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Optimizing the indirect extraction of prokaryotic DNA from soils

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ABSTRACT

The objective of this work was to develop protocols to selectively extract prokaryotic DNA from soils, representative of the whole community, amenable to high-throughput whole genome shotgun sequencing. Prokaryotic cells were extracted from soils by blending, followed by gradient centrifugation. Detergent (sodium deoxycholate) was required for complete dispersion of soil aggregates and detachment of prokaryotic cells from a broad range of soil types. Repeated extractions of a given soil sample were critical to maximize cell yield. Furthermore, cells obtained through repeated extractions captured unique prokaryotic assemblages that would otherwise have been missed in single-pass extractions. DNA was isolated from extracted cells using one of the following treatments: i) lysozyme–SDS–proteinase K (enzymatic) digestion; ii) potassium ethyl xanthogenate digestion; or iii) enzymatic digestion of cells embedded in agarose plugs. In addition, these methods were compared to a commercial bead-beating extraction kit (MoBio UltraClean). Of the indirect DNA extraction methods, plug digestion generated the largest yields (up to 70% of yields obtained by direct DNA extraction) of high-molecular weight DNA (>400 kb). Thus, plug digestion is amenable to large-insert metagenomic library construction and analysis. Comparisons of banding patterns generated by RAPD-PCR and DGGE indicated that sequence composition and inferred community composition of a given extract varied greatly with DNA isolation method. While overall diversity did not change significantly with the cell lysis method, analysis of 16S rRNA gene clone libraries revealed that each extraction procedure produced unique distributions of prokaryotic phyla within the sample population.

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1. Introduction

Soils likely represent the greatest reservoir of biodiversity on the planet. Prokaryotic diversity in soils has been estimated to be three orders of magnitude greater than in all other ecosystems combined (Curtis et al., 2002; Kemp and Aller, 2004). In terms of function, soils and their microbial inhabitants are critical to global biogeochemical cycles including carbon, nitrogen, and phosphorus, which support all other forms of terrestrial diversity. Because of their importance on multiple levels, soils have been the subject of studies in microbial ecology for decades [e.g., (Borneman and Triplett, 1997; Cavigelli et al., 1995; Nunan et al., 2003; Skinner et al., 1952; Skyring and Quadling, 1969; Steffan et al., 1988; Waksman and Woodruff, 1940)]. However, the spatial and temporal heterogeneity of soils, and the complexity of soil chemical and biological characteristics that give rise to such genetic and functional diversity among soil microbiota, also make soils one of the most challenging natural environments for studies of microbial ecology.

The advent of DNA-based techniques, such as PCR amplification of the 16S ribosomal RNA gene (Lane, 1991), has granted new views of prokaryotic diversity by circumventing the requirement of cultivation. Efficient extraction of target DNA is the crucial first step in any DNA-based analysis of soil microbes. While many specific methods for the isolation of prokaryotic DNA from soils have been published [for a review, see (Robe et al., 2003)], the canon of available protocols may be reduced to two general approaches: direct and indirect DNA extraction. Direct extraction involves the *in situ* lysis of cells followed by extraction and purification of DNA. By contrast, indirect extraction procedures initially separate prokaryotic cells from the soil matrix; cells are then lysed *ex situ* and the DNA purified. Studies comparing the yield and quality of DNAs extracted from soils by various direct and indirect methods (Krsek and Wellington, 1999; Maron et al., 2006; Roh et al., 2006; Tien et al., 1999) have demonstrated that each extraction approach has

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specific advantages and disadvantages in terms of DNA yield, purity, and sampling biases, which must be considered in light of the particular experimental goals (Frostegard et al., 1999).

Direct DNA extraction is appropriate if the objective is to characterize the taxonomic diversity of the soil prokaryotic community (Ashby et al., 2007; Fierer et al., 2007; LaMontagne et al., 2003) or the sequence diversity of specific gene sets (Jensen et al., 2000; Tolli and King, 2005; Verhagen et al., 1995). Direct extractions typically provide enough DNA of sufficient quality (*i.e.*, free of inhibitory contaminants) and nominal fragment size that the target gene(s) can be quickly amplified from the resulting mixture of templates. However, different approaches are warranted if the goal is to assess functional diversity, or to establish connections between taxonomy and function.

Several recent publications have revealed the utility of metagenomic libraries in identifying potential connections between taxonomy and function within specific clones (Beja et al., 2000; Beja et al., 2002; Liles et al., 2003; Quaiser et al., 2002). Construction of metagenomic libraries from soil prokaryotic assemblages requires robust methods for the extraction and purification of high-molecular weight prokaryotic DNA. These criteria exclude direct DNA extraction techniques because: 1) DNA released through *in situ* lysis may bind to clays or organic matter (Frostegard et al., 1999), severely limiting their recovery; 2) DNA from direct extractions necessarily includes eukaryotic (Courtois et al., 2001; Frostegard et al., 1999) and extracellular DNAs (Frostegard et al., 1999; Pietramellara et al., 2009), inflating the perceived DNA yield without necessarily providing additional information on the prokaryotic fraction (which has, ostensibly, been the subject of interest for the majority of molecular microbial ecology studies). Furthermore, eukaryotic genomes are far larger than those of prokaryotes, thus, without some type of screening (such as 16S rRNA PCR) the DNA from eukaryotic cells in total soil DNA extractions can significantly reduce the number of prokaryotic sequences within a metagenome; 3) DNA fragments obtained through direct extractions are rarely larger than 20 kb in size (Krsek and Wellington, 1999; Robe et al., 2003), placing a fundamental limit on the establishment of linkages between taxonomy and function.

By contrast, indirect lysis approaches are required for the construction of metagenomic libraries of soil prokaryotic DNA, because indirect extraction provides for the recovery of large, contiguous DNA fragments (Berry et al., 2003; Bertrand et al., 2005). Indirect lysis approaches therefore facilitate the functional analysis of soil prokaryotic assemblages, and also enable connections to be made between taxonomy and function within contiguous clone inserts. Furthermore, DNAs in such extracts are almost exclusively from prokaryotic sources (Courtois et al., 2001). Major disadvantages associated with indirect DNA extraction are the reduction in DNA yield and the potential decrease in sampling efficiency (i.e., the extent to which the phylogenetic diversity of the sample population represents the diversity of the whole community) relative to direct extraction approaches. The bacterial fraction obtained by single-pass cell extraction has been reported at 25–50% of the total community (Robe et al., 2003). Thus, estimates based on single-pass extractions do not account for potential increases in cell recovery resulting from repeated sequential cell extractions. Furthermore, no data are available regarding improvements in the representation of the phylogenetic diversity of cells through repeated extractions.

In this study, we developed methods for obtaining high-molecular weight (≥400 kb), high-purity, prokaryotic DNA from soils, amenable to the construction of metagenomic libraries. The impacts of repeated sequential extractions on cell recovery and sampling efficiency were also evaluated. After optimization of cell extraction, five cell lysis procedures were used to obtain prokaryotic DNA. The main objective of this work was to evaluate potential taxonomic biases imparted by specific lysis procedures. Evaluations were performed using 16S rRNA PCR-DGGE, as well as through comparisons of sequence data obtained from 16S rRNA gene clone libraries.

2. Materials and methods

2.1. Soils

Composite ~500 g soil samples were collected from the A horizons (0–10 cm) of five different soil types (Table 1). An unclassified agricultural clay loam (AGC) was obtained from a no-till farm field planted to wheat in Sykesville, MD; Glenelg silt loam (GSL) was obtained from a forested site in Rock Creek Regional Park, Rockville, MD; CAL loamy sand was obtained from an eucalyptus forest in La Jolla, CA; BWR loamy sand was collected from a field planted to tomatoes at the Be Wise Ranch, Escondido, CA; MBP soil was gathered from Mission Bay Park, San Diego, CA. Each soil sample was homogenized, passed through sterilized 2-mm sieves, and stored at 4 °C for no more than 1 week prior to cell extraction. Air dried samples of each soil were sent to Delaware Soil Testing Lab (University of Delaware, Newark, DE) for physical analyses.

2.2. Extraction of bacteria

2.2.1. Single extractions

Soils (10 g) were suspended in 100 ml of one of the following extraction media: 0.1% (w/v) sodium deoxycholate (SdCh) (Hopkins et al., 1991; Krsek and Wellington, 1999; MacDonald, 1986); 0.1 M sodium phosphate, pH 4.5 with 0.005% sodium dodecyl sulfate (SDS) (Steffan et al., 1988); 10 mM sodium pyrophosphate (Courtois et al., 2001; Lindahl, 1996); potassium citrate buffer [1% (w/v) potassium citrate, 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4] (Williamson et al., 2007); phosphate buffered saline (PBS – 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl) (Courtois et al., 2001; Maron et al., 2006); or 10% (v/v) glycerol (all reagents from VWR International, West Chester, PA, unless otherwise noted); and dispersed in a Waring blender for 3 min, each minute interrupted by 1 min incubation on ice (Lindahl and Bakken, 1995). Prokaryotic cells were separated from soil particles by high-speed centrifugation on Nycodenz cushions (Accurate Chemical, Westbury, NY) (Maron et al., 2006). Blender supernatants (~25 ml) were loaded onto Nycodenz cushions (7 ml; 1.3 g ml^{-1}) in 25 × 89 mm Polyallomer centrifuge tubes (Beckman Coulter, Fullerton, CA) and centrifuged at 10,000 × g in a Beckman SW32 rotor for 20 min at 4 °C (Lindahl and Bakken, 1995). Cells were recovered from the interface between the blender supernatant and the Nycodenz cushion with a sterile pipette (Courtois et al., 2001).

Table 1
Global soil properties.

Soil	Sand (%)[a]	Silt (%)[a]	Clay (%)[a]	OM[b]	%W[c]	pH[a]	CEC[d]
GSL	45	39	16	12.8	56.5	4.9	27.8
AGC	35	37	28	5.5	48.6	7.1	15.0
CAL	77	18	5	5.3	2.76	6.4	20.3
BWR	80	13	7	1.2	11.4	7.6	11.1
MBP	72	23	5	20.8	109.1	7.2	52.7

[a] Average of duplicate samples.
[b] Organic matter content by loss-on-ignition, dry weight basis.
[c] Gravimetric water content, average of triplicate samples.
[d] Cation exchange capacity at pH 7.0, meq 100 g^{-1}.

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