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Testing of primers for the study of cyanobacterial molecular diversity by DGGE

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Abstract

Denaturing Gradient Gel electrophoresis (DGGE) is a PCR-based technique which is widely used in the study of microbial communities. Here, the use of the three specific 16S rRNA cyanobacterial specific primers CYA359F, CYA781R(a) and CYA781R(b) on the assessment of the molecular diversity of cyanobacterial communities is examined. Assignments of the reverse primers CYA781R(a) and CYA781R(b) with cyanobacterial strain sequences showed that the former preferentially targets filamentous cyanobacteria whereas the latter targets unicellular cyanobacteria. The influence of the GC clamp position on the forward or on reverse primer and the use of the two reverse primers separately or in equimolar mixture were investigated. Three environmental samples were subjected to amplification with 6 combinations of primers. The 6 banding patterns as well as the sequences of the bands extracted were analysed and compared. In addition, to assess the effect of the position of the GC clamp, the melting profiles of the sequences of *Aphanizomenon flos-aquae* PMC9707 and *Synechococcus* sp. MH305 were determined, with the GC clamp in the 3′ or 5′ position. Results showed that the use of two separate amplifications allowed a more complete study of the molecular diversity of the cyanobacterial community investigated. Furthermore, similar richness and identical phylogenetic assignments of extracted bands were obtained irrespective of the positioning of the GC clamp.

Keywords: 16S ribosomal RNA; Denaturating gradient gel electrophoresis; Cyanobacteria; Molecular diversity

1. Introduction

16S rRNA PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis) is one of the most frequently used technique to assess the genetic diversity of microbial communities (Muyzer, 1999; Ercolini, 2004). This method allows the separation of small DNA fragments (maximum size of 1000 bp) of the same length but of different sequence according to their melting properties (Nollau and Wagener, 1997). Indeed, fragments with only one single base substitution can be separated with this technique (Myers et al., 1985). Typically, so as to prevent a complete denaturation of the double-stranded fragments, a GC clamp is added to the 5′ end of the forward primer (Myers et al., 1985; Sheffield et al., 1989; Casamayor et al., 2000; Sekiguchi et al., 2001; Lyautey et al., 2005). However, the effect of the position of this GC clamp on the forward or reverse primer on the quality of the patterns obtained has not previously been investigated.

The numbers, positions and intensities of the DGGE bands obtained can be used to determine the diversity of natural samples (Muyzer, 1999; Garcia-Pichel et al., 2003; Lyautey et al., 2005). Several biases which may

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cause pitfalls in the interpretation of the DGGE patterns have been reported, like heteroduplex formation, amplification errors, the presence of multiple copies of 16S rRNA operons (Speksnijder et al., 2001; Wintzingerode et al., 1997), co-migration of different DNA fragments (Sekiguchi et al., 2001) and the presence of several melting domains in a DNA fragment (Kisand and Wikner, 2003). Nevertheless, some of these problems can be solved by the excision of the bands followed by reamplification and sequencing, as the identity of the organisms can be ascertained if the DNA fragment analysed is polymorphic.

Nübel et al. (1997) have designed the cyanobacterial primers CYA359F (forward), CYA781R(a) and CYA781R(b) (reverse) for specific amplification of a 379 bp 16S rRNA gene sequence. CYA781R(a) and CYA781R(b) differ by two polymorphic bases situated at positions 7 and 23 (5′ to 3′). These primers have the advantage of giving a PCR product which corresponds to variable regions V3 and V4, and contains significant information for phylogenetic assignments (Yu and Morrison, 2004). Indeed, they have been used unmodified (e.g. Casamayor et al., 2000; Garcia-Pichel et al., 2001; Abed et al., 2002; Geiß et al., 2004) or slightly adapted (Zwart et al., 2005) for numerous DGGE studies investigating cyanobacterial diversity in environmental samples.

In the present study, we aim to determine (1) the variation in the banding profiles caused by the position of the GC clamp on the forward or reverse primer, and (2) the combination of the primers designed by Nübel et al. (1997) which allows an optimum investigation of the diversity of the cyanobacterial community. To achieve this, we analysed and compared the DGGE fingerprints obtained with the six pairs of primers CYA359FGC-CYA781R(a), CYA359FGC-CYA781R(b), CYA359FGC-CYA781R(a+b), CYA359F-CYA781RGC(a), CYA359F-CYA781RGC(b), CYA359F-CYA781RGC(a+b), where GC means the GC clamp. In addition, as many as possible DGGE bands were sequenced to assess the diversity of the sequences obtained.

2. Materials and methods

2.1. In silico match of the reverse primers CYA781R(a) and CYA781R(b)

Using the Probe Search tool of the software package ARB (Ludwig et al., 2004) with an alignment of 1124 cyanobacterial strain sequences, the theoretical matches of the reverse primers CYA781R(a) and CYA781R(b) were investigated.

2.2. Sampling and DNA extraction

Environmental samples were collected at the Esch-sur-Sûre reservoir in North Luxembourg. The typical genera detected microscopically in this site during spring and summer were *Anabaena*, *Aphanizomenon* and *Planktothrix*. In August, populations of *Limnothrix* and *Snowella* have been reported. *Woronichinia* have been detected in autumn whereas no cyanobacteria have been observed during winter (Willame et al., MS in prep.).

Samples were collected by filtration of 500 ml water on 0.2 μm-pore-size filters (Supor, Pall Life Science, USA). The filters were stored in 2 ml lysis buffer (40 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris HCl pH 8.3) (Giovannoni et al., 1990) and immediately frozen at −20 °C. Three environmental samples were collected for the comparison of the primer pairs, 0ES27 (04/07/2000), 0ES32 (08/08/2000) and 0ES39 (26/09/2000). DNA was extracted from the filters as described by Wilmotte et al. (2002) with modifications as follows. For the enzymatic lysis step, a volume of 30 μl of proteinase K (20 mg ml^{-1}, MBI Fermentas, Lithuania) was used. Two milliliters of hot phenol (Sigma, USA)/chloroform/isoamylalcohol (Merck, Germany) (25:24:1) was added and incubated for 10 min at 56 °C. During the precipitation step, nucleic acids were precipitated from the supernatant (divided in several Eppendorf tubes) by addition of two volumes of ethanol and incubated for two hours at −20 °C. The tubes were centrifuged for 20 min at 16,000 g and after extraction, DNA was subjected to a purification step using the Prep-A-Gene DNA Purification Kit (Biorad, USA).

2.3. PCR reactions

As a direct amplification of the DNA with the Nübel primers gave a weak signal (data not shown), a semi-nested PCR reaction was performed so as to increase the sensitivity of the DGGE analysis. The 16S rRNA fragments were first amplified using the primers CYA359F and 23S30R (Taton et al., 2003). The second PCR reaction was performed with CYA359F and CYA781R(a) and/or (b), with the GC clamp on the forward or reverse primer. All the primers used are listed in Table 1 and were synthetized and purified by Gel PAGE (Polyacrylamide Gel Electrophoresis) at Eurogentec (Belgium). For the first PCR, 0.5 μl of the isolated DNA was added to 49.5 μl of the amplification mixture, giving rise to final concentrations of 1 X Super Taq Plus buffer, 1 mg ml^{-1} BSA (bovine serum albumin), 200 μM dNTP mix, 0.5 μM of each of the forward and reverse primers and