extracted with acid phenol and a mixture of acid phenol (pH 5.0)/chloroform/isoamyl alcohol (25:24:1, v/v). Nucleic acids were precipitated with 3 M sodium acetate (pH 5.5) and isopropanol. DNA was purified by precipitation with CsCl followed by purification using Wizard clean-up system (Promega, USA). Absorbency measurements at A_{260} and A_{260} were determined with a GeneQuant RNA/DNA calculator (Pharmacia, Sweden) and a small-volumes quartz cuvette to calculate concentration (1 A_{260} unit = 50 µg ml^{-1} double-stranded DNA) and the A_{260}/A_{280} purity ratio of DNA samples [12]. DNA quality (size) was checked by electrophoresis in 0.8% horizontal agarose gel run in 0.5% TBE buffer and stained with 0.9 µg ml^{-1} of ethidium bromide [13].

PCR Amplification

A I-µ! 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 each primer, 200 µM each dNTP, 5 nl of 10 x Stoffel buffer (Perkin-Elmer, USA), 5 U of *AmpliTaq* 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 50 µl. The primers for PCR were specific for conserved bacterial 16S rDNA sequences [14]. 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 [15]. PCR amplification was performed for 40 thermal cycles in a touchdown scheme [11] 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 T_A for 1 min, and primer extension at 72°C for 1 min. In the first 10 cycles, T_A decreased by 2°C every second cycle from 65°C in the first cycle to 57°C in the 10th. 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 [13].

Results and Discussion

To isolate bacterial DNA from soil, the cells have to be separated from colloids of the soil and efficiently lysed followed by the extraction and purification of high molecular weight DNA. Additionally, this DNA must be free from inhibitors for molecular biological manipulations to be performed. In polluted soils, an important class of such inhibitors is represented by heavy metals. Testing several polluted soils enabled the applicability of the DNA extraction methods to be examined.

DNA extracted with all methods described here was greater than 10 kb in size (Fig. 1). Kuske et al. [16] using

hot-detergent treatment, freeze-thaw cycles, and bead mill homogenization also recovered DNA greater than 12 kb in size. Clegg et al. [17] using the lysozyme, SDS and freeze-thaw lysis protocol obtained microbial DNA in the size range 10 to 20 kb. In comparison, the bead beating methods proposed by Yeates et al. [18], and Cullen and Hirsch [19] have yielded DNA greater than 20 kb in size. Although the spun columns purification of DNA used in this study resulted in the DNA molecules shorter than 20 kb, this has not affected the efficiency of PCR analysis. However, higher molecular weight DNA was desirable for PCR as the greater the size of the DNA, the less likely the formation of chimeras during PCR [2].

From 100 g of soil, Steffan et al. [4] extracted 9 µg DNA g^{-1} soil, and Yeates et al. [18] obtained 15 to 23.5 µg g'soil, while from smaller soil samples ranging from 500 mg to 2 g in size, yields of 2.8 to 30 µg DNA g^{-1} soil have been reported [16,17,19-21]. The direct methods used in this study generated 26. 6 to 78.0 µg DNA g^{-1} soil (Table 1), making them at least as efficient as the methods previously reported. Modification of Saano and Lindstrom method [9] by additional bead beating step did not result in higher yield of DNA extracted. In addition, the level of soil pollution with heavy metals did not affect the efficiency of DNA extraction from the soils by the methods used (Table 1). For the indirect RNA/DNA method used here, yields of 5.1 to 13.5 µg DNA g^{-1} soil was obtained from bacterial fraction separated from 3-g soil samples. In comparison, Ovreas and Torsvik [22] required 60-g samples of organic or sandy soil to extract 27 and 9 µg DNA g^{-1} soil from bacterial fraction, respectively. Various recoveries of DNA from the bacterial fraction may result from differences in soil type, the numbers of bacteria and the methods used for the bacteria separation and DNA extraction.

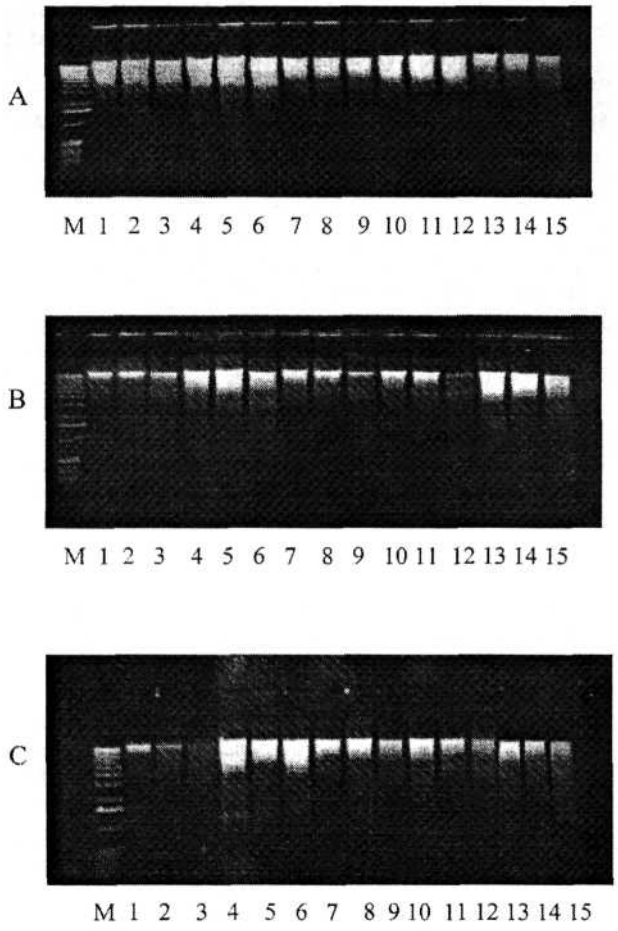

Fig. 1. DNA extracted from PS soils contaminated with heavy metals and uncontaminated Flevo soil. DNA was extracted from the soils by three methods: (A) the direct of Saano and Lindstrom (1995), (B) the direct modified by the addition of bead beating step, (C) the indirect RNA/DNA, respectively. Tracks are as follows: (M) 1-kb DNA ladder marker (Gibco BRL); (1, 2, 3) PS1 soil DNA crude, pure and Wizard pure, respectively; (4, 5, 6) PS2 soil DNA crude, pure and Wizard pure, respectively; (7, 8, 9) PS19 soil DNA crude, pure and Wizard pure, respectively; (10, 11, 12) PS22 soil DNA crude, pure and Wizard pure, respectively; (13, 14, 15) Flevo soil DNA crude, pure and Wizard pure, respectively.

Table 1. Quantities of DNA [a] (µg g^{-1} soil) extracted from different soils by three methods.

Soil	Direct method	Medified direct method	Indirect RNA/DNA method
PS1	53.1 ± 15.4	78.0 ± 28.9	13.5 ± 3.7
PS2	41.5 ± 17.6	39.6 ± 10.4	8.7 ± 4.7
PS19	26.6 ± 1.3	27.7 ± 10.2	11.0 ± 6.3
PS22	31.3 ± 14.4	41.1 ± 17.2	5.1 ± 2.9
Flevo	33.7 ± 14.6	34.0 ± 8.6	7.1 ± 3.9

[a] Values are means ± standard deviations.

The purity of DNA was tested using UV absorbency at different wavelengths. The DNA samples did not exhibit high absorbency ratios (i.e. 1.1 to 1.3 A260/A280) and they were even lower than the A26a/A28o ratio (i.e. 1.5) of DNA extracted from another heavy metal polluted soil, as reported by Yeates et al. [18]. Humic acids, proteins and polysaccharides are common contaminants of DNA extracted from soil and cells [1]. That is why extensive purification is required to reduce inhibitory effect on DNA polymerase and amplify a PCR product. Tsai and Olson [7] found that heavy metals also contribute to inhibitory effects of DNA polymerases. However, Yeates et al. [18] demonstrated that a PCR product from DNA contaminated with humic acids and heavy metals could be obtained without the use of special purification steps. In this study, bacterial 16S rRNA genes were also successfully amplified; however, the extracted DNA was not of high purity, and contaminated soil could be a source of metal inhibitors. It was possible to detect the 450 bp 16S rDNA products of PCR amplification (Fig. 2). However, the dilution of target DNA by 1:10 and 1:50 resulted in some faint bands after PCR. These bands were especially noticed at the 50-fold dilution of the template DNA samples extracted by the direct and indirect RNA/DNA method (data not shown). It is possible that the dilution decreased the number of amplifyable fragments of target DNA, hence *Tag* polymerase, adversely affected by soil

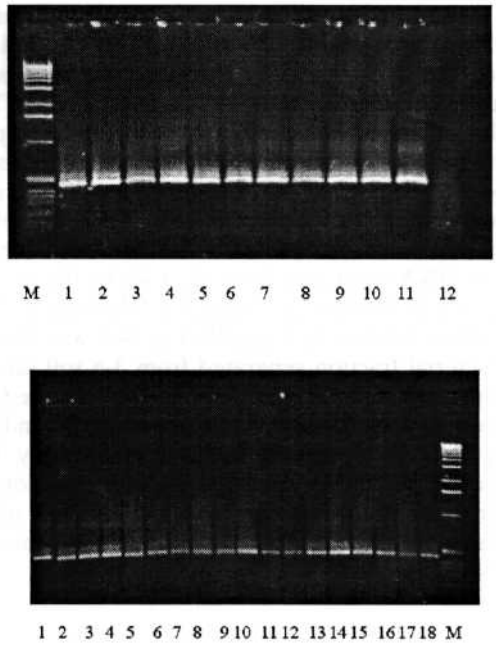

Fig. 2. PCR amplification of DNA extracted from PS soils polluted with heavy metals and unpolluted Flevo soil. DNA was extracted from the soils by the following methods: (a) the direct by Saano and Lindstrom (1995), (b) the direct modified by the addition of bead beating step, (c) the indirect RNA/DNA. The top gel shows results of the method a; M, 1-kb DNA ladder marker (Gibco BRL); lanes 1 and 2, PS1; lanes 3 and 4, PS2; lanes 5 and 6, PS19; lanes 7 and 8, PS22; lanes 9 and 10, Flevo; lane 11 *Burkholderia cepacia* P2; lane 12, negative control. The bottom gel shows results of the method b (lanes 1 to 9) and method c (lanes 10 to 18). Lanes 1, 2, 10 and 11, PS1; lanes 3, 4, 12 and 13, PS2; lanes 5, 6, 14 and 15, PS19; lanes 7, 8, 16 and 17, PS22; lanes 9 and 18, Flevo; M., 1-kb DNA ladder marker (Gibco BRL).

contaminants, was unable to amplify the templates efficiently. PCR amplification of extracted DNA is a good indicator of purity of the sample, as *Taq* polymerase can be inhibited by humic acid and heavy metal contamination [6, 7].

In conclusion, both direct methods and indirect RNA/DNA method have been successfully applied for extraction of DNA from soil and bacterial cells ready for PCR amplification of eubacterial community 16S rDNA. The addition of a bead beating step to the direct procedure of the DNA extraction did not increase yields of DNA obtained. The level of soil contamination with heavy metals did not decrease the contents of total soil community DNA and those of bacterial fraction. In addition, the concentrations of heavy metals in the soils did not inhibit PCR reaction as compared with the unpolluted soil. The results indicate that the methods can be used with contaminated soils to provide enough quality DNA from the soil microbial community for PCR amplification, a prerequisite of modern techniques for studying microbial ecology.

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