Modified T-RFLP Methods for Taxonomic Interpretation of T-RF

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Terminal restriction fragment length polymorphism (T-RFLP) is a method that has been frequently used to survey the microbial diversity of environmental samples and to monitor changes in microbial communities. T-RFLP is a highly sensitive and reproducible procedure that combines a PCR with a labeled primer, restriction digestion of the amplified DNA, and separation of the terminal restriction fragment (T-RF). The reliable identification of T-RF requires the information of nucleotide sequences as well as the size of T-RF. However, it is difficult to obtain the information of nucleotide sequences because the T-RFs are fragmented and lack a priming site of 3'-end for efficient cloning and sequence analysis. Here, we improved the T-RFLP method in order to analyze the nucleotide sequences of the distinct T-RFs. The first method is to selectively amplify the portion of T-RF ligated with specific oligonucleotide adapters. In the second method, the termini of T-RFs were tagged with deoxynucleotides using terminal deoxynucleotidytransferase (TdT) and amplified by a second round of PCR. The major T-RFs generated from reference strains and from T-RFLP profiles of activated sludge samples were efficiently isolated and identified by using two modified T-RFLP methods. These methods are less time consuming and labor-intensive when compared with other methods. The T-RFLP method using TdT has the advantages of being a simple process and having no limit of restriction enzymes. Our results suggest that these methods could be useful tools for the taxonomic interpretation of T-RFs.

Keywords: 16S rRNA, microbial diversity, oligonucleotide adapter, TdT, T-RFLP

Characterization of the microbial community structure in natural ecosystems is one of the important tasks of environmental microbiology and microbial ecology. Traditional methods to study microbial diversity depend on the cultivation of microorganisms. However, very small proportions of microbial communities are known to be cultivable [5]. Molecular biological techniques provide a powerful approach, and have been applied to the study of microbial communities in the natural ecosystem [6, 21]. In particular, the analysis of the microbial diversity is largely based on 16S rRNA genes. Construction of clone libraries of 16S rDNAs amplified from environmental samples provides the comprehensive analyses of the bacterial diversity in an ecosystem [23]. However, the screening of these clone libraries is a time-consuming and laborious work, especially when several samples have to be analyzed.

Genomic fingerprinting techniques, which are based on the detection of 16S rRNA pools, have been used for the analysis of microbial communities; these include denaturing gradient gel electrophoresis (DGGE) [1, 18], single-strand conformation polymorphism (SSCP) [9], and terminal restriction fragment length polymorphism (T-RFLP) [11]. Smalla et al. [24] demonstrated that DGGE, SSCP, and T-RFLP analyses provided similar results on the bacterial community composition of the four soil samples.

T-RFLP is a sensitive and reproducible method [17] and has been used successfully for comparing microbial communities in soil [13], seawater [3], and activated sludge [22] samples. T-RFLP has several advantages over other methodologies in monitoring the complex microbial communities [14]. First, T-RFLP has a greater resolution power than either DGGE or SSCP. Second, the size of T-RF can be compared with the T-RFs calculated from the supporting sequence databases in order to derive phylogenetic inference.

However, the connection of T-RFLP data to phylogenetic information is limited. One of the major limitations is the variation between T-RF length of the database and observed T-RF length (T-RF drift) [7]. Moreover, a single T-RF can represent several genera in many cases. In addition, the limited number of sequences with phylogenetic affiliations is presented in the database, so some T-RFs generated from environmental samples cannot be identified as a member of the database [2]. These limitations directly contribute to
misidentification of T-RFs using comparison of observed T-RF length to the databases. Therefore, T-RFs of the community fingerprint should be identified by their information of nucleotide sequences, rather than by comparing with the T-RFs sizes derived from the databases. Although several authors attempted cloning and sequencing of T-RFs [15, 27], these methods have the problems of being labor intensive, and time-consuming, and having a limitation of restriction enzymes.

To overcome these problems, we developed two convenient methods for the taxonomic interpretation of T-RFs. The first one is a variation of preexisting methodologies using the oligonucleotide adapter, which is available for the T-RF containing a sticky end (complementary single-stranded end). The second one is a new technique of blunt-end cloning employing terminal deoxynucleotidyl transferase (TdT). This method is useful for the analysis of T-RF, which has a blunt end.

**Materials and Methods**

**Reference Strains and Activated Sludge Samples**
The bacterial strains used in this study are *Acinetobacter calcoaceticus* (KCTC 2357T), *Bacillus megaterium* (KCTC 3007T), *Bacillus subtilis* (KCTC 3135T), *Burkholderia cepacia* (KCTC 2966T), and *Enterobacter pyrinius* (KCTC 2520T). Strains were incubated in nutrient broth (peptone, 5 g/l; beef extract, 3 g/l) at 30°C for 1 day. Activated sludge samples, AS and AC, were collected from a sewage treatment plant and an industrial wastewater treatment plant in Korea, respectively.

**DNA Extraction**
The bacterial DNAs of the reference strains and activated sludge samples were prepared by the bead mill homogenization method [10]. The extracted genomic DNAs were purified with an UltraClean kit (MoBio, U.S.A.), electrophoresed in an agarose gel (0.8%), and stored at −20°C.

**PCR Amplification for T-RFLP**
16S rRNA fragments were amplified by PCR using two eubacterial primers 27FB (*E. coli* numbering 8–27: 5'-AGAGTTTGATCMTGGCTCAG-3') and 785R (*E. coli* numbering 785–804: 5'-ACTACCTG-GGTATCTGATC-3') [8]. The 27FB primer was biotinylated at the 5'-end to separate terminal restriction fragments (T-RFs) from the other digested fragments. PCR was carried out with 50 µl of reaction mixture containing 1× PCR buffer (100 mM Tris-HCl, 400 mM KCl, 1.5 mM MgCl2, 500 µg/ml BSA, pH 8.3), 160 µM of each dNTP, 0.3 µM of each primer, 1.5 unit of *Taq* DNA polymerase (Genenmed, Korea), and 10–15 ng/µl of template DNA. An initial denaturation step (3 min at 95°C) was followed by 30 cycles consisting of denaturation step (30 s at 95°C), annealing step (30 s at 58°C), and extension step (1 min at 72°C), and final 10 min extension step at 72°C. The PCR products were purified with an UltraClean kit (MoBio).

**Physical Capture T-RFLP Method**
Purified PCR products of 16S rRNA were digested with 5 units of restriction endonuclease, HhaI or HaeIII (TaKaRa, Japan), for 5 h at 37°C. The biotinylated T-RFs were selectively isolated from the digested fragments by using Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs) and a Magnetic Separation Stand (Promega, U.S.A.) following the manufacturer’s instructions. To denature double-stranded T-RFs (dsT-RFs), the samples were soaked in 0.2 N

**Figure 1.** Schematic diagram of the modified T-RFLP methods for nucleotide sequencing.
A. T-RFLP method using the oligonucleotide adapter; B. T-RFLP method using TdT.