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# 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 on 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 tailed with deoxynucleotides using terminal deoxynucleotidyl transferase (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

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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 2357$^T$), *Bacillus megaterium* (KCTC 3007$^T$), *Bacillus subtilis* (KCTC 3135$^T$), *Burkholderia cepacia* (KCTC 2966$^T$), and *Enterobacter pyrinus* (KCTC 2520$^T$). 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'-AGAGTTTGATCMTGG-CTCAG-3') and 785R (*E. coli* numbering 785–804: 5'-ACTACCRG-GGTATCTAATCC-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 $MgCl_2$, 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.) by following the manufacturer's instructions. To denature double-stranded T-RFs (dsT-RFs), the samples were soaked in 0.2 N

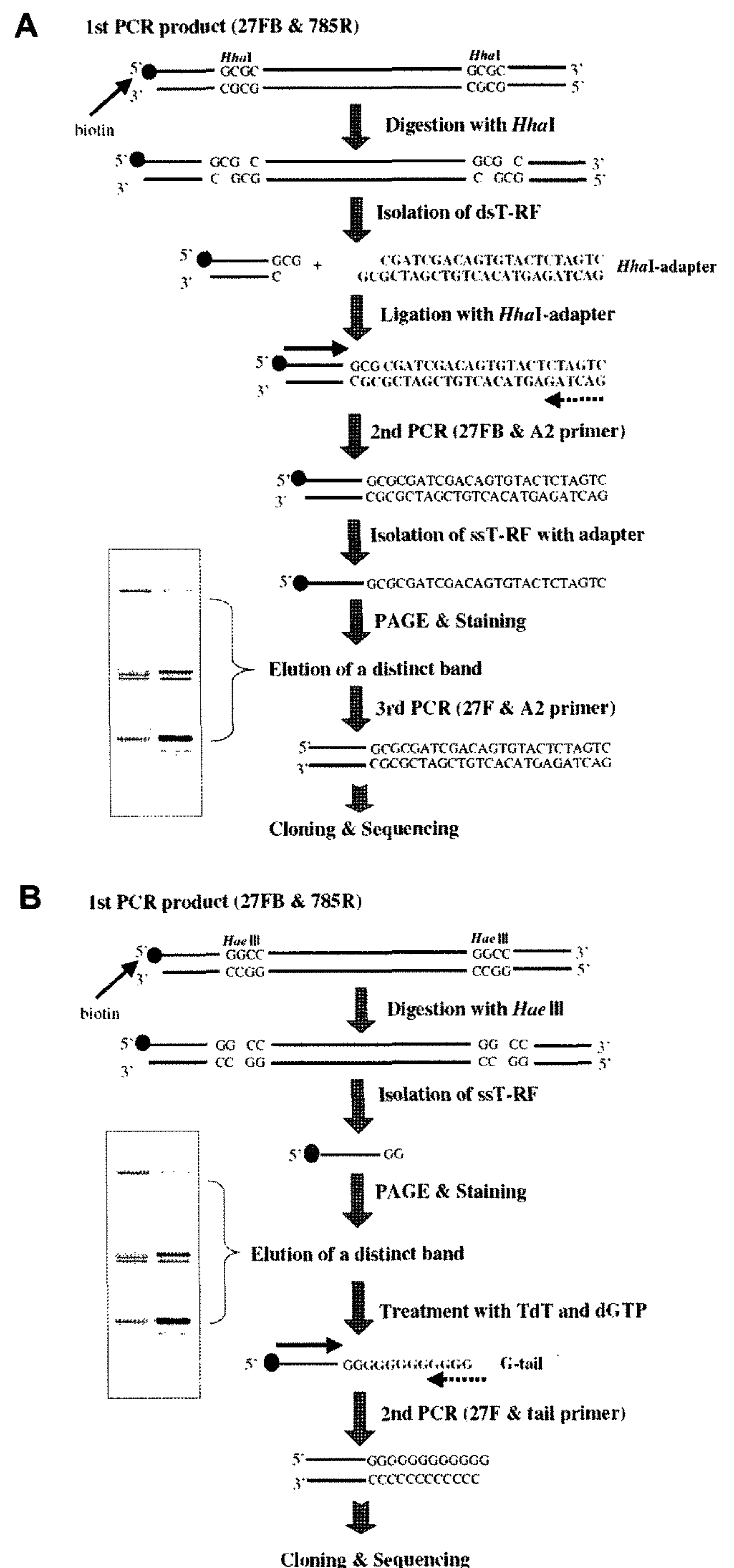


**Fig. 1.** Schematic diagram of the modified T-RFLP methods for nucleotide sequencing.
**A.** T-RFLP method using the oligonucleotide adapter; **B.** T-RFLP method using TdT.

NaOH for 5 min at room temperature. The biotinylated single-stranded T-RFs (ssT-RFs) binding with SA-PMPs were harvested in a Magnetic Separation Stand (Promega). The biotinylated ssT-RFs were mixed with 25% $NH_4OH$ and incubated at 65°C for 10 min to release ssT-RFs from SA-PMPs. $NH_4OH$ was removed by using a vacuum microcentrifuge (Hanil Science Industrial Co., Korea) and the obtained T-RF pellets were resuspended in distilled water. T-RFLP patterns were analyzed by electrophoresis on a 6% polyacrylamide gel (acrylamide:bisacrylamide=19:1; 7.0 M urea;1×TBE). After mixing with an equal volume of loading dye buffer (95% formamide, 10 mM NaOH, 20 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol FF), ssT-RFs samples were heated for 3 min at 95°C, and chilled on ice prior to electrophoresis. After electrophoresis at 1,900 V for 2 h, silver staining was performed according to the manufacturer's instructions of the Silverstar Staining Kit (Bioneer, Korea).

**Modified T-RFLP Method Using the Oligonucleotide Adapter**

The oligonucleotide adapter was used to improve the T-RFLP method in this study (Fig. 1A). The adapter was designed to restore the original HhaI site and primer sequences at the 3'-end of T-RF. The adapter was made of A1 primer (5'-CGATCGACAGTGTACTCTA-GTC-3') and A2 primer (5'-GACTAGAGTACACTGTCGATCGCG-3'). After addition of equimolar amounts of both primers, the reaction mixture was incubated for 10 min at 95°C and 20 min at room temperature in order to make the oligonucleotide adapter [26].

The dsT-RFs were selectively isolated from the HhaI-digested fragments of PCR product as described above with the exception of a 0.2 N NaOH treatment process. The dsT-RFs were ligated with 5 pmol of the oligonucleotide adapter and 2 units of T4 DNA ligase (KOSCO, Korea) for 2 h at 16°C. After ligation, those ligates were selectively amplified with 27FB and A2 primers. An initial denaturation step (3 min at 95°C) was followed by 30 cycles consisting of a 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 ssT-RFs were isolated from the PCR product of ligates, separated by electrophoresis on a polyacrylamide gel, and detected by the silver staining technique. In order to analyze the nucleotide sequences of T-RFs, the intense T-RF bands were eluted and re-amplified with 27F and A2 primers as described above. The PCR products were ligated into the pGEM-T vector (Promega) and cloned with *E. coli* XL1-Blue according to the manufacturer's instructions. The nucleotide sequences of the cloned T-RFs were achieved using the BaseStation DNA Fragment Analyzer (MJ Research, U.S.A.).

**Modified T-RFLP Method Using Terminal Deoxynucleotidyl Transferase (TdT)**

Terminal deoxynucleotidyl transferase (TdT) was used for another improved T-RFLP method in this study (Fig. 1B). HaeIII-digested ssT-RFs of *Acinetobacter calcoaceticus*, *Bacillus megaterium*, *Bacillus subtilis*, and *Burkholderia cepacia* were eluted from the polyacrylamide gel prepared by the physical capture T-RFLP method described above. The 3'-OH termini of eluted ssT-RF were tailed with dGTP using TdT. The TdT reaction was carried out with 50 μl of mixture containing 1×TdT buffer (12 mM potassium phosphate, pH 7.2, 30 mM KCl, 0.2 mM 2-mercaptoethanol, 50% glycerol), 0.01% BSA, 0.5 mM dGTP, 13 unit of TdT (TaKaRa), and ssT-RF. The mixture was incubated at 37°C for 30 min. Tailed ssT-RF was amplified with 27F and tail primer (5'-CCCCCCCCCCCC-3'). The "C" homopolymeric sequence of the primer was complementary to the "G" tail added by TdT and the fragment end in a site digested by HaeIII. An initial denaturation step (3 min at 95°C) was followed by 30 cycles consisting of a denaturation step (30 s at 95°C), annealing step (30 s at 50°C), and extension step (1 min at 72°C), and final 10 min extension step at 72°C. The PCR products were electrophoresed in an agarose gel (2%, 0.5×TBE), and cloned and sequenced as described above.

## RESULTS

**Modification of T-RFLP Method Using the Oligonucleotide Adapter**

The amplified fragment length polymorphism (AFLP) technique was applied to the T-RFLP method in order to analyze the nucleotide sequences of the specific T-RFs in this study. In the AFLP technique, restriction fragments can be amplified by using the enzyme specific adapter without knowledge of the nucleotide sequences [26]. In this study, the oligonucleotide adapter was used for the amplification

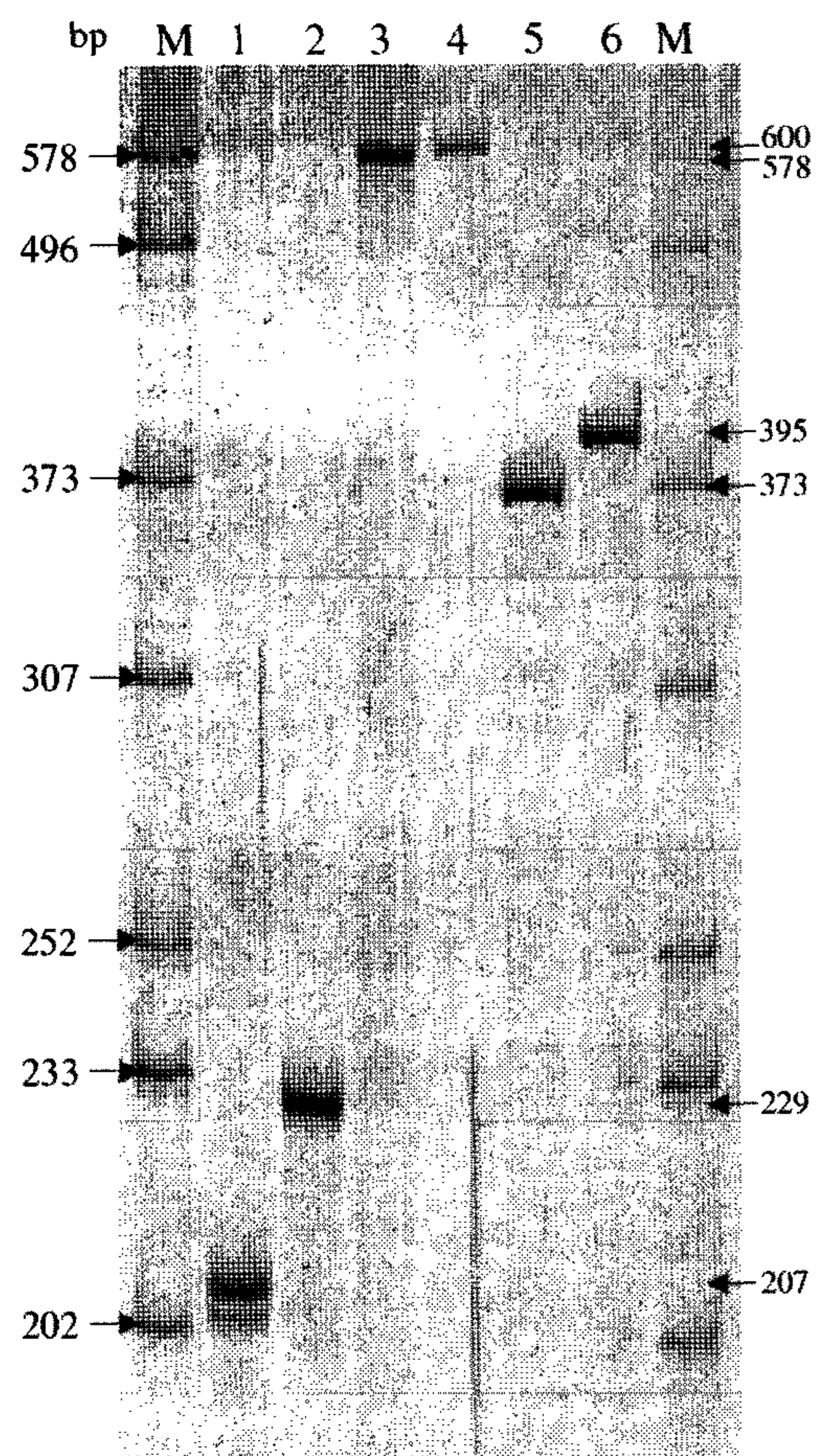


**Fig. 2.** Polyacrylamide gel electrophoresis of the HhaI T-RFs generated from the reference strains.

Lanes: M, size marker; 1 and 2, *Acinetobacter calcoaceticus*; 3 and 4, *Bacillus megaterium*; 5 and 6, *Enterobacter pyrinus*. The HhaI T-RF and adapter-ligated HhaI T-RF are shown in odd-numbered lanes and even-numbered lanes, respectively.

of T-RF that had lost the information of the 3'-end primer (Fig. 1A). The modified method involves eight steps: 1) PCR and restriction enzyme digestion, 2) isolation of double-stranded T-RFs from the restriction fragments, 3) ligation with the oligonucleotide adapter, 4) selective amplification with 27FB and A2 primers, 5) isolation of single-stranded T-RFs (ssT-RFs), 6) gel electrophoresis of ssT-RFs, 7) elution and PCR of distinct ssT-RF band, and 8) cloning and sequencing of T-RF.

We evaluated this method using three reference strains, *A. calcoaceticus*, *B. megaterium*, and *E. pyrinus*, and a restriction enzyme (HhaI) producing sticky ends (Fig. 2). The length of HhaI T-RF of *A. calcoaceticus*, *B. megaterium*, and *E. pyrinus* was 207, 578, and 373 bp, respectively (Fig. 2, lanes 1, 3, and 5). The length of adapter-ligated HhaI T-RF of *A. calcoaceticus*, *B. megaterium*, and *E. pyrinus* was 229 bp, 600 bp, and 395 bp, respectively (Fig. 2, lanes 2, 4, and 6). The size of adapter-ligated HhaI T-RF was 22 bp longer than that of T-RF in all reference strains owing to the oligonucleotide adapter ligation. We could elute and re-amplify this adapter-ligated HhaI T-RF (data not shown). This technique was also used to identify the bacterial groups of the major T-RF bands obtained from two sludge samples, AS and AC (Fig. 3 and Table 1). The sizes of adapter-ligated HhaI T-RFs generated from the sludge samples were 22 bp longer than those of T-RFs as observed in the experiment of reference strains (Fig. 3). The T-RF profile of the AS sample revealed strong bands at 207 bp and 205 bp, whereas the AC sample had the bands of 216 bp and 207 bp. The adapter-ligated major T-RFs were eluted from the polyacrylamide gel and re-amplified with 27F and A2 primers. These four bands were 229 bp and 227 bp of the AS sample, and 238 bp and 229 bp of the AC sample (Fig. 3, lanes 3, 4, 7, and 8). We constructed the clone libraries of these PCR products and analyzed 16S rRNA sequences of the T-RFs (Table 1). Nucleotide sequences of three clones generated from the 205 bp T-RF or 207 bp T-RF of the AS sample were different from each other and displayed sequence similarity of 86.9–98.7% and 80.4–96.1%, respectively. The similarity of nucleotide sequences derived from the 207 bp T-RF or 216 bp T-RF of the AC sample was 97.2–97.6% and 98.2–99.5%, respectively.

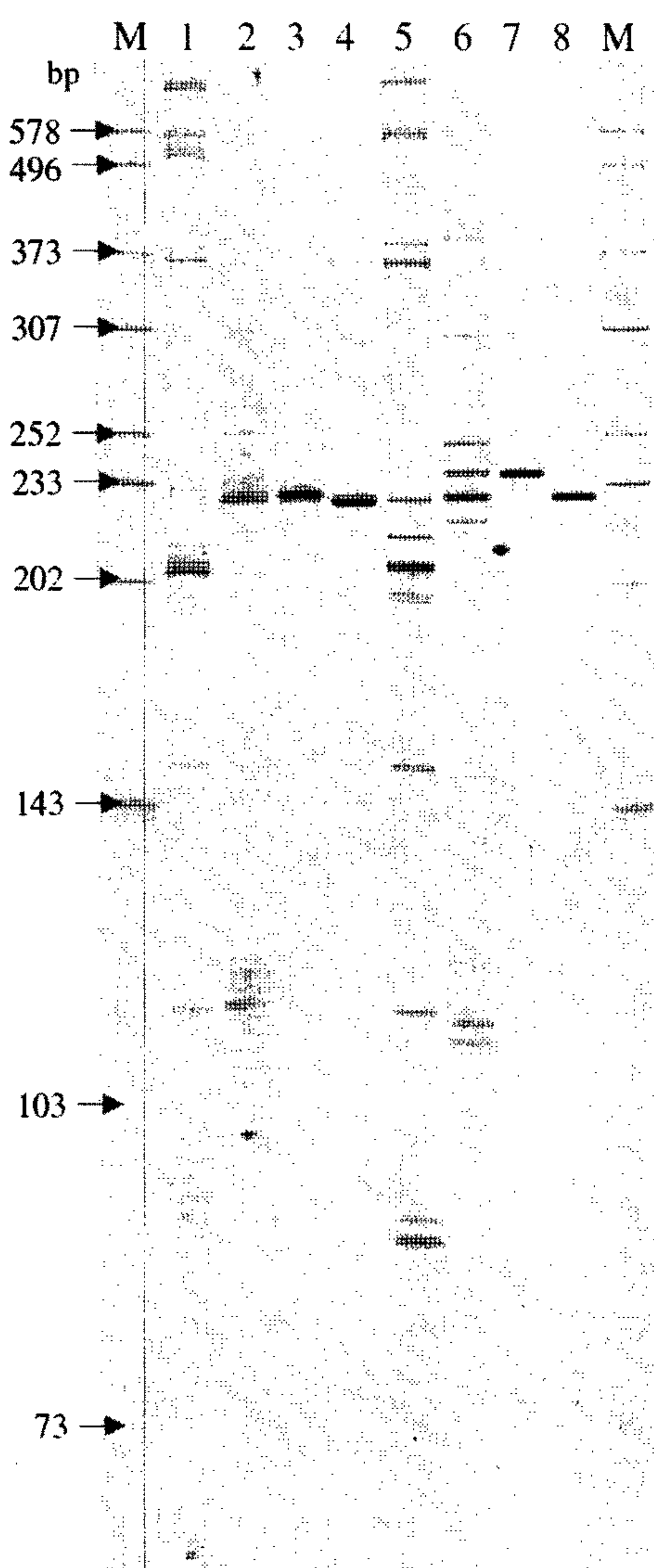


**Fig. 3.** Polyacrylamide gel electrophoresis of the HhaI T-RFs generated from the activated sludge samples.
Lanes: M, size marker; 1 and 5, the HhaI T-RFs from AS and AC, respectively; 2 and 6, the adapter-ligated HhaI T-RFs from AS and AC, respectively; 3 and 4, the PCR products of two major bands on lane 2; 7 and 8, the PCR products of two major bands on lane 6.

BLASTN search of 16S rRNA sequences obtained from T-RFs showed that β-Proteobacteria predominated in the bacterial communities of the two sludge samples. 16S rRNA sequences of T-RFs were closely related to the unclassified and uncultured bacterium with the exception of the T-RF band of 207 bp isolated from the AS sample. The nucleotide sequences of this band were related to the genera *Dechlorimonas* and *Pseudomonas* according to phylogenetic inference derived from the database using the size of 207 bp. The genera confirmed by sequence analysis corresponded with the putative genera deduced from the size of dominant T-RF bands, except for the band of 216 bp isolated from the AC sample. The HhaI T-RF of 216 bp in the AC sample was expected to be a member of *Enterococcus* (Firmicutes), *Legionella* (γ-Proteobacteria), *Shewanella* (γ-Proteobacteria), or *Xanthomonas* (γ-Proteobacteria). However, 16S rRNA sequences of this T-RF were related to a member of the CFB group.

## Modification of T-RFLP Method Using Terminal Deoxynucleotidyl Transferase (TdT)

In this study, another method for analysis of nucleotide sequences of T-RFs was an application of using terminal deoxynucleotidyl transferase (TdT) (Fig. 1B). The TdT is a template-independent DNA polymerase that is capable of

**Table 1.** Taxonomic information of the T-RF clones based on 16S rRNA sequences identified by BLAST search.

| Length of T-RF (bp) | Putative bacterial genus[a] | Clone[b] | BLAST results | | |
|---|---|---|---|---|---|
| | | | Closest microorganism (Accession No.) | Similarity (%) | Phylum |
| 205 | *Achromatium*, | AS-1 | Uncultured eubacterium clone F10.16 (AF495386) | 99 | Unclassified |
| | *Aquaspirillum*, | | *Dechloromonas* sp. MissR (AF170357) | 92 | β-Proteobacteria |
| | *Comamonas*, ***Dechlorimonas***, | AS-2 | Uncultured bacterium SJA-109 (AJ009484) | 89 | β-Proteobacteria |
| | *Delftia*, | AS-3 | Uncultured eubacterium clone F13.35 (AF495431) | 99 | Unclassified |
| | *Pseudomonas*, etc. | | *Dechloromonas* sp. MissR (AF170357) | 93 | β-Proteobacteria |
| 207 | *Acinetobacter*, | AS-4 | *Pseudomonas* sp. AEBL3 (AY247063) | 91 | γ-Proteobacteria |
| | *Alteromonas*, | AS-5 | *Dechloromonas* sp. PC1 (AY126452) | 95 | β-Proteobacteria |
| | *Aquaspirillum*, | AS-6 | *Dechloromonas* sp. CL (AF170354) | 99 | β-Proteobacteria |
| | *Burkholderia*, | AC-1 | Uncultured eubacterium clone F13.29 (AF495425) | 93 | Unclassified |
| | ***Comamonas***, | | *Comamonas terrigena* (AF078772) | 91 | β-Proteobacteria |
| | ***Dechlorimonas***, | AC-2 | Uncultured eubacterium clone F13.29 (AF495425) | 95 | Unclassified |
| | *Nitrosomonas*, | | *Comamonas terrigena* (AF078772) | 94 | β-Proteobacteria |
| | ***Pseudomonas***, | AC-3 | Uncultured eubacterium clone F13.29 (AF495425) | 93 | Unclassified |
| | *Rhodocyclus*, etc. | | *Comamonas terrigena* (AF078772) | 92 | β-Proteobacteria |
| 216 | *Enterococcus*, | AC-4 | Uncultured gold mine bacterium D17 (AF337873) | 93 | Unclassified |
| | *Legionella*, | | Uncultured Bacteroidetes bacterium (AY214765) | 91 | CFB group |
| | *Shewanella*, | AC-5 | Uncultured gold mine bacterium D17 (AF337873) | 92 | Unclassified |
| | *Xanthomonas* | | Uncultured Bacteroidetes bacterium (AY214765) | 90 | CFB group |
| | | AC-6 | Uncultured gold mine bacterium D17 (AF337873) | 93 | Unclassified |
| | | | Uncultured Bacteroidetes bacterium (AY214765) | 92 | CFB group |

[a]Putative name was deduced from the RDP database by the length of T-RF. The bold letters indicate putative bacterial genera that corresponded with the genera confirmed by nucleotide sequence analysis.

[b]Nucleotide sequences of three clones were analyzed for each clone library of a distinct T-RF. AS: clones derived from AS sample; AC: clones derived from AC sample.

catalyzing the elongation of a DNA strand by the addition of nucleotides from the surrounding solution. In this study, TdT was used to add a homopolymeric tail to the 3'-end of the

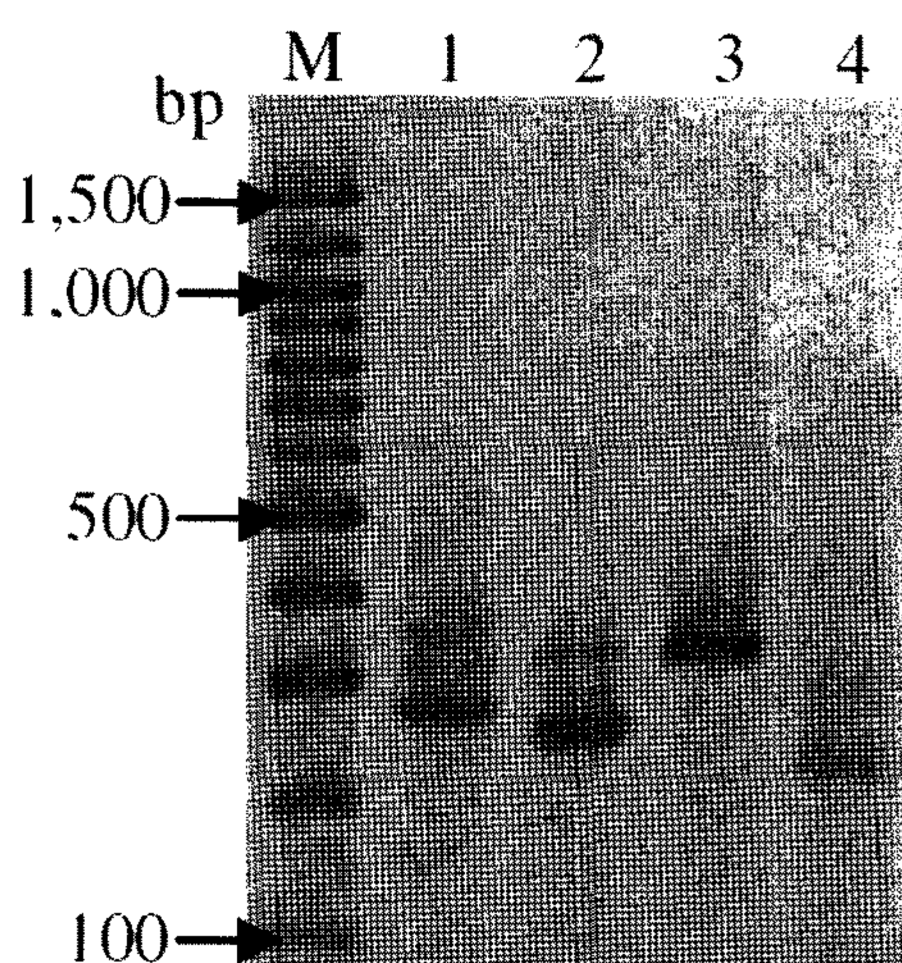


**Fig. 4.** Agarose gel electrophoresis of PCR product of d(G)n-tailed HaeIII T-RFs.

Lanes: M, size marker; 1, *Acinetobacter calcoaceticus*; 2, *Bacillus megaterium*; 3, *Bacillus subtilis*; 4, *Burkholderia cepacia*.

T-RF lost information of the 3'-end primer. The modified method involves six steps: 1) PCR and restriction enzyme digestion, 2) isolation of single-stranded T-RFs (ssT-RFs) from the restriction fragments, 3) gel electrophoresis of ssT-RFs, 4) elution and homopolynucleotides tailing using TdT, 5) PCR of the tailed T-RF, and 6) cloning and sequencing of the T-RF.

This method was evaluated with the reference strains *Acinetobacter calcoaceticus*, *Bacillus megaterium*, *B. subtilis*, and *Burkholderia cepacia*, by using a restriction enzyme (HaeIII) producing blunt ends. The size of the HaeIII-digested T-RF from *A. calcoaceticus*, *B. megaterium*, *B. subtilis*, and *Burkholderia cepacia* was 252 bp, 233 bp, 307 bp, and 202 bp, respectively. These T-RFs of the four strains were eluted from a polyacrylamide gel and a homopolymeric tail was added at the site of the 3'-end by using TdT. The $d(G)_n$-tailed T-RFs were amplified with 27F and tail primers (Fig. 4). The size of amplified T-RFs was 10 bp longer than those of T-RFs of the four reference strains owing to the $d(G)_n$-tail and the use of tail primer.

We were also able to isolate and amplify the HhaI-digested T-RFs of reference strains *A. calcoaceticus* and *E. pyrinus*, which had a complementary single-stranded end (data not shown). The PCR products were cloned and

the nucleotide sequences of the T-RFs were analyzed. The nucleotide sequences were the same as presented in RDP and GenBank databases (GenBank Accession Nos., AJ888984 and AJ010486).

## DISCUSSION

Molecular biological approaches are used to characterize bacterial populations in the field of microbial ecology, and which include 16S rRNA gene cloning and genomic fingerprinting techniques [21]. 16S rRNA cloning is the method to combine construction of a clone library, analysis of the nucleotide sequences, and phylogenetic analysis. It is time-consuming and cumbersome because it requires a large number of clones for good estimation of microbial diversity. Genomic fingerprinting techniques are valuable tools for the characterization of complex microbial communities in the environment. T-RFLP and DGGE methods of genomic fingerprinting technique have been frequently used to examine the microbial diversity of environmental samples and to monitor changes in microbial communities.

The potential of T-RFLP and DGGE to analyze microbial diversity was compared by several authors. Moeseneder *et al.* [16] reported that both T-RFLP and DGGE showed the same clustering topology for the samples taken from the South and North Aegean Sea. However, they found a larger number of OTUs with T-RFLP than with DGGE. T-RFLP and DGGE were also applied to unravel *pmo*A (particulate methane monooxygenase) diversity in bacterial communities, which associated with the roots of submerged rice plants [4]. The *pmo*A-based methanotroph diversity detected by T-RFLP was higher than that by DGGE. Nunan *et al.* [20] reported that T-RFLP had greater resolving power than DGGE, although the bacterial community structure analyzed by these two methods was similar in the sample of rhizosphere soil.

However, the advantage of DGGE is that it can be coupled with additional approaches (elution, re-amplification, cloning, and nucleotide sequence analysis of a selected band) to identify a distinct band. Sequencing of a specific band in T-RFLP is very difficult, although several methods were suggested for resolving this problem. Kim *et al.* [8] reported that the major T-RFs could be amplified with eubacterial primers 27F and 263R (*E. coli* numbering 246–263: 5'-TTACCCCACCAACTAGCT-3') [8]. Nevertheless, this method is not available for the T-RF less than 255 bp. Mengoni *et al.* [15] developed a rapid screening method of specific T-RFs based on oligonucleotide adapter ligation. However, this method requires many clones for identification of a specific T-RF because whole T-RFs are eluted from an agarose gel and cloned. Widmer *et al.* [27] were able to isolate and identify a specific T-RF on an Elchrom-1200 gel by using the oligonucleotide adapter. However, this method is not able to isolate a specific T-RF before the size of the T-RF is obtained by using an automated DNA sequencer. Moreover, both methods are effective against the only restriction enzymes producing complementary single-stranded ends. Other methods for cloning and identifying T-RFs used a modified PCR primer in the technique of physical capture T-RFLP, which was labeled with biotin and had an additional restriction site at the 5'-end [2, 19]. This physical capture of T-RFLP profiles showed very similar banding patterns when compared with those obtained by fluorescent T-RFLP profiles. However, this method resolved fewer T-RFs than fluorescent T-RFLP because T-RFs were electrophoresed on an agarose gel [2].

Here, we have developed two new methods to analyze the nucleotide sequences of T-RFs. The oligonucleotide adapter and terminal deoxynucleotidyl transferase (TdT) were applied to the physical capture T-RFLP method. In this study, we succeeded in sequencing the major T-RFs generated from the reference strains and activated sludge samples by using the two modified T-RFLP methods. Most of the T-RF nucleotide sequences obtained from the activated sludge samples were related to the sequences of uncultivated bacteria presented in GenBank. This result suggests that the culture-independent molecular biological technique such as T-RFLP is an important method to characterize bacterial communities in the environment. Bacteria belonging to the class β-Proteobacteria were predominant in both AS and AC sludge samples, as presented [12, 25]. In the AS sample, the similarities between the nucleotide sequences of three clones constructed from a single band of T-RF were not high. It suggests that a single band in the T-RFLP profile may be generated from several species or genera. The sequences obtained from the T-RF band of 216 bp was not related to the taxonomic groups predicted by *in silico* restriction of sequences in the RDP database. These results confirm that the nucleotide sequence of the T-RF is more important for obtaining taxonomic information of the microorganisms responsible for a distinct T-RF.

Our methods have some advantages in comparison with the previously reported techniques. In our methods, the specific T-RF was eluted and re-amplified from the polyacrylamide gel with higher resolution. Thus, we would accomplish the direct sequencing of a T-RF needing taxonomic interpretation, without a large clone library. Our methods have the advantage of a more simple and rapid processing compared with other methodologies and does not need special equipments or screening of a relatively large size of clone library. In particular, the method based on TdT is able to use the restriction enzymes (such as HaeIII) producing a blunt end as well as a sticky end (such as HhaI). Therefore, we suggest that these methods could be useful tools for determining the taxonomic information of T-RFs.

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