ADAPTATION OF MICROBIAL FINGERPRINT METHOD FOR ANALYSING OF ENVIRONMENTAL SAMPLES

PH.D. THESIS

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SZEGED
2018

INTRODUCTION AND OBJECTIVES

One of the great challenges of the 21st century is to improve the state of the environment. Initially remediation programs were focused only on the assessment of polluting sources that threaten soil and groundwater. Later the key position of soil as a habitat and as the site of biogeochemical cycles was recognised, this led to the research and development of soil management methods that are efficient, cost-effective while at the same time environmentally friendly and sustainable. With the advancement of remediation technologies, biotechnology has also undergone major development. In the first two thirds of the last century, traditional microbial culture techniques were used for the isolation of degrading microbes. Researchers around the '90s were first faced with the problem that not all soil bacteria are culturable. The large number of unculturable prokaryotes compelled researchers to develop and utilize non-culture-based molecular biological techniques.

Today, PCR-based techniques are inevitable in the determination of microbial compositions, as it provides the opportunity to obtain information about microorganisms and their properties that cannot be studied with classical microbiology and culture techniques. However, the obtained result depends to a great extent on sampling, sample preparation and the measurement method. It is particularly important to consider this in procedures where the result obtained during the experiment is determined by several successive steps. For instance PCR-DGGE assay used for the determination of microbial compositions is a multi-step method. The DGGE pattern is fundamentally influenced by the method of DNA isolation (efficiency of cell disruption, purity and amount of the DNA sample, etc.).

For this reason we would like to answer following questions:

1. *Which kind of DNA extraction gives the best diversity in PCR-DGGE pattern?*

For this reason, in the first part of my research, the extent to which the method used for DNA isolation in soil samples influenced the eubacteria-specific DGGE pattern was investigated.

In the course of our experiments, the effect of a stronger chemical lysis for cell disruption on the obtained DGGE pattern was compared to the most frequently used kit (Mo Bio Power Soil Kit) to determine if there is a detectable difference in between the dominant bacterial strains of the examined soil sample.

2. *Whether polymerase can influence on PCR-DGGE pattern and on the detected microbial diversity?*

In the second part of my research, the effect of modifying the amplification conditions in PCR-DGGE assays was investigated, particularly the effect of the DNA polymerase enzyme

used for amplification on DGGE pattern. Since there is little evidence in the literature for differences in the pattern depending on the different amplification ability of the enzymes, the comparison was based on the most commonly used *Taq* DNA polymerase, *Phusion* DNA polymerase that has high accuracy and is tolerant of various inhibitors, and *KOD* polymerase, a lesser known and used enzyme that is capable of copying large C/G rich templates.

3. *How does affect PCR inhibitors on DNS polymerases?*
4. *Which kind of DNA polymerase can give the best PCR-DGGE's pattern of environmental samples?*
5. *How does affect PCR inhibitors on PCR-DGGE pattern?*

We investigated differences in amplification efficacy of DNA polymerases using multi-template which important during analysis of microbial diversity. Experiments on the synthetic template were suitable to investigate the effect of the inhibitory components on the DGGE pattern.

EXPERIMENTAL

For DNA isolation from soil samples, two commercially available DNA isolation kits -Mo Bio PowerSoil® DNA Isolation Kit and AquaGenomic™ solution- were used, according to the manufacturer's instructions, and a method developed for mycobacterial DNA isolation was also used. Methods were modified by using sterile water for DNA elution in the last step of the isolation to avoid the effect of different buffers.

Three DNA polymerase enzymes were used in the experiments. The 16S ribosomal RNA coding gene was PCR amplified by eubacteria specific primers (EubB (27F) and EubA (1552R)). For the DGGE patterns the V3 variable region (~ 200 bp length) of the 16S RNA gene with "GC-clamp" was amplified, 16S rDNA PCR products were used as templates, 341F-GC and 534R primers were used for the amplification. In the reactions KOD (*Novagen*) and Taq (*Fermentas*) and Phusion (*Finnzyme*) DNA polymerases were used according to the manufacturer's instructions or with minor modifications (with identical primer and enzyme concentrations). The 1.5 kb 16S rRNA gene was verified by running the PCR product on 1% agarose gel, and the 200 bp 16S rRNA gene V3 gene by using a 2% agarose gel.

Denaturing gradient gel electrophoresis was performed on 8% polyacrylamide gel with a denaturing gradient of 30-70% and of 30-60%. The gel was run in a BioRad DCode Universal Mutation Detection System at 60 °C, 150 V, in 1x TAE buffer for 4 hours. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/mL) for 20 minutes and then irradiated with UV light and documented with VisionWorks® LS 5.5.0 software.

For the preparation of the synthetic consortium, Mo Bio PowerSoil® DNA Isolation Kit was used to isolate DNA from reference bacterial cultures. 16S rDNA PCR from purified DNA was performed. The remaining template DNA and oligonucleotide were separated from PCR products using an EZ-Spin Column PCR Products Purification Kit. Individual fragments were ligated into a pJET 2.1 blunt cloning vector.

The reaction mixture was incubated for 20 minutes at room temperature (22 °C) and directly transformed into chemically competent *Escherichia coli* DH5α cells: 200 μL of recently thawed competent cells were added to 10 μL of ligation reaction mixture and incubated for 30 minutes on ice followed by a 40 seconds long heat shock at 42 °C and after 5 minutes on ice, 800 μl SOC solution (2% tripton, 0.5% yeast extract, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose, 10 mM NaCl, 2.5 mM KCl) was added, and the mixture was incubated for 30

minutes at 37 °C. The cells were then plated onto LB medium containing 100 μg/mL ampicillin. The plasmid from cells that grew on the antibiotic containing media was purified. Pure plasmids containing the specific sequences were mixed in the same ratios. The plasmids were stored in *E. coli* competent cells in a strain collection at -80 °C.

PCR inhibition assays were performed with humic acid, soil extract, blood and blood serum. To prepare a soil extract, 1 g of potting soil was suspended in 10 mL of KLA buffer (50 mM Tris, pH 9.5, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$ and 0.1% Tween) and incubated for 30 min at 75 °C, centrifuged at 12,000 g for 20 minutes, and the supernatant was stored at -20 °C until use. The whole blood was untreated blood containing 5 mM EDTA (anticoagulant). For blood serum, the blood was left at room temperature (22 °C) for 30 minutes, after coagulation it was centrifuged at 2000 g for 10 minutes and the supernatant (blood serum) was separated.

The inhibitory sensitivity of heat-stable DNA polymerases was examined using 16S rRNA gene-specific PCR. The PCR reaction mixture contained 5 ng genomic DNA templates isolated from the soil samples and the reaction was performed using humic acid concentrations, in a range of 0 to 0.6 μg/mL. For the inhibition experiments with known bacterial strains, 1:1 plasmid mixture was prepared from plasmids containing 16S rRNA genes of the synthetic consortium. From the plasmid mixture, the 16S rRNA gene was amplified with the 27F/Eub-1552R primers and with the cloning vector (pJET1.2/blunt) primers. The inhibitory concentration was determined from the reaction inhibition of the two PCRs. To investigate the effect of inhibitors on the DGGE pattern, the amplicons were examined on the 8% polyacrylamide gel with a 30-60% denaturing gradient.

The preparation of DNA oligonucleotides for molecular work was carried out by Integrated DNA Technologies, DNA sequencing by Microsynth, the Sequencing Platform of the Hungarian Academy of Sciences Biological Research Center and Xenovea Ltd.

NOVEL SCIENTIFIC RESULTS

T1. The method developed for mycobacterial DNA isolation can be efficiently used to examine the bacterial population of soil samples.

1.1. Based on PCR-DGGE patterns by Käser method (for mycobacterial DNA isolation), from soil samples a greater bacterial diversity can be detected compared to the commercially available DNA purification kits for soil samples, particularly in case of G/C rich templates.

1.2. Metagenomic sequencing results have confirmed that the Käser method is more suitable for extracting *Actinobacterium* DNA from the soil samples.

T2. We proved the benefit of using *KOD* DNA polymerase in a PCR-DGGE assays.

2.1. The KOD DNA polymerase enzyme is able to amplify G/C rich templates, which are difficult to amplify, more efficiently than *Taq* and *Phusion* DNA polymerase.

2.2. *KOD* DNA polymerase gives a sharper pattern due to high fidelity compared to *Taq* and *Phusion* polymerisation, which fundamentally determines the software evaluation of the DGGE pattern.

2.3. We have confirmed that in the PCR-DGGE assays the DNA polymerase enzyme used for amplification has an influence on the resulting pattern.

T3. We developed a method for preparing a DGGE marker containing known sequences

3.1. Using plasmids containing 16S rRNA genes, we were able to successfully generate a PCR-DGGE marker with a desired sequence composition.

3.2. We have developed and applied a methodological procedure that allows selection of arbitrary sequences to allow PCR-DGGE to be used as a quick quality control method.

T4. We were the first to prove that *KOD* DNA polymerase was highly resistant to the presence of humic acid during PCR.

4.1. Based on our results, in amplification from genomic DNA, *KOD* DNA polymerase is able to amplify the desired amplicon with 16 times higher (5 μg/mL) humic acid concentration compared to *Phusion* (~ 0.3 μg/mL) and *Taq* (<0.2 μg/mL) DNA polymerases.

T5. *KOD* DNA polymerase is less sensitive to soil-derived inhibitor components.

5.1. Endpoint PCR experiments with synthetic consortiums have confirmed that *KOD* tolerates the presence of humic acid at an order of magnitude greater than that of *Phusion* polymerase.

5.2. Inhibition experiments confirmed that *KOD* is highly resistant to the presence of soil-derived inhibitors, even in the presence of 10% soil extract.

T6. We were the first to prove that the PCR-DGGE pattern was influenced by the presence of PCR inhibitors.

6.1. In the presence of soil and blood PCR inhibitors, the PCR-DGGE pattern changes due to partial amplification. Based on the results, it can be stated that although the *Phusion* DNA polymerase enzyme is able to tolerate blood inhibitor components at a higher concentration (up to 20 v/v %) based on endpoint PCR results, however, multi-template amplification products do not return the original population composition.

6.2. Based on our results, the inhibitor sensitivity of the DNA polymerase is insufficient to determine whether an enzyme is suitable for the examination the microbial composition of inhibitor-containing DNA samples. In experiments with the three polymerase enzymes and four PCR inhibitors, the amount of PCR inhibitor in the DGGE pattern produced by the synthetic consortium did not decrease the intensity of the individual bands in the pattern with correlation to the inhibitor. The presence of inhibitors resulted in a template preference, thus, amplification of some sequences was inhibited to a greater extent.

PUBLICATIONS

1. **Balázs M**, Rónavári A, Rutkai E, Kiss I, Szvetnik A **Investigation of effects of different DNA Polymerases on PCR-DGGE patterns in an artificial microbial consortium** under review in *International Biodeterioration and Biodegradation*-ben (2018)

2. **Balázs M,** Rónavári A, Németh A, Bihari Z, Rutkai E, Bartos P, Kiss I, Szvetnik A **Effect of DNA polymerases on PCR-DGGE patterns** *International Biodeterioration & Biodegradation* 2013, 84, pp. 244–249

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3. Rónavári A., **Balázs M.**, Tolmacsov P., Molnár Cs., Kiss I, Kukovecz A., Kónya Z. **Impact of the morphology and reactivity of nanoscale zero-valent iron (NZVI) on dechlorinating bacteria**. *Water Research*, 2016, 95:165-173

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2. **Effect of DNA polymerases on DGGE patterns.**
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SCIENTOMETRIC DATA

Peer-reviewed papers total:	9
Cumulative impact factor:	17,194
Independent citations total:	90