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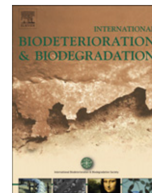

Effect of DNA polymerases on PCR-DGGE patterns

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PCR-based molecular biological techniques became fundamental in the analysis of microbial communities. One of the most popular techniques is PCR-DGGE wherein the outcome is greatly influenced by the DNA amplification process. A large number of different factors were described as key elements for successful amplification, however the potential effect of the DNA polymerase itself has not been analyzed in detail. In this study, three different DNA polymerases were tested in PCR-DGGE analysis of complex microbial communities. The wildly used *Taq* was compared to two highly processive and accurate DNA polymerases. According to the results, the utilization of different DNA polymerases indeed influences the produced fingerprints in PCR-DGGE analysis. KOD DNA polymerase presented the best performance, and was also found to have remarkable resistance against humic acids.

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1. Introduction

PCR-based fingerprinting methods (DGGE/TGGE, SSCP, T-RFLP, ARISA) brought a remarkable advancement in microbial community analysis, since traditional laboratory cultivation approaches capture only about 1% of the population diversity (Staley and Konopka, 1985; Amann et al., 1995; Torsvik et al., 1996). Polymerase chain reaction coupled with denaturing gradient gel electrophoresis (PCR-DGGE; Muyzer et al., 1993) has been used widely to determine the structure of microbial communities in many research fields, including bioremediation, biodeterioration, food analysis, clinical microbiology, forensics and molecular archeology (Ercolini, 2004; Lerner et al., 2006; Landy et al., 2008; He et al., 2008; Pandey et al., 2009; Cámara et al., 2011; Matussek et al., 2011).

PCR-DGGE analysis is a multi-step procedure containing extraction of total DNA from the sample, PCR amplification with specific primers, separation of amplicons based on sequence differences (DGGE), and analysis of obtained patterns. Weaknesses and important limitations of the technique are well known and have already been reviewed by several authors (Muyzer and Smalla, 1998; Muyzer, 1999; Nakatsu, 2007). The general opinion is that DNA preparation and the amplification steps have the greatest influence on final results, thus represent the sources of most bias.

Purity and quantity of template DNA was found to be crucial for efficient and reproducible PCR-DGGE analysis (Niemi et al., 2001). However, the efficiency of DNA extraction greatly depends on the sample, as well as on the chosen extraction procedure. Additionally, soil and environmental samples usually contain various levels of humic acids or other potential PCR inhibitors (Tebbe and Vahjen, 1993). Therefore, the quality of obtained DNA often needs further improvement, although the efforts to remove PCR inhibitors and produce high-quality template decreases the quantity of DNA, which can be finally reflected in the variation of the PCR-DGGE pattern. In consequence, soil and other environmental samples are considered as challenging targets of PCR-based fingerprinting analysis.

The outcome of PCR-DGGE is highly determined by the amplification circumstances (Lahr and Katz, 2009). Artificial products can arise during PCR due to the formation of heteroduplexes and chimeras, as well as by the introduction of point mutations and deletions (Wintzingerode et al., 1997; Speksnijder et al., 2001; Qiu et al., 2001; Kanagawa, 2003). The PCR bias to 1:1 (Suzuki and Giovannoni, 1996), the difference in primer binding energy (Polz and Cavanaugh, 1998) or primer mismatch (Hongoh et al., 2003) can cause differential or preferential amplification of certain rRNA genes by PCR which distorts the relative abundance of the members of the community. On the other hand, certain additives (e.g., BSA, DMSO, betaine, etc.) were reported to enhance amplification in the

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presence of inhibitors or GC-rich templates (Rees et al., 1993; Varadaraj and Skinner, 1994; Kreader, 1996).

Much of the above highlighted difficulties may be prevented using DNA polymerases having superior characteristics. High fidelity amplification could decrease the occurrence of introduced mutations, a processive DNA polymerase would perform better on low-amount or GC-rich templates, and an inhibitor resistant enzyme would be able to handle contaminated templates as well. In spite of the availability of such DNA polymerases, *Taq* DNA polymerase is utilized in the large majority of the publications on PCR-based community analysis and only a minority of the studies was performed with other enzymes. Out of the small group of other DNA polymerases applied for PCR-DGGE, Phusion and KOD were used most often, thus, in the present study we chose these enzymes to be compared with *Taq*. Phusion, a *Pyrococcus*-like enzyme is commercialized as the highest fidelity DNA polymerase available (with an error rate of 4.4×10^{-7}) possessing remarkable speed as well (www.finnzymes.com). The *Thermococcus kodakarensis* KOD DNA polymerase is also a high fidelity proofreading enzyme characterized with high processivity. It was successfully used for the amplification of various templates originating from contaminated environmental samples or GC-rich genomic DNA in our laboratory (Bihari et al., 2011; Papp et al., 2012). Similarly, Hoshino and Morimoto (2008) reported that KOD performed better than other polymerases with soil DNA templates in PCR in their preliminary experiments, although experimental results were not presented.

Considering the above details, important and basic questions were raised regarding the utilized DNA polymerases in PCR-DGGE. First, is there a difference in the produced patterns of DNA polymerases bearing varying abilities? Second, which is recommended for the analysis of “difficult” environmental samples and what are the advantages of its use?

To answer these questions, in the present work we aimed to test the potential advantages of highly accurate and processive DNA polymerases in PCR-DGGE. The performance and humic acid resistance of KOD and Phusion DNA polymerases were compared to the generally utilized *Taq* in the analysis of complex environmental soil samples.

2. Materials and methods

2.1. Preparation of DNA and PCR conditions

Total DNA was prepared from a hydrocarbon (soil 1), a heavy metal polluted (soil 2) and a pristine soil sample (soil 3) originating from three Hungarian bioremediation sites (see soil textures in Table S1). The isolation of DNA was performed with the PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc.) according to the manufacturer's suggestions. DNA quantity was measured with a NanoDrop ND-1000 spectrophotometer.

The three DNA polymerases compared were: *Taq* (Fermentas EP0402; with KCl buffer) KOD Hot Start (Novagen; 71086) and Phusion (Finnzymes F-530S; with HF buffer). PCRs were performed on a PTC 200 thermalcycler PCR System (MJ Research), the PCR programs are summarized on Table 1. Annealing temperature for Phusion was calculated with the modified Breslauer's method (Breslauer et al., 1986) by Finnzymes' Tm calculator (http://www.finnzymes.com/tm_determination.html).

The 16S rDNA fragments were amplified using the eubacteria-specific primer pair (5′-AGAGTTTGATCMTGGCTCAG-3′ and 5′-AAGGAGGTGATCCANCCRCA-3′) described by Suzuki and Giovannoni (1996). 5 ng of the isolated DNA was used as template in each 30 μl PCR reaction. The set of PCRs always included negative and positive controls. The PCR products (2 μl) were separated by agarose gel electrophoresis (1%) and detected with ethidium bromide staining.

The amplification of V3 region of the 16S rDNA gene and the attachment of GC-clamp was carried out using the primers 5′-ATTACCGCGGCTGCTGG-3′ and 5′- CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGGCCTACGGGAGGC, as described by Muyzer et al. (1993). The V3 specific PCR was performed in 50 μl total volume; 2 μl was analyzed by electrophoresis on 2% agarose gel prior to DGGE. All PCR reactions were performed at least in triplicate.

In our experiments two different amplification mixtures were tested, as circumstances of the amplifications differ for the three DNA polymerases, such as buffer components, primer concentration and enzyme unit applied. In the first set of amplifications all components were set to the optimum according to the manufacturer's recommendations. Second, since KOD's reaction buffer contains the least amount of primers and enzyme, the other two reactions were adjusted corresponding to that of KOD's, so as to test the performances of DNA polymerases between circumstances as similar as possible. Therefore these reactions contained 0.02 U μl^{-1} of the appropriate DNA polymerase and 0.3 μM of each primer.

2.2. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using the DCode Universal Mutation Detection System (Bio-Rad Laboratories) as described in the instructions of the manufacturer. Polyacrylamide gels (8% of a 37:1 acrylamide–bisacrylamide mixture in 1 × TAE buffer), with a gradient of 30–70% denaturant, were made with a gradient maker (Bio-Rad Laboratories). 100% denaturing acrylamide was defined as 7 M Urea and 40% formamide. Equal volumes of PCR reactions (25 μl) were loaded on gels, and run for 4 h at 150 V in 0.5 × TAE buffer at a constant temperature of 60 °C. Gels were stained with ethidium bromide (0.2 μg ml^{-1} in 1 × TAE) for 20 min. DGGE separation of different PCR reactions was performed in several replicates to check reproducibility.

2.3. Detection and statistical analysis of DGGE patterns

The DGGE patterns were digitized with a CCD camera and the VisionWorks®LS Image Acquisition and Analysis Software (UVP). The digital images were then analyzed by the Phoretix 1D Pro gel analysis software (TotalLab): numbers of bands per lane were

Table 1
Summary of the utilized PCR conditions.

Gene	DNA polymerase	1 cycle	30 cycles			1 cycle
		Initial denaturation (°C/s)	Denaturation (°C/s)	Anneling (°C/s)	Extension (°C/s)	Final extension (°C/s)
16S rRNA gene	*Taq* (Fermentas)	94 °C/60	94 °C/60	55 °C/60	72 °C/60	72 °C/300
	KOD (Novagen)	95 °C/120	95 °C/20	55 °C/10	70 °C/40	70 °C/300
	Phusion (Finnzymes)	98 °C/30	98 °C/10	60 °C/30	72 °C/45	72 °C/300
V3 region	*Taq* (Fermentas)	94 °C/60	94 °C/30	55 °C/30	72 °C/30	72 °C/300
	KOD (Novagen)	95 °C/120	95 °C/20	55 °C/10	70 °C/10	70 °C/300
	Phusion (Finnzymes)	98 °C/30	98 °C/10	60 °C/30	72 °C/30	72 °C/300

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