[SI-2]

Comparison of Terminal-restriction Fragment Length Polymorphism (T-RFLP) Analysis and Sequencing of 16S rDNA Clones in marine sediments

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Abstract

Terminal-restriction fragment length polymorphism (T-RFLP) analysis has been optimized by using in vitro model community composed of genomic DNAs of known bacterial strains and has been applied to assess the bacterial community structure in marine sediments. The specific fluorescence-labeled terminal restriction fragments (T-RFs) between 39 and 839 base long specifying each strain were precisely measured for known bacterial strains. The addition of a co-solvent (dimethylsulfoxide or glycerol) into PCR reactions has reduced differential PCR amplification. Comparative bacterial community structure was investigated for pristine and polluted sediments. A complex T-RFLP pattern showing complex bacterial community structure was obtained in the pristine sediment, whereas simple T-RFLP pattern (low bacterial diversity) was shown in polluted sediments where caged aquaculture has been conducted for several years. The results of T-RFLP analysis were compared with that of cloning and sequencing 16S rDNA clones from the same sediments. Sequence analysis of 16S rDNA clones (72) of the pristine sediment revealed a diverse collection of lineages, largely of the class *Proteobacteria* (6% alpha subdivision, 46% gamma subdivision, 13% delta subdivision, and 3% epsilon subdivision), *Nitrospina* (8%), high G+C gram positive (8%), *Verrucomicrobia* (7%), and Planctomycetes (6%) In the contaminated sediments, 17 (59%) of the 16S rDNA clones (29) were related to *Campylobacter* and symbiont of *Rimicaris exoculata* belonging to epsilon subdivision of *Proteobacteria*. The results obtained indicated that T-RFLP analysis is a rapid and precise technique for comparative bacterial community analysis.

Introduction

Defining the diversity and structure of natural microbial communities through the quantification of their constituent populations has been a long-standing challenge on microbial ecology. Cultivation as an approach for the description of naturally occurring microbial communities has serious limitations. The majority (typically >90 to 99%) of bacteria in nature cannot be cultivated by using traditional techniques. The application of molecular approaches to characterize bacterial communities has overcome the requirement for prior cultivation of community members. Various molecular methods to assess microbial diversity have been developed without need for isolation and cultivation of microorganisms. Among them, terminal restriction fragment length polymorphism (T-RFLP) analysis is regarded as a robust and reproducible methodology for the rapid analysis

of microbial community structure in different samples and for the study of dynamics and changes in community structure in response to changes in prevailing physicochemical parameters. T-RFLP analysis measures the size polymorphism of termial restriction fragments (TRFs) from a PCR amplified marker. It is an assemblage of at least three technologies including comparative genomics/RFLP, PCR, and nucleic acid electrophoresis. The use of fluorescently tagged primer limits the analysis to only the TRFs of the digestion. The T-RFLP pattern produced from amplified 16S rDNA is a simplified representation of the bacterial community in natural environments. In this study, T-RFLP analysis has been optimized by using in vitro model community composed of genomic DNAs of known bacterial strains and was applied to assess the bacterial community structure of marine sediments in comparison with cloning and sequencing.

Materials and Methods

Microbial model community

Mixture of bacterial genomic DNAs was used as a model community. Used bacterial strains are *Arthrobacter globiformis* 168 DSM 20124 (T) (KCTC 9101), *Bacillus megaterium* DSM 32 (KCTC 3007), *Bacillus subtilis* KCTC 3135, *Escherichia coli* ATCC 25922 (KCTC 1682), *Pseudomonas aeruginosa* DSM 50071 (KCTC 1750), and *Sphingomonas* KH3-2. These strains except for KH3-2 were obtained from KCTC (Korean Collection for Type Cultures, Daejeon, Korea) and were cultured in small scale (2 ml) according to the KCTC manual. Strain KH3-2 isolated in our Lab. was cultured using Zobell 2216e medium (Bacto peptone 5 g, Bacto yeast extract 1 g, $FePO_4$ 10 mg, aged sea water 750 ml, distilled water 250 ml, pH 7.0) at 30°C. Genomic DNAs were extracted using Wizard genomic DNA kit (Promega).

Marine sediments

Marine sediment samples were collected using Grab sampler from aquaculture area in southern coast of Korea, August 1998. Three kinds of marine sediment samples were pristine (D-1; non-contaminated), moderately contaminated (B-1) and heavily contaminated (E-1). The sediments samples were aliquoted (0.5 ml) into sterile eppendorf tube and were freezed on dry ice before storage at -70°C.

DNA extraction

Total DNAs from marine sediment samples were extracted by mechanical method for avoiding selective cell disruption. A bead beating protocol (Kuske et al., 1998) was adapted with modifications.

T-RFLP analysis

A forward primer 27F (5'-AGA GTT TGA TCM TGG CTC-AG3') and a reverse primer 1522R (5'-AAG GAG GTG ATC CAN CCR CA- 3') were used for amplification of 16S rDNA. The fluorescent primer (27F-Hex) was prepared by 5'-end labeling with phosphoramidite fluorochrome, 5-hexachlorofluorescein (Perkin Elmer, USA). PCR mixture contains 1× buffer (50 mM Tris-HCl, 0.1% TritonX-100, 1.5 mM $MgCl_2$), 0.2

mM dNTPs, 0.2 mM each primer, 10-100 ng of template, and 2.5 U of *Taq* DNA polymerase (Takara) in 50 ul reaction volume. PCR amplifications were done by an initial denaturation step of 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1min, and extension at 72°C for 2 min. The reaction was completed by a final extension at 72°C for 7 min. During the optimization of PCR conditions using model community, various PCR conditions differing in cycle number, annealing temperature, and additive co-solvents were tested for alleviation of preferential PCR. The PCR products were purified with the Wizard PCR purification kit (Promega). Each digest mixture contained 5 μl of cleaned PCR products, 5 units of restriction enzymes (Promega), and the respective restriction buffer at 1 × and was filled up to a final volume of 20 μl with deionized water. After restriction reaction, excess primers and salts were removed by ethanol precipitation. The precise lengths of the T-RFs were determined by electrophoresis with a model 377 automatic sequencer (Applied Biosystems Instruments). Lengths of fluorescently labeled T-RFs were determined by comparison with internal standards (Rox 2500; ABI) by using GeneScan analysis software (version 3.1).

16S rDNA cloning and sequencing

The 16S rDNAs amplification was performed in three samples (B-1, D-1, and E-1) as described above condition without primer labeling. PCR product was ligated into pGEM-T Easy vector (Promega) and transformed into competent *E. coli* JM109 cells. The 16S rDNA transformants were chosen at random and were sequenced by using automatic sequencer ABI 377. The prediction of TRF size of each clone was determined from those sequences. Distribution of TRFs in each samples were compared between T-RFLP analysis and clone sequences.

Results and Discussions

Comparison of T-RFs size between predicted and observed results

The expected size of terminal-restriction fragments (TRFs) for 16S rDNA of each strain with tetrameric restriction enzymes (*Alu*I, *Hae*III, *Hha*I, *Msp*I, *Rsa*I, and *Nru*I) was compared with those of observed T-RFs from the experiment using each genomic DNA (Table 1). Experimentally observed size of the T-RFs derived from various restriction enzymes coincided with that of expected T-RFs in the range from –4 to +3 base difference. Extra TRFs with larger size and lower intensity were observed in *Arthrobacter globiformis* and *Bacillus subtilis*. This may be derived from either partial digestion of amplified products or heterogeneity of rDNA sequences in a single microorganism such as *B. subtilis*. It was observed that *B. subtilis* has 10 copies of heterogeneous 16S rRNA gene per genome.

Optimization of T-RFLP analysis in model community

T-RFLP analysis by *Hha*I has been conducted with model community composed of equivalent quantity of each bacterial genomic DNAs. Although the expected T-RF peaks for each bacterial strain were simultaneously appeared, peak height or relative peak area was differed so much. Among community members, the T-RF specifying *A. globiformis* was the least. The effect of PCR condition varying annealing temperature (50, 55,

and 60°C) and cycle number (25, 30, 35 cycles) on the T-RFLP analysis has been studied. Optimized condition for PCR amplification was annealing temperature at 55°C and 30 cycle numbers, though much improvement was not obtained. The information of genome size and number of 16S rRNA gene copy per genomes was available only for *B. subtilis, E. coli*, and *P. aeruginosa*. The theoretical ratio of 16S rRNA gene copy number per equivalent genomic DNAs and its relative peak area of three components could be calculated and compared with that of experimental results (Table 2). Supposed that non-preferential PCR was obtained, the peak height ratio of *B. subtilis, E. coli*, and *P. aeruginosa* should be 3.541:2.246:1.000, respectively. The observed relative peak heights of *E. coli*, and *P. aeruginosa* were reduced compared to the calculated values. But the peak for *B. subtilis* was increased (Table 2).

Effect of co-solvents as an additive on PCR

Upon a model community composed of genomic DNAs from three strains (*B. subtilis, E. coli*, and *P. aeruginosa*), effect of additive co-solvents during PCR on T-RFLP profile was analyzed. The addition of solvents (glycerol, dimethyl sulfoxide, and mixture of solvents) into PCR has increased the intensity of *P. aeruginosa* in T-RFLP analysis (Fig. 1). Among them, addition of 5% DMSO and 10% glycerol was seemed to reduce preferential amplification. In following experiment, addition of 5% DMSO was selected for T-RFLP analysis of complex natural microbial community.

T-RFLP analysis of natural environments and comparison with sequencing clones

A complex T-RFLP profile showing complex bacterial community structure was obtained in the pristine sediment (sediment D-1), whereas simple T-RFLP pattern (low bacterial diversity) was shown in polluted sediment (E-1) where caged aquaculture has been operated for several years. In the sediment (B-1) of aquaculture area where aquaculture cage were constructed recently, the T-RFLP pattern was similar to that of D-1 except for the appearance of one unique peak (T-RF; 549/551 bp) designated as 'a' in Fig. 2. The peak 'a' was the most abundant in sediment, E-1. The 'a' peak may be an indicator for organic contamination from excessive feed. To confirm the results of T-RFLP analysis were compared with that of cloning and sequencing 16S rDNA clones from the same sediments (Fig. 2). Consequently, the T-RFLP profile of sediment D-1 was very similar with profiles derived 16S rDNA clones.

Comparison of bacterial community structure

Sequence analysis of 16S rDNA clones (No. 72) of the pristine sediment (D-1) revealed a diverse collection of lineages, largely of the class *Proteobacteria* (6% alpha subdivision, 46% gamma subdivision, 13% delta subdivision, and 3% epsilon subdivision), *Nitrospina* (8%), high G+C gram positive (8%), *Verrucomicrobia* (7%), and Planctomycetes (6%). In the contaminated sediments (E-1), 17 (59%) of the 29 16S rDNA clones were related to *Campylobacter* and symbiont of *Rimicaris exoculata* belonging to epsilon subdivision of *Proteobacteria*. In the sediment of B-1 (newly constructed aquaculture area), the community structure was similar to that of D-1 but distinctive epsilon subdivision of *Proteobacteria* of E-1 sediment was also present in high percentage (25%).

Conclusions

Terminal-restriction fragment length polymorphism (T-RFLP) analysis has been optimized using in vitro model community composed of genomic DNAs of known bacterial strains. The preferential amplification of 16S rDNA was alleviated by the addition of co-solvents (glycerol and DMSO) during PCR. T-RFLP analysis has been applied to assess the bacterial community structure in marine sediments. A complex T-RFLP pattern showing complex bacterial community structure was obtained in the pristine sediment, whereas simple T-RFLP pattern (low bacterial diversity) was shown in polluted sediments where caged aquaculture has been conducted for several years. The results of T-RFLP analysis were consistent with that of cloning and sequencing 16S rDNA clones from the same sediments. Sequence analysis of 72 16S rDNA clones of the pristine sediment revealed a diverse collection of lineages, largely of the class *Proteobacteria* (6% alpha subdivision, 46% gamma subdivision, 13% delta subdivision, and 3% epsilon subdivision), *Nitrospina* (8%), high G+C gram positive (8%), *Verrucomicrobia* (7%), and Planctomycetes (6%). In the contaminated sediments, 17 (59%) of the 29 16S rDNA clones were related to *Campylobacter* and symbiont of *Rimicaris exoculata* belonging to epsilon subdivision of *Proteobacteria*. These results indicated that T-RFLP analysis is a rapid and precise technique for comparative bacterial community analysis. T-RFLP analysis could be used for differentiation of microbial communities, for comparison of the relative phylotype richness and structure of communities, and for identifying specific microorganisms in a community.

References

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Table 1. Observed and expected lengths of 5'-terminal restriction fragments (5'-TRFs) for bacterial strains in model microbial community

Strain	Observed (expected) 5'-TRF(s) in base [a]				
	*Alu*I (AG▼CT)	*Hae*III (GG▼CC)	*Hha*I (GCG▼C)	*Msp*I (C▼CGG)	*Rsa*I (GT▼AC)
Arthrobacter globiformis DSM20124[T]	**201**, 237 (201)	229 (229)	474 (472)	67 (67)	459 (456)
Bacillus megaterium DSM32	75 (74)	234 (234)	581 (578)	167 (167)	461 (458)
Bacillus subtilis KCTC3135	74 (73)	310 (309)	241 (240)	145 (145)	**458**, 477 (456)
Escherichia coli KCTC1682	75 (75)	38 (39)	373 (373)	497 (496)	430 (427)
P. aeruginosa DSM50071	73(72)	38 (39)	155 (155)	143 (143)	649 (644)
Sphingomonas sp. KH3-2	220 (220)	239 (239)	82 (82)	150 (150)	839 (839)

[a]; Bold numbers denote main peak.

Table 2. Effect of genome size and copy number of 16S rRNA genes in the T-RFLP analysis

Strain	Genome size (kb)	16S rRNA gene copy number per genome	Ratio of 16S rRNA gene copy per equivalent each genomic DNA and percentage of predicted relative peak height	Observed peak height and percentage of peak height	Deviation from predicted value (%)[a]
B. subtilis	**4,165**	**10**	**3,541 (52)**	**815 (66)**	**▲27**
E. coli	**4,595**	**7**	**2,246 (33)**	**303 (25)**	**▼24**
P. aeruginosa	**5,900**	**4**	**1.000 (15)**	**113 (9)**	**▼40**

a; ▲ increase; ▼ decrease.

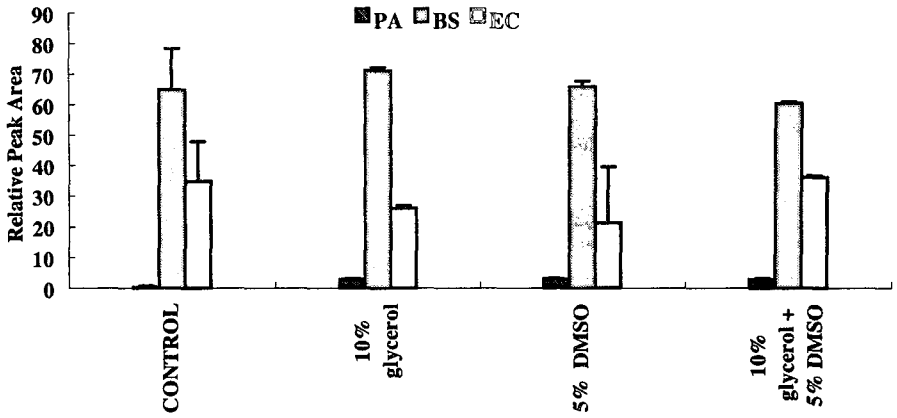

Fig. 1. Effect of additive solvents on peak height and relative peak area in the T-RFLP analysis.

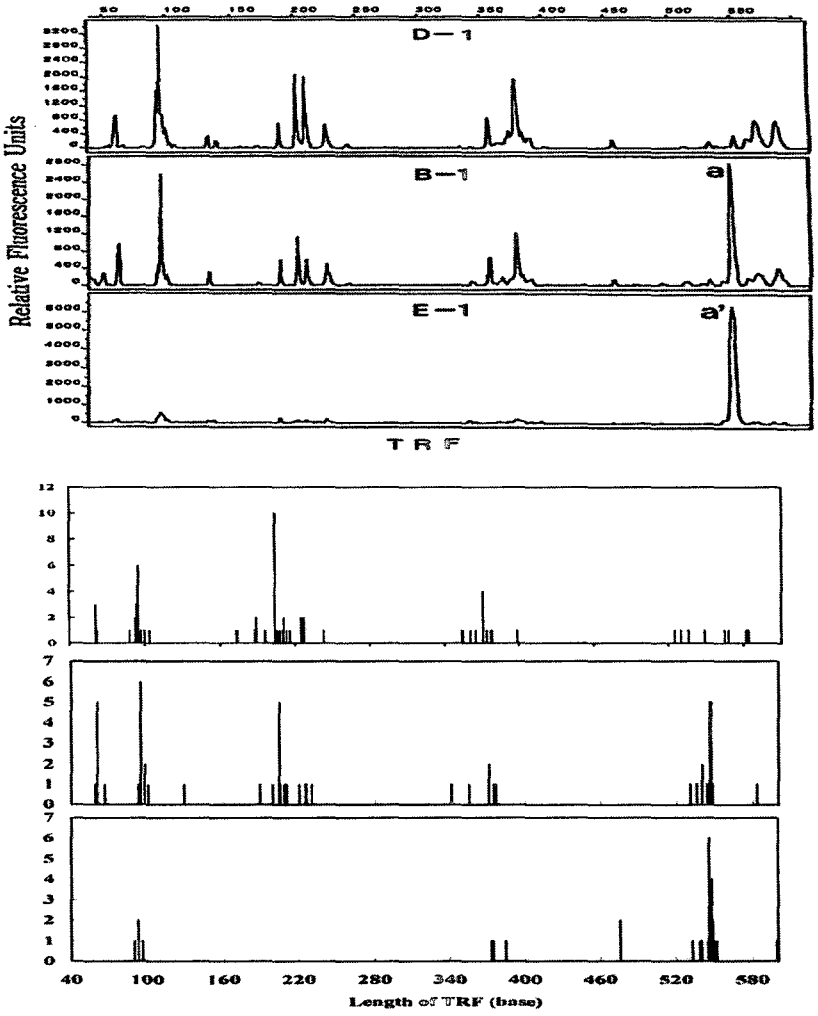

Fig. 2. Comparison of the 5' T-RFLPs (*Hhal*) electropherograms of 16S rDNAs amplified from total DNA and T-RFLP profile deduced from obtained clones in marine sediments, B-1 (moderately contaminated), D-1 (not contaminated as control), and E-1 (heavily contaminated).