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Note

PCR-based community structure studies of Bacteria associated with eukaryotic organisms: A simple PCR strategy to avoid co-amplification of eukaryotic DNA

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ABSTRACT

PCR primers targeting conserved regions of the SSU rRNA gene are commonly used in bacterial community studies. For microbes associated with eukaryotes, co-amplification of eukaryotic DNA may preclude the analysis. We present a simple and efficient PCR strategy to obtain pure bacterial rDNA amplicons from samples predominated by eukaryotic DNA.

During the past two decades, culture-independent methods have become commonly applied for studying the composition of Bacteria in samples (Cardenas and Tiedje, 2008; Tringe and Hugenholtz, 2008). Examples of such methods are Denaturing Gradient Gel Electrophoresis (DGGE), Terminal Restriction Fragment Length Polymorphism (T-RFLP), sequencing of SSU ribosomal DNA (rDNA) clone libraries, and more recently barcoded amplicon 454-sequencing. They all involve a step with PCR amplification of extracted DNA. Typically, fragments of the SSU rRNA (small subunit ribosomal RNA) gene are amplified. PCR primers should ideally target most taxa of Bacteria. Further, the resulting amplicons need to be of a length suitable for further analysis, and the DNA sequence of the amplified region should be informative in phylogenetic or diversity analysis. These factors limit the possibilities in primer design.

In the SSU rRNA gene of Bacteria, two conserved regions corresponding to *E. coli* gene nucleotide positions around 320–355 (e.g. primer 338F; 5′-actcctacgggaggcagcag-3′) and 505–535 (e.g. primer 518R; 5′-attaccgcggctgctgg-3′) have been extensively used for generating PCR products for DGGE analysis (Ahn et al., 2006; Klammer et al., 2008; Muyzer et al., 1993). These primers efficiently amplify an approximately 200 bp fragment for most bacterial taxa. However, the primer sequences exhibit similarity to conserved regions in eukaryotic SSU rRNA genes (Fig. 1), with 100% sequence homology for the 518R primer sequence to a conserved region in the eukaryotic SSU rRNA gene.

When studying composition of Bacteria in or on eukaryotic organisms, a separation of nucleic acids from Bacteria and Eukarya may be impossible, and the nucleic acid extracts to be examined often predominantly consist of eukaryotic DNA. This may cause problems when using conserved SSU rRNA primers, as previously emphasized by Huys et al. (2008).

We used the primers 338F-GC (for sequence, see legend to Fig. 2) and 518R to study the composition of Bacteria associated to cod larvae (*Gadus morhua*; 4–60 days after hatching) by DGGE. Total DNA was extracted using the Blood and Tissue Kit (Qiagen). PCR reactions were run for 35 cycles (95 °C 30 s, 50 °C 30 s, and 72 °C 60 s) with 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 μM of each primer, and Taq polymerase and reaction buffer from VWR. Assuming that the total DNA extracts from the cod larvae samples were predominated by eukaryotic cod DNA, we included a control where DNA from Bacteria-free cod muscle was used as template. This control PCR reaction efficiently amplified cod DNA and resulted in a distinct PCR product slightly smaller than 200 bp (Fig. 2A). PCR products obtained from 6 individual cod larvae samples were subjected to DGGE analysis together with the amplicon from sterile cod muscle DNA (Fig. 3A). A band corresponding to the cod muscle PCR product appeared to dominate all the cod larvae DGGE profiles. Sequencing (see band in frame of Fig. 3A) revealed that the band represented an approximately 170 bp *Gadus morhua* SSU rDNA fragment, resulting from primer binding to the SSU rDNA regions shown in Fig. 1.

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	↓338	↓518
Escherichia (Poteobacteria)	ccag*actcctacgggaggcagcag*tggggaat	cgtg*ccagcagccgcggtaat*acg
Bacillus (Firmicutes)	..........a.....	.....
Flavobacterium (Bacteroidetes)	..........a.....	.....
Planctomyces (Planctomycetes)	......a.....t...t.....c.a....	.....
Spirochaeta (Spirochaetes)	..........ctaa....	.....c...
Mycoplasma (Tenericutes)	..........at.....	t.....a
Akkermansia (Verrucomicrobia)	......a.....t.....c.a....	t.....a
Rubidibacter (Cyanobacteria)	...t.....	.....
Streptomyces (Actinobacteria)	.....	.....
Fusobacterium (Fusobacteria)	..tt.....	.....
Isochrysis (Haptophyceae)	...c.-...-.a..a.....gc.c.t.a	g.....t.c
Microsporidium (Fungi)	...cg-...-.a..ac.....gc.c...a	g.....c
Salvinia (Viridiplantae)	...c.-...-.a..a.....gc.c.c.a	g.....t.c
Gadus (Metazoa)	...c.-...-.a..a.....gc.c.c.a	g.....t.c

Fig. 1. Sequence alignment of two conserved regions of the SSU rRNA gene for diverse organisms from Bacteria and Eukarya. Sequences shown: *Escherichia coli* (Brosius *et al.*, 1978), *Bacillus lentus* (AB021189; Goto et al., 2000), *Flavobacterium limicola* (AB075230; Tamaki et al., 2003), *Planctomyces limnophilus* (X62911, Liesack et al., 1992), *Spirochaeta africana* (X93928, Zhilina et al., 1996), *Mycoplasma synoviae* (X52083), *Akkermansia muciniphila* (AY271254, Derrien et al., 2004), *Rubidibacter lacunae* (EF126149, Choi et al., 2008), *Streptomyces arenae* (AJ399485), *Fusobacterium nucleatum* (AE009951, Kapatral et al., 2002), *Isochrysis galbana* (AJ246266), *Microsporidium* sp. DP-1-19 (AF394528; Refardt et al., 2002), *Salvinia natans* (X90413, Kranz and Huss, 1996), and *Gadus morhua* (AF518205). Dots indicate identical positions. Arrows above the alignment indicate the nucleotide positions in the *E. coli* SSU rRNA gene. Sequences corresponding to primers 338F and 518R are shown in italics.

To avoid the co-amplification of eukaryotic SSU rDNA we used a nested PCR strategy. For the external PCR amplification we used the primer Eub8F, 5′-agagtttgatcmtggctcag-3′ (Weisburg et al., 1991) and the broad coverage primer 984yR, 5′-gtaaggttcytcgcgt-3′ (Wang and Qian, 2009). None of these primer sequences are 100% identical to conserved sequences in the eukaryotic SSU rRNA gene, and therefore this first PCR amplification should enrich the fraction of bacterial SSU rDNA. The 338F-GC/518R primer set was used for the internal PCR amplification to obtain a product suitable for DGGE analysis. For both the external and internal PCR, 25 PCR cycles were used (95 °C 30 s, 50 °C 30 s, and 72 °C 60 s). For the rest, the conditions were as described above, except that an annealing temperature of 53 °C was used in the internal PCR. This protocol succeeded in avoiding amplification of cod DNA (Fig. 2B). To investigate whether the nested PCR protocol could affect the composition of the PCR product, we used a template with assumingly low amounts of eukaryotic DNA (faecal samples from sea bass) with the nested and the regular PCR protocols and compared the products on DGGE (Fig. 3C). The DGGE profiles were similar (Bray Curtis similarity of 0.87). DGGE profiles obtained for the 6 individual cod larvae samples with the nested PCR protocol is shown in Fig. 3B. Comparison to the profiles shown in Fig. 3A (regular PCR, same individual samples) shows that the band representing the 18S rDNA is absent when the nested protocol is used. Further, the co-amplification of eukaryotic DNA seems to greatly affect the DGGE profiles; a number of bands observed in Fig 3B are missing in the DGGE profiles in Fig. 3A. For each of the cod larvae samples we calculated the Bray Curtis similarity between the band patterns obtained for the two PCR protocols, and the average similarity was only 0.33.

We have successfully applied this strategy to study bacterial communities in samples from diverse eukaryotic organisms, like sea bass (*Dicentrarchus labrax*), mangrove killifish (*Kryptolebias marmoratus*), rotifer (*Brachionus* 'Nevada'), brine shrimp (*Artemia franciscana*), mouse (*Mus musculus*), Siberian sturgeon (*Acipenser baerii*), and microalgae (*Nannochloropsis* sp.).

It is important to consider the problem of co-amplification of eukaryotic SSU rDNA when using primers targeting conserved sequences of the bacterial SSU rRNA gene. As shown in the present work, PCR primers commonly used in DGGE could seriously bias the composition of the resulting amplicons. This problem is also relevant to other PCR-based techniques for studying the composition of Bacteria. The PCR strategy suggested here offers an efficient and simple solution to the problems associated to community analysis of Bacteria in samples with high portions of animal or plant DNA.

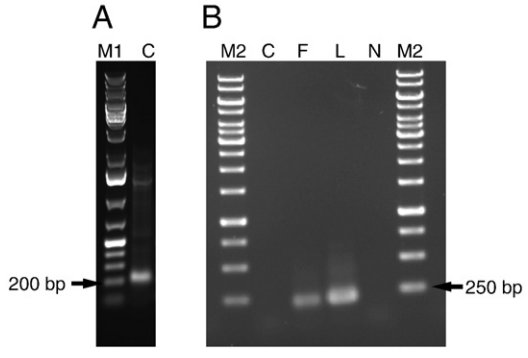

Fig. 2. Agarose gels showing (A) PCR products obtained using DNA isolates from Bacteria-free cod muscle (lane C) as template, with primers 338F-GC (cgcccgccgcgcgcggcgggcggggcgggggcacgggggg actcctacgggaggcagcag) and 518R and the regular PCR protocol. M1: GeneRuler 1 kb Plus DNA Ladder (Fermentas). (B) PCR products obtained with the nested PCR protocol (primers Eub8F/984R in the external PCR, and primers 338F-GC/518R in the internal PCR) and templates isolated from sterile cod muscle (lane C), sea bass faeces (lane F), cod larva (lane L), and a non-template control (lane N). M2: GeneRuler 1 kb DNA ladder (Fermentas).

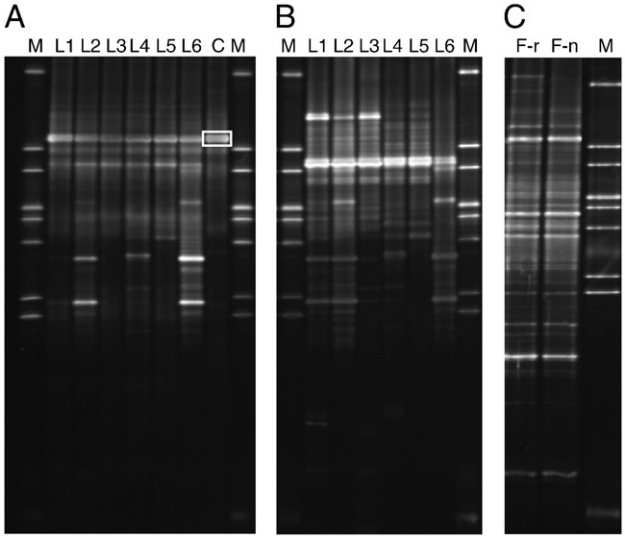

Fig. 3. DGGE gels (8% acrylamide, 35–55% denaturing gradient) with PCR products obtained from (A) DNA from individual cod larvae (lanes L1–L6) and sterile cod muscle (lane C) by a regular PCR protocol with primers 338F-GC and 518R, and (B) DNA from individual cod larvae (lanes L1–L6) by a nested PCR strategy with primers Eub8F and 984R in the external PCR, and primers 338F-GC and 518R in the internal PCR. C) A template with low amounts of eukaryotic DNA (faeces from sea bass) was used for comparison of the nested (lane F-n) and the regular (F-r) PCR protocols. M: a marker produced from a template consisting of DNA from pure cultures of 9 different bacterial strains.

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