Extraction of DNA from soil

Patrick Robe a,*, Renaud Nalin a, Carmela Capellanob, Timothy M. Vogel c, Pascal Simonetc

a LibraGen, 11, bd Einstein, 69100 Villeurbanne, France
b Aventis Pharma, centre de recherche de Vitry-Alfortville, 13, quai Jules Guesde B. P. 14, 94403 Vitry sur Seine cedex, France
c Center for Microbial Ecology, UMR CNRS 5557, University Claude Bernard LYON I, 43, boulevard 11 Novembre 1918, 69622 Villeurbanne cedex, France

Received 21 March 2003; accepted 20 May 2003

Abstract

There is an increased interest in the extraction of nucleic acids from various environmental samples, since molecular techniques allow less biased access to a greater portion of uncultivable microorganisms. Two strategies have been developed to improve DNA recovery in terms of yield, purity and unbiased representation of the microbial diversity. The first approach consists of the direct extraction of nucleic acids from soil through in situ cell lysis followed by DNA purification. The alternative approach is based on the separation of bacteria from the soil particles followed by cell lysis and then DNA purification. Several published methods describe the recovery of highly purified nucleic acids that are well-suited for molecular purposes even though a new challenge concerns the recovery of large bacterial DNAs essential for functional investigation of gene clusters and biosynthetic pathways. This review presents an overview of the available methods to achieve this challenging objective.

Keywords: Soils; DNA extraction; Direct lysis; Indirect extraction; Metagenome; Bacteria

1. Introduction

Prokaryotes are the most ubiquitous organisms on earth, represented in all habitats, including soil, sediment, marine and terrestrial subsurface, animals and plant tissues and play a key role in the biogeochemical cycles of the biosphere and represent an enormous reservoir of novel valuable molecules for health or industry. The vast majority of enzymes and antimicrobial products have been isolated from microorganisms cultivated on artificial media in the laboratory.

Soil appears to be a major reservoir of microbial genetic diversity [11] and may be considered as a complex environment. This extreme complexity results from multiple interacting parameters including soil texture and structure, water content, pH, climatic variations and biotic activity. Soil texture and structure are mainly determined by sand, silt, clay and organic matter content through the organization of micro- and macro-aggregates. Microorganisms are heterogeneously distributed inside microaggregates and in macroporosities outside microaggregates [19,54]. Moreover, microorganisms strongly bind with soil particles (including clay) through a variety of binding mechanisms [4] that reduce access to the whole bacteria.

Microbial ecology studies have provided a better understanding of (i) the structure of microbial communities and the evolution of these communities under various climatic, biotic and xenobiotic conditions and (ii) the activities of microorganisms. For several years, investigations of microbial diversity were mainly based on isolation and laboratory cultivation of bacteria which lead to underestimation of the true diversity. Laboratory cultivation relied on a succession of critical steps that increase the bias: (i) soil dispersion in order to dislodge cells from inner soil compartments and from soil surface particles; (ii) centrifugation-based separation of the bacteria from soil and organic particles; (iii) plating of bac-
terial suspension on various solid nutrient media; (iv) isolation and characterization of individuals. Laboratory cultivation approaches remain of great interest when a selective enrichment targets isolates for their ability to metabolize specific substrates or to degrade deleterious xenobiotic compounds. However, only bacterial isolates for which optimal cultivation criteria have been determined can be recovered by this approach.

The emerging use of molecular biology in microbial ecology has revealed that less than 1% of the microorganisms present in numerous environments are readily cultivable [2]. Molecular analysis of bacterial diversity in complex environments has relied on DNA/DNA hybridization, reassociation kinetics studies [66] and mainly on 16S rDNA amplification, (for review see Ref. [74]). However, these methods require DNA extracts free of the numerous inhibitory contaminants commonly found in environmental samples (for review see Ref. [75]) and must exhibit an unbiased sampling of the investigated bacterial community.

The aim of this review is to present an overview of soil DNA extraction methods. Two approaches have been developed for extracting nucleic acids from soil samples. One is the direct extraction of nucleic acids from soil samples after in situ cell lysis which is then followed by DNA purification [46]. The second approach separates the cell fraction from soil particles first. Then, this bacterial fraction is lysed and the nucleic acids purified [10,23,67]. Both approaches have advantages and disadvantages related to DNA yields, DNA purity for molecular purposes, and the unbiased representation of the entire microbial diversity [10,58].

2. Direct lysis extraction method

The direct in situ lysis extraction method has been widely used during the last decade. This method, which assumes complete in situ lysis of all microorganisms, generally provides the highest DNA yields within acceptable processing times.

All published techniques derived from the original procedure of Ogram et al. [46] are summarized in two critical steps. The disruption of the microbial cell wall is the first step leading to the release of all nucleic acids from all bacteria, theoretically independent of the cell wall sensitivity to lysis, the location of bacteria in microstructures, and their interactions with soil particles. In the second step, nucleic acids are separated from soil particles.

2.1. Cell lysis

Currently, three types of cell lysis (or membrane disruption) are used alone or in combination: (i) physical-, (ii) chemical- and (iii) enzymatic-disruption.

Physical treatments, which destroy soil structure, tend to have the greatest access to the whole bacterial community, including bacteria deep within soil microaggregates. The most commonly used physical disruption methods are freezing–thawing or freezing–boiling [12,43,71] and bead-mill homogenization [37,41,42,63]. When testing a “bead beating” method, Bürgmann et al. [7] established that DNA yields increased with longer beating times, higher speed and reduced extraction buffer volumes but at the cost of DNA shearing.

Other methods, which use mortar mill grinding [64], grinding under liquid nitrogen [73,77], ultrasonication [48,51] and microwave thermal shock [47] have also been reported. Frostegard et al. [17] observed that drying soil before grinding greatly improved the lysis efficiency.

In general, physical methods have shown efficiencies for disruption of vegetative forms, small cells and spores [43] but they often result in significant DNA shearing [35,62].

Chemical lysis either alone or in association with physical methods have been used extensively. Probably the most common chemical is the detergent sodium dodecyl sulfate (SDS) which dissolves the hydrophobic material of cell membranes. Detergents have often been used in combination with heat-treatment and with chelating agents such as EDTA, Chelex 100 [21,28] and diverse Tris buffer or sodium phosphate buffers [29]. In several studies, increasing the EDTA concentration increased the strength of extraction and lysis buffers resulting in higher yields, but lower purity of isolated nucleic acids. This result leads to the conclusion that the choice of buffer is a compromise between the expected DNA quantity and the required DNA purity. In a comparative study, Miller et al. [42] reported that the Chelex 100 treatment had detrimental effect by decreasing the DNA yield. A rapid protocol was reported by Selenska and Klingmüller [60] based only on gentle shaking of soil slurry in a SDS–sodium phosphate buffer at 70 °C. Nucleic acids recovered from this “gentle procedure” were on average approximately 25 kbp.

Addition of cetyltrimethyl-ammonium bromide (CTAB) and polyvinylpolypyrrolidone (PVPP) has also been reported [29,44,77]. Both CTAB and PVPP can partially remove humic compounds, but (contrary to CTAB) PVPP resulted in DNA loss [77]. CTAB forms insoluble complexes with denatured proteins, polysaccharides and cell debris [59]. PVPP was shown to be ineffective during cell lysis, but efficient when used as a spin column during the nucleic acids purification step [29].

Another denaturing agent, guanidine isothiocyanate has been used for mRNA extraction from seeded soils [47,71] but has [71] not proven valuable when extracting DNA from soil samples [37,42].

Many protocols including an enzymatic lysis have been developed. Lysozyme treatment is one of the most common [6,37,55,64,70]. Improvement in DNA purity may result from the lysozyme hydrolytic action on glycosidic or other humic components bonds. Another enzyme, achromopeptidase, was used to improve the lysis of the recalcitrant Gram positive Frankia [61] and another, proteinase K, was used to digest contaminating proteins [37,50,77].
2.2. Nucleic acid extraction and purification

Several methods for separating and purifying nucleic acids from soil components have been investigated. A major soil component, humic acid, inhibits restriction enzyme digestion of DNA, the polymerase chain reaction [64] and also alters the results of quantitative membrane hybridizations by lowering the expected hybridization signal [1]. The phenolic groups in humic acids denature biological molecules by bonding to amides or are oxidized to form a quinone which covalently bonds to DNA [76]. In most studies, following groups in humic acids denature biological molecules by lowering the expected hybridization signal [1]. The phenolic alters the results of quantitative membrane hybridizations by digestion of DNA, the polymerase chain reaction [64] and also soil component, humic acid, inhibits restriction enzyme digestion from soil components have been investigated. A major

2.3. Toward a universal lysis extraction method?

Although numerous direct lysis extraction protocols resulting from complex combinations of physical, chemical, and/or enzymatic methods to purify nucleic acids have been published application has been limited to only a few samples. Therefore, the individual contribution of each step to the final success of DNA recovery cannot be clearly evaluated. Even when evaluation is attempted, the positive or negative effects of extraction parameters is difficult to extrapolate to other samples. However, some comparative studies have contributed to better understanding the value of extraction and/or purification steps when applied to previously characterized sample soil samples [10,17,25,33,42,77].

Unbiased nucleic acid-based access to bacterial diversity in environmental samples requires several conditions:

1. All bacteria must be released from soil compartments. This includes those bacteria which are strongly adsorbed on soil colloids or those located within the inner microporosity of soil aggregates [54]. Hattori [19] observed that cultivable Gram-positive bacteria were more abundant in soil macropores whereas Gram-negative bacteria predominated in micropores. Physical disruption methods such as bead-mill homogenization and freezing–thawing helped homogenize soil rendering confined bacteria available for lysis treatments [17], and thus lead to better DNA yields.

2. Lysis of recalcitrant organisms such as Gram-positive cells, spores and small bacteria requires harsh treatments that may result in DNA shearing of lysis-sensitive bacteria.

3. Nucleic acid extraction should be performed as soon as possible after sample collection since storing samples at 4 °C for several weeks can result in the degradation of the large molecular-weight DNA fraction [65].
Although recovery of large DNA fragments (40–90 kb) using gentle lysozyme–SDS-based methods was reported [29], satisfactory DNA yields and unbiased DNA extraction require the introduction of mechanical lysis treatments which commonly result in DNA shearing. In most studies, direct extraction did not lead to the recovery of DNA fragments larger than 20 kb. Some applications, such as construction of metagenomic libraries, require the extraction of large DNA fragments that are rarely obtained by direct extraction methods.

### 3. Bacteria extraction method

Bacterial extraction was first reported by Faegri et al. [16] and Torsvik and Goksoyr [69] and is based on the following sequential steps: (i) dispersion of soil particles, (ii) separation of the cells from soil particles by centrifugation according to sedimentation velocities, buoyant density or both, (iii) lysis of extracted cells and (iv) DNA purification [4].

#### 3.1. Soil dispersion methods

As for the direct lysis extraction approach, dispersion of the soil sample by physical and/or chemical methods is a critical step because of the location of microorganisms within soil aggregates and because of bacterial adhesion to soil particles. Waring blender dispersion has been widely used [4,16]. Other methods have been tested, such as sonication [53], mild dispersal by shaking [72], and rotating pestle [36]. When compared, waring blender and rotating pestle proved to be the most efficient dispersion methods for large and small soil sample volumes, respectively [36].

Chemical dispersal has often been used in combination with mechanical methods [36]. Cation exchange resins (Chelex 100) have proven efficient for soil dispersion [28]. Bakken [3] did not find a significant dispersion difference between hexametaphosphate and distilled water. Many other chemical agents have been evaluated: specific detergents (sodium cholate and sodium deoxycholate), which interact with bacterial lipopolysaccharides [40], polyethylene glycol (PEG) and SDS which dissolve hydrophobic material [63], PVPP which removes humic acids [63]. However, chemical agents may have adverse side effects. For example, the metabolic state of soil bacteria as measured by cellular adenosine triphosphate levels was negatively affected when a combined SDS–Chelex chemical treatment was applied [4]. Preservation of bacterial integrity during cell separation seems to be essential in order to prevent released DNA to be degraded by physical, chemical and/or enzymatic processes.

#### 3.2. Centrifugation-based cell separation

Separation of bacteria from soil particles according to sedimentation velocities was first described by Faegri et al. [16]. This method is based on two successive centrifugations: first a low speed centrifugation ranging from 500 × g to 1000 × g lasting 2–15 min, respectively, in order to remove (sediment) soil debris, fungal mycelia and heavy soil particles [16]. Optimal conditions for low-speed centrifugation is a compromise between cell recovery efficiency and elimination of contaminating material [63]. A subsequent high-speed centrifugation of the cell-containing supernatant produces the bacterial fraction. Chains and clusters of bacteria and bacteria attached to soil particles might be easily lost during the first centrifugation. Holben et al. [23] established that approximately 10% of the total bacteria present in the soil were released per round of homogenization-separation. According to these authors, the bacterial fraction released after a single round is apparently as representative of the total bacterial population as after multiple rounds. Several rounds of extraction may, however, greatly improve total cell recovery.

Supernatants resulting from the low speed centrifugation might still contain non-cellular material and soil contaminants such as humic acids. Flocculation of the cell debris and clay particles using CaCl2 resulted in a considerable decrease in cell recovery [28]. An alternative high-speed centrifugation method based on density gradient centrifugation, was developed [3] in order to separate bacteria according to their buoyant density. Several multi-gradient media have been tested such as Percoll, metrizamide and Nycodenz [4] but the use of Nycodenz provided the best results. The dispersed soil suspension is deposited onto Nycodenz cushion (p = 1.3 g ml⁻¹) and then centrifuged at high speed, commonly 10,000 × g. Bacteria will settle on top of the Nycodenz “cushion”, and organic and mineral particles of greater density will sediment to the bottom of the tube. Extraction efficiency (number of extracted bacteria as a percentage of total bacteria in the soil suspension), was between 6% and 25% [39] and from 20% to 50% [4]. The organic matter and clay content of the soil will affect the cell extraction efficiency [36] confirming that considerable attention needs to be paid to soil dispersion procedures (as described above). Even if most bacteria have densities below 1.12 g ml⁻¹ and should be recovered on the Nycodenz cushion, many bacteria are strongly attached to dense soils particles, such as clays and will sediment through the gradient. Although the bacterial fraction will still contain humic material and soil particles having the same density as the bacteria [68], centrifugation on Nycodenz gradient allows recovery of a relatively clean bacterial fraction as compared to low speed centrifugation methods. A similar method described by Pillai et al. [49] was based on sucrose density gradient centrifugation.

#### 3.3. Nucleic acid separation and purification

Bacterial DNA can be extracted from the purified cellular fraction using numerous physical, chemical and enzymatic procedures as mentioned above. Cesium chloride–Ethidium bromide equilibrium density centrifugation has been successfully used to recover pure DNA [28,65] of large size (at least 48 kb) [9,23].
Torsvik [67] developed a protocol in which the bacterial lysate was purified by passage through a hydroxyapatite column. A high urea concentration was used to disrupt hydrogen bonds between DNA and humic acids. Steffan et al. [63] observed that the removal of humic acids by PVPP greatly improved the DNA purity but lowered the DNA recovery slightly. CsCl density centrifugation and hydroxypatite column chromatographic purification improved the DNA purity, as measured by the spectrophotometric A260/280 and A260/230 ratio, but both caused the loss of DNA [63].

Gentle lysis methods combined with CsCl density purification recovered highly purified nucleic acids with improved sizes reaching more than 100 kb (Robe, unpublished data). The DNA size remains limiting for functional genomic approaches where the exploration of gene clusters and biosynthetic pathways through cosmid and bacterial artificial chromosome (BAC) cloning requires DNA greater than 200 kb.

Embedding bacteria in agarose plugs before performing a gentle bacterial lysis recovered DNA fragments of a few hundred kilobases with limited mechanical shearing. This approach was used to establish libraries of BAC clones containing large DNA fragments from several microorganisms including Methanosaeta thermophila [14], Bacillus cereus [57], Mycobacterium tuberculosis [5] and Pseudomonas aeruginosa [13]. An integrated approach combining centrifugation-based cell separation from soil particles, in plug lysis and pulsed field gel electrophoresis (PFGE) has been successfully applied to non-culturable bacteria from environmental samples [45]. DNA fragments recovered using this method are more than 300 kbp in size and of adequate purity for further molecular cloning procedures [45]. This method has already lead to the construction of a soil fosmid metagenomic library containing more than 3.5 Gbp providing access to metagenomic functional genetics (Nalin and Robe, unpublished results).

4. Comparative evaluation of direct and indirect methods

In a comparative study between several extraction methods, Leff et al. [33] established that the bead-beating-SDS method of Ogram et al. [46] allowed the greatest DNA yield but resulted in increased DNA shearing when compared to the comparatively gentle freezing-thawing lysozyme method described by Tsai and Olson [70]. Compared to these two direct extraction methods, the indirect extraction method of Jacobsen and Rasmussen [28] based on centrifugation recovery of cell fraction before lysis and CsCl DNA purification, gave the lowest DNA yield but showed the highest DNA purity with a low degree of DNA shearing. Tien et al. [65] reported that the direct lysis methods produced similarly DNA yield at least a 10-fold greater than the indirect extraction methods.

The main disadvantage of cell fractionation-based methods is that the recovered bacterial fraction represents only 25–50% of the total endogenous bacterial community [4]. In return direct extraction lysis has been assumed to recover more than 60% of the total theoretical bacterial DNA [43]. Miller et al. [42] reported DNA recovery efficiencies ranging from 86% to more than 100%, based on direct counts of endogenous microorganisms [42]. These values probably overestimate the recovery efficiencies, resulting from the eukaryotic and extracellular DNA recovery and the underestimation of bacteria by fluorescent direct counts.

Even though indirect extraction methods typically result in lower humic acid contamination than direct extraction methods, both approaches rely on subsequent purification steps in order to produce nucleic acids usable for molecular purposes. When comparing both direct and indirect methods, Steffan et al. [63] established that the degree of purity required for restriction endonuclease digestion of recovered DNA varied with the enzyme used. While PstI did not require further hydroxyapatite purification after CsCl purification, target DNA restriction by EcoRI was only observed with DNA recovered by cell fractionation method using the most extensive CsCl-hydroxyapatite purification. Endonuclease restriction by SseI failed for both extraction methods. Clearly, comprehensive knowledge of the sensitivity of molecular methods to inhibitory effect of contaminants coextracted with environmental DNA is a prerequisite when establishing an extraction and purification strategy.

Denaturing gradient gel electrophoresis (DGGE) was used by several authors to detect differences between extraction methods [22,29,37]. The DGGE band pattern was affected by the extraction procedure as indicated by the variation of band intensity [29]. Such sources of bias were also observed by Maarit Niemi et al. [37] when comparing DGGE community profiles in rhizosphere soils samples.

Ribosomal intergenic spacer analysis (RISA) of soil microbial diversity also revealed that both abundance and apparent members of the bacterial community are affected by the extraction method [38]. By using hybridization of the PCR amplified 16S rDNA gene Courtois et al. [10] did not find significant difference in the spectrum of diversity resulting from the two extraction strategies, the only exception being the gamma-subclass of Proteobacteria.

5. Conclusion

Both direct and indirect extraction methods can recover purified nucleic acids for molecular biology purposes. Direct extraction methods have been developed for several reasons; they are assumed to provide a less biased extraction of microbial DNA, with less laborious methods and higher nucleic acid yields. Direct lysis procedures are preferred when large quantities of nucleic acids are required for DNA-consuming methods, statistically significant detection of non-abundant microorganisms, and when the entire diversity of an environmental sample must be investigated with minimum bias. However, the resulting nucleic acid extracts are commonly sheared and contaminated with humic acids. Fur-
thermore, extracts often contain unknown amounts of extracellular and/or eukaryotic DNA.

Although time-consuming, indirect extraction methods are preferred for targeting prokaryotic communities, when high DNA purity is required for inhibitor-sensitive methods, and when recovery of high molecular weight DNA is necessary.

For both direct and indirect extraction methods, several parameters may be varied to optimize soil dispersion, cell disruption, and separation and purification of nucleic acids. Evidently, there is no single ideal extraction method and the definition of an optimal strategy will rely on several elements:

1. A comprehensive description of soil is necessary.
2. Is co-extraction of extracellular and eukaryotic nucleic acids acceptable and to which limit?
3. Targeted organisms need to be defined (the whole community or a specific taxon or group of taxa).
4. Whether the targeted microorganisms can be easily recovered or whether they are confined inside inner sample aggregates or strongly adsorbed on particle surfaces needs to be considered.
5. The targeted microorganisms sensitivity to lysis procedures needs to be determined.
6. Constraints caused by non-satisfactory DNA quantity and purity will greatly influence the choice of extraction and purification methods, as previously discussed. Undoubtedly, an unbiased description of microbial diversity depends on the optimization of nucleic acid extraction methods. In addition, considerable attention will need to be paid to other major sources of bias introduced by environmental sampling protocols and molecular biology methods.

Acknowledgements

This work was financially supported by the European project TRANSBAC No. QLK3-CT-2001-02242.

References


