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16S rDNA CLONE LIBRARY SCREENING OF ENVIRONMENTAL SAMPLE USING MELTING CURVE ANALYSIS

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ABSTRACT

A new method of screening a 16S rDNA clone library from an environmental sample is demonstrated using the non-specific fluorescent dye SYBR Green for the melting curve analysis of PCR-amplified DNA fragments. It is simple, easy and swift to operate. In addition, it requires little manual operation and the product may be still used for further analysis.

Key Words: 16S rDNA, clone library, melting curve analysis.

I. INTRODUCTION

Molecular techniques have been used widely to reveal microbial community structure and dynamics of the environmental samples. One of these techniques is to generate a clone library from the 16S rDNA fragments of an environmental sample amplified by PCR (polymerase chain reaction) and to sequence individual clones in the library (Zhang and Fang, 2001). However, to sequence all the clones in the clone library is slow, tedious and costly. The alternative is to screen the library and sequence only the representative clones.

The common screening techniques include SSCP (single-stranded conformation polymorphism) (Orita *et al.*, 1989), TGGE (temperature gradient gel electrophoresis) (Riesner *et al.*, 1992), DGGE (denaturing gradient gel electrophoresis) (Muyzer *et al.*, 1993), T-RFLP (terminal restriction fragment length polymorphism) (Liu *et al.*, 1997), and RFLP (restriction fragment length polymorphism) which is also known as ARDRA (amplified ribosomal DNA restriction analysis) (Massol-Deya *et al.*, 1995). However, all of these techniques are time consuming and each has its limitations. For RFLP and T-RFLP, hybridization analysis or sequencing of the PCR products is not possible, because DNA fragments have been digested by the restriction enzyme. For DGGE, TGGE and SSCP, it is difficult to separate DNA fragments over 500 bps and those of similar melting behavior.

Hydrogen bonds, which hold the double-stranded DNA structure, are weakened as temperature increases. Each double-stranded DNA structure has a specific melting temperature (T_m) that is defined as the temperature at which 50% of the DNA becomes single stranded. T_m is primarily dependent upon the length of the double-stranded DNA, degree of GC content, DNA sequence, and degree of complementarity between the two strands. It may be used for the detection and identification of individual DNA. It can be measured by the simple, rapid, and reliable melting curve analysis (MCA). MCA has been used for the detection and identification of known pathogenic bacteria (Shrestha *et al.*, 2003), hemoflagellates (Nicolas *et al.*, 2002), RNA viruses (Beuret, 2004), and the strain typing of plum pox virus (Varga and James, 2005). Samples of these studies often had simple microbial constituents, sometimes of known identities. However, MCA has never been used to screen a clone library from the PCR-amplified 16S rDNA fragments of environmental samples, which often have diverse unknown DNA sequences (Muyzer *et al.*, 1993; Massol-Deya *et al.*, 1995; Liu *et al.*, 1997).

In this study, a method is proposed to screen a clone library of PCR-amplified 16S rDNA fragments of an environmental sample using SYBR Green and MCA. SYBR Green is a non-specific fluorescent dye with a high affinity only for double-stranded DNA, but not for single-stranded DNA. As the PCR-amplified DNA fragments split into single strands during

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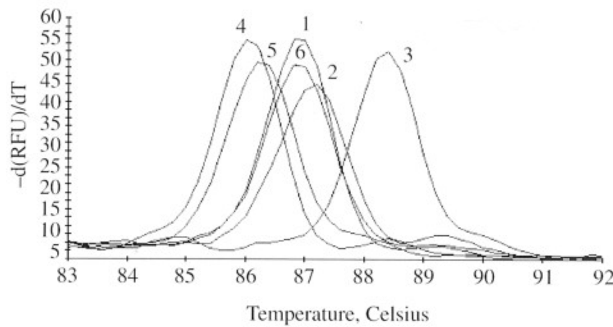

Fig. 1 The melting curves of clones EC-1 to EC-6

Table 1 The PCR product sizes, GC contents and T_m values

Sample	Length (bps)	G + C (%)	T_m (°C) Run 1	Run 2	Average
EC-1	195	56.9	87.0	86.8	86.9
EC-2	170	57.7	87.2	87.2	87.2
EC-3	171	60.2	88.4	88.4	88.4
EC-4	194	53.1	86.0	86.2	86.1
EC-5	169	56.2	86.4	86.4	86.4
EC-6	170	58.2	87.0	86.8	86.9

the MCA, the SYBR Green fluorescent intensity diminishes gradually. As the temperature reaches T_m, the fluorescent intensity is reduced by 50% as half of the double-stranded DNA has become single-stranded.

II. MATERIALS AND METHODS

1. Clone Library and Unique Clones

A clone library was constructed from a phenol-degrading community using the PCR fragments amplified with a primer set of EUB8F (5'-AGAGTTTGATCMTGGCTCAG-3') (Weisburg *et al.*, 1991) and UNIV1392R (5'-ACGGGCGGTGTGTRC-3') (Ferris *et al.*, 1996). According to the sequencing result of the clones in this clone library, there were six unique sequences represented by six environmental clones (EC). These six clones were selected to demonstrate the proposed method.

2. PCR with SYBR Green and Melting Curve Analysis

PCR was performed in 96-well 0.2-ml thin-wall PCR plates using the iCycler IQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with iQ SYBR Green Supermix (Bio-Rad) using the primer set of 341F/518R. Cycling conditions began with an initial hold at 95°C for 3′, followed by 40 cycles consisting of 95°C for 30″ and 55°C for 30″, then with 95°C for 1′ and 55°C for 1′. iQ SYBR Green Supermix contains SYBR Green which is a non-specific fluorescent dye with a high affinity for double-stranded DNA.

At the end of the PCR reaction, to confirm that a single PCR product was detected and to discriminate the clone type, MCA was performed on the iCycler IQ over the range 52 to 98°C by monitoring iQ SYBR green fluorescence with increasing temperature (0.2°C increment changes at 10 s intervals). All sample measurements were performed in duplicate. After completion of the PCR amplification and MCA, data were analyzed with the iCycler 3.0 version software (Bio-Rad).

3. Accession Numbers

The nucleotide sequence data reported in this paper have been submitted to the GenBank, EMBL and DDBJ databases, and have been assigned the following accession numbers: AY861460-5.

III. RESULTS AND DISCUSSIONS

Results showed that each clone produced only a single PCR product without any primer dimmers, as illustrated in Fig. 1. The T_m values were measured according to the melting curves. The PCR products of six EC are different in size, GC content, and T_m value, as summarized in Table 1. Fig. 1 illustrates that the T_m of EC-2 (87.2°C), EC-3 (88.4°C) and EC-4 (86.1°C) differed clearly from one another and can thus be unambiguously separated. Although clones EC-1 to -5 differed as little as 0.2°C from one another, these five clones could still be clearly identified from the MCA, as illustrated in Fig. 1. However, clones with the same T_m cannot be identified individually by MCA as shown by EC-1 and -6 in Fig. 1.

These results suggested that most of the clones could be discriminated and screened for their genotypes using the proposed method by the PCR with SYBR Green followed by MCA. This method has a few advantages over other clone screening methods. First, the PCR-MCA method is easy to operate with just an additional step after PCR. Second, this method is rapid, taking about 2 h for as many as 96 clones in a plate. Third, this method requires little manual operation and thus has high degrees of reproducibility. Lastly, the PCR product after MCA analysis can be used directly for further analysis, such as sequencing and ligation, which cannot be achieved by any other methods. However, the PCR-MCA method also has its limitation, just like other screening techniques, that is, it cannot separate PCR products with the same T_m. Further improvements of this method are needed.

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