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Chapter 13

Application of PCR-DGGE Fingerprinting in Molecular Ecology of Marine Microbial Symbionts

Zhiyong Li*

Marine Biotechnology Laboratory, School of Life Sciences and Biotechnology and Key Laboratory of Microbial Metabolism, Ministry of Education, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, P.R. China.

Abstract

It is now well recognised that only a small fraction of microorganisms can be isolated and characterized, especially marine microbial symbionts because of the difficulty in cultivation. The application of molecular biological methods to study the diversity and ecology of microorganisms in natural environments has been practiced since the mid-1980s. Molecular biological techniques such as genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community, and are now frequently used in molecular ecology to explore the microbial diversity and to analyse the structure of microbial communities. Denaturing gradient gel electrophoresis (DGGE) of PCR amplified ribosomal DNA fragments has been introduced into microbial ecology in 1993. Combined with PCR amplification of marker genes or their transcripts, DGGE can give a direct display of the predominant constituents in microbial assemblages with the advantage of easiness, reproducibility, reliability, and speed. PCR-DGGE fingerprinting is suitable for revealing the diversity of uncultured marine microbial symbionts, comparing and monitoring the dynamic change of the community structure of marine microbial symbionts. In this chapter, the theoretical aspects of PCR-DGGE fingerprinting and its application in the molecular ecology of microbial symbionts of marine organisms such as sponge, coral, ascidians, bryozoans and algae are introduced.

* Corresponding author: E-mail: zyli@sjtu.edu.cn; Tel.: (+86)21-34204036.

1. Introduction

It is now well recognised that only a small fraction of microorganisms can be isolated and characterized in the laboratory. Marine microbial symbionts are much more difficult to cultivate because of their special environmental conditions. So, a cultivation-dependent approach is difficult for revealing the microbial diversity of marine microbial symbionts. Meanwhile, media used for the isolation and cultivation of microorganisms are selective and hence give a biased view of microbial community structure. Therefore, culture-independent molecular strategies are required to supplement the classical culture-based techniques.

The application of molecular biological methods to study the diversity and ecology of microorganisms in natural environments has been practiced since the mid-1980s. Since that time, many new insights into the composition of uncultivated microbial communities have been gained. Culture-independent molecular biological techniques such as genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community, and are now frequently used in molecular ecology to explore the microbial diversity and to analyse the structure of microbial communities. In 1993, genetic fingerprinting technique, denaturing gradient gel electrophoresis (DGGE) of PCR amplified ribosomal DNA fragments was introduced into microbial ecology by Muyzer et al.(1993) to profile community complexity of a microbial mat and bacterial biofilms. At present, PCR-DGGE fingerprinting has been widely used to study microbial communities in various habitats. In the case of marine microbial ecology, in 1995, Muyzer et al. (1995) used DGGE analysis of PCR amplified rDNA fragments to provide information on the genetic diversity of microbial communities found around hydrothermal vents. Sequencing of excised DGGE bands revealed sequences similar to those of members of the genus *Thiomicrospira*, sulfuroxidizing bacteria. In this chapter, we will mainly introduce the application of PCR-DGGE fingerprinting in the diversity revelation, dynamic monitor and detection of special microbes of marine microbial symbionts.

2. Theoretical Aspects of PCR-DGGE Fingerprinting

Denaturing gradient gel electrophoresis (DGGE) is a method by which fragments of DNA of the same length but different sequence can be resolved electrophoretically, allowing the separation of a heterogeneous mixture of PCR amplified genes on a polyacrylamide gel. The separation of DGGE DNA fragments of the same length but with different sequences is based on the decreased electrophoretic mobility of a partially melted doublestranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide). The melting of DNA fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Once a domain with the lowest melting temperature reaches its melting temperature (*Tm*) at a particular position in the denaturing gradient gel, a transition from a double stranded to a partially melted molecule occurs. The protruding single strands practically cause a halt of the molecules at that position. Sequence variation within such domains causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different

positions in the gel. 100% of the sequence variants can be detected in DNA fragments up to 500 bp by the attachment of a GC rich sequence (GC clamp) that acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands to one side of the DNA fragment. A sequence of guanines (G) and cytosines (C) varying between 30 and 50 nucleotides is added to the 50 end of one of the PCR primers, coamplified and thus introduced into the amplified DNA fragments (Muyzer et al.1998).

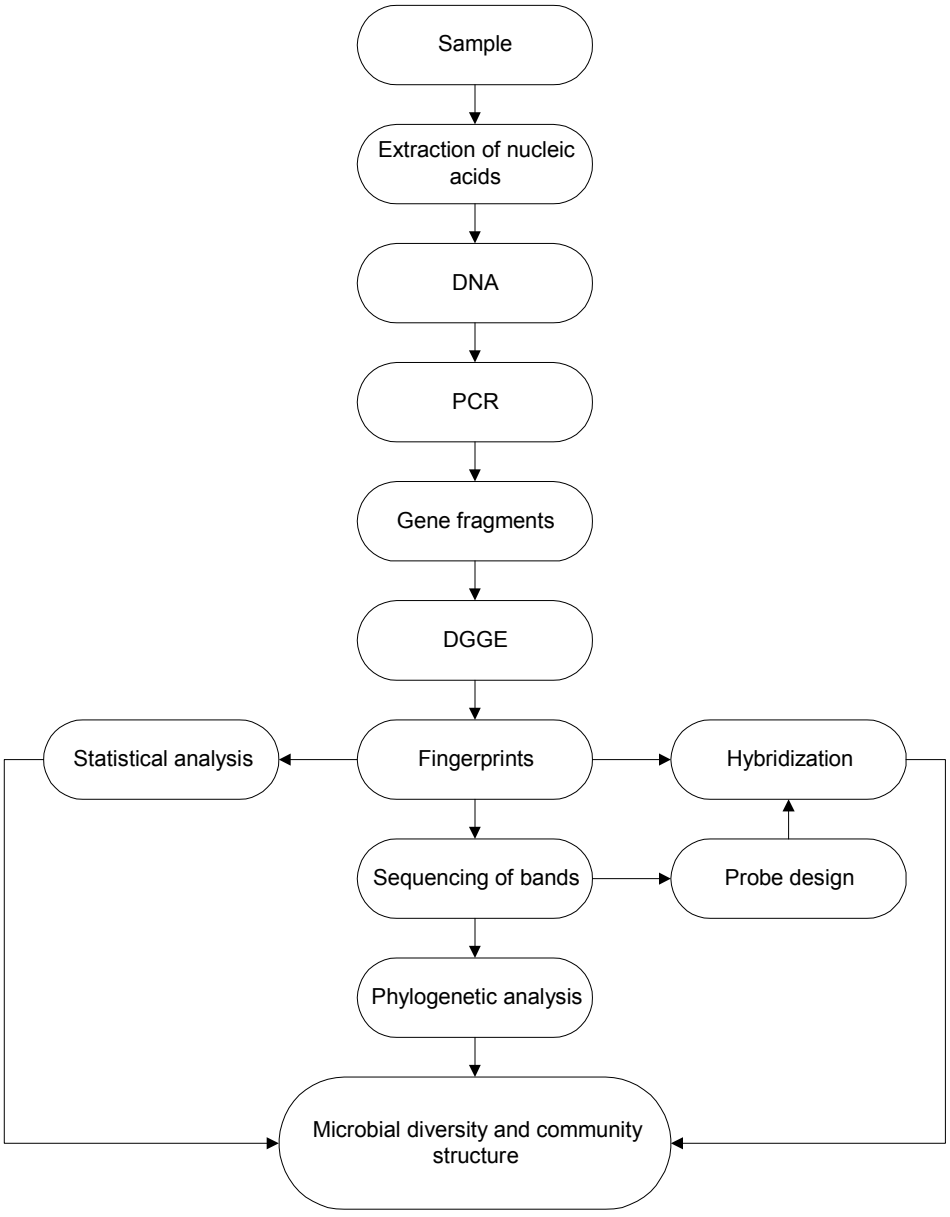

Figure 1. Flow diagram of PCR-DGGE fingerprinting analysis of microbial community.

Amplification of DNA extracted from mixed microbial communities with primers specific for 16Sr RNA gene fragments, 18Sr RNA gene fragments or ITS fragments result in mixtures of PCR products. The obtained different sequences with the same size by PCR can be separated in polyacrylamide gels containing a gradient of DNA denaturants. As the PCR products proceed through the gel, the denaturing conditions gradually become stronger, therefore PCR products with different sequences start melting at different positions (i.e. at different denaturant concentrations) in the gel. DNA bands in DGGE profiles can be visualised using ethidium bromide or silver staining. DGGE profiles can be blotted onto nylon membranes and hybridised with a radioactively-labelled oligonucleotide probe. Individual bands may be excised, reamplified and sequenced to give an indication of the composition and diversity of the microbial community. A general overview of PCR-DGGE fingerprinting of microbial community is shown in Figure 1. For a more comprehensive overview of the detailed technique of PCR-DGGE fingerprinting the reader is referred to Schäfer et al.(2001).

3. The Application of PCR-DGGE Fingerprinting in Molecular Ecology of Marine Microbial Symbionts

3.1. The Revelation of Microbial Community Structure and Diversity

Many marine macroorganisms such as sponges are known to acquire symbiotic microorganisms from the external environment (horizontal acquisition) or inherit their symbionts from the parent colony (maternal or vertical acquisition). Most of the marine microbial symbionts are uncultured, so, culture-independent PCR-DGGE fingerprint-based molecular methods have been widely used in the revelation of marine symbiotic microbial community structure and diversity. For instance, a stable and specific bacterial community was shown to be associated with the Mediterranean sponge *Chondrilla nucula* regardless of sampling time and geographical region (Thiel et al.2007a). As for four South China Sea sponges, *Stelletta tenuis*, *Halichondria rugosa*, *Dysidea avara*, and *Craniella australiensis*, sponge host-specific bacteria including Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria were detected (Li et al.2006).

Besides sponge, the structure and composition of microbial communities inhabiting the soft coral *Alcyonium antarcticum* were investigated using DGGE by Webster et al.(2007). Diverse microbial communities including Alpha- and Betaproteobacteria, Bacteroidetes, Firmicutes, Actinomycetales, Planctomycetes, and Chlorobi and bacteria from the functional group of sulfate-reducing bacteria were revealed. The photobiology and distribution of dinoflagellates in the genus *Symbiodinium* was investigated for eight common reef coral species over a depth range of 1–25 m on a coral reef in Belize (Warner et al. 2006). PCR-DGGE of the internal transcribed spacer (ITS) 2 region revealed marked differences in host specificity and depth zonation for certain symbiont types.

Diversity of intratunical bacteria in the tunic matrix of the colonial ascidian *Diplosoma migrans* was also revealed using PCR-DGGE (Wichels, 2005). DGGE profiles based sequence data generated five different subgroups of intratunical proteobacteria such as

Ruminococcus flavefaciens, *R. flavefaciens*, *Pseudomonas saccherophilia*, *Flavobacterium* symbiont.

Species- and site-specific bacterial communities associated with four encrusting bryozoans from the North Sea, Germany, were investigated by Kittelmann et al.(2005). DGGE of PCR-amplified 16S rRNA gene fragments of associated bacteria displayed specific bacterial community profiles in the examined species *Aspidelectra melolontha*, *Conopeum reticulum*, *Electra monostachys* and *Electra pilosa*.

Culturable and nonculturable bacterial symbionts in the toxic benthic dinoflagellate *Ostreopsis lenticularis* was revealed by Ashton et al.(2003). DGGE analyses of extracted *Ostreopsis* associated bacterial DNAs indicated that there were three culturable and four non-culturable associated bacteria including *Pseudomonas*/*Alteromonas*, *Pseudomonas*/*Alteromonas* and *Acinetobacter*.

In the case of eukaryotic symbionts, phylogenetic analysis of sequence data from eukaryotic DGGE analysis revealed that the communities present in Antarctic sponges fell into diatom and dinoflagellate clusters with many sequences having no known close relatives. In addition, seven eukaryotic sequences that were not detected in seawater samples or other sponge species were observed in Antarctic sponge *Kirkpatrickia varialosa* (Webster et al.2004). DGGE analysis indicated that fungal communities differ among different sponge species (Suberites zeteki and Mycale armata) and also vary between sponges and seawater (Gao et al. 2008).

The diversity of dinoflagellate endosymbionts living with cnidarians was examined using denaturing gradient gel electrophoresis (PCR-DGGE) fingerprint analysis of the rDNA internal transcribed spacers (ITS) 1 and 2 (La Jeunesse et al.2008). Detection and identification of fungi intimately associated with the brown seaweed *Fucus serratus* were carried out by Zuccaro et al.(2008). The predominant DGGE bands obtained from healthy algal thalli belonged to the *Lindra*, *Lulworthia*, *Engyodontium*, *Sigmoidea/Corollospora* complex, and *Emericellopsis/Acremonium*-like ribotypes.

3.2. The Monitor of Spatial and Temporal Variability of Marine Microbial Symbionts

Distinct bacterial communities were found to inhabit the endosome and cortex of sponge *Tethya aurantium Pallas* 1766 by Thiel et al.(2007b), where a new phylotype of *Flexibacteria* was recovered only from the sponge cortex, while *Synechococcus* species were present mainly in the sponge endosome.

Using a combination of the internal transcribed spacer region 2 (ITS2) and denaturing gradient gel electrophoresis (DGGE), Macdonald et al.(2008) assessed the cladal and subcladal variability of *Symbiodinium* in the widely distributed species *Stylophora pistillata* along a latitudinal transect in southeast African waters which extended into high latitude locations, it appears that there is a shift in symbiont distributions with increasing latitude.

Frade et al.(2008) identified three symbiont genotypes with distributions that reveal patterns of host specificity and depth-based zonation using the same technique as above. Contrasting with variation over depth, strong functional within-colony uniformity in symbiont diversity was found. Relating symbiont distributions to measured physical factors (irradiance,

light spectral distribution, temperature), suggests depth-based ecological function and host specificity for *Symbiodinium* ITS2 types, even among closely related coral species.

PCR-DGGE fingerprinting has been used to monitor changes in bacterial communities when examining the feasibility of growing sponges in aquaculture systems (Mohamed et al.2008a; 2008b). DGGE analysis revealed that the diversity of the bacterial community of *M. laxissima* increased when sponges were maintained in aquaculture and that bacterial communities associated with wild and aquacultured *M. laxissima* were markedly different than those of the corresponding surrounding water (Mohamed et al.2008a). As for marine sponge *Ircinia strobilina*, according to DGGE analysis, populations affiliated with Beta- and Deltaproteobacteria, *Clostridia*, and *Planctomycetes* emerged in sponges maintained in aquaculture (Mohamed et al.2008b).

Highly stable symbioses in response to seasonal environmental situations among western Atlantic brooding corals were revealed by Thornhin et al.(2006a) using DGGE of the internal transcribed spacer 2 (ITS2) region. Multi-year, seasonal genotypic surveys of coral-algal symbioses reveal prevalent stability or post-bleaching reversion (Thornhill et al.2006b).

PCR-DGGE fingerprint-based molecular technique is also used to investigate how the microbial symbionts are acquired by their hosts. For example, the bacterial community profiles of adults, larvae, and juvenile Caribbean demosponge *Ircinia felix* were compared by Schmitt et al.(2007), as a result, it was shown that in *I. felix*, vertical transmission of microorganisms through the larvae is an important mechanism for the establishment of the sponge-microbe association.

3.3. The Detection of Special Marine Microbial Symbionts

A highly integrated, morphologically diverse bacterial community is associated with the dorsal surface of *Alvinella pompejana*, a polychaetous annelid that inhabits active high-temperature deep-sea hydrothermal vent sites along the East Pacific Rise (EPR). PCR amplification of the community with spirochete-specific primers used in conjunction with DGGE analysis identified two spirochete phylotypes (Campbell et al.2001). *Candidatus Endoecteinascidia frumentensis*, was found specifically associated to *E. turbinata* from the Caribbean and has also been found to be associated with *E. turbinata* from the Mediterranean (Govind 2007).

In the study of Webster et al. (2008), DGGE detected multiple sequences that were exclusively present in diseased sponges. A Deltaproteobacteria sequence with high homology to a coral black band disease strain was detected in all sponge lesions and was absent from all healthy and unaffected regions of diseased sponges. Based on 16S rRNA gene sequence analysis, only the diseased sponges were found to contain sequences belonging to the Epsilonproteobacteria and Firmicutes, and there was a much greater number of Bacteroidetes sequences within the diseased sponges. In contrast, only the healthy sponges contained sequences corresponding to the cyanobacteria and the healthy sponges were dominated by Chloroflexi and Gammaproteobacteria sequences. PCR-denaturing gradient gel electrophoresis was used to detect the epiphyte community on marine macroalgae by Ohkubo et al. (2006). Two phylotypes of Acaryochloris, a chlorophyll d-containing cyanobacterium, were found not only on red macroalgae but also on green and brown macroalgae.

4. Concluding Remarks

Combined with PCR amplification of marker genes or their transcripts, PCR-DGGE fingerprinting can give a direct display of the predominant constituents in microbial assemblages with the advantage of easiness, reproducibility, reliability, and speed and is suitable to reveal the microbial community, especially to compare and monitor the temporal dynamics or spatial variation of the complex microbial community structure. But, there are some limitations of PCR-DGGE fingerprinting. For instance, the PCR-DGGE fingerprinting is based on nucleic acid extraction, so a major limitation is quantitative recovery of nucleic acids from environmental samples. Selectivity in PCR amplification of rRNA genes is another source of bias that can affect the results of PCR-DGGE fingerprinting. Small differences in the sequence of universally conserved regions may result in selective amplification of some sequences, particularly when primer annealing is at high stringency (Head et al.1998). Even so, at present, PCR-DGGE fingerprinting technique is still an efficient technique in microbial ecology.

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