A novel retrieval system for nearly complete microbial genomic fragments from soil samples

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Abstract

The construction of a complex genomic library is one of the comprehensive ways to study a complex bacterial community and to access the variety of metabolic pathways present in the rich soil environment. In this report, we developed a new protocol whereby we are able to retrieve nearly complete microbe genomic fragments from soil samples, which are employed to generate a metagenomic library for visualizing the basic scaffolding of the soil microbial community. The use of direct cell lysis within soil-embedded agarose plugs, along with a double-size selection, enabled us to successfully isolate pure and high-molecular weight DNA (0.1–1 Mb) without the need for any further purification. A metagenomic library containing 1.2 Gbp of DNA in total was constructed. Furthermore, analysis of the microbial community structure using 16S rDNA partial sequences found the dominant phylotypes to consist of *α-Proteobacteria* and *Actinobacteria*, which are similar to those seen in forest and agricultural soils, and numerous uncultured microbes from a wide variety of bacterial taxa as well. In conclusion, this study presents a novel protocol for generating a metagenomic library that carries much larger and diverse DNA fragments from soil bacteria that will be applied for the reconstruction of soil microbial genomes and the discovery of novel habitat-specific pathways.

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1. Introduction

Sequence data from microorganisms provide scientists with a blueprint for rapidly assessing organism-specific pathways and microbial network regulation on the basis of comparative genomics. According to previous studies, the sequenced microbial genomes only present a small piece of information of total microbiota in the environment (Torsvik and Ovreas, 2002). Therefore, the construction of metagenomic libraries via the direct cloning of genomic fragments isolated from soil samples has become an essential step in retrieving the collective genomes of environmental microflora, as opposed to traditional genome sequencing projects that required the isolation of a single species from laboratory cultivation. However, the complexity and heterogeneity of the biotic and abiotic

components of soil are the largest obstacles for building a well-organized soil-based library (Rondon et al., 2000; Daniel, 2005).

Separating whole DNA from the soil mixtures is first challenge to overcome before construction of a highly representative library. Many investigators have attempted to optimize DNA recovery from soils by implementing severe physical treatments, such as bead mill homogenization and ultrasonication to lyse the microbial cells (Steffan et al., 1988; Kuske et al., 1998). Such treatments apparently reduce the size of the DNA fragments to sizes of 5 to 10 kb or even less. Another two groups (Zhou et al., 1996; Krsek and Wellington, 1999) showed that a SDS-based cell lysis, in combination with grinding and freezing-thawing, resulted in higher DNA yields with sizes of about 20–40 kb from most gram-positive bacteria. All of these protocols have to accompany with DNA purification for eliminating humic substances which seriously inhibited the following operations, e.g. DNA cloning (Tsai and Olson, 1992). However, as those stated above, many of the steps required for cell lysis and DNA purification result in severe

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DNA shearing. To improve DNA recovery size and purity, this study focuses on the development of a new strategy by modifying the traditional genome sequencing project library protocol (Zimmer and Verrinder Gibbins, 1997; Osoegawa et al., 1998). Accordingly, we obtain high-molecular weight (HMW) DNA fragments from soil mixtures that can be used in the construction of large-insert libraries.

To date, two major methods for making soil DNA libraries exist. One utilizes large-insert cloning vectors, such as bacterial artificial chromosome (BAC) (Rondon et al., 2000), fosmids (Quaiser et al., 2002; Quaiser et al., 2003; Voget et al., 2003; Treusch et al., 2004), or cosmids (Brady et al., 2002; Courtois et al., 2003). The other involves small-insert expression-cloning, *i.e.* short fragments of soil DNA was cloned to expression vector for library screening (Henne et al., 1999; Majernik et al., 2001; Riesenfeld et al., 2004), or random shotgun sequencing libraries (Tyson et al., 2004; Venter et al., 2004; Tringe et al., 2005). Two research groups (Tyson et al., 2004; Venter et al., 2004), have succeeded in building a metagenome library using a shotgun approach and have reconstructed all or certain dominant microbial genomes from a very simple microbial consortia. However, this successful example only happened in this extreme simple case. Tringe and her colleagues (Tringe et al., 2005) have demonstrated that less than 1% of the nearly 150,000 reads generated from a high-diversity soil sample exhibited overlap with reads from independent shotgun library clones. Consequently, this is an immense challenge for computer sequence assembly, especially in cases where low-abundance community members and genomes with high frequency of polymorphic variations, insertions and/or deletions are present. In addition, evidence for horizontally transferred genes and genomic islands in prokaryotic genomes has become more prevalent in the last decade or so (Dobrindt et al., 2004). The presence of alien genes or fragments in the genome, which can be up to 100 kb in length, would seriously affect any prediction of the genetic composition when assembling the reads or sequences from a small-insert library. In contrast, large-insert libraries raise the ratio for isolating overlapping fragments in assemblies that have extremely diverse genomes while also eliminating interference from extensive genomic rearrangements. Furthermore, paired-end sequences from each clone provide information about the orientation and scaffolding of the assembled genome fragments and how they should be linked together (Hallam et al., 2004). These larger DNA fragments also offer a more precise statistical analysis of the *G+C* content, codon usage, and oligo-nucleotide frequencies, all of which aid in species identification and linking specific metabolisms and microbial communities together (Teeling et al., 2004; Chen and Pachter, 2005). According to the above comparisons, large-insert library provides a better way to acquire reliable and integral sequence information for further applications.

Here, we described a new extraction system that successfully overcame the most challenging issue, *i.e.*, separation of genetic materials from the complex biotic and abiotic components present in orchard soil in Taiwan, and is capable of deriving high-purity and diverse HMW DNA (0.1–1 Mb). Using this DNA, we then constructed a metagenome BAC library whose

average size was about 90 kbp and subsequently surveyed the phylogenetic distribution among the library. The reservoir containing HMW DNA fragments will be employed to elucidate the habitat-specific metabolisms represented within the soil samples as well as the genes involved in the generation of novel natural products.

2. Materials and methods

2.1. Bacteria strains and plasmids

E. coli strain TransforMax™ EC300™ and BAC vector CopyControl™ 2 pCC1BAC™ vector, copy number of which can be controlled by an inducer, were purchased from Epicentre (USA). Chloramphenicol (Sigma, USA) was added to all the media at a concentration of 12.5 µg/ml.

2.2. Soil sampling and preservation

The soils from an orchard were collected from three places in Tainan, Taiwan. A sterile spoon was employed to collect approximately 100-g samples from the upper 2 to 5 cm of the soil at each site near the roots of the trees. All of the samples were lyophilized, ground to a fine powder with a mortar and pestle, and then stored at –20 °C.

2.3. HMW DNA extraction from soil

One gram of soil was mixed with 1 ml of suspension buffer (10 mM Tris–HCl, 1 M NaCl, 50 mM EDTA) and 1 ml of 1% low-melting agarose (Fig. 1). The suspension was kept at 37–40 °C to prevent the agarose from solidifying. The slurry-agarose mixture was then pipetted into molds (Pharmacia). After setting at 4 °C, three plugs were transferred to a 2-ml Eppendorf tube, 1.5 ml lysis buffer (50 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, 0.2% (w/v) sodium deoxycholate, 0.5% (w/v) 20 cetyl ether, 0.5% (w/v) lauroylsarcosine sodium salt and 5 mg/ml lysozyme) were added and the tubes were stored at 37 °C for 24 h with gentle horizontal agitation (50 rpm). The lysis buffer was then replaced with 1.5 ml digestion buffer (100 mM Tris–HCl, 500 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1.5 mg/ml protease K and 0.5% (w/v) SDS) and the tubes were stored at 50 °C for 24 h with a gentle horizontal agitation (50 rpm). The plugs were then collected and washed with 50 volumes of wash buffer (10 mM Tris–HCl [pH 8.0], 100 mM EDTA) and then several times with ice-cold 0.5× TBE buffer. Next, the plugs were secured against the teeth of a comb for an electrophoresis unit using several drops of melted agarose, and cooled down to 50 °C. Afterwards, more agarose was poured into the tray around the plugs and allowed to solidify. The HMW DNA was separated by pulsed-field gel electrophoresis (PFGE) using a Gene Navigator system (Pharmacia) set at 175 V, with a 1–14-s pulse, for 18 h at 8 °C in 0.5× TBE buffer. DNA fragments larger than 240 kb were compressed into a small region at the top of the gel. This band was cut out and prepared for the second round of PFGE, which was done using a 165 V, with a 5-s pulse, for 16 h at 8 °C

Fig. 1. Schematic showing the process to extract HMW DNA directly from soil without DNA purification. During dialysis, the soil contaminants and cell debris was removed from the soil plugs. Afterwards, PFGE separation was used to isolate high quality environmental genomic DNA.

in 0.5× TBE buffer. After staining, the HMW DNA band was excised from the gel and the DNA fragments were isolated using electroelution following the method described by Strong

et al., (1997), except that the TAE buffer was replaced with 0.5× TBE buffer and the procedure was run at 120 V for 10 h at 8 °C. The HMW DNA was then precipitated with 30% polyethylene glycol 8000 and 1.5 M NaCl (PGE buffer) for 30 min on ice. The sample was centrifuged for 20 min and the supernatant was removed. Finally, the DNA was dissolved in 10 mM Tris–HCl (pH 8.0) buffer.

Other extraction methods were examined for comparison. The soil DNA kit used in this study was the UltraClean Soil DNA Kit from MO-BIO Laboratories. The SDS-based DNA extraction method followed that of Zhou et al. (1996), and the resulting DNA fragments were purified by Sepharose CL-4B (Pharmacia) as described by Jackson et al. (1997).

2.4. BAC library construction

After concentrating the HMW DNA isolated from soil samples, partial digestion was carried out with an appropriate amount of *Hind* III (Roche) and 1× BSA for 15 min at 37 °C. Ligations were performed in 50-μl reactions containing 65 ng of partially digested DNA, 50 ng of the linear and dephosphorylated pCC1BAC vector (1:10 molar ratio of insert:vector), ligase buffer and 5 U of T4 DNA ligase (Fermentas) at 16 °C overnight. After inactivating the ligase, the ligation products were desalted and condensed by drop-dialysis using a 0.025-μm membrane filter suspended on PEG buffer. Two microliters of the ligation reaction were employed to electroporate 20 μl of *E. coli* TransformMax EC300 cells using Gene Pulser II (Bio Red). Electroporation conditions were 12.5 kV/cm, 100 Ω and 25 μF. After transformation, the cells were resuspended in 500 μl of SOC medium and incubated for 1 h at 37 °C. The broth was plated on the LB agar medium containing 12.5 μg/ml chloramphenicol, 40 μg/ml X-gal and 0.4 mM IPTG and incubated at 37 °C overnight. White recombinant colonies were placed into 96-well plates containing LB freezing buffer (Osoegawa et al., 1998) and stored at –80 °C.

2.5. Phylogenetic relationship analysis of 16S rDNA

A mixed pool of recombinant plasmids was prepared from each 96-well plate. To do this, each of the clones was grown overnight in 2 ml LB, with chloramphenicol added, and the plasmids from each was isolated using the protocol of Rondon et al. (1999) and mixed together. This pooled DNA was used for 16S rDNA PCR amplification with primers 530F, 1492R specifying to *Bacteria* and *Archaea* 16S rDNA gene (Hugenholtz and Goebel, 2001). The library was constructed and the clones with partial 16S rDNA gene were sequenced by an ABI 377-96 DNA sequencer (Perkin-Elmer).

The nucleotide sequences of the partial 16S rDNA fragments were analyzed using the Clustal X program to obtain a preliminary list of the closest phylogenetic neighbors. Distance matrices and phylogenetic trees were constructed using the Jukes–Cantor algorithm and neighbor-joining. The branching consistency in the phylogenetic trees were evaluated using 100 randomly resampled data points produced by the SEQBOOT program, and finally bootstrap values were calculated using the

Fig. 2. Characterization of the new extraction method with different soils. DNA was isolated from: Lane 1, sandy clay loam; Lane 2, sandy loam; Lane 3, loam. Major bands were concentrated toward the top of each lane due to limitations in PFGE. The results are shown after the first round of phase gel electrophoresis. The PFGE was performed at 180 V, with 1–25 s pulses, for 18 h at 8 °C in 0.5× TBE buffer.

CONSENSE program. The programs for phylogenetic analysis are included in the PHYLIP package (Version 3.6) and executed using the free on-line Server Interface (<http://bioweb.pasteur.fr/seqanal/phylogeny/phytip-uk.html>).

All of the partial 16S rRNA sequences obtained from agricultural soil samples (3370 sequences) and forest soil samples (219 sequences) were downloaded from the NCBI

website and compared with the 16S rDNA library generated in this study. To accomplish this, two programs, Classifier and Library Compare, from the RDP II website were used (<http://rdp.cme.msu.edu/index.jsp>).

2.6. Sequence deposition

The sequences of the partial rDNA clones in this paper were submitted to GenBank under the following accession number: GenBank EU151801–EU151840.

3. Results

3.1. A new approach to extraction of HMW DNA from soil

A new soil DNA extraction system (Fig. 1) was developed in this study for two major reasons: to maintain the integrity of the genomic DNA, a problem encountered by several previously reported extraction methods (Steffan et al., 1988; Zhou et al., 1996; Kuske et al., 1998; Krsek and Wellington, 1999); and to generate a sample that is highly representative of the soil microbe structure using a direct lysis method without the need to collect biomass (Krsek and Wellington, 1999; Quaiser et al., 2002; Treusch et al., 2004).

PFGE analysis of the DNA fragments purified with the new method found that the sizes ranged from 15 kb to more than 1000 kb in length (Fig. 2), demonstrating that this protocol does not seriously disrupt the genomes as they are being purified. To determine if this is true for different soils, the relationship between the DNA extraction efficiency and the type of soil was evaluated using soil samples taken from three independent sites and were roughly classified as sandy clay loam, sandy loam and loam soils (Fig. 2). Although the electrophoresis results found that the amount of DNA extracted from the sandy loam and loam soils was greater than that of the sandy clay loam, these differences could be due to the soil characteristics of the sandy clay loam, which has a much smaller pore size than the other two soil types.

3.2. Evaluation of various DNA extraction procedures

The new extraction system was compared with two other popular procedures commonly employed to extract soil DNA (Table 1). Method I, the soil DNA isolation kit, was very convenient and the protocol was quickly finished, but the DNA

Table 1
Comparison of three different methods used to extract DNA from agricultural soils

Method ^a	Time	Purity ^b		Additional purification	Concentration µg/g (dry weight)	Molecular weight
		A ₂₆₀ /A ₂₃₀	A ₂₆₀ /A ₂₈₀			
I	1 day	1.95±0.03	2.03±0.06	No	9.6±1.1	10–20 kb
II	2 days	0.97±0.12	1.45±0.09	Yes	12.5±0.7	30–50 kb
III	4 days	1.81±0.07	1.89±0.03	No	19.2±1.8	10–1000 kb

^a Method I: UltraClean soil DNA isolation Kit (MO BIO); Method II: the method reported from Zhou et al. (1996); Method III: DNA extraction by embedding soil in agarose.

^b Ratio of A₂₆₀/A₂₈₀ >1.7 is indicative of pure DNA. Ratio of A₂₆₀/A₂₃₀ >2 is indicative of pure DNA, whereas a low ratio is indicative of phenol (humic) contamination (Steffan et al., 1988).

Fig. 3. Comparison with different DNA extraction methods from soil by PFGE analysis used at 180 V, with 1–25 s pulses, for 18 h at 8 °C in 0.5× TBE buffer. Lane 1, UltraClean soil DNA isolation Kit (MO BIO); Lane 2, the method reported from Zhou et al. (1996); Lane 3, DNA from the same method as Lane 2 but purified further with a spin column packing Sepharose CL-4B gel. Lane 4, DNA extracted using the method developed in this study; Lane M, MidRange PFG markers.

quality was not adequate for BAC vector cloning. Owing to bead mill homogenization employed in this kit, the genomic DNA was significantly sheared in the process (Fig. 3; Table 1). The average size of the fragments was about 10 kb, which is too short for a large-fragment library, even though the DNA yields

were approximately 10 µg DNA per gram dry soil without any further purification. Method II, modified from Zhou et al. (1996), had a greater yield (12.5 µg DNA per gram dry soil) and produced larger DNA fragments (average ~45 kb). Unfortunately, a significant amount of humic substances were coextracted with the DNA during the organic solvent extraction step. The A_{260}/A_{230} ratio for DNA purified using this method was 0.97 ± 0.12 , which is significantly lower than the ratios obtained by other methods (Table 1). Furthermore, both the size and concentration of the DNA fragments decreased considerably when they were subsequently purified with a Sepharose CL-4B gel filtration column (Fig. 3). Method III involved extracting the soil DNA using soil plugs. Not only was the amount of humic substances present significantly lower when compared with that extracted by Method II, the size distribution of the fragments was also much greater, with a large portion of the isolated fragments being 100 kb or more in size. Furthermore, the DNA yields (19.5 µg DNA per gram dry soil) were almost twice those obtained by the soil DNA isolation kit (Fig. 3; Table 1).

3.3. Construction and analysis of BAC library

DNA fragments isolated by the new method were employed to construct a BAC library as described in the Materials and methods. By using electroporation, transformation efficiency reached 10^6 cfu/µg for the large-insert plasmids, with 98% of the recombinant colonies. A total of 12,000 colonies were isolated for further analysis. Restriction analysis of 120 randomly selected clones (1% of the library) using *NotI* found that each clone had an average insert size of 90 kb (Fig. 4). Consequently, the BAC library included approximately 1.2 Gbp of soil microbial genomic DNA.

3.4. Phylogenetic analysis of soil DNA library

To further evaluate the soil BAC library constructed with the newly developed system, a PCR-based rDNA sequence

Fig. 4. PFGE of randomly selected soil metagenomic library clones digested with *NotI*. The arrow indicates the BAC vector. The gel was electrophoresed for 18 h at 160 V, 8 °C and with 1–10 s pulses. Lane M, Lambda Ladder PFG marker.

Fig. 5. Phylogenetic relationships of partial 16S rDNA sequences from unknown soil clones and previously identified bacteria present in the NCBI database. The distance matrices and phylogenetic trees were calculated using the Jukes–Cantor algorithm and the neighbor-joining method. The percentage shown for each branch is from the 100 bootstrap resamplings that support each topological element in the neighbor-joining analyses. Unknown sequences are represented by a clone number followed by their closet relative with percent identity. Database sequence accession numbers for know organisms are indicated. The names of the major taxa are shown in boldface on the right-hand side. The scale bar indicates the substitutions seen per sequence position.

analysis was performed using *Bacteria* and *Archaea*-specific 16S rDNA primers (Hugenholtz and Goebel, 2001). A total of 40 random PCR products, representing partial 16S rRNA gene segments, were amplified from the BAC library pools and sequenced. All of these clones were from the domain *Bacteria*, i.e., no *Archaea* sequences were found, and corresponded to more than nine phyla. With a 98.5% sequence identity cutoff when aligned with BLASTN against the NCBI nucleotide database, it was found that the 40 clones belonged to distinct bacterial ribotypes (Fig. 5), including numerous uncultured microbes from a wide variety of bacterial taxa (Amann et al., 1995; Rossello-Mora and Amann, 2001).

The phylogenetic affiliation of these partial 16S rRNA sequences is illustrated according to their phylogenetic (Fig. 5) and taxonomic distribution (Fig. 6). The results in Fig. 5 agree with the previous finding (Hugenholtz et al., 1998) that α - *Proteobacteria*, *Acidobacteria*, *Actinobacteria* and *Chloroflexi* are broadly seen in soil environments. However, the 16S rDNA sequences isolated show that a large percentage of these clones are most similar to uncultured or unclassified bacteria. For example, one sequence clone (Z142) had a 99% similarity to the uncultured *Acidobacteria bacterium* AY922041. These results clearly demonstrated that a large portion of the soil organisms, and their metabolisms, still abide in unknown territory. It is also noteworthy that several sequences were from gram-positive bacteria, showing that this method can recover DNA from a widely variety of microbiota, including hard-to-lyse gram-positive bacteria.

To further understand the microbe taxonomic distribution, comparison was made with those from similar environments, i.e., agricultural soil and forest soil (Fig. 6). According to the sequences from the three pools, about 12 major phylum groups were seen, with the dominant bacterial species being *Proteobacteria* and *Actinobacteria*. All three soil types had similar microbial community profiles, but a pairwise comparison of each with the Pearson correlation coefficient found the microbes present in the orchard soil to be more similar to those found in the agricultural soil ($r=0.97$) than the forest soil ($r=0.48$). Furthermore, almost 20% of the 16S rRNA clones identified in this study were unclassified bacteria, which were much higher than the value seen for the forest soil and only slightly more than that for the agricultural soil. The similarities in these profiles suggested that this method is capable of recovering DNA from most of the soil microbiota without the presence of any significant experimental biases.

4. Discussion

In Taiwan, orchard soils are commonly exposed to different insecticides and fertilizers in order to increase productivity. This has resulted in a dramatic alteration of the soil structure and nutrition sources, and directly influenced the microbial metabolism and community structure (Borneman et al., 1996). Therefore, creating a soil library to reconstruct microbial genomes will help interpret environmentally-orientated metabolisms and provide insight into the population structure of the

Fig. 6. Comparison of the phylogenetic diversity present in the three collections when the 16S rRNA gene is used as a phylogenetic marker. The relative distribution of microorganisms from different major phylogenetic groups was classified with the Classifier program and Library Compare program based on the 16S rRNA sequence divergence. The 16S rRNA pools from the forest soil and agricultural soil were gathered from the NCBI database. To compare the microbial communities present in each of the three pools, the relative proportion of each phylotype is shown. The left-top chart expands the results from *Proteobacteria* into its representative classes.

Table 2

Using the Lander–Waterman Model^a to calculate the coverage ratio of a metagenome library for soil microbial communities

Total sequenced length (Gbp) ^b	Coverage (C) ^c	Probability of a base without sequencing ($P_0=e^{-C}$) ^d	% not sequenced	The total length of gap (Ge^{-C}) ^e (Mb)	% sequenced (1– P_0)
1.2	0.1×	0.90	90	10,800	10
12	1×	0.37	37	4440	63
36	3×	0.05	5	100	95
96	8×	0.0003	0.03	3.6	99.97

^a Lander–Waterman Model: Followed by Lander and Waterman, 1988.^b In this study, we assumed $G=12$ Gbp. (3000 genomes per gram in soil) (Hallam et al., 2004; Torsvik and Ovreas, 2002).^c Coverage $C=LN/G$, LN: number of bases sequenced, G : the length of total genomes.^d Poisson formula: $P_k=(C^k * e^{-C})/k!$, C : average coverage times, k : the number of times a base was sequenced practically. P_k : probability of bases has been sequenced by k times. In this Table, $k=0$, so $P_0=e^{-C}$.^e The total length of gap=(% not sequence)*(total genomes)= Ge^{-C} .

microbes present (Hallam et al., 2004; Tyson et al., 2004; Venter et al., 2004; Tringe et al., 2005).

As such, the major goal in this study was to develop a method for retrieving nearly complete genomes from members within soil microbial communities, most of which cannot be isolated by traditional culture methods, and to implement them within a larger-insert soil library. This was achieved in this study through the use of the agarose plugs. During dialysis, most of the soil contaminants, cellular proteins and cellular debris diffuse out of the plugs while the HMW DNA remain trapped within the plug and are subsequently isolated using PFGE (Fig. 1). In a comparison with other methods for isolating DNA from soil samples, it was found that the newly developed direct lysis method yields at least a 10-fold total DNA recovery more than the cell extraction method (Steffan et al., 1988), separating bacteria from soil particles via a nycodenz gradient (Berry et al., 2003) or suspension in buffer (Quaiser et al., 2002). It was suggested that this new method can be employed to generate a better representative library. Furthermore, HMW DNA was isolated from three different soil types showing that this method is versatile (Fig. 2), but the results also indicated that the characteristics of the soil may have an effect on the final amount of DNA recovered.

Several research groups have reported on the use of shotgun sequencing methods (Hallam et al., 2004; Tyson et al., 2004; Venter et al., 2004; Tringe et al., 2005) to retrieve DNA fragments and construct libraries. However, owing to the huge and complicated microbial community present in the soil, it is difficult to back construct complete genomes using such approaches. This is demonstrated using the Lander–Waterman model (Lander and Waterman, 1988) (Table 2). A minimum of $9.6 * 10^7$ clones (with a 8× coverage, averaging 1 kb per clone) are required to completely reconstruct the soil microbial genomes where 3000 different species in 1 g of soil are present (Torsvik and Ovreas, 2002). However, for certain rare genomes (<1%) present in the microbiota, at least a 100× coverage is needed, meaning 1200 Gbp or $1.2 * 10^9$ clones. In contrast, large fragments, such as those seen in this study, provide more sequence information for annotation while also improving the accuracy of predicting different genomic characteristics, such as the $G+C$ content, codon usage, and oligo-nucleotide frequencies (Teeling et al., 2004; Chen and Pachter, 2005) and the

possibilities of obtaining multiple phylogenetic markers, such as 16S rDNA, RecA/RadA, EF-Tu, EF-G, HSP60/70, and RpoB/C. The data will further aid in linking each BAC clone to the possible species for reconstruction of the soil microbial genomes.

The phylogenetic analysis of these rDNA sequences provides a straightforward method for exploiting the genetic diversity within the uncultured microbial community. According to the alignment results (Fig. 5), a significant portion of the rDNA sequences corresponded to uncultured and unclassified bacteria, especially in *Acidobacteria* and *Chloroflexi*. *Acidobacteria* is a newly recognized phylum of bacteria and only three species: *Acidobacterium capsulatum*, *Geothrix fermentans* and *Holophaga foetida* have been cultivated thus far (Hugenholtz et al., 1998; Quaiser et al., 2003). Both phyla, *Acidobacteria* and *Chloroflexi*, are poorly represented by sequences from cultivated organisms and, consequently, little is known about their general physiological properties. However, our phylogenetic analysis found that large DNA fragments from several strains of these phyla are included in our BAC library and, as such, it should be possible to use the genomic sequences to further evaluate and identify the physiological characteristics of these specific species.

In summary, we have developed a new system using a direct cloning method to carefully extract HMW soil DNA that can be used to construct large-insert metagenome libraries. As seen in the results presented here, the libraries provide unique and highly divergent sequences that can be used as a primary blueprint for reconstructing microbial community genomes and as a platform to discover novel natural products. Furthermore, they can serve as a link between the genetic identities and functions of uncultured microbial communities, helping us to understand the complex networks present within the structure of a microbial community, its environmentally oriented metabolisms and the soil ecosystem as a whole.

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