Journal of Bacteriology and Parasitology

Reyes-Escogido et al., J Bacteriol Parasitol 2012, 3:6
DOI: 10.4172/2155-9597.1000147

Rapid Communication Open Access

A Versatile Metagenome Purification Method to Identify Uncultivable Bacteria by Denaturing Gradient Gel Electrophoresis (DGGE) from Sediments and Soils

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Abstract

Here, we report a versatile metagenome purification method to identify uncultivable species from sediments and soils by nested-PCR-DGGE according to 16S rDNA. This combination of methods uses enzymatic lysis *in situ*, polyvinylpyrrolidone (PVP), Chelex 100, glass bead-silica gel and chaotropic salts, with the advantage that it can be applied to different soils.

Keywords: Metagenome purification; Agriculture; Costal lagoon; Hypersaline soils; DGGE; rDNA 16S

Metagenomics is the study of the genomic repertoire of all the organisms living in a particular environment and their activities as a collective [1]. Metagenomes have been purified from soils and other niches, with an estimated 10^3-10^7 species/g [2,3]. To determine the diversity of a microbial community, a common gene present in all species, such as the 16S ribosomal gene (16S rDNA), is amplified by PCR and their amplicons are separated by DGGE, or can be used to generate libraries [4,5]. Since, each amplicon is derived from one genome; their sequences provide taxonomic information and the physiological connections of every species within the community. Therefore, the sequences of the primers, metagenome purity and integrity are essential factors for biodiversity determinations.

A common protocol or commercial kit for metagenome purification from soils or sediments has not been published yet. However, all of them aim to 1) obtain the majority of genomes to represent actual diversity, 2) keep the integrity of each genome, and 3) eliminate humic substances which can inhibit PCR [3,4].

Several protocols were combined to obtain a general method for metagenome purification from sediments of coast lagoons with different salinity concentrations, forests with petrified waterfalls or geysers and soils employed as garbage collector from agricultural and livestock sector (papaya harvest, henequen production and cattle farm). Such method was used to determine the presence of *Lactobacillales* and *Firmicutes* strains with nested-PCR-DGGE.

To prevent cellular lysis by osmotic change and to eliminate debris and humic acids in hypersaline samples, 0.5 g of sediments were homogenized/washed in 5 ml TEN Buffer (100 mM Tris-HCl, pH 7.5; 10 mM EDTA, pH 8; 200 mM NaCl) and centrifuged at 4,000 g for 5 min. All other sediments were washed 4 times in TE buffer (Buffer TEN without NaCl). Cell lysis was carried out in situ [6], the sample was resuspended in 1 ml of TET buffer (100 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8; 1% Triton X-100 (W/V) with 500 µg/ml hen egg white Lysozyme (USB)) and was heated in a micro-wave oven [7] at 300 Watts for 5 seconds (applying >400 Watts may result in soil blow ups), and incubated twice for 5 min at 55ºC. Proteinase K (500 µg/ml; Invitrogen) and 0.05 ml of SDS at 10% (W/V) were added and incubated as before.

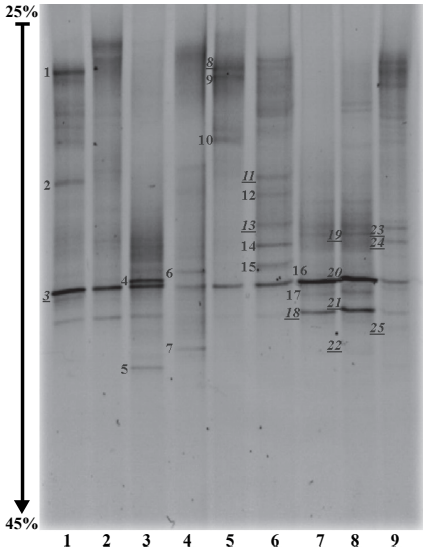

Figure 1: Bacterial communities were determined by nested PCR-DGGE. Soil samples were from hypersaline sediment (lane 1), lagoons (2-4), garbage collectors from agricultural sectors (5-6), forest with petrified waterfalls and geiser (7-8) and cattle farm (9). Geographic localizations are described in Table 1. The numbers indicate the sequenced amplicons. The amplicons reported in the GenBank appear in italics and underlined. The arrow shows the direction of the urea gradient.

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Received August 06, 2012; **Accepted** August 29, 2012; **Published** August 30, 2012

Citation: Reyes-Escogido L, Rodríguez-Buenfil I, Valdés J, Kameyama L, Cárdenas OS, et al. (2012) A Versatile Metagenome Purification Method to Identify Uncultivable Bacteria by Denaturing Gradient Gel Electrophoresis (DGGE) from Sediments and Soils. J Bacteriol Parasitol 3:147. doi:10.4172/2155-9597.1000147

	Ecological niche	Site	µg/ml	[2]Amplicon number	Phylum
1	Salt works drainage holes	[1]Rio Lagartos	52	1,2,*3	Firmicutes 42%.
2	Lagoon	[1]Rio Lagartos	61.7	N.D.	N.D.
3	Lagoon	[1]Celestún	63	4-5	N.D.
4	Sediment rich in birds feces	[1]Celestún	136.7	6-7	N.D.
5	Papaya waste	[1]Yucatán	191.8	*8, 9, 10	*Acidobacteria* 9%.
6	Soil to elaborate compost from henequen residues	[1]Hunucmá	148.5	*11,12, *13,14,15	*Chlorobi* 15%, *Proteobacteria* 45%.
7	Forest with petrified waterfalls	[2]Hierve el agua	128.1	16, 17,*18	*Proteobacteria* 41%.
8	Geisers	[3]Los Azufres	196	*[1]19-22	*Aquificae* 17%, *Lentisphaerae* 1%. *Firmicutes* 40%, *Proteobacteria* 56%
9	Cattle farm	[1]Yucatán	189	*[1]23-25	*Actinobacteria* 8%, *Proteobacteria* 52%. *Firmicutes* 37%

[1]Yucatán, [2]Oaxaca [3]Michoacán states.
*-[1]*Sequences reported to GenBank.
N.D. Not Determined.
[2]See Figure 1.

Table 1: Metagenome samples settlements, yields and taxonomical determinations.

RNase A (50 mg/ml; USB) was mixed and incubated for 5 min at 55ºC. Samples were then centrifuged at 10,000 g for 10 min. To precipitate the SDS, the solution was chilled on ice for 10 min and centrifuged at 11,000 g for 5 min at 4ºC. Metagenome was precipitated with 0.3 vol. of high salt buffer (0.8 M sodium acetate, 1.2 M NaCl) and 0.7 vol. of isopropanol for 10 min at -20ºC and centrifuged. The pellet was washed with 0.5 ml 80% ethanol and dried.

To separate the metals and humic acids, the pellet was resuspended in 0.7 ml TE Buffer-4% PVPP (W/V) [8], and microwave-incubated twice at 400 watts for 3 s followed by an incubation at 55ºC for 10 min. Samples were then centrifuged at 5,000 g for 5 min at 25ºC. The recovered solution was mixed with 40 mg of chelex-100 (Sigma W/V) [9], and was incubated and centrifuged as just described. Then, 0.7 ml of binding solution (4M Guanidanine thiocyanate, 50 mM Tris-HCl, pH 7.0; 20 mM EDTA, pH 8.0) and 0.05 ml of silica mix (glass bead 425-600 µm, silica gel 63-200 µm, 60 Å, 550 m^2/g and 5-25 µm, 60 Å, 0.75 cm^3/g, 30:50:20 g in water), were added and mixed gently for 5 min and centrifuged at 10,000 g for 1 min (the following centrifugations were identical). The pellet was washed with 0.5 ml of wash buffer 1 (ethanol and binding solution 7:3 vol.) and centrifuged. Glass-silica pellet was resuspended in 0.6 ml of clean solution (1 M Guanidanine thiocyanate, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4, 70% ethanol) and centrifuged. The metagenome was recovered with 100 µL of water at 70ºC for 2 min and then was centrifuged.

The average metagenome concentration in hypersaline and coast sediments was 60 µg/mL, whereas for other soils ranged from 2 to 3 times more (Table 1). To determine the metagenome performance, we monitored *Lactobacillales* and *Firmicutes* strains diversity by means of nested PCR-DGGE with the regions V1-V2 from rDNA 16S genes. In the first PCR, most 16S rDNA genes were amplified with 10 ng of each metagenome and 0.5 U of Deep Vent DNA Polymerase, as reported by Reyes-Escogido et al. [10]. The amplicons were purified with QUIAGEN PCR columns, according to the manufacturer. 2 µL from the first PCR were used for the nested PCR with 20 pmol of primers UPT1 to tail 1 [10] and RvT12GCLAMP to tail 2 primers, which include the GC clamp suggested for DGGE [11]. In this work the GC clamp was 36 bp (5' CGGCGGGGCGGGGGGGCGGGCGG GCGGGGCGGGGCCNTTACCTCACCAACTARCTAATNC 3') and the PCR conditions were 30 cycles of at 95°C for 15 s; followed by 55°C for 15 s, and 72°C for 45 s. The amplicons were purified as previously described and separated in a 6% acrylamide gel with a 25-45% urea gradient at 100 Volts for 16 h at 60°C. The gel was stained with 2X syber green for 5 min. The gel showed a specific amplicon pattern for hypersaline coast sediments and waste soils, whereas the forest geyser soils had similar patterns (Figure 1).

25 amplicons were selected, re-amplified and purified as for nested PCR, to be sequenced by MACROGENE (Korea) using UPT1 primer. The BLAST with Ribosomal Data Base Project, indicated that 3 of them (from in extremis soils and in cattle farms) were ~42% homologous to *Firmicutes*; 4 were 41-56% homologous to *Proteobacteria*; and the homology of 5 amplicons was lower than 17%. The sequence from the other 13 amplicons was not determined (Table 1).

The differences with other protocols or commercial kits account for the use of this method for diverse soils. The microwave heat-enzymatic-Chelex 100 treatments released complete genomes eliminating the need of phenol-chloroform extractions. Furthermore, PVPP and glass silica beads removed most contaminants that inhibit PCR-DGGE. Therefore, with this strategy bacterial diversity can be determined fast and without the requirement of any special equipment.

Acknowledgements

This work was supported by the Fondo Sectorial de Investigación Ambiental grant FOSEMARNAT-2004-01-280 (to FJMP).

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