Study on the Effective Method of Optimizing PCR-DGGE by adding PCR enhancers

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Abstract. PCR-based denaturing gradient gel electronic (PCR-DGGE) has become a frequently used method in the determination of bacteria community in water, soil and other habitats. However, the precision and the integrality of microbiology community by this approach should be reinforced and the key for this is optimization on PCR for which is the basis of PCR-DGGE. Aiming at this, PCR enhancer combinations were applied in both two amplification rounds of PCR-DGGE in this study. The results showed that many new bands were produced and originally weak bands were intensified after the addition of PCR enhancer combinations, particularly DBD-2 (1% DMSO, 0.4 M Betaine, 1mM DTT) in PCR amplification buffer system. Virtually all newly appeared bands in DGGE were probably derived from the bacteria with high GC% content (60%). Thus the major optimization on PCR-DGGE was PCR enhancer may contribute to decreasing annealing temperature and improving the PCR product yields of the high-GC%-contented bacteria. In conclusion, PCR enhancers could remarkably improve the amplification efficacy, particularly for using the complex and tiny environmental DNA or weaker bands from DGGE as PCR template, and furthermore increase the detected bacterial species in PCR-DGGE. Therefore we strongly proposed that the PCR enhancer combination be routinely applied in PCR-DGGE for the bacterial community determination.

Keywords: Denatured gradient gel electronic; microbial diversity; aerobic anoxygenic phototrophic bacteria; 16S rDNA; pufM.

1. Introduction
In recent years, whole-community fingerprinting approaches, especially DGGE (denaturing gradient gel electronic) have been used to study complex bacterial communities and to estimate the diversity and relative representation of individual bacterial taxonomic units within the total detectable bacterial communities [1, 2]. DGGE appeared to be one of the best molecular community fingerprinting techniques in terms of predicting the actual Shannon-Wiener diversity index, richness, and evenness [3,
4]. Additionally, DGGE supports the identification of community members because the amplification products can be recovered and sequenced [4, 5]. This may explain why DGGE has become the most frequently used method of molecular community fingerprinting. The benefit of this approach is that a molecular fingerprint of the community structure is generated for each soil, water and other habitats [2, 6], and its effect is dependent on the quantity and quality of PCR products [7]. Due to the high Tm demand of DGGE, usually an GC-clamp added at the 5’ end of the primers, which has been a reason to restrict the PCR. Improvements of the PCR conditions can be achieved by modifying the classical reaction conditions, for example, by performing “touchdown” PCR, consisting of a stepwise reduction of the annealing temperature for each cycle [8], or by the use of modified DNA polymerases for carrying out “hot-start” reactions [9]. The Nest-PCR has been widely used for the DGGE [7] and a lot of new primers have been designed to enhance the effect of PCR [10, 11]. Also some research tried cDNA to optimize the DGGE and find more species of microbes. However, there existed a common theme that degraded the approach of PCR-DGGE and was overlooked for a much longer time: many weak bands in DGGE could not be successfully re-PCR-amplified and sequenced and the represented bacterial species by them were escaped away [12]. PCR-DGGE was the method basing on PCR and therefore overcoming amplification problems in an efficient and economic way was extremely significant. PCR enhancers may be served for this, which functioned in enhancing the specificity and/or the yield of the PCR. The most prominent PCR enhancing additives that are currently used are either betaine [13], small sulfoxides like dimethyl sulfoxide (DMSO) [14], small amides like formamide [15] or reducing compounds like β-mercaptoethanol or dithiothreitol (DTT) [8]. There were some research indicate some kind of enhancers to promote the efficiency of PCR, however, the study of the enhancer applied to PCR-DGGE is still in absence. Other paragraphs are indented (BodytextIndented style).

As well known, some gene has been widely used as molecular marker for taxonomic classification and diversity assay, such as 16S rDNA for prokaryotic microorganisms [16] and the gene puFM for aerobic anoxygenic phototrophic bacteria (AAPB), which is responsible for encoding the M-subunit of the photosynthetic reaction center [11, 17]. This study analyzed and compared the community structure and diversity of bacteria or AAPB in soil and water using PCR-DGGE between with and without the addition of PCR enhancers, aiming to find out an efficient and economic pathway for improving the precision and integrality of community structure analysis by PCR-DGGE.

2. Methods and Materials

2.1. Sample and Genomic extraction

The environmental microbial genomic DNA was extracted from surface soil of biological soil crust (sampled from the Desert Hunsandaco, North China) and surface water (sampled from the Lake Ulansuhai, North-western China), respectively.

Total environmental DNA extraction of water sample was as followed by the description of Boström et al (2004): filtered 200 ml water sample onto 0.22 μm membrane (Millipore), then crushed the membrane, gathered into the 10 -ml tubes; after Freeze-thaw twice, lysozyme treatment with 1.5 ml of lysis buffer (200 mM Tris-HCl, pH 8.0; 1.5 mg lysozyme; 125 mM EDTA), incubated at 37 ℃ for 30 min; after addition of 0.25 ml of proteinase K lysis buffer (to a final concentration of 1.25 % w/v SDS, 300 mg proteinase K, 70 mM NaCl), the tubes were incubated at 37 ℃ for 15 min followed by another 60 min at 55 ℃ with regular rotation of the tubes; subsequently, the lysis mix was transferred to 1.5 ml microcentrifuge tubes and extracted twice with one volume of chloroform/isopropanol (24:1); the DNA was precipitated (1 vol isopropanol; 0.4 vol 7.5 M ammonium acetate), and the pellet was washed with 70 % (v/v) ethanol; the DNA pellet was resuspended in sterile, double-distilled water.

Soil sample Genomic was extracted by the method as describe by Miller. Firstly, soil sample was freeze-thawed, treated with lysozyme (1.5 ml of lysis buffer with the composition of 200 mM Tris-HCl, pH 8.0; 1.5 mg lysozyme; 125 mM EDTA), incubated at 37 ℃ for 90 min; then added 0.25 ml of proteinase K lysis buffer (to a final concentration of 20 % SDS-phenol, 300 mg proteinase K, 70 mM NaCl), incubated at 65 ℃ for 60 min. subsequently, mixed with ice-cold ethanol and 20 mg of glycogen,
centrifuged. The mixture was then allowed to stand overnight at −20 °C. The pellets were washed with 200 ml of ice-cold 70 % (vol/vol) ethanol, air dried, and resuspended in 50 ml of sterile distilled water. The extracted DNA was ran on 0.8 % agarose gel and stored at −20 °C before use.

2.2. PCR of 16S rRNA and pufM genes

Primers used in this study were as shown in Table 1. PCR amplifications of partial 16S rDNA genes were performed as described by Schäfer [18]. Cycling conditions were as follows: initial denaturation step at 95 °C for 5 min, then denaturation step at 95 °C for 1 min, annealing step was started at 65 °C for 1 min and declined in 0.5 °C steps for each cycle until a temperature of 55 °C was reached, the elongation step at 72 °C for 1 min. Subsequently, 10 additional cycles were performed with a constant annealing temperature of 55 °C. Each 50 μL PCR reaction mix contained 5 μL PCR buffer, 4 μL dNTP mixture (2.5 mM each), 50 pmol of each primer, 50–100 ng template DNA, and 1 unit Easy-Taq DNA polymerase (Transgene, China).

The amplifications of pufM gene were performed with the cycling conditions were as follows [11]: an initial 4-min denaturation step at 94 °C, followed by a three-step cycle consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 48 °C and 1 min of extension at 68 °C. The cycle was repeated 35 times and the reaction concluded with a 10-min extension at 72 °C. The reaction mixture contained 5 μL PCR buffer, 5 μL dNTP mixture (2.5 mM each), 60 pmol of each primer, 50–100 ng template DNA, and 1.5 unit Easy-Taq DNA polymerase (Transgene, China) in a total volume of 50 μl.

Optimization experiments were carried out by the adding PCR enhancer to the reaction buffer, including PCR enhancer combination of either None (no any enhancer added), DBD-1(2% DMSO, 0.8 M Betaine and 2 mM DTT), DBD-2 (1% DMSO, 0.4 M Betaine, 1 mM DTT), DB-1(5% DMSO, 1.25 M Betaine), or DB-2 (2% DMSO, 0.5 M Betaine) referred from the description of Ralser and Sahdev.

Table 1. Primers used in this study.

| PRIMERS | SEQUENCE | GC% |
|---------|----------|-----|
| GC-F341 | GCACGGGCGGCCTACGGGAGGCAGCAG | 68% |
| F341    | GCACGGGCGGCGCCGGGCGGGGGCGGGG | 69% |
| R907    | GGTAATCAATTCCTTTGAGT | 37% |
| UNIF    | GGTAATCAATTCCTTTGAGT | 24% |
| GC-WAW  | GCCGCCGCCGCGGCGGGGGGGGGGGGGGGGGGGGGGGGGGGG | 83% |
| WAW     | AYNGCRAACCACCGGCCCA | 60% |

2.3. Denaturing gradient gel electrophoresis (DGGE)

Approximately 300 ng of PCR amplified products of 16s rDNA and pufM from each sample were separated by DGGE using a denaturing gradient of 45 %–60 % (for 16s rDNA) and 30 %–60 % (for pufM) as described previously by Sanchez et al. (2007) and Cottrell et al. 2010, respectively. Electrophoresis was performed at 60 °C for 20 h at 100 V using a DCode DGGE apparatus (Bio-Rad, Hercules, CA, USA). DGGE gels were stained with silver nitrate solution (0.2%) for 15 mins [19]. Images of the DGG-E gels were recorded and analyzed using Kodak (Rochester, NY, USA) Gel Logic 100 electrophoresis documentation.

2.4. Band isolation, clone and sequence analysis

Individual special bands from DGGE gels of both 16S rDNA and pufM genes were carefully excised using sterile razor blades, placed in 1.5-ml micro centrifuge tubes containing 40 μl of 1×Tris-EDTA buffer, and stored for 24 h at 4 °C to elute the DNA out of the gels. A volume of 1 μl of the elution from the DGGE band was used for re amplifying with non-GC-clamped primers following by the same PCR
programs described above. Then cloned the sequence and sequenced in BGI company (Beijing, China). The source organism of the 16S rDNA sequence was classified by the CLASSIFIER of RDP [20]. Similarity of the obtained 16S rDNA and pufM gene sequences were compared to those stored in GenBank using the basic local alignment search tools, BLASTn and BLASTx [21], respectively. Phylogenetic analysis was performed in the method of Neighbor-joining through the software of MEGA (Ver. 5.1) based on Jukes-Cantor model [22].

3. Results and discussion

3.1. Amplification of 16S rDNA and pufM

The amplification in PCR-DGGE was never an easy case, in which the primer with GC-clamp was used, leaded to high (G+C) content appeared in sequence, weak band was hardly recovered enough DNA as the template in the regular PCR and resulted in the failure of sequencing the band sequence. For overcoming these limitations, in the amplification of 16S rDNA and pufM gene the combinations of PCR enhancers were added into reaction buffer in this study, along with using classic touch-down PCR of one optimization condition. PCR products obtained with the GC-clamped primer (first round PCR) were electrophoresised in agarose gel. As show in Fig.1.

The results showed that the combination of DBD-2 highest promoted the yields of PCR products of both 16S rDNA and pufM from soil or water. DBD-1 obtained a similar promoting effect on PCR amplification to that of DBD-2 with the exception of no evident effect on the amplification 16S rDNA from soil sample, of which the reason may be the high concentration of the PCR enhancers would refrain the reaction. Other combinations or one single of PCR enhancer had less improvement or even no PCR products observed on the gel. For a particular GC-rich sequence, both DMSO and betaine additive could greatly improve target product specificity and yield during PCR amplification [23, 24]. But for the more complex community structure analysis, our results showed that another PCR enhancer DTT should be added beside DMSO and betaine and proper concentration should also be considered, especially for the amplification of 16S rDNA from soil sample. Lots of high GC fragment could not be correctly amplified, either because the PCR products were unspecific or the poor yield of the amplicons [23]. No matter the composition of the target sequence itself, GC-clamp added to the primer in PCR-DGGE would no doubly increase the GC content in PCR products and therefore difficulted the PCR amplification. Utilization of the PCR enhancers may be helpful in decreasing the formation of the secondary structure and thereby reducing the melting temperature of both primers and templates [23, 24, 25]. Moreover, some inhibitory contaminant may be neutralized, which presented in PCR reaction buffer or the DNA template solution [8]. However presently, much less information was known about the PCR enhancer in improving the bacterial community structure or diversity analysis. In PCR amplification for community structure analysis, various template sequences existed and the cases, which made the amplification more difficult, may happen more often, like incorrectly paired and varied melting temperature. The results of this study revealed that the PCR enhancer mix could effectively enhance the efficacy of PCR amplification of all bacteria (16S rDNA) or a particular group of AAmpB (pufM) with GC-clamped primers.
3.2. **Denaturing gradient gel electrophoresis (DGGE)**

Excluded no obvious PCR products were seen in the agarose gel, other products were run in DGGE and silver staining was used for which had higher sensitivity, and the gel could be preserve for a longer time than other nucleic acid dye staining. The DGGE fingerprint of 16S rDNA and pufM genes clearly indicated the variations between samples with and without enhancer used. As show in Fig.2.

**Figure 1.** Agarose gel electrophoresis of the first round PCR products of 16S rDNA and pufM genes. Top symbols represent the PCR enhancer combinations, NONE (no any enhancer added), DBD-1(2% DMSO, 0.8 M Betaine and 2 mM DTT), DBD-2 (1% DMSO, 0.4 M Betaine, 1mM DTT), DB-1(5%DMSO, 1.25M Betaine), or DB-2 (2% DMSO, 0.5 M Betaine). Left symbols indicate amplified genes and their originated samples with S and W representing the water and biological soil crusts samples, respectively.

**Figure 2.** DGGE analysis of bacterial 16S rDNA (A) and pufM(B) from the samples. The signs of lanes are of the PCR enhancer combinations, NONE (no any enhancer added), DBD-1(2% DMSO, 0.8 M Betaine and 2 mM DTT), DBD-2 (1% DMSO, 0.4 M Betaine, 1mM DTT), DB-1(5%DMSO, 1.25M Betaine), or DB-2 (2% DMSO, 0.5 M Betaine). A and B show the fingerprint of 16S rDNA gene and pufM gene respectively, and the band numbers are indicated below the gel.
More band numbers were detected and thicker intensity of the corresponding band was observed after PCR enhancers especially DBD-2 used. Compared with no enhancer additive, the band number of 16s rDNA has increased from 19 to 20 (water sample) and 14 to 20 (soil sample) respectively. The band richness of pufM has increased from 5 to 7 (water sample) and 6 to 7 (soil sample). DBD-2 increased additional four bands and one band of 16S rDNA, respectively from soil and water (Fig. 2A), which meant PCR enhancer would improve the efficacy up to 24% in the band number. In the analysis of AAPB diversity of water and soil with DGGE, the increased band number was of 1 to 2, in which DB-2, DBD-2 and DBD-1 produced similar DGGE fingerprint profiles. Combined Fig.1 and Fig. 2, it’s easy to see that higher yield of products from first round PCR with GC-clamped primers would produce more band numbers and more thickened band intensity in DGGE fingerprint profile. Generally the microbial community of soil was more complex than that of water and so the whole bacteria than one particular group like AAPB. Also it’s common that more inhibitory compounds may be included in environmental genomic DNA extracted from soil than that from water. Microbial population number and diversity of biological soil crusts in desert were relatively lower than those of other types of soil, such as sludge [26, 27]. Beside soil sample of biological soil crusts, we also checked the improving effect of PCR enhancer used in the surface sludge of Lake Ulansuhai, one hypereutrophic freshwater lake with sludge up to 1 m in depth, in which bacterial richness was high and the concentration of complex organic compounds was heavy [28]. And better improvement effect (band numbers increased from 7 to 16) was achieved with the utilization of PCR enhancer combination of DBD-2. As show in Supplementary Fig. 1.

For highlighting the improvement was not only confined in the use of a certain polymerase EasyTaq, a very cheap one made in China, three more expensive and widely used commercial polymerase Taq, namely rTaq (TakaRa, Japan), LA Taq (TakaRa, Japan) and Taq DNA Pol (Fermentas, US), were also tried in this study and very similar results were produced as Fig. 1 and Fig. 2 (DGGE profile shown in Supplementary Fig. 1).

### 3.3. Reamplification of excised bands, clone and sequence

Attempts to reamplify and sequence the excised DGGE bands would shed some light on the nature of community DGGE patterns. However there was a problem existed in reamplification that was hardly to extracting weak bands and re-PCR successfully [12, 29]. To understand more for additional appear-ed bands with PCR enhancers used, we excised these bands, reamplified and sequenced. There were 9 bands, 5 for 16S rDNA (Band 1 to Band 5) and 4 for pufM bands (Band 6 to Band 9), were respectively cloned, sequenced, further analyzed with the online tools of RDP and/or BLAST. Band 4 was obviously weaker band than other selected bands. The application with PCR enhancer combination of DBD-2 and increased diluted template solution, heavy band of PCR products was produced and succe-essfully sequenced; yet with no optimization and dilution, it failed to see any PCR products on agarose gel (Fig. 2). But as to the newly increased band on DGGE, a possible cause was the formation of heteroduplex [30]. However, the chimera analysis showed that all sequenced additional bands in this study were not heteroduplexed and represented the single-gened variants.

According to the phylogenetic affiliations of bands, the added bands after PCR enhancers applied were all derived from Proteobacteria. Band 1 related to Rhodobacter sp. TSE4, an unauthoritatively identified bacterium, with a homology of 98% by BLASTn, but it was assigned to Catellibacterium another member of Rhodobacter-clad so classified the cause was the result of RDP classifier and its similarity analysis between type strains. Band 2 belonged to a more familiar genus Escherichia. Band 3, Band 4 was highest related to DGGE band HelMes_D40, which is partial sequence of uncultured marine bacterium [31] and divided in Seohaeicola, which was isolated from Yellow Sea [32]. Band 5 shared a highest sequence similarity with a cultured species Hydrogenophaga defluvii (99%) and so belonged to the genus Hydrogenophaga. Band 2,3 respectively belonged to uncultured gamma proteob -obacterium and planctomycete. Based on the similarity analysis of pufM by BLASTx, both Band 6 and Band 8 were originated from the members of Acidiphilium, which was acidophilus and strictly aerobic photosynthetic bacteria”. Band 7 and band 9 were possibly derived from Rosebacter of α-subclass and Roseateles of subclass proteobacteria, which were important groups of aerobic anoxygenic
photosynthetic bacteria in aquatic environments [33]. Most of the related bacteria were found widely distributed and abundant in nature environments. Although the GC contents of the obtained band sequences from this study were within the range of 51% to 57%, those of genomic DNA of their highly related microbes were mostly high as to 63%-71%, just with only one exception of lower GC content (55%) of Escherichia. Thus we suggested that the improvements of PCR enhancer in PCR-DGGE are accredited majorly to easy the PCR amplification using the genomic DNA comprised high GC content as template. As shown in Table 2 and Supplementary Fig. 2.

Table 2. Phylogenetic affiliations of the sequenced additional DGGE bands with PCR enhancers used.

| Band sequence | Possible affiliated taxonomic genus and their genomic GC content | Blast Hitsa / Accession no. / Similarity |
|---------------|-------------------------------------------------------------|------------------------------------------|
| Band 1        | Rhodobacter 64-69(55) clone SLE32H / GU390191 / 99%         | Rhodobacter sp. TSE4 / HM156123.1 / 98%  |
|               | clone 16saw46-1b02.p1ka / HE582445 / 99%                   | Escherichia coli strain DP 1001 / JF828029 / 100% |
| Band 2        | Escherichia 50-53(55) clone TG105 / JX186578 / 100%        | Burkholderia fungorum strain Uz1101 / JX133206 / 100% |
| Band 3        | Burkholderia 62-69(54) DGGE band HelMes_D40 / FM210397 / 91% | Seohicola saemankumensis strain SD-15 / NR_044437 / 91% |
| Band 4        | Seohaeicola 63 (53) clone C09-10 / FJ844077 / 99%          | Hydrogenophaga defluvii / AM942546 / 99% |
| Band 5        | Hydrogenophaga 63-70(55) clone WHP232 / GU458852 / 88%    | Acidiphilium rubrum / AB005218 / 84%     |
| Band 6        | Acidiphilium 66-70(51) clone WHP258 / GU458847 / 100%      | Acidiphilium cryptum / AB005220 / 95%    |
| Band 7        | Roseobacter 56-62(52) clone PROSOPE_8 / GQ468949 / 95%     | Roseobacter sp. NP30 / EU196351 / 87%    |
| Band 8        | Acidiphilium 66-70(52) clone PROSOPE_50 / GQ468980 / 94%   | Roseateles depolymerans / AB028938 / 90% |
| Band 9        | Roseateles 68-71(57) |

4. Conclusion
PCR-DGGE has been the most commonly used molecular fingerprint in the analysis of the microbial community structure and the integrity and precision of its resulted composition are closely related with two rounds PCR. In first round PCR before running DGGE, GC-clamped primers have to been utilized, making some target sequences especially with high GC content are hardly to be amplified, and additionally species in lower abundance are not able to yield enough PCR products to be showed and exercised on DGGE gel. In second round PCR, reamplification of exercised DGGE band, to acquire enough products for sequencing from weak band is the most outstanding challenged task. More polymerases and/or template may enhance the yields of PCR products, yet these may introduce more bias. Therefore better quality and more yields of PCR products is the key to success for PCR-DGGE performance.

This study demonstrates that PCR enhancer combination of DBD, comprised cost-effective DMSO, betaine and DTT with proper concentration (1% DMSO, 0.4 M Betaine, 1mM DTT) reaches tradeoff between quality and yield, producing many newly appeared bands on DGGE and no heteroduplex after sequencing. Moreover, more complicated community structure and/or more inhibitory contaminants contained, better improvement effects in PCR-DGGE would be obtained after optimized with the PCR enhancer combination. Therefore we strongly propose that PCR enhancer combination must be included
in PCR reaction system in the microbial community structure analysis by the approach of PCR-DGGE as well as gene library method.

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