Separation of 16S rRNA PCR Products From a Model Community Using Temporal Temperature Gradient Gel Electrophoresis

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Introduction

Identification and characterization of bacterial species from membrane biofilms has been a microbiological challenge. A vast majority (>90%) of bacterial cells from membrane biofilms cannot be cultivated by standard microbiological techniques, which have traditionally used culture-dependent methods. In addition, culture-based identification methods may be affected by the physiological state of the cells while growing on a specific medium. As a consequence of these limitations, microbial populations from membrane biofilms have not currently been well characterized.

Several molecular approaches now provide powerful culture-independent techniques that can be used for identification and characterization of a multispecies consortia (i.e., membrane biofilm community structure). One strategy is based on a combination of polymerase chain reaction (PCR), restriction fragment length analysis, and/or DNA sequencing to characterize multispecies consortia from a membrane biofilm (Avaniss-Aghajani et al. 1996). A limitation of this approach is that DNA sequencing is expensive and laborious when dealing with a multispecies consortia. Additionally, restriction fragment length analysis requires fragment pattern matches against a large database (Ribosomal Database Project, RDP) (Maidak et al. 1999), which contains more than 6,000 organisms, to identify a list of potential organisms in the mixture. This can result in spurious pattern matches because a combination of patterns from two or more bacteria may resemble unrelated restriction fragment patterns found in the RDP. In order to confirm the existence of an organism in the consortium, analysis must be conducted on PCR products of individual isolates. A direct approach to solving this problem is to first separate a mixture of PCR products by using temporal temperature gradient gel electrophoresis (TTGE).

In the commonly used denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman et al. 1979), electrophoretic separation is performed at high temperature in the presence of a chemical gradient. In TTGE, the denaturing environment is formed by a constant concentration of denaturant in the gel in combination with a gradual increase in temperature over the course of the separation. Thus, by using TTGE, one avoids pouring chemical gradient gels (Zoller et al. 1998).

We have developed a strategy for using restriction fragment length analysis to characterize multispecies consortia. First, total DNA is isolated and used as a template for PCR amplification of 16S rRNA genes with universal primers targeting a hypervariable region (500 bp) of the 16S rRNA. This hypervariable region shows enough differences in DNA sequences between organisms that it can be used accurately to identify a species (Avaniss-Aghajani et al. 1994). The mixture of amplified hypervariable regions is separated by TTGE using the DCode™ universal mutation detection system (Bio-Rad Laboratories, Inc.). The separated PCR products are then excised out of the temperature gradient gel and reamplified using a fluorochrome-tagged universal primer. The tagged products are sequenced or digested with a suite of restriction endonucleases. Fragment lengths are analyzed by capillary electrophoresis to obtain fragment patterns that are matched against the RDP database for identification. This approach was used with a model community, demonstrating identification of individual organisms as components of the consortium.

Methods

The model community consisted of seven bacteria: *Serratia marcescens* (ATCC 13880), *Pseudomonas aeruginosa* (ATCC 25330), *Pseudomonas mendocina* (ATCC 25411(T)), *Pseudomonas putida MT-2* (ATCC 33015), *Flavobacterium aquatile* (ATCC 11947), *E. coli* K12 (ATCC 25404), and *E. coli* K12 (ATCC 29947). Total genomic DNA was extracted from the model community according to the lysozyme/sodium dodecyl sulfate (SDS) lysis protocol described by Leddy et al. (1995), with the modification that the pellet was subjected to three cycles of freezing in dry ice/ethanol and thawed out at 65°C following the addition of 5 M NaCl. Crude DNA was further purified by using a commercially available plasmid purification system.

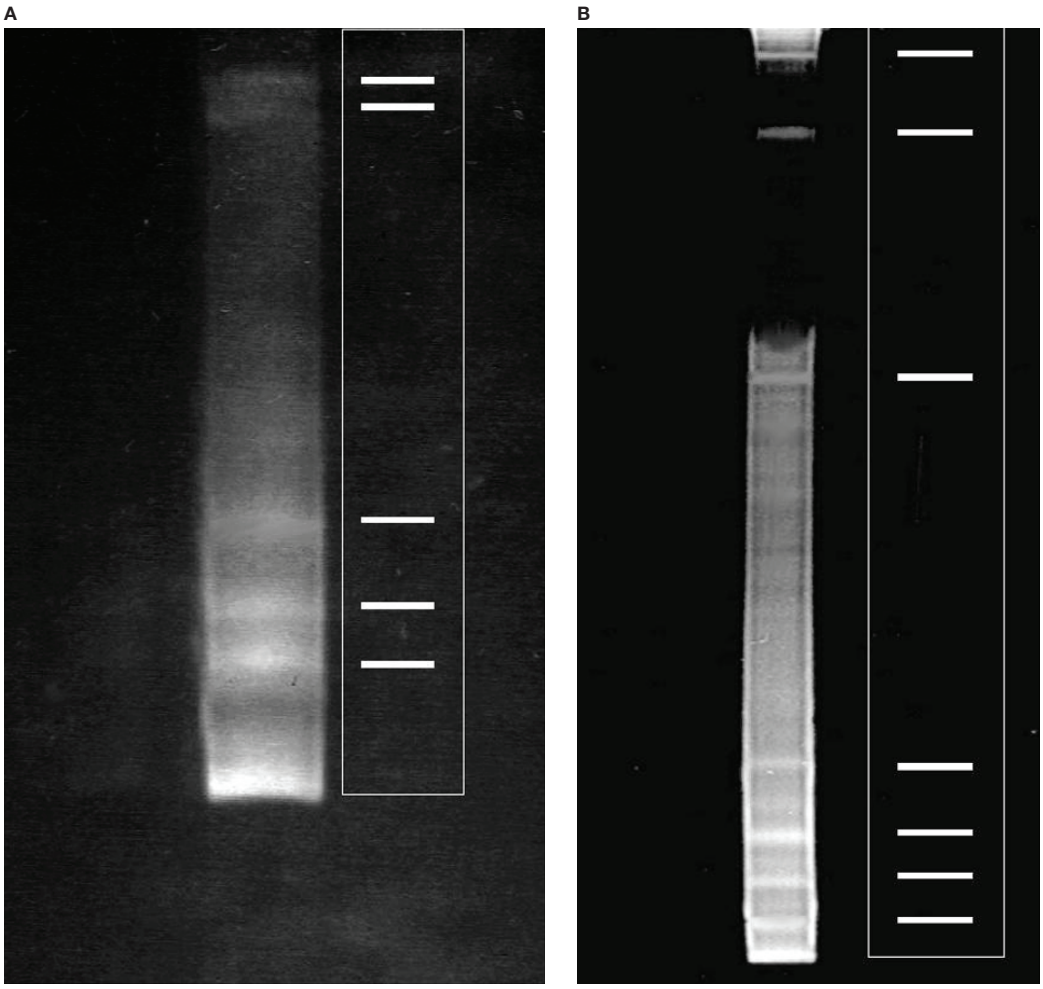

Fig. 1A. Separation of 16S rRNA PCR products from a seven-member model community. A, A 1 mm thick 16 x 16 cm gel, containing 6 M urea and 40% acrylamide/bis (37.5:1) in 1.25x TAE buffer, was run at room temperature and stained with SYBR Green I in 1.25x TAE buffer for 30 min. Horizontal lines indicate location of bands. **B**, Using TTGE, PCR products from *Serratia marcescens*, *Pseudomonas aeruginosa*, *Pseudomonas mendocina*, *Pseudomonas putida*, *Flavobacterium aquatile*, *E.coli* K12 (25404), and *E.coli* K12 (29947) were separated at a temperature denaturation range of 23.1–30°C with a ramp rate of 0.4°C/hr. Horizontal lines indicate location of bands.

PCR amplification of the mixture of 16S rRNA hypervariable regions was performed using the GeneAmp PCR reagent kit with AmpliTaq Gold DNA polymerase (Applied Biosystems). A 50 ng aliquot of target DNA was suspended in 100 µl of reaction buffer which contained 1.5 mM $MgCl_2$ PCR buffer II, 200 µM each of dNTP, and 0.5 µM of each of the universal eubacterial primers. Although a GC-clamped version of the universal primers was recommended for separation by TTGE, we obtained satisfactory results without GC clamps. DNA amplification was performed with a thermal cycler using the following program: a hot start at 95°C for 11 min followed by 35 cycles consisting of denaturation (60 sec at 94°C), annealing (60 sec at 55°C), and extension (3 min at 72°C), with a final extension at 72°C for 10 min.

The amplified products were subjected to TTGE on 1 mm thick 16 x 16 cm gels containing 40% acrylamide/bis (37.5:1; Bio-Rad) and 6 M urea in 1.25x TAE buffer. A 40 µl aliquot of the PCR product was mixed with 40 µl of gel loading dye (Bio-Rad) and electrophoresed on the DCode system at 120 V for 17.5 hr with a temperature denaturation range of 23.1–30°C and a temperature ramp rate of 0.4°C/hr. Following electrophoresis, the gel was stained with 1x SYBR Green I (Molecular Probes, Inc.) in 1.25x TAE buffer for 30 min. Gel bands were imaged under ultraviolet (UV) transillumination and excised from the gel. The excised bands were incubated overnight at 37°C in a solution containing 0.3 M NaCl and 3 mM Tris (pH 7.6) to elute the DNA (Rolleke et al. 1996). The residual acrylamide was removed by standard procedures. The supernatant was precipitated with absolute ethanol overnight at –20°C and the pellet resuspended in 10–15 µl of TE buffer, then reamplified using 0.5 µM of each of the universal eubacterial primers, one of which was labeled at the 5' end with Hex Amidite fluorochrome (Applied Biosystems).

Results

The PCR products of the seven-member model community were initially run on a 40% acrylamide gel with no temperature gradient (Figure 1A). As anticipated, based on the size of the PCR products of the organisms in the model community (Table 1), five PCR products (five bands) were resolved. Since the two PCR products from *E. coli* were the same size, they would be expected to comigrate through the acrylamide gel. The same was true for *Pseudomonas aeruginosa* and *Pseudomonas putida* PCR products.

Table 1. Observed lengths (base pairs (bp)) and T_m (°C) values of PCR products for organisms in seven-member model community.

Organism	Size (bp)	T_m Value
E. coli K12 (25404)	486	69.4
E. coli K12 (29947)	486	Not available
Serratia marcescens	484	69.6
Pseudomonas aeruginosa	481	69.1
Pseudomonas putida	480	68.7
Pseudomonas mendocina	479	68.3
Flavobacterium aquatile	472	67.0

Separation on the TTGE gel is based on both size of the PCR product and different melting behavior. Since the melting temperature (T_m) values were different for each of the PCR products (see Table 1), they should denature at different points along the gel, resulting in separate bands on the TTGE gel even though the PCR products were similar in molecular weight. When the PCR products of the model community were run on a TTGE gel, seven separate PCR products (seven bands) were resolved (Figure 1B). This suggested that the individual PCR product of each organism in the model community was separated by TTGE. Even though the PCR products were similar in size, as long as their T_m values were significantly different (see Table 1, T_m values for *E. coli* K12, *Pseudomonas aeruginosa*, and *Pseudomonas putida*), separation occurs on the TTGE gel.

Discussion

Cloning and sequencing of PCR products of the 16S rRNA hypervariable region can be successfully employed to confirm the presence of specific bacteria in a consortium such as a membrane biofilm. However, for this strategy to work and produce statistically reliable results, numerous clones must be analyzed (100 or more). Restriction fragment pattern analysis is far simpler; however, with mixtures, this method yields highly ambiguous results due to spurious matches. These ambiguities are prevented if the PCR products are initially separated by using TTGE. This separates by both molecular weight and also the unique thermal denaturation characteristics of each PCR product determined by their base pair sequences. Different sequences will result in DNA bands in different positions on the gradient gel where the PCR product begins to halt. As a result, a single PCR product may be recovered. This method is a powerful, easily reproducible, reliable technique for separating mixtures of PCR products prior to further characterization by restriction pattern analysis (or sequencing).

References

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Bulletin 2449 Rev B US/EG 06-0373 0408 Sig 0308