



Use of PCR-DHPLC with fluorescence detection for the characterization of the bacterial diversity during cassava (*Manihot esculenta* crantz) fermentation

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Genet. Mol. Res. 13 (1): 1304-1313 (2014)

Received December 19, 2012

Accepted May 17, 2013

Published February 28, 2014

DOI <http://dx.doi.org/10.4238/2014.February.28.2>

ABSTRACT. Denaturing high-performance liquid chromatography (DHPLC) has been described as a suitable method to study DNA polymorphisms. Here, cassava (*Manihot esculenta* Crantz) fermentation liquor was examined using DHPLC analysis to characterize the bacterial diversity during the fermentation process. GC-clamped amplicons corresponding to a variable region of the bacterial community 16S rDNA were synthesized using polymerase chain reaction (PCR) and then resolved on a base-composition basis using preparative DHPLC. Eluate fractions were collected at random and used as a source of whole community DNA that could be used to determine the bacterial diversity. As a first approach, GC-clamps were removed from the eluted DNA

fragments using PCR to avoid the possible bias these clamps could cause during the construction of clone libraries. As a second approach, a clone library of each eluate sample was constructed, preserving the GC-clamps of the DNA fragments. The first approach generated 132 bacterial rDNA sequences with an average size of 200 bp, 45% of which had similarity to unculturable or non-classified bacteria. The second approach produced 194 sequences identified as Proteobacteria (48%), uncultured or non-classified environmental bacteria (40%) and Firmicutes (12%). We detected a remarkably greater bacterial diversity using the first approach than the second approach. The DHPLC-PCR method allowed for the fast and non-laborious detection of a vast bacterial diversity that was associated with cassava fermentation, and we conclude that it is a promising alternative for the characterization of the overall microbial diversity in complex samples.

Key words: Metagenomics; Fermentation; Bacterial diversity; Denaturing high-performance liquid chromatography (DHPLC)

INTRODUCTION

Denaturing high-performance liquid chromatography (DHPLC) has been pointed out as a rapid, reliable, and highly sensitive method for screening microbial rDNA amplicons for clinical (Hurtle et al., 2003) and industrial (Hutzler et al., 2010) applications. DHPLC has also been used as an accessory method in the clinical diagnosis of human diseases such as Von Willebrand syndrome (Kakela et al., 2006) and cancer (Li et al., 2008).

In the microbial ecology field, DHPLC was recently proven to be a useful method. It has been used, for instance, in the study of marine bacterial diversity (Barlaan et al., 2005), in the analysis of the human gut microbiome during antibiotic therapy (Goldenberg et al., 2007), in the monitoring of bacterial populations during the treatment of urogenital infections (Domann et al., 2003), and in the determination of endodontic infection-related microbiota (Jacinto et al., 2007).

Endophytic microorganisms are important in agriculture and are regarded to be ubiquitous, i.e., they are found in virtually all cultivable plants including soy (Hung et al., 2007; Li et al., 2008), beans (Trujillo-López et al., 2006), corn (Rijavec et al., 2007), cassava (Teixeira et al., 2007), potato (Rasche et al., 2009), rice (Mattos et al., 2008), and peanut (Ibáñez et al., 2009). Cassava (*Manihot esculenta* Crantz) storage roots have been widely used for human and animal nutrition, mainly in tropical regions. Industrial processing of cassava results in a number of products such as distilled beverages and biofuels (Choi et al., 2010; Zhang et al., 2010). Like other agronomic crops, cassava may be colonized by different soil microorganisms, such as mycorrhizal fungi and diazotrophic bacteria (Balota et al., 1999; Teixeira et al., 2007).

Because biotechnological processes that are mediated by microorganisms are of industrial interest, the development of alternative methods to study the microbial community composition and dynamics is of prime importance.

MATERIAL AND METHODS

Collection and preparation of samples

Cassava (*M. esculenta* Crantz) roots were obtained from a local farmer in Belém, PA, Brazil, and they were treated as follows: initial wash with tap water and neutral detergent; immersion in 70% ethanol for 1 min, in 2% hypochlorite for 6 min, in 70% ethanol for 30 s, and a final wash with sterile distilled water. A 50- μ L aliquot of the final washing water was spread onto potato dextrose agar medium and incubated at 37°C for up to 7 days to confirm the superficial sterility of the cassava roots.

Following asepsis, the roots were kept immersed in distilled water (1:2, w/v) for 7 days at room temperature. Every 12 h, a 100-mL aliquot of the fermented broth was taken and filtered under a vacuum through sterile 0.22- μ m nitrocellulose membranes (Whatman, USA). At the end of filtration, the membranes were frozen in buffer containing 1 M NaCl, 1 M Tris-HCl, and 100 mM EDTA for DNA preservation.

DNA extraction

The biomass collected on the membranes was used as a source of metagenomic DNA. The Ultra Clean™ Soil DNA kit (MoBio Laboratories, Inc., USA) was used. The membranes were cut into small pieces and submitted to the bead-beating step for 10 min with 1.0 mL bead suspension buffer and 60 μ L buffer S1. From that step on, the manufacturer instructions were followed.

PCR conditions

A fragment of approximately 1.5 kb of the 16S rRNA gene was amplified using primers 8F (5'-AGAGTTTGAATYMTGGCTCAG-3') and 1407R (5'-GACGGGCGGTGTGTACAAG-3') (Domann et al., 2003). The PCR (50 μ L) mixture comprised the following: 5 pmol of each primer, 1.25 mM of each deoxyribonucleotide triphosphate, 1.5 mM magnesium chloride, 1.0 U Taq DNA polymerase (Invitrogen, Brazil), and 50 ng template DNA. Reaction conditions were as follows: 1 cycle of denaturation at 95°C for 6 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min; and a final elongation cycle at 72°C for 5 min.

An aliquot of this amplicon was used as the template in a second PCR in which a fragment covering the variable region V3 of the 16S rDNA was amplified. GC-clamps of 40 bp were added to PCR primers to introduce a high melting temperature domain to each of the target molecules, which was shown by Wurzbürger et al. (2003) to help resolve similar fragments on a denaturing gradient electrophoresis gel. PCR optimization was done by testing a GC-clamped set of forward primers that was described by Barlaan et al. (2005), namely FP1, FP2, FP3, FP4, FP5, and FP6, combined with the 534R universal primer, in the amplification of 2 distinct samples that were collected on the first day at 12 and 24 h. Once we determined the best primer combination, it was used to amplify the 16S rDNA from all other metagenomic DNA samples throughout the experiment.

PCR was performed on a MasterCycler thermocycler (Eppendorf, Germany) in 1X

buffer (200 mM Tris-HCl, pH 8.0 and 500 mM KCl). The reaction mixture (60 μ L) contained the following: 5 pmol of each primer, 1.25 mM of each deoxyribonucleotide triphosphate, 1.5 mM magnesium chloride, 1.0 U Taq DNA polymerase (Invitrogen), and 50 ng template DNA. PCR conditions consisted of 1 cycle of denaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 40 s, and elongation at 72°C for 1 min; and a final elongation cycle at 72°C for 15 min.

DHPLC separation conditions

The separation of amplicons was performed in a Transgenomic WAVE 4500 system controlled by a Navigator Software version 2.0.0 and equipped with DNASep cartridges with alkylated non-porous polystyrene-divinylbenzene copolymer microspheres, WAVE HSD (High-Sensitivity Detection Module), and Fragment Collector FCW-200 (Transgenomic, USA).

Fragments were resolved on a WAVE system at a constant flow of 0.9 mL/min. The best column temperature was determined through a screening in which we tested temperatures ranging from 55° to 74°C on a 0.5°C-step basis. The reverse phase loading mixture was as follows: 65% buffer A [0.1 M triethylamine/acetate (TEAA), pH 7.0] mixed with 35% buffer B (25% acetonitrile in 0.1 M TEAA, v/v, pH 7.0). A gradient was performed starting with a mixture of 60.0% buffer A and 40.0% buffer B. At the end point (17 min), the mixture was 28.0% buffer A and 72.0% buffer B. Sequences were detected with a fluorescence detector HSX-3500, and chromatogram analyses were performed with the Navigator™ software, version 2.0.0 (Transgenomic).

Peak collection strategy

An aliquot of rDNA amplicons collected from the WAVE system was analyzed using 2 different approaches. The first approach consisted of re-amplifying the sequences to remove the GC-clamps prior to cloning and sequencing; this was accomplished through the use of primers FP1 (Barlaan et al., 2005) (no clamp) and 534R (Muyzer et al., 1993) in a PCR (50 μ L) composed of 5 pmol of each primer, 1.25 mM deoxyribonucleotide triphosphate, 1.5 mM magnesium chloride, 1.0 U Taq DNA polymerase (Invitrogen), and 50 ng template DNA. PCR conditions consisted of 1 cycle of denaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 40 s, and elongation at 72°C for 1 min; and a final elongation cycle at 72°C for 15 min.

In the second approach, DHPLC fractions were directly cloned and sequenced. This was done in order to verify the existence of bias caused by a second PCR, which could lead to a bias in the operational taxonomic unit determination, thus affecting the accuracy of the microbial diversity estimation.

Cloning and sequencing

DHPLC samples and PCR products without the GC-clamp were ligated into the pGEM®-T Easy Vector (Promega Corp., USA) according to the manufacturer instructions, and used to transform electrocompetent DH5 α *Escherichia coli* cells (Stratagene, USA) through a Cell-Porator *E. coli* Electroporation System (Life Technologies, GIBCO BRL, USA). Transformant cells were

plated onto semi-solid LB agar (DIFCO) that was amended with 100 mg/mL ampicillin (sodium salt, EMS, Brazil) and 40 mg/mL X-gal (GE HealthCare, USA). Recombinant clones were detected by the blue/white screening system. Recombinant plasmids were isolated using alkaline lysis miniprep and purified with MAGV N22 membranes (Millipore, USA).

The selected clones were sequenced with the Dye Terminator Cycle Sequencing Kit for MegaBACE using M13 forward and reverse primers (Invitrogen). The samples were read in an automatic DNA sequencer (MegaBACE 1000 DNA Analysis Systems, GE Healthcare).

Data analysis

Ribosomal RNA gene sequences were analyzed using BioEdit 7.0.5.3 (Hall, 1999) and ClustalW Multiple Alignment Tool (Edgar and Batzoglou, 2006). The identification of microorganisms was performed using the online tool ARB-Silva (Pruesse et al., 2007). Partial 16S rRNA sequences have been deposited in the GenBank database under accession Nos. GU480124 to GU480449.

RESULTS

The combination of primers FP3 and universal primer 534R for the amplification of the variable region (V3) of the 16S rRNA gene was considered to be the best suited for our goals because it generated the most defined and diverse peaks with good resolution, as seen on DHPLC. Thus, this primer pair was chosen for subsequent bacterial diversity analysis. The PCR using FP3/534R yielded amplicons with an average size of 194 bp. According to Barlaan et al. (2005), amplification of the V3 region is suitable for the analysis of microbial communities using DHPLC.

The DHPLC separation conditions were optimized, and a constant column temperature of 64.5°C worked the best for our samples, resulting in the partial denaturation of DNA and sharp peaks. Similar to Wagner et al. (2009), we did not obtain baseline separated peaks, but this was already expected considering the complexity of our samples.

Twelve peaks corresponding to 16S rDNA (Figure 1) were collected at random. Two aliquots of each peak to be sampled were collected. For each peak, 1 aliquot was cloned straight away and the other aliquot was re-amplified with regular (non-clamped) primers to remove the GC-clamp prior to cloning.

As previously mentioned, as a first approach, we prepared a clone library of each of the peak aliquots that had been submitted to a second PCR to remove the GC-clamp. Around 10 clones were selected from each library for sequencing, excepting peak X, from which 30 clones were sequenced because of the apparent higher diversity of the bacterial population. Thus, 140 clones were selected, and 132 of them were successfully sequenced.

As a second approach, we prepared clone libraries of each peak using sequences that still contained the GC-clamp. A total of 194 clones were successfully sequenced.

The microbial diversity, as assessed through DHPLC using the 2 above-mentioned approaches, is presented in [Tables S1](#) and [S2](#), which are available in the Supplementary material.

Even though some hits that were identified in the first approach corresponded to the same species as those that were identified in the second approach, their accession number was different in some cases, suggesting that there are base-sequence differences between them.

Exclusive species were detected in each library, which is possibly a consequence of the different approaches that were used.

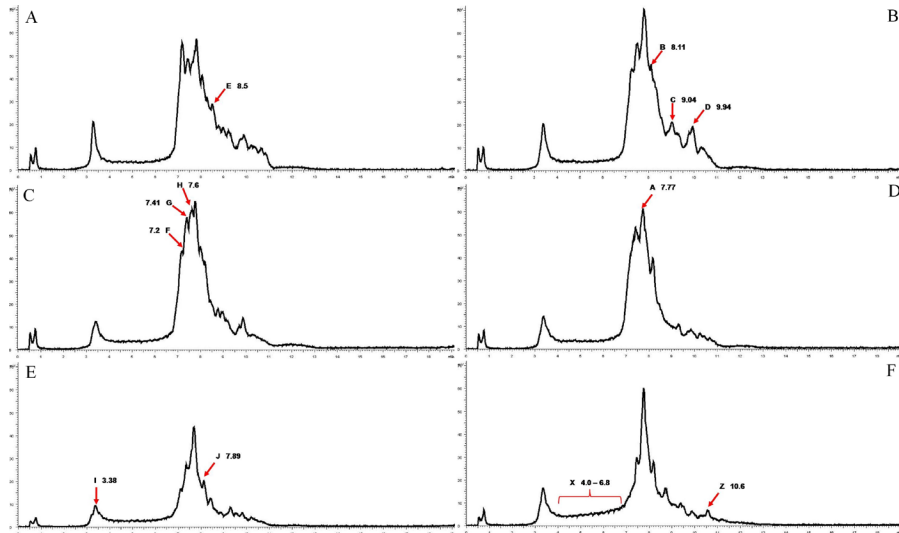


Figure 1. DHPLC of amplified 16S rDNA isolated from samples of cassava-fermented broth. Chromatograms represent the bacterial diversity at different times of collection. Arrows indicate the peaks that were chosen at random and named after the fermentation time (on a daily basis), as follows: (A) day 2, peak E; (B) day 3, peaks B, C and D; (C) day 4 (84 h), peaks F, G and H; (D) day 4 (96 h), peak A; (E) day 5, peaks I and J; (F) day 7, peaks X and Z. Samples from days 1 and 6 were not included in this study.

The first approach (the one in which the GC-clamp was removed before cloning) produced 132 bacterial rDNA sequences with an average size of 200 bp, 45% of which have some degree of similarity to 16S rDNA sequences from the database that are identified as environmental bacteria, either uncultivable or non-classified. We also identified sequences belonging to the Proteobacteria (29%), Firmicutes (22%), Bacteroidetes (3%), and Actinobacteria (1%), as shown in Figure 2.

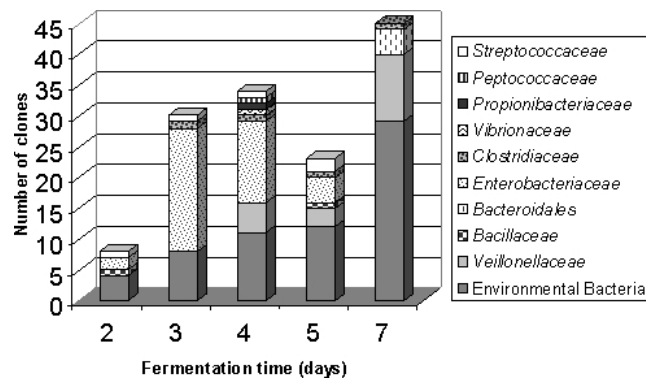


Figure 2. Taxonomic profile of bacteria from cassava-fermented broth as detected through PCR-DHPLC using an approach in which the GC-clamp was removed before cloning the 16S rDNA fragments.

The second approach produced 194 sequences that were identified as Proteobacteria (48%), uncultured or non-classified environmental bacteria (40%), and Firmicutes belonging to the Clostridia class (12%), as shown in Figure 3.

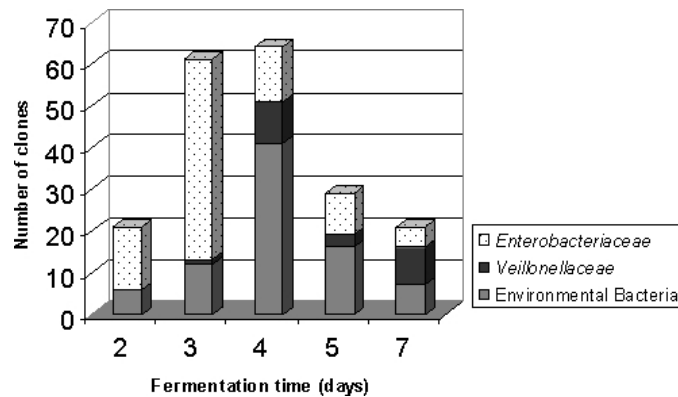


Figure 3. Taxonomic profile of bacteria from cassava-fermented broth as detected through PCR-DHPLC using an approach in which the GC-clamp was maintained in the 16S rDNA clone libraries.

Because we used 2 approaches, we believe that it would be better to show the general diversity taking the 2 results together than showing the individual results. These results are shown in Figure 4.

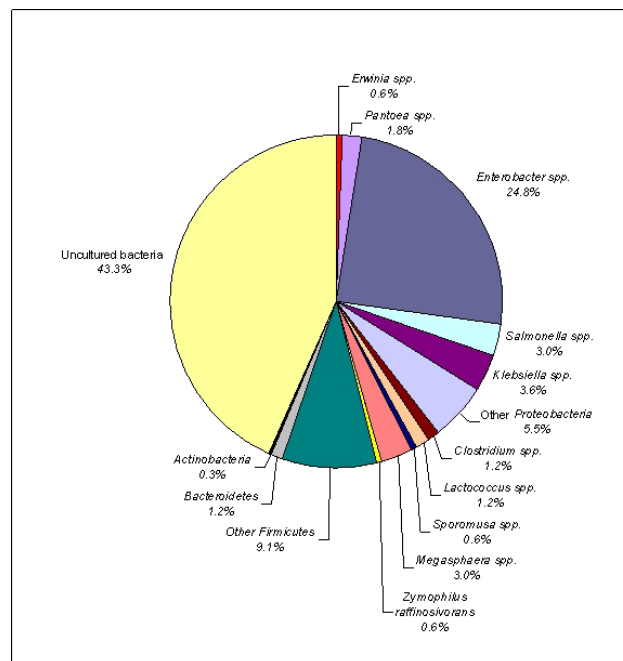


Figure 4. Diversity of endophytic bacteria from the cassava fermentation broth, as detected using PCR-DHPLC. The estimates of the two approaches are presented together. Bacteria were grouped at genus level.

DISCUSSION

We describe a new approach for the identification of endophytic bacterial strains based on genetic variation. We used DHPLC to monitor the bacterial communities that were present for 7 days of cassava fermentation. We used PCR and cloning as accessory methods and the 16S rRNA gene as a genetic marker. We determined the best analytical conditions for DHPLC to allow the discrimination of the endophytic bacteria that were present in cassava fermentation broth. The best temperature for 16S rDNA fragment resolution was 64.5°C when the WAVE system was operated on the universal linear mode, which agrees with previously published data (Barlaan et al., 2005).

It has been postulated that the use of PCR-based molecular techniques for the study of microbial ecology has an inherent disadvantage of possibly generating bias that could affect the measure of taxonomic diversity, for instance, by selectively amplifying some sequences. Furthermore, the use of GC-clamped primers in PCR is considered a prime requirement for DHPLC analysis of amplified sequences, and this could possibly result in a further bias in the sequence analysis. In spite of this, no studies have yet been conducted to evaluate the influence of this clamp on the subsequent analysis of the DHPLC-separated fragments.

Here, we evaluated how the removal of the GC-clamp influenced cloning and diversity estimation. We observed important differences in the bacterial diversity at the genus and species levels when 2 distinct approaches were used, namely, when we removed the GC-clamp from the DHPLC-separated sequences before cloning and sequencing (first approach), and when we did not (second approach) (Figures 2 and 3). We demonstrated with this experiment that a second PCR to remove the GC-clamp before cloning results in a larger number of taxonomically different clones and contributes to an increase in the sensitivity of the method because a greater diversity was observed at the phylum and family levels (Figure 2) than in the approach where the GC-clamp was maintained (Figure 3).

According to our results, a high proportion of the 16S rDNA that was isolated from the cassava-fermented broth corresponded to non-classified environmental bacteria. These unidentified sequences increase dramatically in frequency towards the end of the experiment (Figure 2); it is remarkable that these endophytic organisms remain unclassified because they possibly play a crucial role in cassava fermentation kinetics and on the overall fermented product quality.

Considering the 2 approaches, the phylum that was the most frequently detected during the cassava fermentation process was the Proteobacteria (39%), mainly those representatives belonging to the Gamma and Alpha subdivisions. The most frequently identified family was the Enterobacteriaceae, which includes facultative anaerobic bacteria that are able to ferment sugars to produce acids or butanediol (Kerstens et al., 2006). These bacteria were more frequently detected on the 2nd and 3rd days, suggesting an adaptation to the environmental pH.

The greatest diversity of taxa was observed on the 4th day, when 4 distinct peaks were sampled at different fermentation times. A direct correlation between the number of peaks and the number of taxa detected was observed. Sequences that were assigned to the Clostridiales, mainly to the Family Veillonellaceae (formerly known as Acidaminococcaceae), were detected through both approaches from the 2nd until the 7th day.

Representatives of lactic bacteria were detected throughout the whole process, except on the 7th day, mainly when the first approach was used. The second most frequently detected phylum was Firmicutes (16%), while Bacteroidetes and Actinobacteria were less represented using this method.

The presence of representatives of the Firmicutes was significant, suggesting that they play an important role in the cassava fermentation process, as described previously (Meraz et al., 2006). The Firmicutes represented 22 and 12% of the total number of clones that we analyzed through the first and the second approaches, respectively.

Many species that were found using the first approach were also found using the second. For instance, the Veillonellaceae were represented from the 3rd day on, mainly by *Megasphaera cerevisiae*. However, when we used the second approach, only 2 known groups (Enterobacteriaceae and Veillonellaceae) and environmental non-classified bacteria could be detected. Our results not only show that the sensitivity for bacterial diversity determination was greater when we used the first approach but also show that the greatest relative bacterial diversity occurred on the 4th and the 5th days (Figures 2 and 3). An overall view of the cassava fermentation broth bacterial diversity, as detected through DHPLC, is presented in Figure 4.

DHPLC was efficient in detecting a vast bacterial diversity that was associated with cassava fermentation. Additionally, it proved to be a non-laborious, fast, and efficient method; thus, it is a promising alternative for the identification and monitoring of microbial diversity in complex samples.

ACKNOWLEDGMENTS

Research supported by Fundação Amazônia Paraense (FAPESPA), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

[Supplementary material](#)

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