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DNA extraction from soil

1. Introduction

One gram of soil represents an immense biochemical gene library producing diverse genetic instructions, which is present on Earth since almost 4 billion years. There are sufficient DNA in 1 g of soil to extend over a distance of 1,598 km. However, this is certainly an underestimate for fertile soils.

It is relevant to consider that soils present some of the most difficult challenges to the development of suitable extraction and purification procedures. The complex matrix of soil harbours a variety of substances that inhibit the activity of polymerases and restriction enzymes or interfere with hybridization and detection methods (Steffan et al., 1988; Demeke and Adams, 1992; Tsai and Olson, 1992). It is noticeable that even if several published protocols exist for NA extraction from soil, they markedly diverge even for the purification step and there is no agreement as to the most effective method of purification and none of these has been shown to be robust and general enough to be accepted by the scientific community as a standard protocol (Schneegurt et al., 2003).

Relevant to underline are the consequences of DNA extraction and purification methods on the information about microbial diversity and community structures (e.g. ribotype number and abundance) obtained by molecular analyses as for example genetic fingerprinting techniques.

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2. DNA EXTRACTION FROM SOIL

2.1 *Soil sampling strategy*

A relevant aspect that could influence the results of nucleic acid (NA) based analysis to study the soil microbial community structure and activity is related to the sampling strategy in terms of representativity.

Soil is a structured, heterogeneous, discontinuous system characterized by the domination of the solid phase with organisms living in discrete microhabitats called *hot spots*, representing a small proportion (generally lower than 5%) of the overall available space (Nannipieri et al., 2003). The chemical, physical and biological characteristics of these microhabitats differ both in space and time. The consideration of these aspects together with those related to soil characteristics (texture, pH, etc.) are essential to plan an efficient sampling strategy (Remenant et al., 2009; Hirsch et al., 2010).

Soil sampling, transport, storage and treatment (i.e. drying and sieving) prior to extraction is important for those interested in studying the microbial ecology of native communities in order to maintain the *representativity of the samples*, avoiding any alteration.

Soil sampling physically disrupts the soil structure in a way that can alter its microbial community. Furthermore, the storage of samples in refrigerator (4°C), even for few hours, can cause shifts in the microbial community structure (Tien et al., 1999).

Archiving recommendations (Boone et al., 1999; Campbell et al., 2009; Hirsch et al., 2010) indicate that air-dried soils can be stored at room temperature for an indeterminate length of time, depending upon the intended use. However fresh soils that are to be archived should be stored at -20°C or, better, -80°C, immediately after initial molecular evaluation to possibly reduce subsequent DNA degradation.

One of the challenging aspects of soil sampling in the field is to limit contamination of the samples but, even if sterile materials are used, the accumulation of exogenous material and consequentially exogenous organisms, for example by windblown and/or flooding, is practically inevitable.

2.2 *DNA extraction methods*

Two approaches have been developed for extracting nucleic acids from soil: the *direct extraction* by cell lysis and the *indirect extraction* by cell extraction

from soil and successive DNA extraction from cells. The cell lysis and the successive DNA extraction and purification procedures are in common of both extraction strategies.

2.2.1 Direct DNA extraction

The disruption of the microbial cell wall is the first step leading to the release of nucleic acids from microbes. The efficiency of this step is influenced by cell wall characteristics that depend on the type of microorganisms (bacteria, fungi), the strains (gram positive, gram negative bacteria), the metabolic status ("k" and "r" strategy; growth or stationary phase; spore), the size (microbacteria and spore), morphology (rods, cocci), and the location of bacteria in soil (free or attached to soil particles and /or inside microstructures).

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

The most commonly used *physical* cell lysing methods are thermal shock, like freezing–thawing, freezing–boiling (Degrange and Bardin, 1995; More et al., 1994; Tsai et al., 1991), mortar mill grinding under liquid nitrogen (Volossiouk et al., 1995; Zhou et al., 1996), ultrasonication and microwave treatment (Orsini and Romano-Spica, 2001).

The *mechanical* cell lysing methods are based on *bead beating*. This method, although it does not give the best yield and quality of DNA molecules, is capable to obtain higher numbers of bands on DGGE (Denaturing Gradient Gel Electrophoresis) gels with respect to other lysing methods (de Lipthay et al., 2000).

The *chemical* cell lysing methods are based on the use of chemicals, including a range of compounds which are used to permeabilise and thus to enhance enzymatic attacks and/or physical rupture (Nandakumar et al., 2000). The specificity (at strain or species level) for some of these compounds is possibly a problem and their effects may also depend on the growth rate of the cells (Watt and Clarke, 1994). The most commonly used chemical to lyse bacterial cells is the detergent sodium dodecyl sulfate (SDS), which dissolves the hydrophobic components of cell membranes. Detergents have often been used in combination with heat-treatment and with chelating agents such as EDTA (*ethylenediaminetetraacetic acid)*, Chelex 100 (Herron and Wellington, 1990; Jacobsen and Rasmussen, 1992) and diverse Tris buffer or sodium phosphate buffers (Krsek and Wellington 1999). The choice of the buffer is a compromise between quantity and quality of extracted DNA.

Several commercial kits are available for extracting DNA from soil (Ultra Clean Soil DNA kit, MoBio; FastDNA® SPIN Kit for soil, BIO101; etc.). FastDNA® SPIN Kit is based on a mechanical lysis with ceramic and silica beads in a bead-beater (FastPrep Instrument), that efficiently lyses all microorganisms including eubacterial spores, endospores and gram-positive bacteria. A rich bibliography is available on the comparison of various soil DNA extraction kits (Martin-Laurent et al., 2001).

2.2.1.1. Pre-lysis treatments

Several authors have reported that lysis efficiency is negatively correlated with the clay, humic substances and also with the Fe-Al oxy/idroxy content of soil. To avoid these limitations and thus to increase the DNA extraction yields and to reduce the number of requested DNA purification steps, several pre-lysis treatments have been proposed (Frostegard et al., 1999; Pietramellara et al., 2001; Takada-Hoshino and Matsumoto, 2004; He et al., 2005; Dong et al., 2006; Lakay et al., 2007; Saeki et al., 2008; Ceccherini et al., 2009; Saeki and Sakai, 2009).

To determine the sensitivity of the selected direct extraction method of DNA from soil Schneegurt et al. (2003) proposed an efficient assay based on the seeding of soil samples prior to the extraction with known amounts of selected bacteria that contain a kanamycin-resistance gene abundant in nature, verifying the presence in the extracted solution and then calculating the correspondent number of cells. From the comparison between the initially added cells and those determined after DNA extraction it is possible to determine the efficiency of the selected direct soil DNA extraction method.

2.2.1.2. DNA purification

There are several strategies to purify nucleic acids (NA) (for review see Robe et al., 2003). The choice of the purification method depends on the soil type, on the presence of (co-extracted) contaminants, but also on the goal of the investigation, considering one crucial fact: the higher the number of performed purification steps, the higher the risk of losses of target NA.

Moreover, care should be taken during the whole process of sample handling to avoid that contamination by exogenous substrates as dust, pollen, glove powder, etc., that could exert inhibitory effects on downstream NA analysis (Wilson, 1997).

To avoid DNA degradation after its extraction from cells, the inactiva-

tion of nucleases is a crucial step within the purification procedure, with reagents like Guanidine isothiocyanate ($C_2H_6N_4S$), Aurintricarboxylic acid (ATA, $C_{22}H_{14}O_9$), E*thylenediaminetetraacetic acid* (EDTA, $C_{10}H_{16}N_2O_8$) and Formamide + NaCl. ATA is reported by several authors as the most efficient DNase and proteinase inhibitor (Hallick et al., 1977; Marstorp and Witter, 1999).

Concerning DNA contaminants, the main ones for prokaryotic DNA are represented by its constitutional molecules such as proteins and polysaccharides, eukaryotic DNA, RNA, and by soil components such as clay minerals and humic acids (Pietramellara et al., 2007). These contaminants could interfere with successive molecular techniques such as PCR-based methods (Tebbe and Vahjen, 1993; Robe et al., 2003). The inhibition of Taq polymerase by co-extracted contaminants could be overcome by adding bovine serum albumin (BSA) to the PCR reaction mixture. The magnesium concentration of the PCR reaction mix was also carefully standardized as it may fluctuate according to primer combination and sample source (Kreader, 1996).

2.2.2 Indirect DNA extraction

Despite several commercial kits for direct DNA extraction from soil, no one is available for indirect DNA extraction from soil. This discrepancies is confirmed by the low number of available protocols for indirect DNA extraction from soil.

As already mentioned above, the indirect DNA extraction method is characterized by dispersion of soil particles and separation/extraction of the cells from soil particles through their sedimentation velocities, followed by lysis of extracted cells and DNA purification. Pioneers of this strategy have been Faegri et al. (1977) and Torsvik and Goksoyr (1978).

2.2.2.1. Dispersion of soil particles

To obtain an optimal cell extraction from soil, the dispersion of the soil sample by physical and/or chemical methods is fundamental. This is a crucial step due to the presence of a relevant part of the bacterial community bound on soil particles and/or located inside soil aggregates. Fundamental is also the preservation of the integrity of extracted bacterial cells (Robe et al., 2003). One of the most relevant aspects that characterises the indirect extraction of soil DNA is related to the yield of extracted DNA and to its representativity

of the bacterial community. These aspects generally represent the main disadvantage with respect to the direct extraction approach.

Physical dispersion methods have been tested, like waring blender (Faegri et al., 1977; Bakken and Lindahl, 1995), sonication (Ramsay, 1984), mild dispersal by shaking (Turpin et al., 1993), and rotating pestle (Lindahl and Bakken, 1995), with the former and the latter approach found to be the most efficient ones for large and small scale extraction, respectively (Lindahl and Bakken, 1995).

Chemical dispersal methods have often been used in combination with mechanical methods (Lindahl and Bakken, 1995). Cation exchange resins (Chelex 100) have proven to be efficient for soil dispersion (Jacobsen and Rasmussen, 1992). Other chemical agents utilized to separate bacterial cells from soil particles are sodium cholate and sodium deoxycholate that interact with bacterial lipopolysaccharides (McDonald, 1986), *polyethylene glycol* (*PEG*) and *sodium dodecylsulfate* (*SDS*) that dissolve hydrophobic material, polyvinyl polypyrrolidone (PVPP) which removes humic acids (Steffan et al., 1988), hexametaphosphate, and finally distilled water.

2.2.2.2. Cell extraction from soil

The extraction of intact cells prior to cell lysis and nucleic acid extraction is attractive because it reduces the problems related to contamination by interfering co-extracted substances (humus) and losses of nucleic acids by sorption to soil colloids; drawbacks of the indirect approach are a low extraction efficiency, and that they are complex and thus time consuming.

Robe et al. (2003) pointed out the risk of loss of bacteria present in clusters or attached to soil particles during the low speed centrifugation. An alternative method based on cell buoyant density was proposed by Bakken (1985) that permits to discriminate bacteria by density gradient centrifugation utilizing a multi-gradient media such as Percoll, Metrizamide, Sucrose and Nycodenz, with the latter providing the best results (Pillai et al., 1991; Unge, 1999; Berry et al., 2003). This technique is also capable to eliminate organic and mineral particles, characterized by a greater density respect to bacterial cells, permitting the recovery of a relatively clean bacterial fraction as compared to low speed centrifugation methods (Lindahl and Bakken, 1995).

Another cell-soil separation technique is the immunomagnetic capture, based on the capture of microbial cells by microscopic magnetic beads coated with monoclonal or polyclonal antibodies (Morgan et al., 1991; Fluit et al., 1993; Porter, 1998).

However it is important to underline that the yield and quality of extracted cells depend on the choice of the most suitable method according to the soil characteristics.

2.2.2.3. DNA extraction and purification

The extraction of DNA from cells previously isolated from soil (indirect extraction) and its successive purification methods are similar to those above reported for the direct DNA extraction from soil, but with differences coming from managing a pure cell suspension, permitting a higher lysis efficiency and a marked reduction of the required purification steps due to a drastic reduction of organic and inorganic debris. Theoretically, only organic debris mainly composed by cell wall residues and DNA constitutional molecules (proteins, polysaccharides and lipids) should be present.

2.2.3 Comparative evaluation of direct and indirect DNA extraction methods

The bacterial fraction recovered from soil represents only 25–50% of the total endogenous bacterial community, whereas with the direct extraction it has been assumed to recover more than 60% of the total bacterial DNA (More et al., 1994). There is no *perfect* extraction method and the definition of an optimal strategy requests a comprehensive description of soil, a limited co-extraction of extracellular and eukaryotic DNA, the definition of target organisms (the whole community or a specific taxon or group of taxons) and their sensitivity to lysis procedures due to physical characteristics (cell wall composition) and/or addressing in soil (free or attached to soil particles and inside inner sample aggregates), and is evaluated in terms of quantity and quality (molecular weight and purity) of the extracted DNA.

The capacity to appreciate the efficiency of the selected extraction methods seems to vary in relation to the selected method of analysis. Martin-Laurent et al. (2001), characterizing the soil microbial community by Ribosomal Intergenic Spacer Analysis (RISA), evidenced that both abundance and apparent members of the bacterial community were affected by the selected extraction method. In contrast, Courtois et al. (2001), using hybridization of the PCR amplified 16S rDNA gene, did not find significant differences in the spectrum of diversity resulting from the used extraction strategies.

Concerning the main advantage of direct DNA extraction with respect to

indirect extraction, related to higher yield and representativity of the total soil microbial community, seems to be reduced.

Some interesting improvements in culture methods have recently been made (Balestra and Misaghi, 1997; Kaeberlein et al., 2002). For example, Zengler et al. (2002) proposed an impressive method based on the combination of single-cell encapsulation and flow cytometry (or fluorescence-activated cell sorting, FACS) that enabled cells to grow with nutrients that are present at environmental concentrations, and detected microdroplets containing microcolonies of so far uncultured microorganisms.

A very promising strategy to imporve our capacity to cultivate microorganims was proposed by Bomar et al. (2011) that hypothesized to utilize the High-throughput sequencing of RNA transcripts (RNA-seq) of uncultured microorganisms to characterize their metabolisms in order to design an appropriate medium for their cultivation.

2.2.4. DNA extraction for the meta-genomic approach

The DNA size (molecular weigh) remains limitative for functional meta-genomic approaches where the exploration of gene clusters and biosynthetic pathways through cosmid and bacterial artificial chromosome (BAC) cloning requires DNA larger than 200 kb. Regarding cloning Gabor et al. (2003) have evaluated the efficiency of DNA extracted from soil by a direct and an indirect extraction method, based on protocols of Zhou et al. (1999) and Holben et al. (1988), respectively. The results clearly evidenced that even if the direct extraction method yielded higher amounts of DNA with respect to the indirect extraction method, the representativity in terms of richness and evenness was similar between the two extraction methods with a significant reduction of the amount of co-extracted eukaryotic DNA. These considerations became more significative to increase the efficiency of the indirect DNA extraction method as reported by Duarte et al. (1998) in order to reach the theoretical maximum cell extraction from different soils by a method based on shaking with gravel in pyrophosphate buffer.

Several authors have reported that embedding soil or bacteria in agarose plugs prior to bacterial lysis permits to recover DNA fragments of high molecular weight (HMW). This approach was used to establish libraries of BAC clones containing large DNA fragments from several non-cultivable bacteria from environmental samples.

Concerning indirect DNA extraction from soil, Liles et al. (2008) pro-

posed an integrated approach combining centrifugation based cell separation from soil particles with low melting agarose plug lysis, purification and pulsed field gel electrophoresis (PFGE), to determine the molecular weight of DNA fragments.

Regarding direct DNA extraction from soil, Yu et al. (2008) set up a direct cell lysis within soil-embedded agarose plugs, along with a double-size selection that permits an isolation of pure and HMW DNA without the need of any further purification.

Both extraction strategies generated pure and HMW DNA ranging from 0.1 to 1 Mb, resulting adequate for further molecular cloning procedures and the construction of soil fosmid and BAC metagenomic libraries.

2.2.5. Determination of amount and quality of extracted DNA

DNA can be quantified spectrophotometrically at 260 nm (Sambrook et al., 1989). It is relevant to note that 260 nm adsorption by UV spectrophotometer detects the amount of nucleotide in solution so do not give direct information on the amount of double strand DNA (dsDNA).

The DNA purity index (IP) can be determined by calculating the absorbance ratio of 260 nm and 280 nm (A_{260}/A_{280}) in terms of protein contamination, and 260 nm and 230 nm (A_{260}/A_{230}) in terms of contaminants such as carbohydrates, ethanol, humic acids. Optimal IP values are within the range of 1.8 and 2.0. It is relevant to note that IP is not an absolute parameter for DNA purity but it is based on the PCR amplificability of the extracted DNA. The level of NA purification degree request is strictly dependent on the different molecular methods utilized to analyse the NA like methods based on PCR or enzyme digestion (Steffan et al., 1988) with high purity request for the successful application of the former technique.

The DNA quantification by fluorometer measurements is based on the specific binding of fluorochromes to dsDNA. In the following paragraph some of the commonly used fluorochromes are described.

A specific reagent for primary aldehydes of the type R-CH_2-CHO is the 3,5 diamino-benzoic acid 2HCl (DABA 2HCl) (Lien and Knutsen, 1976). The antibiotic mithramycin specifically reacts with guanine (dsDNA) but the binding efficiency decreases in presence of nucleoproteins, showing less sensibility with respect to DABA 2HCl. Bisbenzimide H33258 (Hoechst) has a high specificity to adenine and thymine base pairs of the dsDNA molecule (Paul and Mayers, 1982).

A very efficient fluorochrome is the YO-PRO 1 (Molecular Probes Inc., Eugene, Oregon USA); its sensitivity has been found to be more than 400 fold greater than Hoechst 33258. As this fluorochrome binds DNA as well as RNA, their amounts in the same sample can be quantified only after subsample pretreatments with RNase or DNase, respectively, or by measuring the fluorescence of the same sample before and after nuclease treatments (Torsvik et al., 1995).

These fluorochromes, due to specific adsorption to base pairs, specifically detect dsDNA, but do not allow the assessment of the purity of DNA. However they are less sensible to the presence of contaminants in the DNA quantification with respect to the UV technique.

The determination of the molecular integrity and weight of DNA molecules is made by different gel electrophoresis techniques. Small, medium and large DNA molecules can be qualitatively analysed by agarose gel electrophoresis (AGE), capillary electrophoresis (CE), and pulse field gel electrophoresis (PFGE), respectively.

2.3 *Conclusions and future perspectives*

The choice of the DNA extraction method severely affects the picture of the microbial diversity present in a soil sample (Frostegard et al., 1999; Krsek et al., 1999; Martin-Laurent et al., 2001; Schneegurt et al., 2003; He et al., 2005; Sagova-Mareckova et al., 2008; Thakuria et al., 2008; Ascher et al., 2009), moreover de Lipthay et al. (2004) reported how DNA extraction method also influence functional diversity. Philippot et al. (2010) with the intent to ensure comparable data proposed, with support of the French Standards Association and the French Environment and Energy Management Agency, in 2006 to the International Organization for Standardization (ISO) the standardization of a direct soil DNA extraction. The request was rapidly recognized by ISO members so that the ISO 11063 soil quality method to directly extract DNA from soil samples is now being prepared by the Technical Committee ISO/TC 190, Subcommittee SC4, Biological methods. The standard was developed based on the publication made by Martin-Laurent et al. (2001) by different laboratories from seven countries. Six soils collected in different European Countries . It was also decided that since DNA purification is dependent on soil type, only the actual DNA extraction step, which is likely to generate the main differences between methods, would be subject to standardization.

No standardization has been planned for the next future regarding indirect soil DNA extraction; this lack is justified by the paucity of so far available methods of indirect soil DNA extraction.

Concerning the standardization we have to consider the risk to seriously limit the main request of metagenomic approaches, that to obtain the whole genome present in soil. A risk that actually could be partially overcome by combining direct and indirect approaches and also by choosing the most adequate methods within the two extraction categories.

RIASSUNTO

Il recente sviluppo delle tecniche molecolari basate sull'analisi del DNA per studiare le comunità microbiche del suolo, anche la frazione non coltivabile, ha richiesto un grande sforzo per aumentare l'efficienza delle metodiche estrattive con l'intento di ottenere DNA di buona qualità e rappresentativo dell'intera comunità dei microrganismi che abitano il suolo. Nel presente articolo è criticamente riassunto lo stato dell'arte dei sistemi di estrazione del DNA dal suolo.

ABSTRACT

The recent develop of molecular techniques based on the DNA analysis to study the soil microbial community, even the unculturable fraction, has request a great effort to increase the efficiency of the extraction methods in order to obtain DNA of good quality and representative of the whole microbial community inhabiting soil. Here are critically summarized the state of the art of the DNA extraction from soil.

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