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Rapid differentiation of bacterial communities using high resolution melting analysis

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ABSTRACT

Analysis of microbial communities is of broad interest in biology and high throughput sequencing is now the preferred method. However, some studies may not need the level of detail high throughput sequencing provides and its cost may limit the number of samples that can be sequenced. High resolution melting analysis (HRM) of 16S rRNA gene variable regions has been proposed as an efficient and low cost method to prioritize samples for sequencing but more specific primers are needed and its efficacy needs to be confirmed. We tested a more specific pair of primers and compared results concerning the structure of microbial communities in tadpole intestine and feces obtained using HRM, denaturing gradient gel electrophoresis (DGGE) and high-throughput sequencing performed in parallel. All three methods showed similar conclusions concerning the communities and revealed communities that differed among intestinal regions and feces. The improved HRM method targets a shorter amplicon in the V3 region of the 16S rRNA gene and uses non-degenerate primers, both of which increase the sensitivity of HRM. The HRM approach was shown to be as effective as DGGE for comparing microbial communities, is considerably easier to perform, and can be used to assess differences in microbial community structure among a large number of samples before committing to sequencing.

1. Introduction

High throughput DNA sequencing can now provide large amounts of data in analyzing microbial communities. However, the cost associated with using high throughput sequencing presents a problem to those with limited funds or those with large numbers of samples. It would be beneficial to have a rapid, low cost method to screen and prioritize samples before committing to sequencing. Hjelmsø et al. (2014) proposed using high resolution melting (HRM) analysis of PCR amplified 16S rRNA gene variable regions as a way to monitor bacterial community composition in soil samples. They also analyzed samples using DGGE and amplicon sequencing but the results from the three methods were not directly compared. A direct comparison would help determine whether HRM analysis can provide the reliability and resolution needed to differentiate bacterial communities compared to more traditional methods. Hjelmsø et al. (2014) also suggested the need to develop more specific primers targeting shorter gene fragments to improve the sensitivity of the assay.

HRM is performed by denaturing PCR amplicons in the presence of a dsDNA saturating dye by increasing the temperature continually at predetermined increments. The melting behavior of PCR products, reflected by the loss of fluorescence, is continuously monitored and determines the melting profile of each sample. Negative first derivative values of the loss of fluorescence are calculated and used to construct high resolution melting peak profiles (reviewed by Tong and Giffard, 2012).

The use of HRM analysis has several benefits compared to methods such as DGGE. HRM is less time consuming to perform and permits the analysis of large numbers of samples in parallel. It avoids contamination problems as a closed-tube method by which the PCR and subsequent HRM step are performed in the same reaction tube without the need to transfer reagents or PCR products. Additionally, bacterial communities can be differentiated by visualization or analysis of the melting curves/peak profiles without the need for additional data manipulation.

In the present study, we describe a new HRM analysis protocol for differentiating bacterial communities by comparing the 16S rRNA hypervariable V3 region of bacteria isolated from five distinct sections of tadpole intestines and feces. Denaturing gradient gel electrophoresis and high throughput sequencing of the same variable region were performed in parallel to compare the reliability of the assay. Based on the concordance among the results obtained using the three methods we suggest that HRM analysis of the V3 region of the 16S rRNA gene can be used as an effective and reliable way to differentiate bacterial

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communities. In addition to being a useful screening tool before investing in high throughput sequencing service, this technique is especially useful when the objective is simply to detect changes in bacterial community composition without needing to know the identity of community members.

2. Material and methods

2.1. Sample collection and DNA extraction

Three Southern leopard frog tadpoles (*Lithobates sphenocepahala*) were collected from a small pond in Hattiesburg, Mississippi, USA (N31°20.821 W089°22.355), caged together and suspended in the water column of the same pond. Three tadpoles were chosen to provide replicates for use in DGGE and DNA sequencing while technical triplicates of each of the replicates were used to examine the reproducibility of HRM. The tadpoles were allowed one week to feed and then retrieved. Tadpoles were placed individually in small cups containing approximately 200 ml of filter-sterilized pond water for feces collection. After collecting feces using sterile pipettes, the tadpoles were euthanized in a 1% solution of MS-222. The entire digestive tract of each tadpole was removed using sterile blades and forceps. From each tadpole a section of the foregut (For), including the mannicoto glandular, anterior small intestine (ASI), posterior small intestine (PSI), colon (Col) and rectum (Rec) were collected (Pryor and Bjorndal, 2005). The entire colon and rectum were collected from each tadpole whereas approximately 2–3 cm portions of each of the remaining gut regions were collected. DNA extraction of the feces and each gut region, with contents included, was completed using the Powersoil Extraction Kit (MoBio) following the manufacturer's protocol. Extracted DNA was quantified using sample absorbance at 260 nm and diluted to a concentration of 10 ng/μl with UV-sterilized DNase/RNase-free water. Tadpole collection and laboratory procedures were approved by the Mississippi Department of Wildlife, Fisheries, and Parks (Permit No. 0623151) and the University of Southern Mississippi IACUC Committee (Protocol No. 13121202).

2.2. High resolution melting analysis

PCR amplification of the hypervariable V3 region (~200 bp) of the bacterial 16S rRNA gene was performed in a 25 μl reaction volume containing 12.5 μl of EconoTaq PLUS 2 × Master Mix (Lucigen), 1 μl of 10 ng/μl extracted DNA, 2.5 μl each of 5 uM primers 341-F (5′-CCTACGGGAGGCAGCAG -3′) and 518-R (5′-ATTACCGCGGCTGCTGG-3′), 1.25 μl of 20 × EvaGreen (Biotium), 0.5 μl of 25 mM $MgCl_2$ to adjust the final magnesium concentration to 2 mM and 4.75 μl of DNase/RNase free water. Touchdown PCR (Korbie and Mattick, 2008) was found to reduce the amount of background smearing in DGGE gels (below) and thus was also used for HRM analysis to maintain consistency. Initial DNA melting took place at 94 °C for 4 min followed by 20 cycles of melting at 94 °C for 1 min, annealing at 66 °C for 20 s with a 0.5 °C decrease in temperature after each cycle and extension at 72 °C for 30 s. The initial 20 cycles were followed by 10 cycles of melting at 94 °C for 1 min, annealing at 56 °C for 20 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min.

High resolution melting analysis was performed using a Rotor-Gene 6000 thermal cycler (Corbett Life Sciences, now Qiagen). All samples were run in technical triplicates and averaged using the Rotor-Gene software (Replicate View function). Sample fluorescence was acquired from 70 °C to 95 °C at 0.2 °C increments two seconds after each temperature increment had been reached. First derivatives of the change in sample fluorescence over time (− dF/dT) at each 0.2 °C increment between 75 °C and 90 °C were calculated using the Rotor-Gene 6000 Series Software version 1.7. Further processing of the averaged melting peak profile data was performed using Excel spreadsheet software. Once a first derivative value of fluorescence dropped below zero, all subsequent fluorescence values were removed and replaced with zeros. A matrix with rows labeled by sample and columns labeled by each temperature increment was created. The resulting matrix was imported in R and using Bray-Curtis as the distance metric non-metric multi-dimensional scaling was performed using the metaMDS function (vegan) while retaining two dimensions ($k = 2$).

2.3. Denaturing gradient gel electrophoresis

PCR amplification of the hypervariable V3 region of the bacterial 16S rRNA gene was performed in a 25 μl reaction volume as described above except for the omission of EvaGreen dye needed for HRM. The forward primer used contained a 40 bp GC clamp (5′-CGCCCGCCGC-GCGCGGCGGG-CGGGGCGGGG-GCACGGGGGG) at the 5′-end. To minimize background smearing in the gel, a three cycle reconditioning PCR step was included to reduce formation of heteroduplex PCR products (Thompson et al., 2002). The 25 μl reaction contained 3 μl of the PCR product as template and the same PCR reagents described above. Initial melting took place at 94 °C for 4 min followed by 3 cycles of melting at 94 °C for 1 min, annealing at 56 °C for 20 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. This was performed only for samples analyzed by DGGE.

DGGE was performed using the Dcode system (Bio-Rad). Five microliter of polished PCR products were mixed with 1 μl of 6 × loading dye and loaded directly into an 8% acrylamide gel with a gradient of 35 to 60% urea and deionized formamide. The gel was run at 80 V for 16 h in 1 × TAE buffer at 60 °C. To analyze and compare DGGE profiles, DNA bands were first visualized and photographed after staining for 15 min with 1 × GelStar Nucleic Acid Gel Stain (Lonza Group, Ltd) and UV transillumination at 312 nm. The number of DNA bands, their relative location in each lane and abundance (intensity of bands) were determined using Bionumerics 6.5 (Applied Maths NV) and then exported as a spreadsheet for subsequent analysis (described below in Data Analysis).

2.4. 16S rRNA gene sequencing

The V3-V4 region of the bacterial 16S rRNA gene was sequenced commercially (Molecular Research LP) in both directions using Illumina MiSeq and the primers whoi341-F (5′-CCTACGGGNGGCWGCAG-3′) and new805-R (5′-GACTACNVGGGTATCTAATCC-3′). Only quality filtered (Q25) overlapping reads that spanned the entire V3 region were used in the analysis. A minimum similarity threshold of 97% was used in assigning OTUs and for parsing out chimeric sequences using USEARCH Version 8.1 (Edgar, 2013). The relative abundance of each OTU in each sample was used in analyzing bacterial communities. This Targeted Locus Study project has been deposited at DDBJ/ENA/GenBank under the accession KAFO00000000. The version described in this paper is the first version, KAFO01000000.

2.5. Data analysis

The relative first derivative of fluorescence at each temperature increment of the HRM melting peak profiles (Kim and Lee, 2014), the number and relative intensity of each band in each lane of the DGGE image, and the relative abundance of each OTU in each bacterial community were used to construct dis-similarity matrices based on the Bray-Curtis metric. Non-metric multidimensional scaling (NMDS), retaining two dimensions, was performed on all three sets of data using metaMDS function of the vegan package (Jari et al., 2015) in R (Version 3.2.2). DNA sequence reads were trimmed using FASTX-Toolkit (Version 0.0.14) to contain only the hypervariable V3 region so that all three analytical methods compared the same region of the 16S rRNA gene. More similar bacterial communities lie closer in proximity to one another, or cluster, compared to those that are more dissimilar in composition when visualizing the data points in two dimensions (Paliy

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