Application of terminal restriction fragment length polymorphism to environmental biotechnology

T-RFLP 法の環境バイオテクノロジーへの応用

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Chapter 1

GENERAL INTRODUCTION

The potential of microorganisms for environmental biotechnology and fingerprinting techniques of microbial community

SUMMARY

The Molecular biology have been developed rapidly over the last few decades and shown that the diversity of environmental microorganism is greater than initially anticipated. Moreover, more than 99% of the environmental microorganisms are unculturable. These microorganisms have the potential to be utilized as two major aspects of biotic function and biological resources. In either case, to utilize the microorganisms more effectively, it is important for obtaining the microbial community fingerprints. Recently, some molecular methods have been developed for analysis of the microbial community structure of environmental samples. Especially, terminal restriction fragment length polymorphism (T-RFLP) is highly effective in fingerprinting the microbial community structure. Moreover, a combination of cloning and/or statistical analysis with T-RFLP is available for monitoring or screening the samples. However, sufficient studies combined with cloning and/or statistical analysis have not been conducted for monitoring microbial community transition or screening environmental samples. Hence, it is significant that T-RFLP combined with cloning and/or statistical analysis is applied to obtain the microbial community fingerprints for development of environmental biotechnology and utilization of environmental microorganisms.

1.1 INTRODUCTION

The field of microbial ecology was revolutionized by molecular biology techniques, which are independent of cultivation, based on 16S rRNA or DNA gene sequence analysis (25, 41). Before then, scientists could not appreciate the enormous difference between the number of microbes cultured in the laboratory and those present in the environment. The application of universal primers for the direct PCR amplification of 16S rRNA genes from total community DNA combined with cloning and sequencing technologies has revealed that the microbial world is enormously diverse and indicated that more than 99% of environmental microorganisms can not be easily cultivated (8, 26, 38).

The environmental microorganisms have the potential to be utilized as two major aspects of biotic function and biological resources. In terms of biotic function, the environmental microorganisms have been utilized for environmental cleanup such as biological wastewater treatment, biodegradation and bioremediation. We took particular note of the biological wastewater treatment using activated sludge because of one of the most significant biotechnological processes. Understanding the structure of microbial communities in the activated sludge is desirable for correct operation and development of wastewater treatment processes (29, 37). Thus, monitoring community succession is necessary to understand and predict future changes in community structure in natural and managed ecosystems (35). On the other hand, in terms of biological resources, the environmental microorganisms present enormous genetic pool that remains mostly unexploited for biotechnology. Metagenomic analysis, which is the culture-independent genomic analysis of the microbial community, has been developed for the last decades and attempted to obtain novel genes (31). In the

metagenomic approach, the target genes represent a small proportion of the total nucleic acid fraction. Thus, pre-enrichment or screening of the sample thus provides an attractive means of enhancing the positive hit rate (2). Therefore, in either case, to utilize the environmental microorganisms more effectively, it is important for understanding the microbial community, especially in obtaining the microbial community fingerprints.

Recently, some culture-independent molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP) and terminal restriction fragment length polymorphism (T-RFLP) have been developed for analysis of the microbial community structure of environmental samples (5, 16, 23). For obtaining the microbial community fingerprints, rapid profiling procedures are necessary. Although DGGE and SSCP allow the analysis of multiple samples, the community fingerprints obtained by these techniques do not translate directly into taxonomic information. T-RFLP has been shown to be reliable fingerprints of complex bacterial communities (24, 28). Moreover, a combination of cloning and/or statistical analysis with T-RFLP is available for monitoring or screening the samples.

A combination of cloning and T-RFLP provides useful information about monitoring the particular bacteria. Although T-RFLP is suitable for this purpose, it has only been applied to monitor spatial (1) and seasonal changes (18) in the microbial communities, and very few attempts have been made at monitoring daily changes. Meanwhile, a combination of T-RFLP with statistical analysis such as principal component analysis and multidimensional scaling (MDS) analysis provides useful information about visualizing the structure of microbial communities (3). However, there are very few studies on screening the environmental samples in metagenomic analysis by applying for T-RFLP combined with statistical analysis. Hence, it is significant that T-RFLP combined with cloning and/or statistical analysis is applied to obtain the microbial community fingerprints for development of environmental biotechnology and

utilization of environmental microorganisms.

This chapter introduces the potential of the microorganisms for environmental biotechnology such as wastewater treatment processes and metagenomic analysis. Moreover, we describe the principles, advantages, and disadvantages of fingerprinting techniques.

1.2 The potential of the microorganisms for environmental biotechnology

1.2.1 Biological wastewater treatment

Biological wastewater treatment using activated sludge is one of the most significant biotechnological processes with a lot of microorganisms. Especially, biological nitrogen removal processes are important because nutrient salts such as nitrogen cause eutrophication (39). The biological nitrogen removal processes consists of nitrification and denitrification.

Generally, autotrophic bacteria such as ammonia oxidizing bacteria and nitrite oxidizing bacteria play a main role in nitrification. On the other hand, heterotrophic bacteria take an active part in denitrification. Thus understanding the structure and stability of microbial communities is desirable for correct operation of wastewater treatment processes because particular microbial communities are responsible for the success or failure of the process. Many studies have addressed microbial community structure in activated sludge (11, 12). However, very few studies have centered on monitoring of microbial succession and the correlation between the stability of the microbial community and the performance of systems. Hence, monitoring the microbial communities is necessary to understand and predict future changes in community structure in biological wastewater treatment processes.

1.2.2 Metagenomic analysis

Current research indicates that more than 99% of environmental microorganisms can not be easily cultivated (26). Thus, the genomes of uncultured microorganisms represent unexploited resource for novel genes and chemical compounds for the development of novel products (17). Metagenomic analysis, which is the culture-independent genomic analysis of the microbial

community, has been developed for the last decades and attempted to obtain novel genes (31).

Two major strategies have been used to identify novel genes or biocatalysts in metagenomic analysis (13). Function-based approach for novel genes has been performed by detecting of the enzymatic activity on the plates. Recently, functional-based approach has been performed with high-throughput screening techniques such as substrate-induced gene expression screening (SIGEX) (34) and metabolite regulated expression screening (METREX) (40). On the other hand, sequence-based approach has been performed by PCR with oligo primers targeting the conserved region of gene families which were designed on the basis of the sequence information in databases. In the metagenomic analysis, the target genes represent a small proportion of the total nucleic acid fraction. Statistically, for small insert (<10kbp) library, between 10^5 and 10^6 clones need to be obtained for a single positive clone (9). Thus, pre-enrichment or screening of the sample provides an attractive means of enhancing the positive hit rate (2).

Therefore, in either case, to utilize the environmental microorganisms more effectively, it is important for understanding the microbial community, especially in obtaining the microbial community fingerprints.

1.3 The principles, advantages, and disadvantages of fingerprinting techniques

Fingerprinting techniques have been used to easily examine microbial communities. Microbial community analyses using fingerprinting techniques were reviewed in detail in Ikeda *et al*. (10). Briefly, the principles, advantages, and disadvantages of DGGE, SSCP, and T-RFLP are described as follows.

Denaturing gradient gel electrophoresis (DGGE)

Tendency for double-strand DNA to be denatured depends on DNA sequence because connection-strength of double-strand DNA is influenced by GC-content and order of base of DNA sequence. DGGE can detect this difference by a polyacrylamide gel, which usually includes an increasing gradient of denaturants such as formamide and urea (19). Briefly, PCR targeting 16S rRNA gene is performed using special forward primer, which has unique GC-rich base pair (GC-clamp) at the 5' end, to prevent double-strand DNA from being thoroughly denatured. After PCR products are electrophoresed in the denaturing gradient polyacrylamide gel, many bands will be observed (20, 21). Each band represents a microbial species. In addition, DNA sequences of bands, which are sliced from the gel, can be analyzed to examine microbial species (5).

Single strand conformation polymorphism (SSCP)

Secondary structures of single-strand DNAs are determined by the nucleotide sequences. SSCP can detect this difference by a non-denaturing polyacrylamide gel owing to different mobility that depends on the secondary structures of single-strand DNAs (23). After

single-strand DNAs of PCR products, which are produced by alkaline solution or heat/cool conditions, are electrophoresed in the non-denaturing polyacrylamide gel, many bands will be observed (15).

Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is based on differences of digestion sites of DNA sequences by restriction enzyme among different microorganisms (16). Briefly, PCR products using special forward primer, which contains a fluorescent dye such as 6-carboxyfluorescein (6-FAM), are digested with an appropriate restriction enzyme and then electrophoresed in a capillary sequencer to detect only terminal fragments labeled with the fluorescent dye. After electrophoresis, many fragments will be observed. Each fragment represents a microbial species. The size of fragment can be obtained on the basis of internal size standard. In addition, existence of each microbial species will be observed by height or area of each fragment (18).

Comparison of the advantages and disadvantages of the current molecular techniques for microbial community analysis

Molecular techniques based on 16S rRNA gene sequences allow microbial community analyses without cultivation to investigate the community structure, diversity, and phylogeny of microorganisms (32). However, careful interpretation is needed because each physical, chemical, and biological step such as DNA extraction and PCR in the molecular techniques is a source of bias (36). In addition, Forney *et al.* (6) reported in detail molecular microbial ecology. Fingerprinting techniques are briefly described as follows. A prime advantage is relative simplicity and low cost. Since DGGE by gel electrophoresis has not so high resolution, it is difficult to compare the data obtained in different laboratories, whereas T-RFLP by capillary sequencer is powerful for rapidly comparing the microbial diversity owing to high resolution

and high sample throughput.

Furthermore, single DGGE band may represent different microbial species (5, 7, 14). In addition to the detection limit of DGGE, re-amplificaion of the background smear bands (22) and microheterogeneity within 16S rRNA gene sequences (27, 30) were reported as DGGE biases. Meanwhile, T-RFLP has reproducibility and robustness by automatically analyzing samples using capillary sequencer (24). However, appropriate restriction enzymes and reaction condition have to be selected. More than two restriction enzymes should be used for microbial community analysis (4, 33). Microbial diversity may be overestimated because of partially digested T-RFLP fragments by incomplete enzymatic-digestion (24). Other techniques such as clone library are required for further examining microbial communities in addition to T-RFLP because microbial species cannot be identified only by T-RFLP fragments (1). Statistical analysis in addition to T-RFLP also provides useful information about microbial community structure (3).

1.4 OBJECTIVE

The objective of this study is to demonstrate the efficacy of T-RFLP in applying for environmental biotechnology under the following points:

1. Evaluation of monitoring the microbial communities in the wastewater treatment processes through T-RFLP and MDS analysis,

2. Evaluation of screening the environmental samples through T-RFLP and MDS analysis.

In detail, the efficacy of T-RFLP in monitoring the microbial communities in the wastewater treatment processes is described in chapter 2-4. Furthermore, the efficacy of T-RFLP in screening the environmental samples is described in chapter 5 and 6.

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Chapter 2

Monitoring the microbial population dynamics at the start-up stage of wastewater treatment reactor by terminal restriction fragment length polymorphism analysis based on 16S rDNA and rRNA gene sequences

SUMMARY

The microbial population dynamics at the start-up stage of a wastewater treatment reactor was investigated using terminal restriction fragment length polymorphism (T-RFLP) analysis based on 16S rDNA and rRNA gene sequences. The results of fragment peaks suggest that the number and activity of nitrifying bacteria increase in association with the start of nitrification, and the relative ratios of 16S rRNA of these bacteria change prior to those of the 16S rDNA. Furthermore, multidimensional scaling (MDS) analysis has revealed that the 16S rRNA exhibits wider dispersion than the 16S rDNA at the start-up stage, indicating that the diversity of 16S rRNA in the microbial communities is strongly affected by environmental changes.

2.1 INTRODUCTION

Activated sludge systems are widely used as a method of biological wastewater treatment. Microbial populations in the activated sludge may be markedly affected by environmental changes (1, 2, 5, 12). Thus, understanding the microbial population is very important to maintain and develop biological wastewater treatment systems. However, due to their being time-consuming and labor-intensive, cloning and denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA are unsuitable for the monitoring of microbial populations in such systems.

Recently, terminal restriction fragment length polymorphism (T-RFLP) analysis has been shown to be a reliable fingerprint of complex bacterial communities and provides information on the number of different fragments and their relative abundance (6, 9, 11). Due to its high resolution, T-RFLP analysis of 16S rDNA was successfully used to detect changes in the microbial community structure in soil environments, having high levels of microbial species diversity (4, 7). However, there has not been any detailed investigation of the relationship between T-RFLP analyses of 16S rDNA genes and 16S rRNA. On the other hand, several statistical analyses have been applied to the results of T-RFLP analysis. For example, principal component analysis is a preferred method for the reduction of complex data and has been frequently used to facilitate the comparison of microbial communities with data derived from amplified gene restriction patterns (3, 10). Multidimensional scaling (MDS) analysis is also a preferred method for visualizing patterns of time-dependent microbial community shifts based on the results of T-RFLP analysis.

The purpose of this chapter is to evaluate the effectiveness of monitoring microbial

community shifts in activated sludge through T-RFLP analysis based on 16S rDNA and rRNA genes and subsequent MDS analysis for the process control of a biological wastewater treatment system.

2.2 MATERIALS AND METHODS

2.2.1 Reactor operation and sampling

The reactor structure and operating conditions are shown in Fig. 1 and Table 1. The reactor with a working volume of 5.5 *l* was operated with intermittent aeration providing aerobic conditions for 30 min and anaerobic conditions for 60 min. The hydraulic residence time was 20 hs. Initially, the concentration of mixed liquor suspended solids was 3500 $mg \cdot l^{-1}$. The sludge return ratio was kept at 50%. The characteristics of the synthetic wastewater were as follows: total organic carbon, 90 $mg \cdot l^{-1}$; total nitrogen, 40 $mg \cdot l^{-1}$; and total phosphorus, 5.0 $mg \cdot l^{-1}$. Ammonia-nitrogen (NH_4-N), nitrite-nitrogen (NO_2-N), and nitrate-nitrogen (NO_3-N) contents of the effluent at sampling were measured with a TRACCS-800 analyzer (Bran+Luebbe, Tokyo). For the characterization of bacterial communities, 10-ml aliquots of liquid samples were collected on days 0, 3, 7, 9, 13 and 18. The samples were centrifuged at 10,000 × *g* for 5 min, and the resultant pellet was resuspended in 2.0 ml of Tris-EDTA (TE) buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and stored at -80°C.

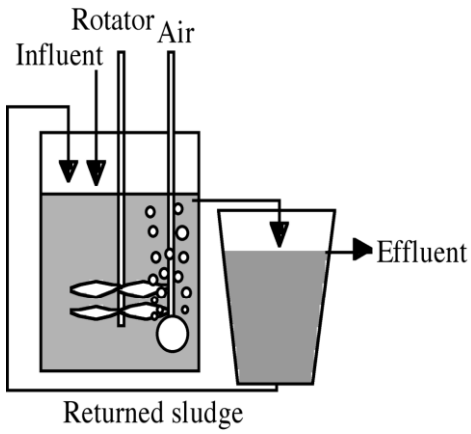

Fig. 1 Schematic diagram of biological wastewater treatment reactor

Table 1 Reactor specifications and operating conditions

Volume (*l*)	5.5
Hydraulic retention time (h)	20
Aerobic condition (min)	30
Anaerobic condition (min)	60
Suspended solids (mg·l^{-1})	3,500
TOC* (mg·l^{-1}) in influent	90
T-N* in influent (mg·l^{-1})	40
T-P* in influent (mg·l^{-1})	5.0

*Total organic carbon, nitrogen, phosphorus, respectively.

2.2.2 DNA extraction

DNA was extracted from samples with the ISOPLANT kit (Nippon Gene, Tokyo), according to the manufacturer's instructions, except for the beadbeating step. DNA was liberated from the cells by ballistic disintegration with 1 g of sterile glass beads (0.1 mm in diameter) in a bead beater at 5000 × *g* for 1 min. The concentration of extracted DNA was adjusted to 10 ng·μl^{-1}.

2.2.3 RNA extraction and reverse transcription

RNA was extracted from samples with the ISOGEN kit (Nippon Gene), according to the manufacturer's instructions, except for the beadbeating step as described above. Then, genomic DNA was removed from extracted RNA with DNase I (Takara Bio, Shiga), according to the manufacturer's instructions. A reverse transcription (RT) reaction of purified RNA (20 ng) was performed using a random primer. Reaction mixtures contained 2.0 μl of RNA template, and 10 mM deoxyribonucleoside triphosphate (Takara Bio), 5 U of RNase inhibitor, 5 × RT buffer, 25 μM of RT primer, 50 U of RT polymerase (ReverTra Ace; Toyobo, Osaka), and RNase-free water to achieve a final volume of 10 μl. The RT reaction mixtures were preheated at 30°C for 10 min, incubated at 42°C for 50 min, and inactivated at 99°C for 5 min. Then, the mixtures were quick-chilled at 4°C for 5 min.

2.2.4 PCR conditions

PCR amplifications of 10 ng of DNA and cDNA using the universal primers 8f and 926r (6) were performed with a GeneAmp PCR System 9700 (PerkinElmer, Wellesley, Massachusetts, USA). Reaction mixtures contained 2.5 μM of each primer, 0.2 mM deoxyribonucleoside triphosphate, 2.5 mM of $MgCl_2$, 10 × PCR buffer (500 mM KCl and 100 mM Tris-HCl, pH 8.3) and 1 U of *Taq* polymerase (Toyobo), and sterilized water to achieve a final volume of 50 μl. PCR was performed under the following conditions: initial denaturation at 94°C for 1 min; 30 cycles of 94°C for 20 s, 53°C for 10 s, and 72°C for 30 s; and a final extension step at 72°C for 1 min. Forward primers for amplification of bacterial 16S rDNAs were 5'-end labeled with 6-carboxyfluoresce.

2.2.5 Purification of PCR product and restriction enzyme digestion

PCR products were verified by electrophoresis on 2% (wt/vol) agarose gels, and by staining with 10 $\mu l \cdot l^{-1}$ ethidium bromide. Then, 40 μl of the products were purified with a Wizard(R) SV Gel and PCR Clean-Up System kit (Promega, Madison, Wisconsin, USA). Aliquots (10 μl) of purified PCR products were digested with *Hae*III [GG'CC] (where the prime shows the site of cleavage), *Hha*I [GCG'C], and *Msp*I [C'CGG] in a water bath for 4 hs at 37°C.

2.2.6 T-RFLP analysis

Aliquots (8 μl) of the digest were purified with standard ethanol precipitation procedures. The precipitate was mixed with 0.3 μl of the internal size standard ROX500 (Applied Biosystems, Foster City, California, USA) and 10 μl of deionized formamide. After denaturing the DNA at 94°C for 5 min and immediate chilling on ice, the fluorescently labeled terminal fragments were analyzed by electrophoresis on an ABI 3100 avant Genetic Analyzer (Applied Biosystems) in GeneScan mode. The injection time was 60 s and the run time was 40 min. To avoid detection

of primers and uncertainties of size determination, terminal fragments smaller than 30 bp and larger than 900 bp were excluded from the analysis. Reproducibility of patterns was confirmed by repeated T-RFLP analyses using the same DNA extracts. Microbial communities were characterized by species richness (the numbers of peaks) and species evenness (the heights of the peaks). Lengths of 16S rDNA terminal fragments were theoretically compared to those of aligned sequences using the TAP T-RFLP function of the Ribosomal Database Project program Beta2, release 7.1 (http://www.rdp.cme.msu.edu/html).

2.2.7 Multidimensional scaling analysis of T-RFLP data

For statistical analysis, T-RFLP data was evaluated using SPSS 11 software (SPSS Inc., Chicago, Illinois, USA). Multidimensional scaling (MDS) analysis was performed on T-RFLP profiles of 16S rRNA and rDNA genes. Application of MDS analysis in this study was solely to aid in the visualization of the relationships between the samples (groupings and relative distances among samples) and not for statistical evaluation.

2.3 RESULTS AND DISCUSSION

2.3.1 Water quality data

The T-N concentration of the influent and respective nitrogen concentrations (NH_4-N, NO_X-N) of the effluent are shown in Fig. 2. Since the insufficiency of the carbon source in the influent caused incomplete denitrification, the accumulation of nitrate was observed, and nitrogen removal efficiency remained at only 40%. The ammonia concentration remained at approximately 15 mg·l^{-1} for the first week, and then decreased to below 5 mg·l^{-1}. On the other hand, the nitrate concentration increased from approximately 10 mg·l^{-1} to 15 mg·l^{-1}. Combining these data demonstrated that nitrification began to occur between day 7 and day 9.

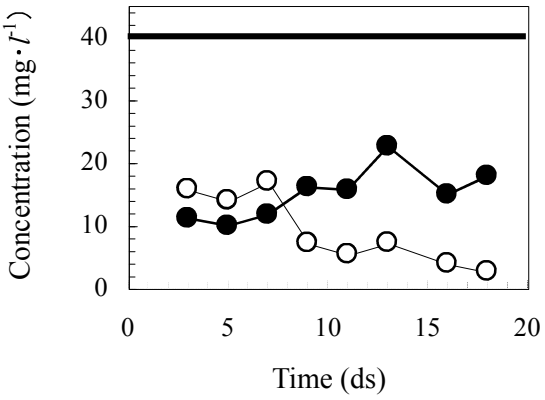

Fig.2 Time course of NH_4-N and NOx-N concentrations in the effluent. Symbols: open circles, NH_4-N; closed circles, NOx-N; solid line, T-N in the influent.

2.3.2 T-RFLP analysis.

The 16S rDNA-targeted T-RFLP electropherograms generated using *Hha*I are shown in Fig. 3a. The results demonstrated that the composition of microbial communities changed with the start of nitrification, probably because the number of bacteria increased at this time. In particular, peaks of 343, 370 and 510 bp became markedly higher on day 13 (370 and 510 bp) and day 18 (343 bp). Identification of these peaks was performed according to Marsh *et al.* (8). Table 2 shows the 5'-end terminal fragments measured from eubacteria digested with three restriction enzymes (*Hha*I, *Hae*III and *Msp*I) of the Ribosomal Database Project (RDP-II). The fragments were sorted first by the lengths of the *Hha*I fragment and then by the lengths of the *Hae*III and *Msp*I fragments. Only *Hha*I digestion products between 368 and 371 bp long are summarized in Table 2. Almost all species can be resolved with two or three digestions. The fragment of 370 bp is found to partly originate from *Nitrosomonas* sp., from the result of collating the RDP-II. This assumption was supported by the electropherograms with *Hae*III and *Msp*I in that the 222-bp (*Hae*III digestion product) and 82-bp (*Msp*I digestion product) products partly originating from *Nitrosomonas* sp. also became markedly higher on day 13 (data not shown). In our study, terminal restriction fragments (T-RFs) were accurately sized to ±2 bp, which was previously described by Liu *et al.* (6). In a similar way, the fragment of 343 bp was found to partly originate from *Nitrobacter* sp, and the fragment of 510 bp was found to partly originate from *Rhodobacter* sp. or *Paracoccus* sp. This fragment could not be identified further because these bacteria generated the same length fragments upon *Hha*I, *Hae*III and *Msp*I digestion (data not shown). The other peaks were not identified by the collation with the RDP-II database. It is well known that *Nitrosomonas* sp. and *Nitrobacter* sp. play prominent roles in the nitrification process of wastewater treatment. Therefore, these results indicate that the relative abundance of these nitrifying bacteria increase in association with the start of nitrification.

Table 2 Sorted display of RDP-II (*Hha*I fragments between 368 bp and 371 bp)

Fragment length (bp)			RDP sequence
*Hha*I	*Hae*III	*Msp*I	
368	63	123	*Thremus thermophilis*
368	227	161	*Streptomyces thermodiastaticus*
369	230	140	*Anaerobranca horikoshii*
369	67	279	*Mycobacterium smegmatis*
369	228	163	*Micrococcus luteus*
369	230	502	*Desulfovibrio desulfuricans*
369	67	279	*Mycobacterium* sp.
369	221	81	*Nitrosomonas* sp.
370	229	163	*Arthrobacter* sp.
370	230	146	*Clavibacter xyli* subsp.
370	67	72	*Mycobacterium thermoresistibile*
370	322	546	*Ureaplasma urealyticum*
370	229	165	*Agromyces mediolanus*
371	39	495	*Enterobacter cloacae*
371	39	494	*Klebsiella* sp.
371	39	494	*Erwinia billingiae*

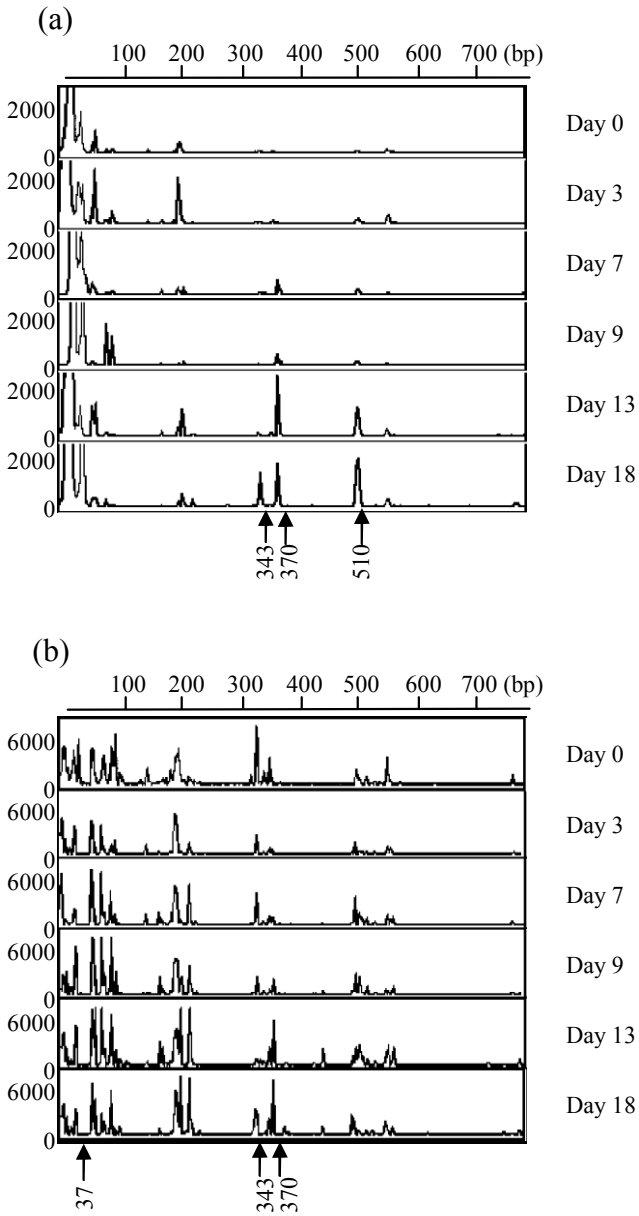

Fig. 3. T-RFLP electropherograms after digestion with *Hha*I (a) 16S rDNA, (b) 16S rRNA.

On the other hand, 16S rRNA-targeted T-RFLP electropherograms with *Hha*I are shown in Fig. 3b. The number of T-RFs in Fig. 3b was threefold higher than that in Fig. 3a. The lower diversity of 16S rDNA indicated that most bacteria existed as a small percentage of the total community in this sample and that they were below the detection limit. This tendency was also

observed by the electropherograms of 16S rRNA with *Hae*III and *Msp*I (data not shown). T-RFs of 16S rRNA in the sample of day 3 were present in low amounts in the original sample, although the T-RFs of 16S rDNA were unchanged. This is probably because the activity of bacteria decreased due to the temporary shock caused by the change in the substrate composition of the influent wastewater. After the first week, the amounts of almost all fragments gradually recovered to the original level because the microbial communities became acclimated to the new substrate composition of the influent wastewater. In particular, the fragments of 37, 343 and 370 bp, which partly originate from *Nitrospira* sp., *Nitrobacter* sp. and *Nitrosomonas* sp., respectively, were markedly higher on day 9 (37 bp), day 13 (370 bp) and day 18 (343 bp). Therefore, these results indicate that the activities of these nitrifying bacteria increase in association with changes in the wastewater quality.

Then, the results of 16S rDNA-targeted T-RFLP analysis were compared with those of 16S rRNA-targeted T-RFLP analysis by calculating the ratio of peak height of selective peaks to total peak heights. The data for *Nitrospira* sp. and *Nitrosomonas* sp. were chosen because the heights of the peaks representing these bacteria markedly increased after the start of nitrification. The relative ratio of each digest was determined by calculating the ratio between the height of each peak and the total height of all peaks except for fragments smaller than 30 bps. Then, the ratios obtained for three restriction enzymes were averaged because levels of microbial diversity could be underestimated using only the result from one restriction enzyme. It was demonstrated that the relative ratios of 16S rRNA increased prior to those of 16S rDNA (Fig. 4). These results indicate that the activity of the microbial species increase and subsequently the number of the microbial species.

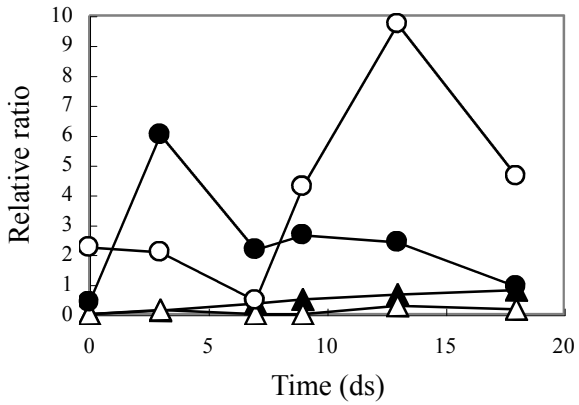

Fig. 4 Relative ratio of peak height. Symbols: open circles, 16S rDNA (370 bp); closed circles, 16S rRNA; open triangles, 16S rDNA (37 bp); closed triangles, 16S rRNA.

2.3.3 Multidimensional scaling analysis

MDS analysis was used to visualize the relationships among the samples based on the results of T-RFLP analysis. The closer the points in the figure are to each other, the more the microbial communities represented by the points are similar. As a result, 16S rRNA data exhibited wider dispersion than 16S rDNA (Fig. 5). These results indicate that the diversity of 16S rRNA in the microbial community is strongly affected by environmental changes. On the other hand, the results of samples on days 7, 9 and 13 demonstrated that the composition of the microbial community changed in association with the start of nitrification. Furthermore, the distance between the points representing 16S rDNA and 16S rRNA also changed. Initially (on day 0), the points representing 16S rDNA and 16S rRNA were close to each other, indicating that the microbial community was in a stable state. However, the distance was greater on day 3, when only the point representing 16S rRNA shifted. This result indicate that at first the activity of microbial members increase, which is also observed in the relative abundances of T-RFs with 16S rDNA and 16S rRNA (Fig. 4). At the start of nitrification, the increased distance was

maintained because the composition of the microbial community changed greatly. However, the distance returned to the original level on day 18, indicating that the microbial community had reached another stable state corresponding to the period of adaptation to the new environment.

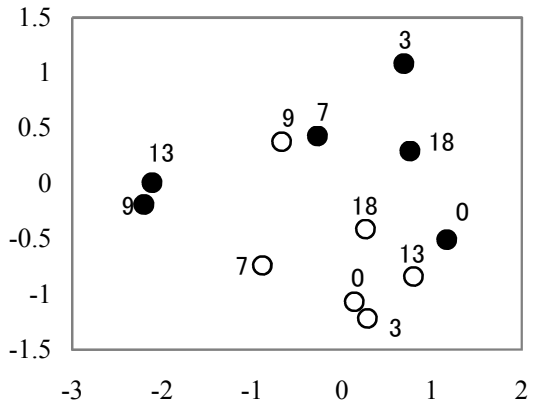

Fig. 5 Multidimensional scaling analysis of T-RFLP results. Symbols: open circles, 16S rDNA; closed circles, 16S rRNA; number represents operating days

2.4 CONCLUSIONS

In this chapter, the microbial population dynamics at the start-up stage of a wastewater treatment reactor was investigated using terminal restriction fragment length polymorphism (T-RFLP) analysis based on 16S rDNA and rRNA gene sequences. The experimental results are summarized as follows.

1. T-RFLP analyses of the 16S rDNA and rRNA genes demonstrate that the relative abundance and activities of nitrifying bacteria increase in association with the start of nitrification.
2. The relative ratios of the 16S rRNA of these nitrifying bacteria increase prior to those of 16S rDNA by calculating the ratio of peak height of selective peaks to total peak heights.
3. MDS analysis suggests that the diversity of 16S rRNA in the microbial community is strongly affected by environmental changes and that the composition of the microbial community changed in association with the start of nitrification.

Therefore, T-RFLP analysis can be useful to monitor microbial communities in wastewater treatment systems and to assess the condition of the reactor for control and improvement of such systems.

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Chapter 3

Molecular analysis of microbial population transition associated with the start of denitrification in a wastewater treatment process

SUMMARY

The objective of this chapter is to determine the bacteria playing an important role in denitrification by monitoring the molecular dynamics accompanying the start of denitrification. The cDNA reverse-transcribed from 16S rRNA was amplified with fluorescent labelled primer for terminal restriction fragment length polymorphism (T-RFLP) analysis and an unlabelled primer for cloning analysis. The terminal restriction fragments (T-RFs) increasing in association with the start of denitrification were determined. These T-RFs were identified by in silico analysis of 16S rRNA sequences obtained from cloning. As a result, it is clearly observed that the bacteria belonging to the genera *Hydrogenophaga* and *Acidovorax* increase in number after the start of denitrification. It is demonstrated that T-RFLP analysis targeting 16S rRNA is appropriate for the daily monitoring of a bacterial community to control wastewater treatment processes. Combination of the results of T-RFLP analysis and 16S rRNA clone library indicate that the bacteria belonging to the genera *Hydrogenophaga* and *Acidovorax* play an important role in denitrification. The results of this chapter provide new insight to the 16S rRNA level of active denitrifying bacteria in wastewater treatment processes.

3.1 INTRODUCTION

Biological wastewater treatment using activated sludge is one of the most significant biotechnological processes with important ecological and economic aspects. In addition to carbon removal, modern nutrient removal plants are designed to eliminate phosphorous and nitrogen compounds from municipal or industrial sewage. Prokaryotic microorganisms catalyze the most important transformations in such wastewater treatment plants.

Various approaches, both culture-dependent (24) and independent (7, 16, 29), have been applied to analyze and compare the microbial community structures of activated sludge samples. However, culture-dependent methods obviously do not show the real bacterial community structure because of the selective pressure imposed by the requirement for growth on a solid substrate.

Recently, the development of culture-independent molecular techniques, such as fluorescence in situ hybridization (FISH) (1), denaturing gradient gel electrophoresis (DGGE) (10), and terminal restriction fragment length polymorphism (T-RFLP) (15) have improved the analysis of the microbial community structure of environmental samples. In several studies, these techniques have been applied to investigate different types of wastewater treatment plants. In particular, many studies have been carried out on bacterial communities associated with nitrification (19, 25). This is because nitrification is the rate-limiting step of nitrogen removal, and the causes of nitrification breakdown events are not always obvious. In addition, nitrifying bacteria belong to a few genera, and thus, it is rather easy to correlate diversity analysis based on the functional gene (amoA: encoding for the active site subunit of the ammonia monooxygenase present in all ammonia oxidizers) with the analysis based on the 16S rRNA

gene.

On the other hand, bacterial communities responsible for denitrification, which is the other primary part of wastewater treatment processes, have not been sufficiently studied, particularly regarding 16S rRNA sequences. In contrast to ammonia and nitrite oxidation, the capability to anaerobically respire using nitrate (or nitrite) is widespread in the bacterial and archaeal domains. Therefore, from the retrieved 16S rRNA sequences, the identification of bacteria that play the main roles in denitrification is very difficult. Thus, it is very effective to analyze denitrifying bacteria using genes encoding nitrite reductases (nirS and nirK) as functional markers (6, 28, 33). However, the database of these genes of pure cultures is not complete. Actually, there are only approx. 80 nirS sequences of pure culture in the DNA Data Bank of Japan (DDBJ) and most part of these sequences belong to a few species such as *Thauera* sp., Marinobacter sp. and Pseudomonas sp. Therefore, the analyses on denitrifying functional genes are hard to correlate with phylogenetic information based on 16S rRNA. Thus, to understand denitrifying bacteria and to manage wastewater treatment processes, analyzing denitrifying bacteria by targeting 16S rRNA is very important.

Monitoring microbial community transition based on 16S rRNA associated with the start of denitrification is an effective approach to determine the bacteria playing important roles in denitrification. Although T-RFLP is suitable for this purpose, it has only been applied to monitor spatial (5) and seasonal changes (17) in the microbial population, and very few attempts have been made at monitoring daily changes.

The objective of this chapter is to determine the key bacteria playing important roles in denitrification by monitoring the microbial population transition associated with the start of denitrification in the activated sludge of an intermittent-aeration reactor by T-RFLP. Furthermore, we established a 16S rRNA clone library from the activated sludge of the reactor to enable a more precise identification of the bacterial species based on 16S rRNA sequences.

3.2 MATERIALS AND METHODS

3.2.1 Reactor operation and sampling

The reactor structure and operating conditions are shown in Fig. 1 and Table 1. The wastewater treatment system was operated with intermittent aeration: aerobic condition for 30 min and anaerobic condition for 90 min. Hydraulic retention time (HRT) was 20 h and shortened to 10 h on day 100. The chemical and biological parameters of the system, including ammonia, nitrite, nitrate, phosphorous and total organic carbon (TOC), were routinely monitored. For the characterization of bacterial communities, 10 ml of liquid sample was collected from the reactor and centrifuged at 10 000 *g* for 5 min. The resultant pellet was resuspended in 2.0 ml of Tris–EDTA (TE) buffer (10 mmol l^{-1} Tris and 1 mmol l^{-1} EDTA, pH 8.0) and stored at -80˚C.

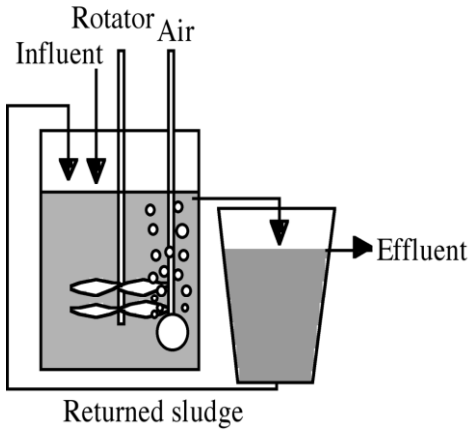

Fig. 1 Schematic diagram of biological wastewater treatment reactor

Table 1 Reactor specifications and operating conditions.

Volume (l)	5.5
Hydraulic retention time (h)	20 , 10**
Aerobic condition (min)	30
Anaerobic condition (min)	90
Suspended solids ($mg \cdot l^{-1}$)	3,500
TOC* ($mg \cdot l^{-1}$) in influent	90
T-N* in influent ($mg \cdot l^{-1}$)	40
T-P* in influent ($mg \cdot l^{-1}$)	5.0

*Total organic carbon, nitrogen, phosphorus, respectively.
**Hydraulic retention time was changed to 10 h on day 100.

3.2.2 RNA extraction and reverse transcription (RT)

RNA was extracted from samples using the ISOGEN kit (Nippon Gene, Tokyo, Japan), according to the manufacturer's instruction, without the bead-beating step described above. After the extracted RNA pellet was resuspended in 100 μl of TNMC buffer (40 mM Tris, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$, pH7.9) containing 20 U of RNase inhibitor (Toyobo, Osaka, Japan) and 50 U of DNase I (Takara Bio, Shiga, Japan), this solution was incubated at 37˚C for 60 min to remove DNA. Then, RNA was purified by applying 100 μl of phenol-chloroform-isoamyl alcohol (25:24:1), following by centrifugation at 15,000 rpm for 10 min. Purified RNA was eluted in 10 μl of 3M sodium acetate and 250 μl of ethanol, and incubated at -20˚C for 20 min. Then RNA was precipitated by centrifugation at 15,000 rpm for 15 min. The RNA pellet was resuspended in 50 μl of RNase-free TE buffer and quantified by UV absorption at 260 nm. The concentrations of all RNA extracts were adjusted to 10 $ng \cdot \mu l^{-1}$. Reverse transcription (RT) reaction of purified RNA (20 ng) was performed using the random primer. Reaction mixtures contained 5 U of RNase inhibitor (Toyobo), 2.0 μl of 5×RT buffer (ReverTra Ace, Toyobo), 10 mM deoxyribonucleoside triphosphate (Takara Bio), 25 μM RT primer, 50 U of RT polymerase (ReverTra Ace, Toyobo), 2.0 μl of RNA template, and 3.5 μl of RNase-free water. The reverse transcription reaction mixtures were preheated to 30˚C for 10 min and incubated at 42˚C for 50 min. After the reverse transcriptase was inactivated at 99 for 5

min, the reaction mixtures were quick-chilled at 4˚C for 5 min. Two microliters of the reaction mixtures was then used as the template for PCR as described below.

3.2.3 PCR conditions

PCR amplifications of DNA and cDNA were performed with a total volume of 50 μl in a GeneAmp PCR System 9700 (PE Applied Biosystems, Calif., USA). Bacterial 16S rDNAs from 10 ng of DNA extract were amplified using the universal primers 8f (2) and 926r (18). Reaction mixtures contained 2.5 μM of each primer, 0.2 mM deoxyribonucleoside triphosphate, 2.5 mM $MgCl_2$, 5 μl of 10×PCR buffer (500 mM KCl and 100 mM Tris-HCl, pH 8.3), 1 U of *Taq* polymerase (Toyobo), and 29.8 μl of sterilized water. After a denaturation step of 1 min at 94˚C, amplification reactions were performed with 30 cycles of denaturation (20 s, 94˚C), primer annealing (10 s, 53˚C), and primer extension (30 s, 72˚C) and a final extension step of 1 min at 72˚C. Forward primers for amplification of bacterial 16S rDNAs were 5'-end labeled with 6-carboxyfluoresce.

3.2.4 Purification of PCR product and restriction enzyme digestion

PCR products were verified by electrophoresis on 2% (wt/vol) agarose gels, followed by 20 min of staining with ethidium bromide (10 $\mu l \cdot l^{-1}$). Fluorescently labeled PCR products (40 μl) were purified with a QIAquick PCR purification kit (Promega). Aliquots (10 μl) of purified PCR products were cleaved for 4 h in a water bath at 37˚C using three different restriction endonucleases in digestions with a single tetrameric enzyme each (for bacterial 16S rDNAs, *Hae*III [GG'CC] [where the prime indicates the site of cleavage], *Cfo*I (an isoschizomer of *Hha*I) [GCG'C], and *Msp*I [C'CGG] (Promega).

3.2.5 T-RFLP analysis

Aliquots (8 μl) of the digest were purified by ethanol precipitation. The precipitate was mixed with 0.3 μl of GeneScan -500 size standard (Applied Biosystems) and 10 μl of deionized formamide. After DNA denaturation at 94˚C for 5 min and immediate chilling on ice, the fluorescently labeled terminal fragments were analyzed by electrophoresis on an ABI PRISM 3100-Avant Genetic Analyzer automated sequence analyzer (Applied Biosystems) in the GeneScan mode. The injection time was 30 s and the run time was 40 min. Reproducibility of patterns was confirmed by repeated T-RFLP analyses using the same RNA extracts. Signals with a peak area contribution below 1% (17) were regarded as background noise and excluded from analysis. Moreover, to avoid detection of primers and uncertainties of size determination, terminal fragments smaller than 30 bp and larger than 900 bp were excluded from further analysis. Reproducibility of patterns was confirmed for repeated T-RFLP analyses using the same DNA extracts. The relative abundance of terminal fragments within the sections was determined by calculating the ratio between the height of each peak and the total height of all peaks within one sample. Lengths of predominant bacterial 16S rDNA terminal fragments were theoretically compared to those found in the clonal library using GENETYX-MAC software (Genetyx, Tokyo, Japan).

3.2.6 Cloning of PCR products

The PCR products from the sample of day 104 were purified by Wizard SV gel and a PCR clean-up system (Promega, Wis., USA) in accordance with the manufacturer’s instruction. The eluted PCR products were cloned using a QIAGEN PCR cloning kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Cell material picked at random was suspended in 20 μl of Insert Check Ready solution (TOYOBO). The amplified inserts were run on 2% agarose gels. The samples including inserts of the estimated size were used for

subsequent sequencing.

3.2.7 Sequencing

The DNA insert was amplified and used as template DNA in a cycle sequencing reaction with a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. The DNA fragments were sequenced with an ABI PRISM 3100-Avant DNA Sequencing system (Applied Biosystems). In total, 121 sequences were determined.

3.2.8 Diversity analysis and phylogenetic analyses

A database search was conducted using BLAST from the DDBJ (DNA Data Bank of Japan). The sequences determined in this study and those retrieved from the database were aligned using Clustal W (32). Phylogenetic trees were constructed using Clustal W and Tree View (20) by the neighbor-joining method (27).

3.2.9 Nucleic sequence accession numbers

The sequences from this study are available through DDBJ under accession numbers AB184978 to AB185018.

3.3 RESULTS

3.3.1 Nitrogen removal characteristics

Time course of nitrogen removal characteristics is shown in Fig. 2. When HRT was 20 h (until day 100), denitrification did not sufficiently occur although NH_4^+ was complementary oxidized to NO_3^-. Total nitrogen concentration in the effluent was approximately 20 mg·l^{-1}, and almost all nitrogen was derived from NO_3^-. After shortening the HRT, denitrification completely occurred. All the remaining nitrogen in the effluent (10 mg·l^{-1}) was derived from NH_4^+, and thus, NO_3^- and NO_2^- did not remain at all.

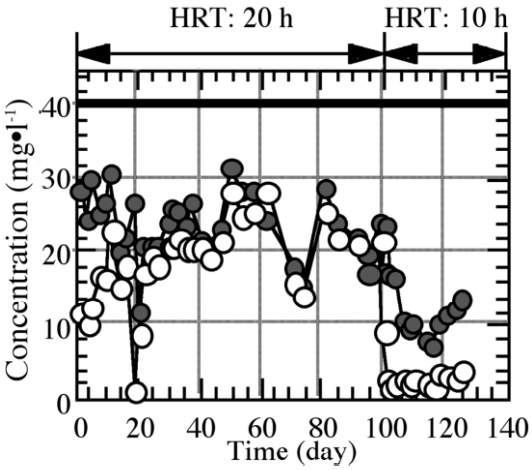

Fig. 2 Time course of NO_3-N and T-N (total nitrogen) concentrations in the effluent. Open circles show NO_3-N, closed circles indicates T-N, and the solid line indicate T-N in the influent

3.3.2 16S rRNA diversity study

To further investigate the community structure in the reactor, cDNA reverse-transcribed from 16S rRNA was amplified with an unlabeled primer set, and then cloned and sequenced. A total

of 119 clones were obtained. Most clone sequences were affiliated with the α- Proteobacteria (37 clones), followed by the β- Proteobacteria (31 clones), and the *Bacteroidetes* (22 clones). As for the other division, 3 clones belonged to *Firmicutes*, 2 clones belonged to *Chloroflexi*, 3 clones belonged to the *Verrucomicrobia* division, 1 clone belonged to *Acidobacteria*, and the other clones were unclassified.

3.3.3 Phylogeny inference of the cloned 16S rRNA sequence

The affiliations of these molecular isolates are depicted in the phylogenetic trees shown in Fig. 3. The trees were constructed using the neighbor-joining method. The genetic distances were calculated using Kimura's two-parameter method. The numbers on the nodes indicate the number of times (percentage) the species (shown on the right) grouped together in 1,000 bootstrap samples. The theoretical length of T-RFs in in silico analysis is shown in parentheses. Scale bar = 10% estimated difference in nucleotide sequence position. Boldface type indicates clones obtained in this study. With respect to the α-Proteobacteria, as shown in Fig. 3a, the 17 clones were closely related with *Sphingomonas* sp. The remaining 19 clones represented nine OTUs, indicating a phylogenetically diverse assemblage of microorganisms in the sample. The 29 clones were affiliated with the β-Proteobacteria. The 18 clones were closely related to *Janthinobacterium lividum*, and the 10 clones were related to *Hydrogenophaga palleronii* and *Acidovorax* sp. AHL 5. The remaining clones were closely related to *Alcaligenes faecalis*. These bacteria had less diversity than the α-Proteobacteria. Interestingly, none of the ammonia oxidizers belonging to the β-Proteobacteria were detected. In the CFB division, 22 of the obtained clones formed seven OTUs. The 12 clones were closely related to clones from gold and diamond mines (Fig. 3b).

Fig .3(a)

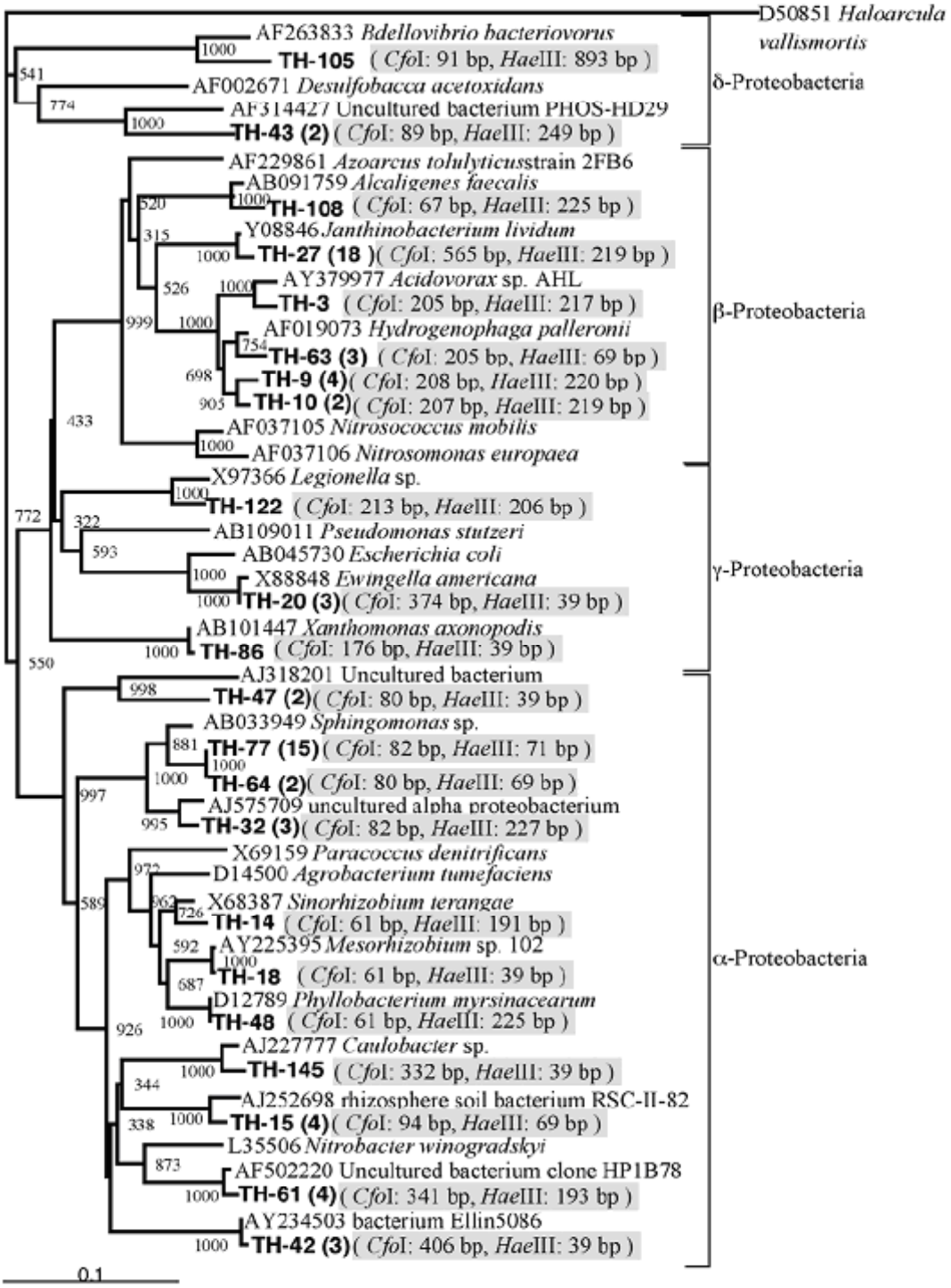
D50851 Haloarcula vallismortis
AF263833 Bdellovibrio bacteriovorus
TH-105 (CfoI: 91 bp, HaeIII: 893 bp)
AF002671 Desulfobacca acetoxidans
AF314427 Uncultured bacterium PHOS-HD29
TH-43 (2) (CfoI: 89 bp, HaeIII: 249 bp)
δ-Proteobacteria
AF229861 Azoarcus tolulyticusstrain 2FB6
AB091759 Alcaligenes faecalis
TH-108 (CfoI: 67 bp, HaeIII: 225 bp)
Y08846 Janthinobacterium lividum
TH-27 (18)(CfoI: 565 bp, HaeIII: 219 bp)
AY379977 Acidovorax sp. AHL
TH-3 (CfoI: 205 bp, HaeIII: 217 bp)
AF019073 Hydrogenophaga palleronii
TH-63 (3) (CfoI: 205 bp, HaeIII: 69 bp)
TH-9 (4)(CfoI: 208 bp, HaeIII: 220 bp)
TH-10 (2)(CfoI: 207 bp, HaeIII: 219 bp)
AF037105 Nitrosococcus mobilis
AF037106 Nitrosomonas europaea
β-Proteobacteria
X97366 Legionella sp.
TH-122 (CfoI: 213 bp, HaeIII: 206 bp)
AB109011 Pseudomonas stutzeri
AB045730 Escherichia coli
X88848 Ewingella americana
TH-20 (3)(CfoI: 374 bp, HaeIII: 39 bp)
AB101447 Xanthomonas axonopodis
TH-86 (CfoI: 176 bp, HaeIII: 39 bp)
γ-Proteobacteria
AJ318201 Uncultured bacterium
TH-47 (2)(CfoI: 80 bp, HaeIII: 39 bp)
AB033949 Sphingomonas sp.
TH-77 (15)(CfoI: 82 bp, HaeIII: 71 bp)
TH-64 (2)(CfoI: 80 bp, HaeIII: 69 bp)
AJ575709 uncultured alpha proteobacterium
TH-32 (3)(CfoI: 82 bp, HaeIII: 227 bp)
X69159 Paracoccus denitrificans
D14500 Agrobacterium tumefaciens
X68387 Sinorhizobium terangae
TH-14 (CfoI: 61 bp, HaeIII: 191 bp)
AY225395 Mesorhizobium sp. 102
TH-18 (CfoI: 61 bp, HaeIII: 39 bp)
D12789 Phyllobacterium myrsinacearum
TH-48 (CfoI: 61 bp, HaeIII: 225 bp)
AJ227777 Caulobacter sp.
TH-145 (CfoI: 332 bp, HaeIII: 39 bp)
AJ252698 rhizosphere soil bacterium RSC-II-82
TH-15 (4)(CfoI: 94 bp, HaeIII: 69 bp)
L35506 Nitrobacter winogradskyi
AF502220 Uncultured bacterium clone HP1B78
TH-61 (4)(CfoI: 341 bp, HaeIII: 193 bp)
AY234503 bacterium Ellin5086
TH-42 (3)(CfoI: 406 bp, HaeIII: 39 bp)
α-Proteobacteria
0.1

Fig.3(b)

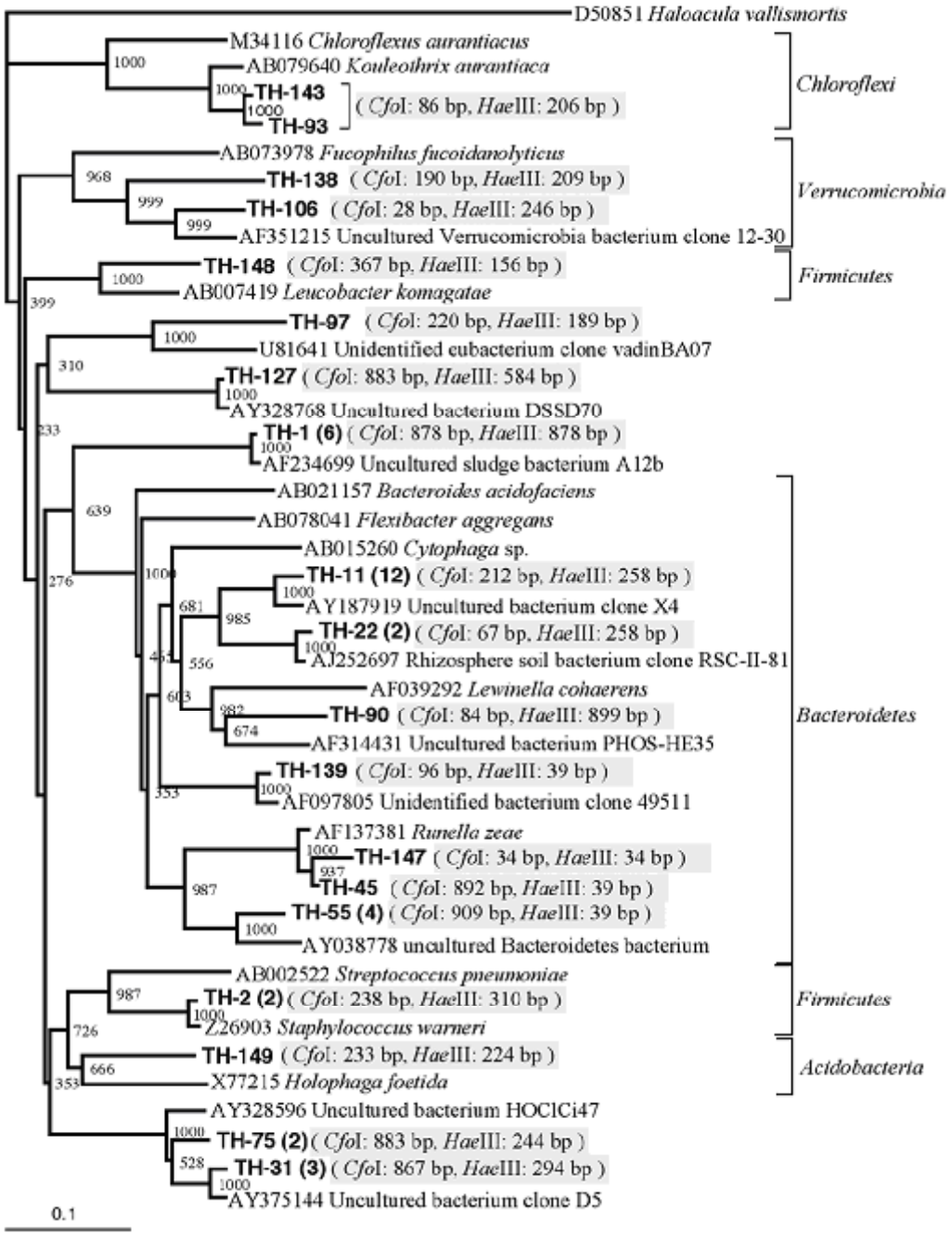

Fig. 3 Phylogenetic dendrogram based on 16S rRNA gene sequences in relation to representive members of *Proteobacteria* (a) and *Bacteroidetes, Chloroflexi*/GNS group, and *Verrucomicrobia* group (b).

3.3.4 T-RFLP analyses

For *Cfo*I and *Hae*III digestion, T-RFLP community fingerprint patterns were in the steady state until the HRT was shortened to 10 h (data not shown). In the case of *Cfo*I digestion, throughout all the periods analyzed, the community fingerprint patterns were characterized by T-RFs with sizes of 76, 204, 208, 341, 564, 884 and 913 bp (Fig. 4a). In this study, T-RFs that increase after the start of denitrification were focused on for the determination of the microbial population associated with denitrification. After the start of denitrification, the peak height of T-RFs with sizes of 204, 341, and 884 bp increased. The time course of the relative abundance of these peaks is shown in Fig. 5a. The relative abundance of T-RFs with sizes of 204, 341 and 884 bp was in the range of 2–4% before the start of denitrification. After the start of denitrification, this increased markedly and reached 5–9%.

On the other hand, for *Hae*III digestion, the predominant peaks were 65, 191, 217, 258, 272 and 884 bp (Fig. 4b). The peak height of T-RFs with sizes of 65, 191 and 884 bp increased with the start of denitrification. The time course of the relative abundance of these three T-RFs is shown in Fig. 5b. The relative abundance of 3–4% increased after the start of denitrification, and reached 5–10%.

3.3.5 Identification of predominant T-RFs

To identify the bacterial groups that correspond to the T-RFs associated with denitrification, 16S rRNA sequences from clones were analysed in silico with respect to *Cfo*I and *Hae*III restriction sites, and the calculated T-RFs showed the expected results (shown in parenthesis in Fig. 3a,b). Some undigested fragments of approx. 900 bp were generated in this study. However, from the results of in silico analysis, it was revealed that a few clones generated undigested fragments. Since the sizes of almost all these undigested fragments were different from each other, it is possible to assign these large T-RFs.

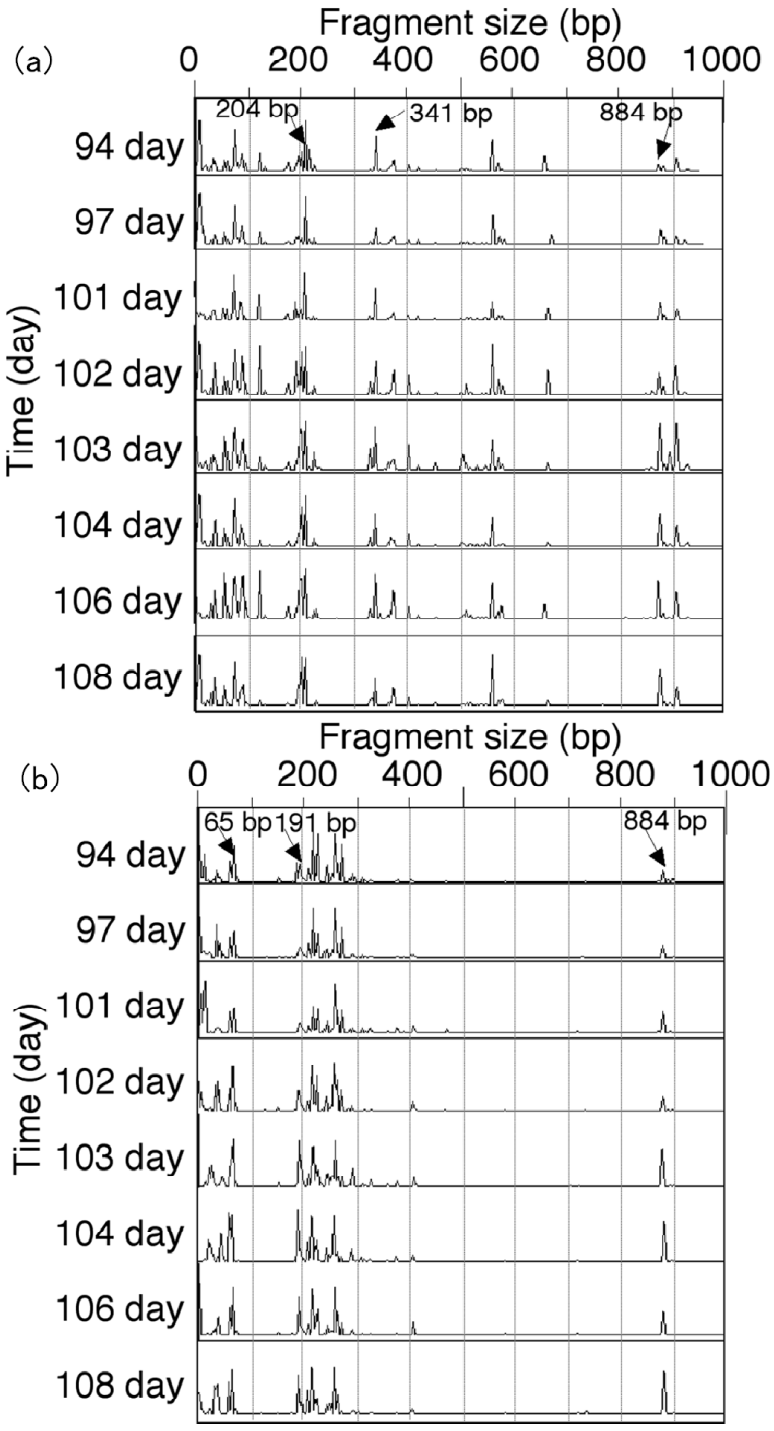

Fig. 4 Transition of bacterial community structure at the start of denitrification as determined by T-RFLP analysis using the restriction enzymes *Cfo*I (a) and *Hae*III (b). After HRT was shortened on day 100, denitrification started.

Rich *et al.* (26) reported that individual T-RFs differed in size by an average of 1.5 bp over a range of 0.3–5.1 bp. Therefore, a gap of less than 4 bp was found acceptable for the identification of the predominant T-RFs in this study. The results of the identification of the T-RFs that increased in number in association with denitrification are shown in Table 2. In the case of *Cfo*I digestion, the 204 bp T-RFs represented the clones TH-3, 9 and 63 affiliated with *Acidovorax* sp. and *Hydrogenophaga* sp., the 341 bp T-RFs represented the clone TH-61 affiliated with uncultured bacteria found to belong to α-Proteobacteria, and the 884 bp fragment that was not cleaved using the enzyme represented the clones TH-127 and 75 affiliated with unclassified bacteria.

Table 2 Comparison of the 16S rRNA genes from the clone libraries with the T-RFs obtained by digestion with *Cfo*I (a)/*Hae*III (b) and with sequences in the DNA Data Bank of Japan

Table 2(a)

Fragment size (T-RFs)	clone	Fragment size (in silico)	Most similar sequence	Phylogenetic group
204 bp	TH-3	204 bp	*Acidovorax* sp. (AY379977)	beta-class of Proteobacteria
	TH-9	207, 208 bp	*Hydrogenophaga palleronii* (AF019073)	beta-class of Proteobacteria
	TH-63	205 bp	*Hydrogenophaga palleronii* (AF019073)	beta-class of Proteobacteria
341 bp	TH-61	341 bp	Uncultured bacterium clone HP1B78 (AF502220)	alpha-class of Proteobacteria
884 bp	TH-127	883 bp	Uncultured bacterium DSSD70 (AY328768)	Unclassified
	TH-75	883 bp	Uncultured bacterium HOClCi47 (AY328596)	Unclassified

Table 2(b)

Fragment size (T-RFs)	clone	Fragment size (in silico)	Most similar sequence	Phylogenetic group
65 bp	TH-15	69 bp	rhizosphere soil bacterium (AJ252698)	alpha-class of Proteobacteria
	TH-64, 67	69, 71 bp	*Sphingomonas* sp. (AB033949)	alpha-class of Proteobacteria
	TH-63	69 bp	*Hydrogenophaga palleronii* (AF019073)	beta-class of Proteobacteria
191 bp	TH-14	191 bp	*Sinorhizobium terangae* (X68387)	alpha-class of Proteobacteria
	TH-61	193 bp	Uncultured bacterium HP1B78 (AF502220)	alpha-class of Proteobacteria
	TH-97	189 bp	Unidentified eubacterium clone (U81641)	Unclassified

On the other hand, as for *Hae*III digestion, 65 bp T-RFs represented the clones TH-15, 63, 64 and 77 affiliated with an uncultured rhizosphere soil bacterium, *Sphingomonas* sp., and *Hydrogenophaga* sp.; 191 bp T-RFs represented the clones TH-14 and 61 affiliated with *Sinorhizobium* sp. and an uncultured bacterium belonging to the α-Proteobacteria; and T-RFs represented the clone TH-97 affiliated with unclassified bacteria. The T-RFs of 884 bp size that increase in association with denitrification (Fig. 5b) could not be identified under this condition (possible deviation of up to 4 bp). From the in silico analysis of the obtained clone library, it was found that the clone TH-1 affiliated with an unclassified clone obtained from nitrifying–denitrifying activated sludge generated 878 bp T-RF. Therefore, this 884 bp T-RF was considered to represent these clones.

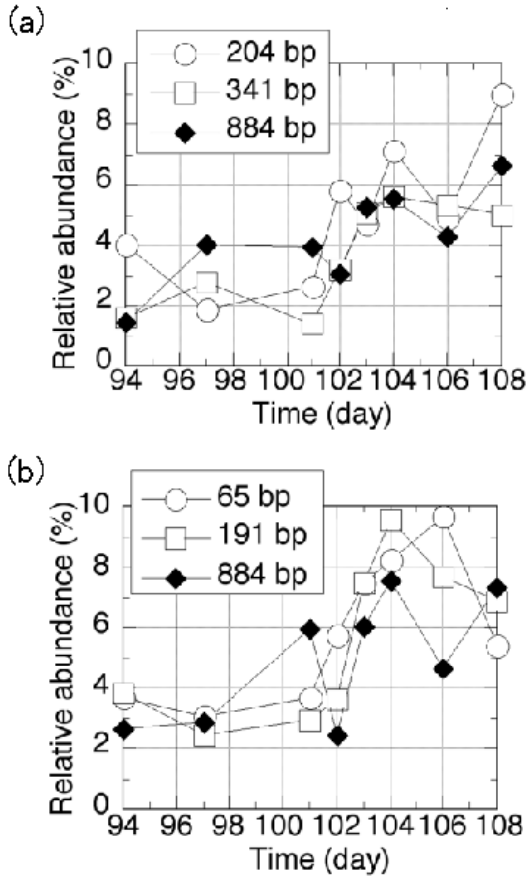

Fig. 5 Time course of relative abundance of key T-RFs for dentrification. (a) *Cfo*I digestion, (b) *Hae*III digestion

3.4 DISCUSSIONS

In this study, the transition of the microbial community structure was monitored by T-RFLP targeting 16S rRNA in detail. The reason rRNA was focused on for the monitoring of microbial population dynamics is as follows. In general, metabolically active cells have a higher ribosome content than those that are in the stationary growth phase (3, 23). Hence, the content of rRNA found in a microbial group represents both metabolic activity and population size. Several studies support this matter. DGGE profiles targeting DNA and RNA from samples obtained from the same environment differed from each other (9, 31). This phenomenon was interpreted as resulting from the difference between the genetic potential (DNA level) and the active portion (RNA level). In this study, our assumption is that DNA and RNA fingerprints are similar when the microbial community structure is in a steady state, but are different when the microbial community is disturbed by environmental change. In our previous study, the microbial population dynamics at the start-up stage of a wastewater treatment reactor was investigated using T-RFLP targeting 16S rDNA and rRNA. The results of fragment peaks suggested that the number and activity of nitrifying bacteria increased with the start of nitrification, and 16S rRNA of these bacteria changed prior to 16S rRNA gene analysis (30). In brief, the activity of the microbial communities increased, and subsequently, the number of microbial species increased. Therefore, targeting rRNA is suitable for short-period monitoring (e.g. daily change) and targeting rDNA for long-period monitoring (e.g. seasonal change). For these reasons, rRNA was targeted in this study; as a result, the transition of the microbial community structure could be sensitively monitored, and thus, this method is suitable for the management of wastewater treatment processes.

As for the comparison between T-RFLP analysis and in silico analysis, the relative abundance of the predominant T-RFs of each method on day 104 is shown in Table 3. These results seem to be reasonable except for the 564 bp (*Cfo*I; Table 3a) and 217 bp (*Hae*III; Table 3b) T-RFs.

Table 3 Comparison between relative abundance of predominant T-RFs obtained from T-RFLP analysis and that obtained from in silico analysis of clonal library (results of *Cfo*I (a) and *Hae*III (b) digestion) on day 104.

(a)

Relative abundance	Fragment size of T-RFs (bp)						
	76	204	208	341	564	884	913
T-RFLP analysis (%)	4.30	7.09	9.13	5.60	4.98	5.49	3.45
In silico analysis (%)	1.68	8.40	10.08	3.36	15.13	2.52	3.36

(b)

Relative abundance	Fragment size of T-RFs (bp)				
	66	191	217	258	884
T-RFLP analysis (%)	8.89	9.50	8.81	8.92	7.52
In silico analysis (%)	7.56	5.04	21.01	11.76	5.04

These fragments were mainly generated from the clones affiliated with the genus *Janthinobadterium* (18/119 clones). Such discrepancies have also been observed in other studies (12, 22) in which no simple explanation for this phenomenon has been made. Egert and Friedrich (8) demonstrated that unexpected T-RFs (pseudo-T-RFs, that is, those not predicted from sequence data and which therefore have to be regarded as false T-RFs) resulted from the presence of at least partly single stranded DNA amplicons unsuitable for the restriction

endonuclease. Thus, these pseudo-T-RFs corresponded to restriction sites downstream of the primary site. Assuming that this phenomenon caused disagreement between the T-RFLP and in silico analysis in this study, these clones have a second restriction site downstream of the primary one and generate pseudo-T-RFs. The fragment size of T-RFs that cleaved at the second restriction site was determined to be 404 bp by *Hae*III digestion. In the case of *Cfo*I digestion, the clones have no second restriction site, and generate a 913 bp fragment. However, the 404 bp T-RF was not observed by *Hae*III digestion (Fig. 4b). In contrast, the 913 bp T-RF was observed (Fig. 4a) by *Cfo*I digestion. From the in silico analysis, it was found that the clone TH-55 generated this T-RF and the results of the T-RFLP and in silico analysis are in good agreement (Table 3a). Therefore, it is reasonable to presume that almost all 913 bp T-RFs were obtained from these clones. Moreover, according to Egert and Friedrich (8), the extent of pseudo-T-RF formation decreased with increasing distance of the terminal restriction site from the 5'-labeled end of the amplicon and few pseudo-T-RFs were generated when the distance was longer than 300 bp. Therefore, the discrepancy in this study did not result from pseudo-T-RFs, but is due to some unavoidable bias associated with cloning in Escherichia coli or from the underestimation of the limited number of rDNA clones sequenced. In any case, the dynamics of the relative abundance of the key T-RFs for denitrification was not significantly affected by these phenomena.

Two restriction enzymes were used in this study. Although the T-RFs that increase in number in association with the start of denitrification were focused on, the dynamics of these T-RFs observed using the two enzymes were different from each other (Table 2a, 2b). The increase in the relative abundance of T-RFs generated from clone TH-63 affiliated with *Hydrogenophaga* and TH-61 was confirmed using both enzymes. However, the results were not in good agreement with each other, which is probably due to the low resolution of either enzyme. For instance, clones TH-9 and 3 generated approx. 204 bp T-RFs with *Cfo*I digestion and approx.

220 bp T-RFs with *Hae*III digestion. This 220 bp T-RF hid in the peak of the clones TH-111 and 13, both of which were predominant clones affiliated with *J. lividum*. For this reason, T-RFLP analysis with even two restriction enzymes is insufficient for the observation of population dynamics, and it is advisable to perform T-RFLP analysis using more restriction enzymes.

The results clearly showed that the metabolic activity of the bacteria belonging to *Hydrogenophaga*, *Acidovorax* and *Sphingomonas* increased with the start of denitrification. In previous studies, these genera have been reported as denitrifying bacteria (11, 13, 21). Accordingly, it is reasonable to assume that they carry out denitrification. It was assumed that these bacteria took up carbon mainly under aerobic condition and did not play a role in denitrification before day 100. Carbon uptake under aerobic condition resulted in the complete consumption of the carbon source for denitrification under anaerobic condition. After day 100, carbon still remained until the end of aerobic condition due to the shorter HRT, which resulted in a sufficient amount of carbon source for denitrification under anaerobic condition. Therefore, these bacteria played an active role in denitrification.

3.5 CONCLUSIONS

In this chapter, the bacteria playing an important role in denitrification was investigated by monitoring the molecular dynamics accompanying the start of denitrification using T-RFLP. The experimental results are summarized as follows.

1. The bacteria belonging to the genera *Hydrogenophaga* and *Acidovorax* increase in number after the start of denitrification
2. Combination of the results of T-RFLP analysis and 16S rRNA clone library indicate that the bacteria belonging to the genera *Hydrogenophaga* and *Acidovorax* play an important role in denitrification

The results of this study provide new insight to the 16S rRNA level of active denitrifying bacteria in wastewater treatment processes. Furthermore, the results obtained in this study show the potential of monitoring the transition of the microbial structure using T-RFLP targeting 16S rRNA for identification of the microbial population associated with denitrification. It is anticipated that genes and transcripts of nitrite reductase (nirS and nirK) will be analyzed by micro autoradiography (MAR) - FISH (14) and/or stable isotope probing (SIP) methods (4), and the combination of the results based on 16S rRNA and functional genes can determine the bacteria that actually perform denitrification in wastewater treatment processes.

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Chapter 4

Long-term monitoring of the succession of a microbial community in activated sludge from a circulation flush toilet as a closed system

SUMMARY

The microbial diversity and community succession of a circulation flush toilet were investigated by terminal restriction fragment length polymorphism (T-RFLP) and cloning analyses. Clone libraries of 16S rRNA gene on day 3 and day 127 were constructed. On day 3, 102 clones were sequenced and *Proteobacteria* and *Bacteroidetes* accounted for 27% and 45%, respectively. On the other hand, *Proteobacteria* increased to 43% and *Bacteroidetes* decreased to 26% of a total of 100 clones on day 127. Moreover, T-RFLP peaks were identified by *in silico* analysis of clone libraries. Consequently, the relative abundances of *Nitrosomonas* increased from 1% to 6% with commencement of nitrification and denitrification. Similarly, relative abundance of T-RFs generated from *Xanthomonas* increased from 3% to 10%. Therefore, these bacteria could play a prominent role in this process. Finally, to reveal the relationship between stability of the microbial community and performance of the system, microbial community succession was visualized by multidimensional scaling (MDS) analysis. As a result, the microbial community structure changed markedly, particularly during the start-up period of the system. The plots then became stable after the start of nitrification and denitrification. This result suggests that the succession of microbial community structure has a correlation with performance of the system.

4.1 INTRODUCTION

Biological wastewater treatment using activated sludge is one of the most significant biotechnological processes with important ecological and economic aspects. Understanding the structure and stability of microbial communities is desirable for correct operation of wastewater treatment processes because particular microbial communities are responsible for the success or failure of the process. Monitoring community succession is necessary to understand and predict future changes in community structure in natural and managed ecosystems.

Recently, some culture-independent molecular techniques, such as denaturing gradient gel electrophoresis (DGGE) (5), temperature gradient gel electrophoresis (TGGE) (24), single-strand conformation polymorphism (SSCP) (22) and terminal restriction fragment length polymorphism (T-RFLP) (14) have been developed for analysis of the microbial community structure of environmental samples. For investigation of changes in community structure in relation to changing physicochemical and biological conditions over space and time, rapid profiling procedures are necessary. Although DGGE, TGGE and SSCP allow the analysis of multiple samples, the community fingerprints obtained from these techniques do not directly translate into taxonomic information. A combination of cloning and T-RFLP analyses provides information about the population dynamics of particular bacteria. Therefore, T-RFLP has an advantage when monitoring bacterial communities, and thus, many researchers have determined microbial community succession in various natural environments by T-RFLP (2, 15, 23).

With respect to wastewater treatment processes, many studies have addressed microbial community structure in activated sludge (10, 11, 31). However, very few studies have centered on statistical analysis of microbial succession and correlation between the stability of the

microbial community and the performance of systems.

The circulation flush toilet can save water and can be used anywhere where there is sufficient the space. In this system, human waste flowing into an activated sludge tank is treated, deodorized and decolorized by ozone, and then water is reused to flush the toilet. Therefore, in this system, water and solid phase did not flow out. Although it is important for reusable water to be deodorized and decolorized sufficiently, nitrite and/or nitrate can be an obstacle to this objective. Therefore, sufficient nitrification and denitrification are also required in this wastewater treatment process.

The major questions address in this chapter include (1) which microorganisms are present in the system and whether they differ from those previously reported, and (2) whether the microbial community is stable, and does the stability of the community relate to the performance of the system. To address these questions, we monitored the microbial community structure for 6 months by T-RFLP targeting 16S rRNA gene. Furthermore, we established a 16S rRNA gene clone library to enable a more precise identification of the bacterial species based on 16S rRNA gene sequences.

4.2 MATERIAL AND METHODS

4.2.1 Reactor operation and sampling

The reactor structure is shown in Figure 1. Dewatered sludge obtained from a livestock excreta treatment process was used as the seed sludge. Sewage from the toilet entered the intermittent aeration tank, and organic and nitrogenous compounds were removed. The volume of the intermittent aeration tank was 3.0 m^3. Then, the liquid phase was separated from the solid phase in the membrane filtration tank. pH in this tank was maintained at 6.5 to 8.0 throughout the experiment. The volume of the filtration tank is 0.5 m^3. The usable area of the membrane is 0.8 m^2 and pore size is 0.4 μm (Kubota). A total of 10 membranes were used in this study. To prevent fouling of the membrane filter, the sludge was aerated in this tank. Excess sludge in this tank was returned to the intermittent aeration tank. Finally, water was decolorized and deodorized by ozonation in the decolorization tank and reused as flushing water. Additionally, an evaporation tank was set for adjustment of the water level in the entire system because water did not flow out. Fifty liters of water evaporated a day in the tank. The total volume of decolorization tank and evaporation tank was 0.8 m^3. The frequency of use of toilets was approximately 240 times for urinating and 40 times for defecating per day. The system was routinely monitored in terms of chemical and biological parameters, including ammonia, nitrite, nitrate and phosphorous. Hydraulic retention time of this system was 2 days. For characterization of bacterial communities, 50-ml liquid samples were collected from the intermittent aeration tank every one or two weeks and stored at -20˚C until DNA extraction. A total of 21 samples were obtained during the 200 days of the experimental period.

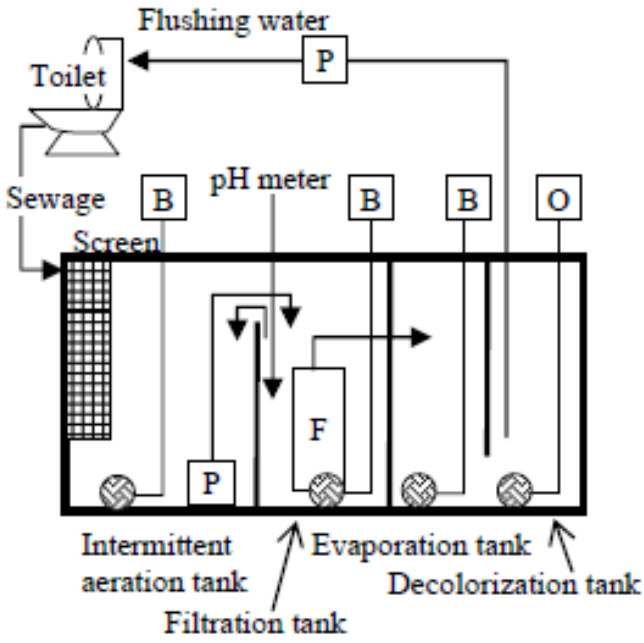

Fig. 1 Schematic diagram of a circulation flush toilet (side view). Total volume of the system is 5.4 m^3. The letters enclosed with squares indicate the following components. B: blower, O: ozone generator, P: pump, F: filtration membrane.

4.2.2 DNA extraction

DNA was extracted from samples with the ISOPLANT kit (NIPPON GENE), according to the manufacturer's instructions, except for the bead-beating step. DNA was liberated from the cells by ballistic disintegration with 1 g of sterile glass beads (0.1 mm in diameter) in a bead beater at 5,000 × g for 1 min. The concentration of extracted DNA was adjusted to 10 $ng \cdot \mu l^{-1}$.

4.2.3 PCR conditions

PCR amplifications of DNA were performed in a total volume of 50 µl in a GeneAmp PCR System 9700 (Perkin Elmer). Bacterial 16S rDNAs from 10 ng of DNA extract were amplified using the universal primers 341f (20) and 926r (21). Reaction mixtures contained each primer at 2.5 µM, 0.2 mM deoxyribonucleoside triphosphate, 2.5 mM $MgCl_2$, 5 µl of 10×PCR buffer (500

mM KCl and 100 mM of Tris-HCl, pH8.3), 1U of *Taq* polymerase (TOYOBO), and 29.8 μl of sterilized water. After a denaturation step of 1 min at 94˚C, amplification reactions were performed with 30 cycles of denaturation (20 sec, 94˚C), primer annealing (10 sec, 53˚C), and primer extension (30 sec, 72˚C) and a final extension step of 1 min at 72˚C. For T-RFLP analysis, the reverse primer for amplification of bacterial 16S rDNAs was 5'-HEX labeled (4, 14, 17).

4.2.4 Purification of PCR products and restriction enzyme digestion

PCR products were verified by electrophoresis on 2% (wt/vol) agarose gels, followed by 20 min of staining with ethidium bromide (10 μl liter^{-1}). Fluorescently labeled PCR products were purified with the QIAquick PCR purification kit (Promega). Aliquots of purified PCR products were cleaved for 4 h in a water bath at 37˚C with three different restriction endonucleases in digestions with a single tetrameric enzyme each for bacterial 16S rDNAs, *Hae*III [GG'CC] [where the prime shows the site of cleavage], *Cfo*I (an isoschizomer of *Hha*I) [GCG'C], and *Msp*I [C'CGG] (Promega).

4.2.5 T-RFLP analysis

Aliquots of the digest were purified by ethanol precipitation. The precipitate was mixed with 0.3 μl of GeneScan -1000 size standard (Applied Biosystems) and 10 μl of deionized formamide. After denaturing of the DNA at 94˚C for 5 min and immediate chilling on ice, the fluorescently labeled terminal fragments were analyzed by electrophoresis on an ABI 3100 *Avant* Genetic Analyzer automated sequence analyzer (Applied Biosystems) in GeneScan mode. The injection time was 30 s and the run time was 40 min. Reproducibility of patterns was confirmed by triplicate T-RFLP analyses using the same DNA extract. Lengths of predominant bacterial 16S rDNA terminal fragments were theoretically compared to clone library generated

in this study using GENETYX-MAC software (Genetyx).

4.2.6 Cloning of PCR products

The PCR products from samples taken on August 21, 2003 and December 23, 2003 were purified by Wizard SV gel and a PCR clean-up system (Promega) in accordance with the manufacturer's instructions. The eluted PCR products were cloned using a QIAGEN PCR cloning kit (QIAGEN) according to the manufacturer's instructions. White colonies of transferring cells picked at random were suspended in 20 μl of Insert Check Ready solution (TOYOBO) containing primers and DNA polymerase. PCR was performed as described above. The amplified inserts were run on 2% agarose gels. The samples including inserts of the estimated size were used for subsequent sequencing.

4.2.7 Sequencing

The DNA insert was amplified and used as template DNA in a cycle sequencing reaction with a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences) according to the manufacturer's instructions. The DNA fragments were sequenced with an ABI PRISM 3100-*Avant* DNA Sequencing system (Applied Biosystems). In total, about 100 sequences were determined.

4.2.8 Diversity and phylogenetic analyses

A database search was conducted using BLAST from the DNA Data Bank of Japan (DDBJ). The sequences determined in this study and those retrieved from the database were aligned using Clustal W (28). A phylogenetic tree was constructed using the njplot program in Clustal W, version 1.7 by the neighbor-joining methods (25).

4.2.9 Analysis of T-RFLP data

Signals with a peak area contribution below 1% (16) were regarded as background noise and excluded from analysis. Moreover, to avoid detection of primers and uncertainties of size determination, terminal fragments smaller than 30 bp and larger than 600 bp were excluded from further analysis. The relative abundance of terminal fragments within the sections was determined by calculating the ratio between the peak height of each peak and the total peak height of all peaks within one sample. The Euclidean distance between each sample was calculated based on the data of peak size and relative abundance. Differences in community structure were assessed graphically using the ordination method of nonmetric multidimensional scaling (MDS) calculated on the basis of Euclidean distance (12). All statistical analyses were performed using SPSS software (SPSS Inc.).

4.2.10 Nucleic sequence accession numbers

The sequences from this study are available through the DDBJ under accession numbers AB196002 to AB196121.

4.3 RESULTS

4.3.1 Water quality data

The time course of NH_4-N and NO_3-N concentrations in the decolorization tank is shown in Fig. 2. The concentration of NH_4-N increased until approximately day 100. Similarly, the NO_3-N concentration increased during this period. Almost all of the nitrogen in the sewage was derived from NH_4 indicating that NH_4^+ was partially oxidized. Consequently, non-oxidized NH_4^+ and NO_3^- resulting from oxidation were accumulated. Nitrate was accumulated because denitrification was not incomplete. The system was improved to enhance the agitation of sludge in the intermittent aeration tank by strengthening aeration on day 100. This improvement appeared to enable the bacteria to use the carbon source easily. Thereafter, denitrification and nitrification began to occur simultaneously (Fig.2a). The pH in the reactor was 6.5 to 8.0 throughout the experiment. The concentrations of PO_4, Cl^-, and SO_4^{2-} increased throughout the experimental period (Fig. 2b). Of these ions, the concentration of chloride ion is the most critical parameter for bacterial growth. The concentration of the chloride ion in the reactor was less than 5,000 mg/L. Mixed liquor suspended solids (MLSS) in the intermittent in the intermittent aeration tank also increased throughout the experimental period. At the end of experiment, MLSS reached to 14,000 mg/l (Fig. 2c). While chemical oxygen demand (COD) in influent varied from 100 to 600 mg/l, COD in the decolorization was below 60 mg/l throughout the experiment (Fig. 2d). This fact indicated that organic carbon was successfully removed in the intermittent aeration tank and filtration tank. Oxidation reduction potential (ORP) in the intermittent aeration tank and the filtration tank widely fluctuated until day 100. However, ORP became stable at -40 mV after day 100.

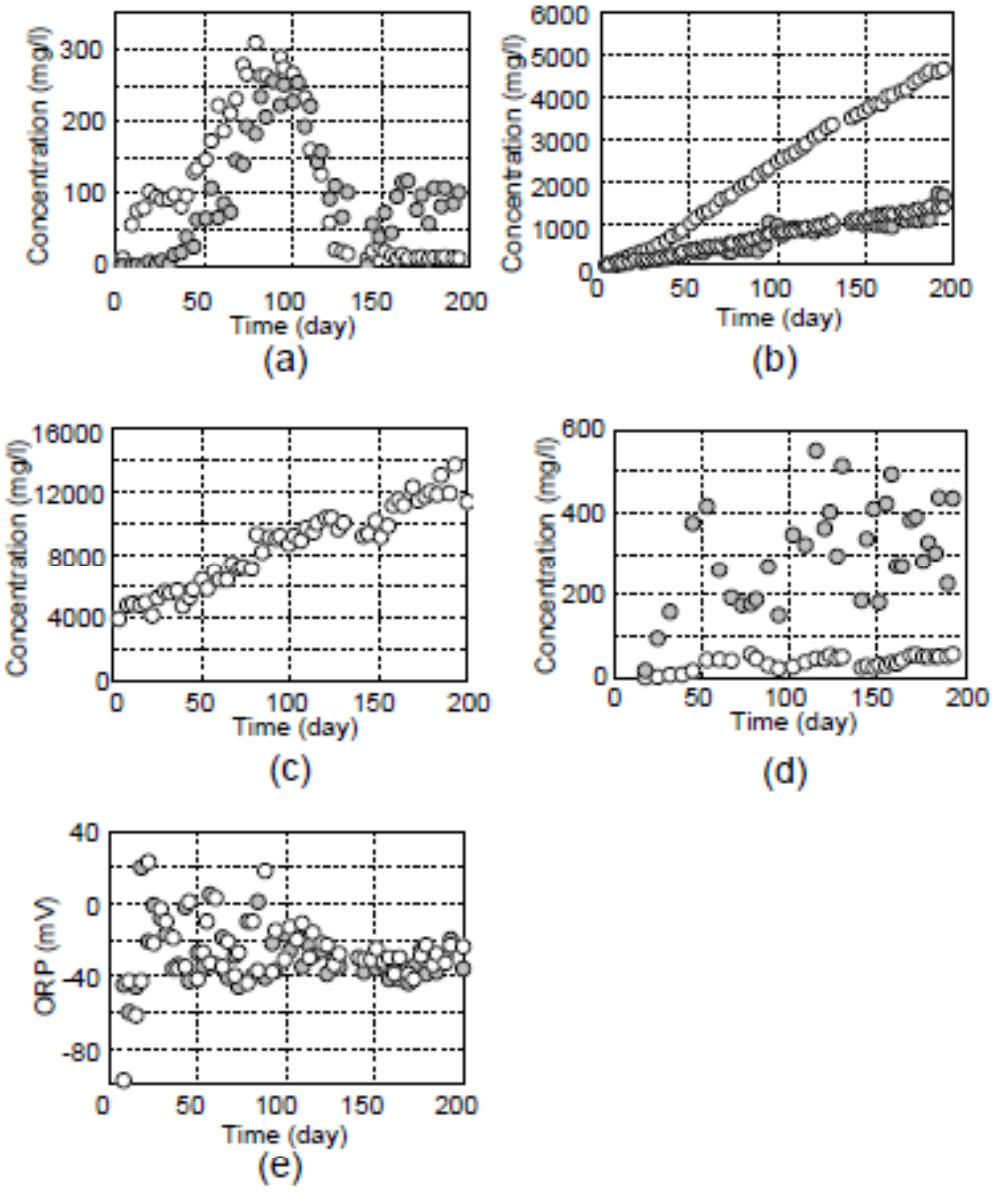

Fig. 2. Time course of water quality. (a) NH_4-N, NO_3-N concentration in the decolorization tank: closed circles indicate NH_4-N and open circles indicate NO_3-N. (b) PO_4-P, Cl^-, and SO_4^{2-} concentration in the decolorization tank: closed circles indicate PO_4-P, open circles indicate Cl^-, and open squares indicate SO_4^{2-}. (c) MLSS in the intermittent aeration tank. (d) COD: closed circles indicate COD in influent, open circle indicate COD in the decolorization tank. (e) ORP: closed circles indicate ORP in the intermittent aeration tank, open circles indicate ORP in the filtration tank.

4.3.2 16S rRNA diversity of microbial communities in the toilet

To investigate the community structure in the reactor, 16S rRNA gene was amplified with an unlabeled primer set, and then cloned and sequenced. A total of 102 and 100 clones were obtained from the samples on day 3 and day 127, respectively (Table 1).

Table 1 Number of the clones obtained in this study

Group	No. of clones	
	day3	day127
Proteobacteria		
α–*Proteobacteria*		
Sphingomonadaceae	3	1
Caulobacteraceae	1	0
Hypomicrobiaceae	0	1
Rhodobacteraceae	0	4
Hypomicrobiaceae	2	0
unclassified α–*Proteobacteria*	1	0
β–*Proteobacteria*		
Nitrosomonadaceae	0	3
Rhodocyclaceae	6	9
Comamonadaceae	1	4
unclassified *Burkholderiales*	5	5
unclassified β–*Proteobacteria*	1	3
γ–*Proteobacteria*		
Xanthomonadaceae	2	7
unclassified γ–*Proteobacteria*	2	4
δ–*Proteobacteria*		
Desulfobacteraceae	1	0
Polyangiaceae	0	1
Clostridiales	1	0
unclassified *Proteobacteria*	2	1
Bacteroidetes		
Sphingobacteriales	38	24
Flavobacteriaceae	0	1
unclassified *Bacteroidetes*	8	1
Actinobacteria	0	3
Acidobacteria	4	6
Firmicutes	1	1
Chloroflexi	10	14
Nitrospira	0	1
Candidate division	3	0
unclassified *Bacteria*	10	6
Total	102	100

As for the sample on day 3, most clone sequences were affiliated with the *Bacteroidetes* (46 clones), followed by the β-*Proteobacteria* (13 clones), and the α-*Proteobacteria* (7 clones). As for the other sample (on day 127), most clone sequences were affliated with the *Bacteroidetes* (26 clones), followed by the β-*Proteobacteria* (24 clones), and the γ-*Proteobacteria* (11 clones). The clone sequences belonging to the *Proteobacteria* increased from 27% (day 3) to 43% (day 127), whereas *Bacteroidetes* sequences decreased from 45% to 26% (Table 1).

4.3.3 Phylogeny inference of the cloned16S rRNA gene sequences

The affiliations of these molecular isolates are depicted in the phylogenetic trees shown in Fig. 3a–d. Thirty-seven clones were affiliated with the *Betaproteobacteria*. These clones represented 27 operation taxonomic units (OTUs), indicating a phylogenetically diverse assemblage of microorganisms in the samples (Fig. 3a). A total of 15 clones belonged to the order *Burkholderiales*. Five of these clones were affiliated with the family *Comamonadaseae*. As for bacteria playing important roles in nitrogen removal from sewage, three clones affiliated with the genus *Nitrosomonas*, representative of ammonia-oxidizing bacteria, were detected in the sample on day 127. Fifteen clones belonged to the family *Rhodocyclaceae*; eight of these were affiliated with the genus *Thauera*, which has been reported as representing denitrifying bacteria in wastewater treatment processes (4, 27).

Thirteen clones belonged to the *Alphaproteobacteria*. Almost all clones obtained on day 3 belonged to the family *Sphingomonadaseae* (three clones) and *Hyphomicrobiaceae* (two clones). By contrast, four of six clones obtained on day 127 belonged to the *Rhodobacterceae*. A total of 15 clones belonged to the *Gammaproteobacteria*, and nine of these belonged to the family *Xanthomonadaceae*. Three clones were obtained for *Deltaproteobacteria*, belonging to *Desulfobacteraceae*, *Polyangiaceae*, and *Clostridiales*.

In the phylum *Bacteroidetes*, 72 clones were obtained and classified into 17 OTUs; 62 of

these belonged to the order *Sphingobacteriales* (Fig. 3c).

Many identical sequences were obtained in this phylum: 23 clones were related to uncultured *Bacteroides* bacterium (AJ318154), and 18 clones were related to uncultured bacterium PHOS-HE35 (AF314431). Almost none of the other 59 clones were closely related to the type strains. These clones belonged to the following phylogenetic lineage: phylum *Actinobacteria*, three clones; phylum *Acidobacteria*, 10 clones; phylum *Firmicutes*, two clones; phylum *Chloroflexi*, 24 clones; phylum *Nitrospira*, one clone; genus Candidate division OP11, three clones (Fig. 3d).

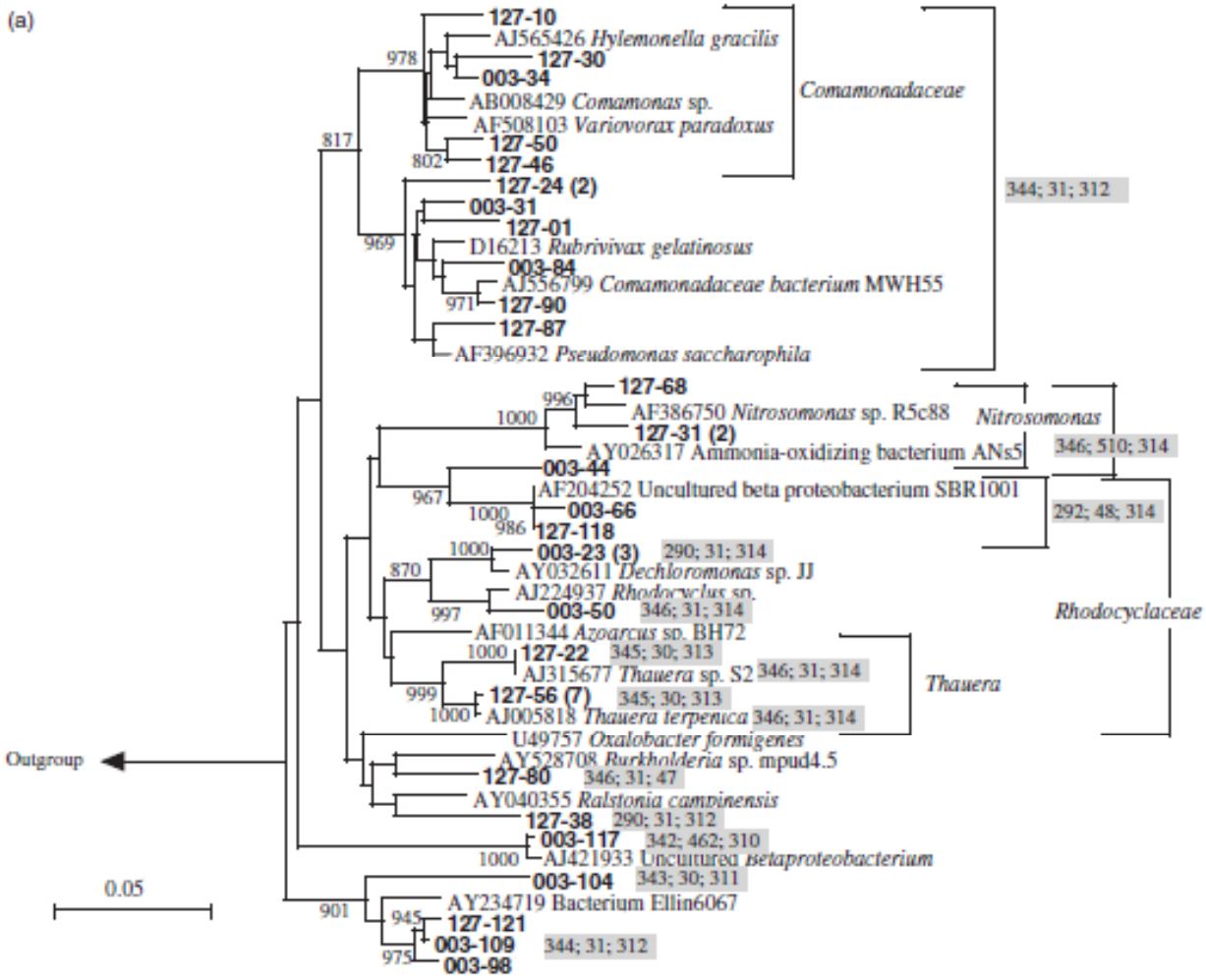

Fig. 3(a) Phylogenetic dendrogram based on 16S rRNA gene sequences in relation to members of the *Betaproteobacteria*

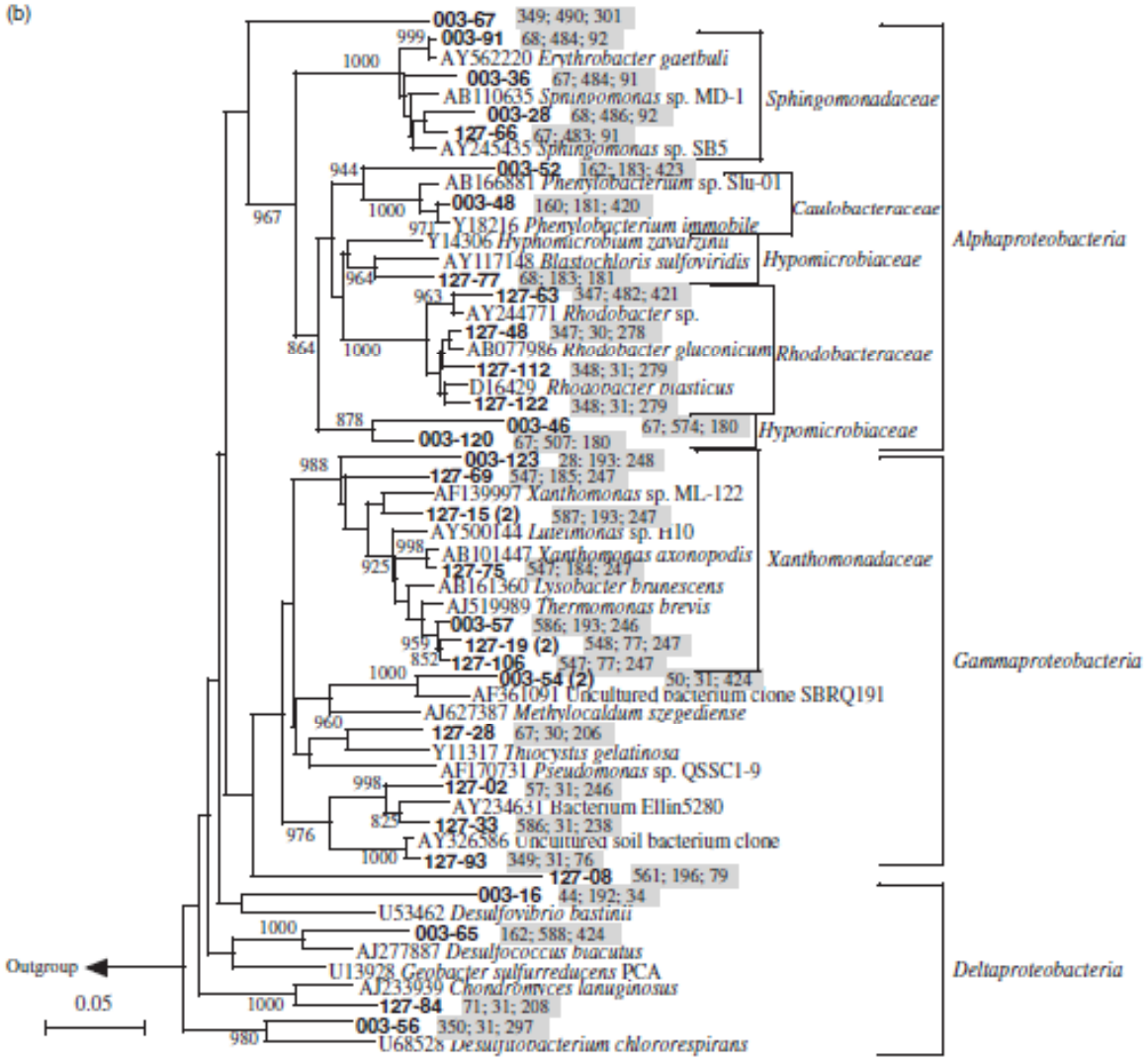

Fig. 3(b) Phylogenetic dendrogram based on 16S rRNA gene sequences in relation to members of *Alpha*-, *Gamma*- and *Deltaproteobacteria*

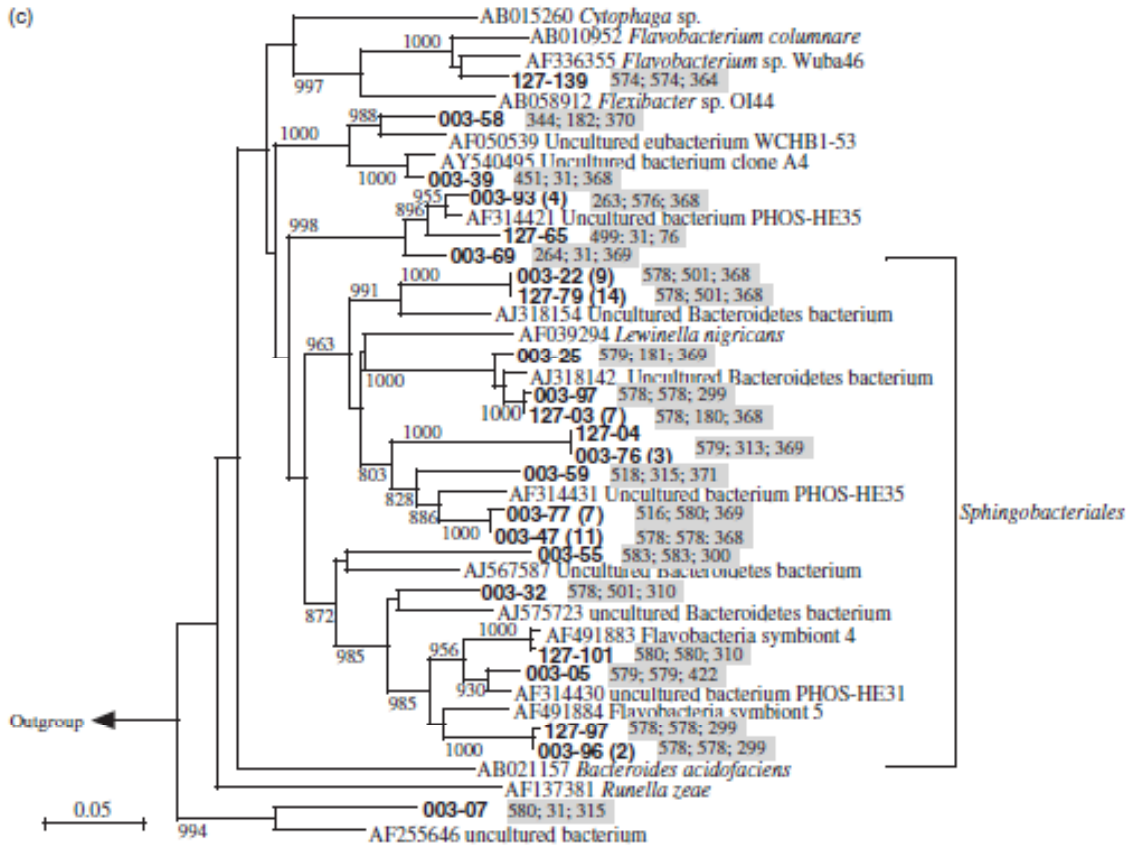

Fig. 3(c) Phylogenetic dendrogram based on 16S rRNA gene sequences in relation to members of the *Bacteroidetes* (c) and other bacteria

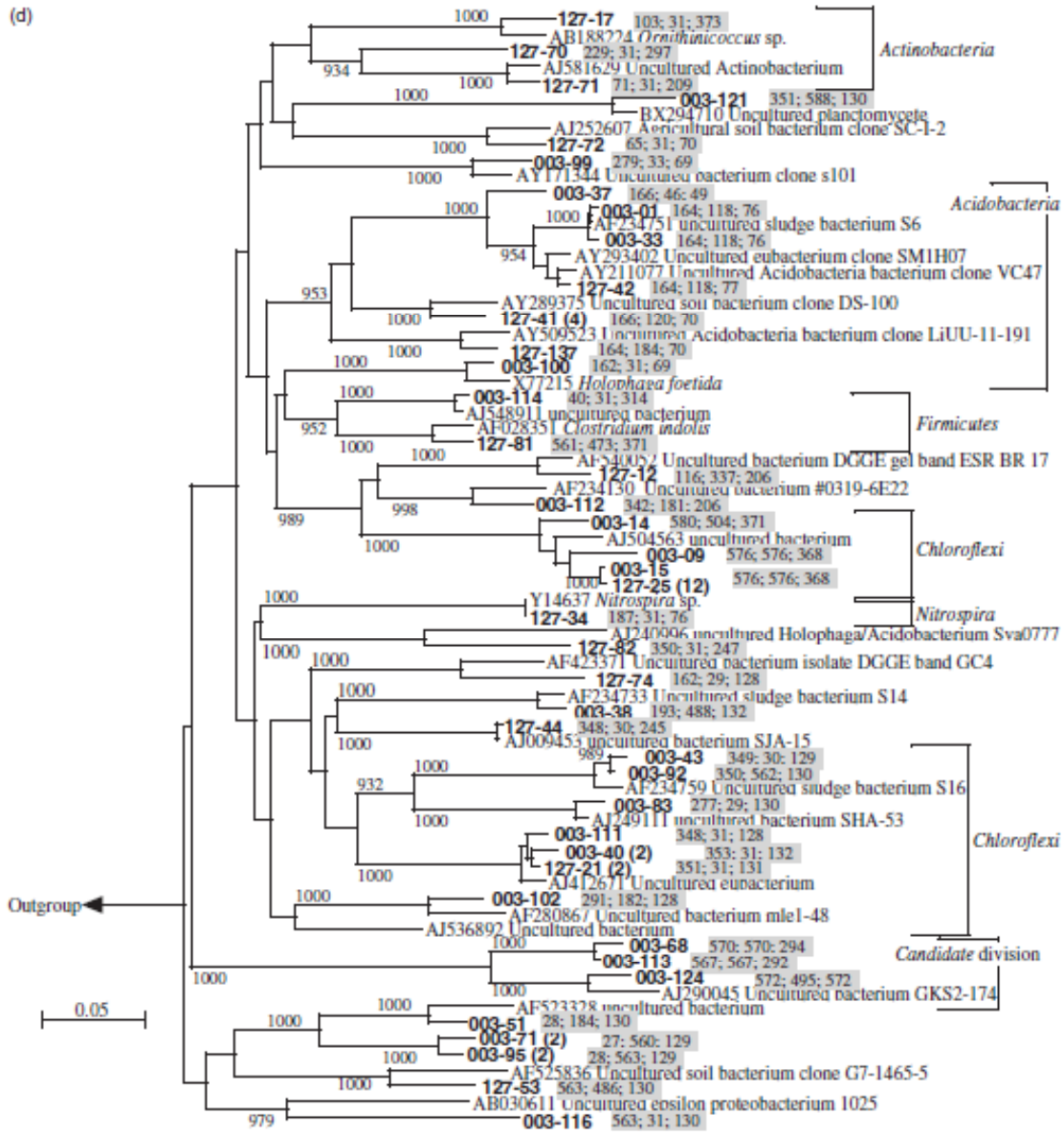

Fig. 3(d) Phylogenetic dendrogram based on 16S rRNA gene sequences in relation to members of the other bacteria

4.3.4 Relative abundance of terminal restriction fragments

Changes in community structure can be seen in detail in Fig. 4, which shows the relative abundance of each fragment over time. The relative abundance of individual peaks did not exceed 8%, indicating that a high microbial diversity existed in the reactor. Following *Cfo*I and *Hae*III digestion, several T-RFs of around 580 bp appeared (Fig. 4a–b). These T-RFs might represent undigested PCR products. There are no peaks for *Msp*I digestion around 580 bp (Fig. 4c). However, *Msp*I digestion generated the fewest number of peaks and could not sufficiently reflect the microbial diversity. In any case, it is impossible that a T-RF from one restriction enzyme attributed to only one phylogenetic group.

The population dynamics of phylum *Bacteroidetes*, *Betaproteobacteria*, family *Xanthomonas*, and genus *Nitrosomonas* were determined by measuring the relative abundance of T-RFs. In general, in T-RFLP analysis, the T-RF fragment size provides phylogenetic information, and the relative abundance provides population information. Although the relative abundance of T-RF did not reflect the accurate population size of a certain member, it has been used for analyzing population dynamics in several studies (15, 30). However, there are some difficulties when bacteria in different phylogenetic positions produce the same size T-RF. Therefore, specific T-RFs representing these bacteria were chosen by in silico analysis of clone libraries with three restriction enzymes.

Bacteroidetes generate 364, 368 and 369 bp T-RFs by *Msp*I digestion. In this study, 72 clones belonging to the *Bacteroidetes* were obtained, and 60 clones generated these T-RFs. The other clones did not generate T-RFs of a specific size. Almost all clones for *Betaproteobacteria* (35/37) generated specific T-RFs of 311, 312, 313 and 314 bp by *Msp*I digestion. All clones belonging to the family *Xanthomonadaceae* specifically generated T-RFs of 547, 548, 586 and 587 bp using *Cfo*I. The clones affiliated with the genus *Nitrosomonas* generated T-RFs 510 bp in size by *Hae*III digestion.

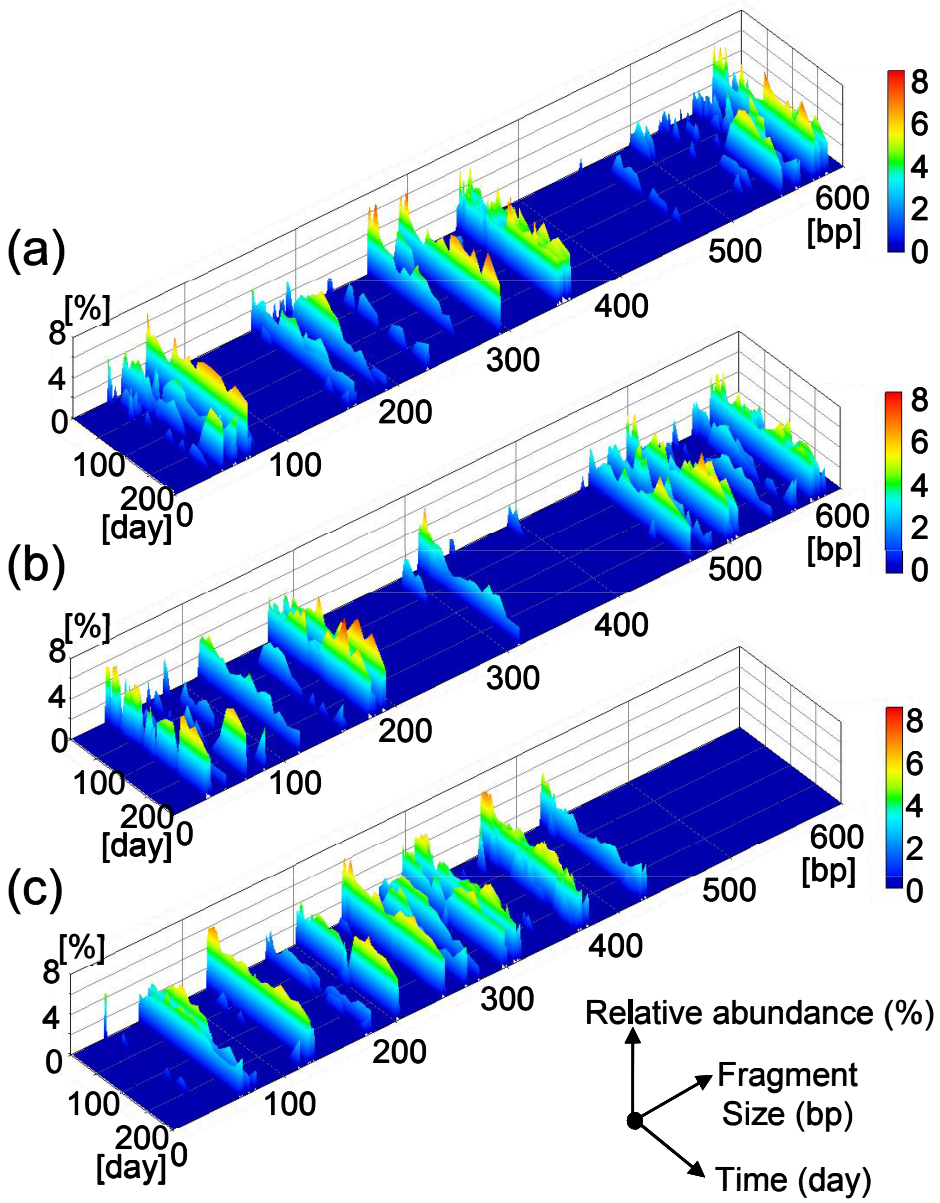

Fig. 4. Time course of relative abundance of bacterial terminal restriction fragments (T-RFs). Graph (a) shows the profile with *Cfo*I digestion, graph (b) with *Hae*III digestion, and graph (c) with *Msp*I digestion.

The population dynamics of these bacteria are shown in Fig. 5. The population of *Bacteroidetes* fluctuated markedly until day 50, and then became stable. The population of *Betaproteobacteria* gradually decreased to 5%, and then increased after day 100 and reached 12%. *Nitrosomonas* exhibited similar behavior: the population decreased to 0% and remained at this level until day 70, and then gradually increased to 7% on day 141. Family *Xanthomonadaceae* could hardly be detected until day 70, and then significantly increased to 10%.

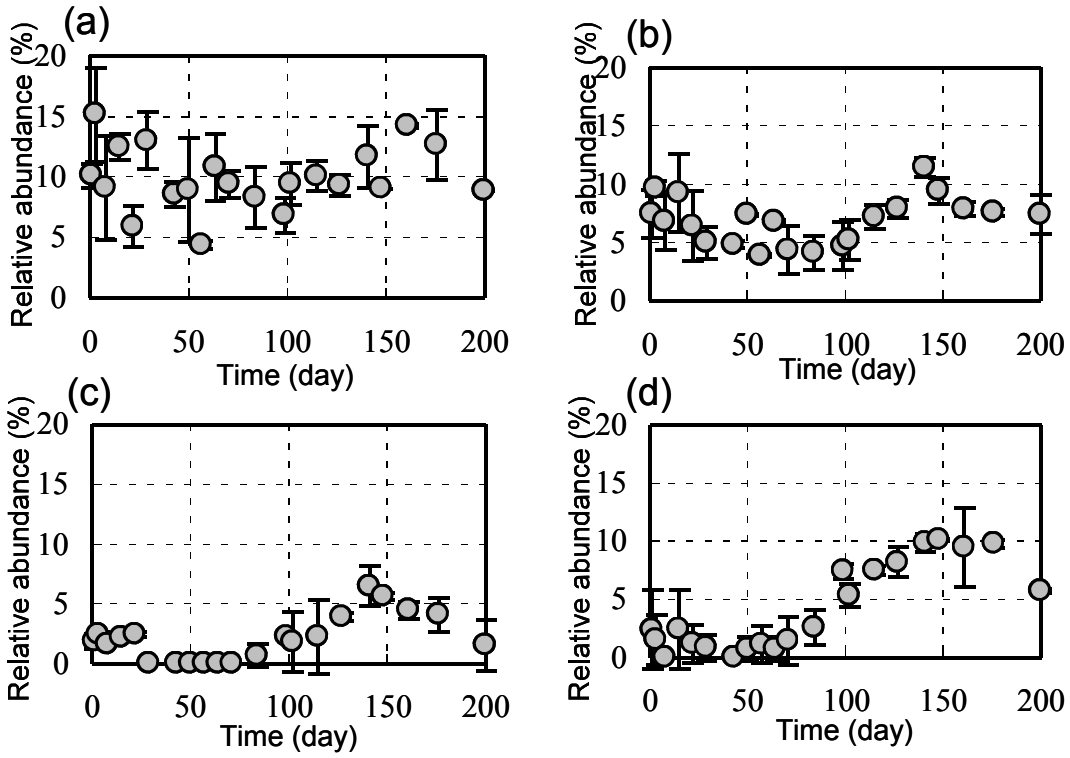

Fig. 5. Time course of relative abundance of terminal restriction fragments representing *Bacteroidetes* (a), *Betaproteobacteria* (b), genus *Nitrosomonas* (c), and family *Xanthomonas* (d). Points and error bars indicate the average and standard deviation based on three experiments. For points without error bars, the value is less than the height of the symbol.

4.3.5 Multidimensional scaling analysis

MDS analysis was used to visualize the relationships among the samples based on the results of T-RFLP analysis. Points close to each other mean that the microbial communities were similar (Fig. 6). In this study, T-RFLP profiles obtained using three enzymes and triplicate experiments were used for MDS analysis (Fig. 6). Three runs of T-RFLP analyses indicated the same MDS pattern of the plots. The two dimensional solution was chosen in this study because it could be interpreted readily and produced an acceptable combination of goodness-of-fit to the data (stress value, 0.336; RSQ, 0.586). This result indicated that the microbial community structure changed in relation to changing physicochemical and/or biological conditions over time. The microbial community structure changed markedly especially during the start-up period of the system. However, the plots started to become similar after day 102, indicating that the microbial community gradually reached a stable level.

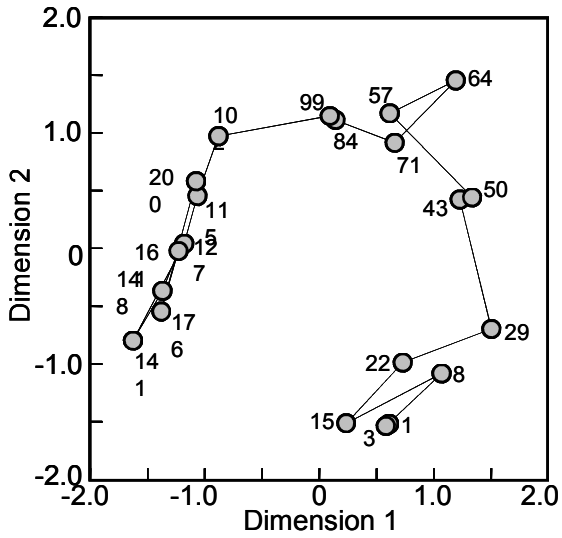

Fig. 6. Nonmetric multidimensional scaling plots of terminal restriction fragment plot results. Numbers beside plots indicate sampling day. Stress value for plots is 0.336.

4.4 DISCUSSIONS

With respect to the results of cloning analysis, the ratio of the clones affiliated with the *Proteobacteria* was lower than the ratio in normal activated sludge for wastewater treatment. However, phylum *Bacteroidetes* clones exhibited a relatively high ratio. *Proteobacteria* have been recognized as a key division in municipal wastewater treatment processes and are the most abundant phylum found (10, 29). This discrepancy might be due to the completely closed circulation flush toilet. In this system, once bacteria have entered, they do not leave, and even bacteria with low activity can be retained easily in the reactor. The cell material of dead cells is then used as a carbon source by the other bacteria. Therefore, it is thought that microbial community structure can be easily affected by the microbial community of influent. The only influent of this system was human waste. However, genus *Bacteroides*, which is well-known as the dominant bacteria in human feces (6, 7, 18), was not detected in this study. Instead, almost all clones belonging to this phylum *Bacteroidetes* fell in the order *Sphingobacteriales*. This result indicates that intestinal bacteria can not survive in this system. In fact, intestinal bacteria were not detected in biological wastewater treatment reactors for life bioregenerative support which only treated human waste (26).

We found no other reports that the order *Sphingobacteriales* were dominant in activated sludge of wastewater treatment process. High rates of *Sphingobacteriales* clones were detected day 3 and seemed to be dominant in the seed sludge. These bacteria were retained in the reactor throughout the experiment and high rates were also detected on day 127, although these could easily decrease in commonly used wastewater treatment processes.

Understanding microbial community succession in a reactor is desirable for optimizing

performance and stability of wastewater treatment plants because wastewater treatment plants often suffer from unpredictable breakdowns in performance. However, conventional methods, such as cloning combined with fluorescence in situ hybridization (FISH) analysis, are very time-consuming, which is a disadvantage when identifying individual bacterial species in a reactor. In this study, identification of T-RFs by comparison with in silico digestion analysis of clone libraries made it possible to investigate the population dynamics of some bacterial species. *Nitrosomonas*, *Xanthomonas* and *Betaproteobacteria* increased their relative abundance after day 100, when denitrification and nitrification started. *Nitrosomonas* are typical ammonia-oxidizing bacteria, and *Betaproteobacteria* have been recognized as an important subdivision in municipal wastewater treatment processes, as described above. Moreover, *Xanthomonas* have been reported to be active denitrifiers in a sequencing batch reactor (19) and heterotrophic nitrifiers in a biological wastewater treatment system treating acrylonitrile, butadiene and styrene resin wastewater (9). Therefore, it is reasonable to assume that these bacteria play an important role in improving the water quality in this system.

T-RFLP is a simple and powerful method for fingerprinting of the microbial community. However, it is necessary to bear in mind that T-RFLP has the drawback of quantitative estimation. This method is subject to a potential PCR bias, which can be excluded only by PCR-independent approaches, e.g. FISH; that is, the relative abundance of T-RF does not necessarily correspond to the real population size. Furthermore, a problem unique to T-RFLP was demonstrated (3); pseudo T-RFs (those not predicted from sequence data and which therefore have to be regarded as false T-RFs) resulted from the presence of at least partly single-stranded DNA amplicons unsuitable for the restriction endonuclease. As these pseudo-T-RFs were not detected in our previous study in which T-RFLP was performed under the same experimental conditions (8), this phenomenon did not have much effect on the results of this study. It is reasonable to suppose that increases and decreases of the relative abundance of a

certain T-RF reflect those of bacteria, although quantitative data should be treated with caution.

Three restriction enzymes were used in this study because the results with one restriction enzyme were insufficient to study the population dynamics of some bacterial species using T-RFLP (13). The bacteria with a phylogenetically close relationship could generate different sizes of T-RFs, or, conversely, phylogenetically distant bacteria could generate identically sized T-RFs. In this study the population dynamics of the family *Xanthomonas* were investigated using T-RFs of 547, 548 and 586 bp by *Cfo*I digestion. As *Hae*III digestion of the family *Xanthomonas* generated many sizes of T-RFs such as 77, 184, 185, 193 and 510 bp, these T-RFs could not be identified. Moreover, bacteria generating T-RFs of 30–31 bp by *Hae*III existed in each phylum. Hence, it is essential that T-RFLP analysis is performed using several restriction enzymes for investigation of the population dynamics of particular bacteria. This is also important for fingerprinting of the microbial community.

Using MDS analysis, microbial community succession was successfully visualized. The microbial community structure changed significantly up until day 100. This is probably because the seed sludge was used originally to treat livestock excreta; some bacteria in the reactor could not survive in the presence of different substrates, or human waste. During this period, it was observed that the plots fluctuated wildly, suggesting that the microbial community structure was relatively unstable. By contrast, after day 100, when nitrification and denitrification started, the microbial community structure fluctuated less. It is not certain whether the stability of the microbial community structure improved the nitrogen removal performance of the system or whether starting the nitrification/dinitrification reaction made the microbial community stable. In any case, it was demonstrated that the stability of the microbial community correlated with the nitrogen removal efficiency of this system. The plots were gathering around the plots of days 148 and 161. Therefore, it is presumed that the observable microbial community is in a steady state around these points.

4.5 CONCLUSIONS

In this chapter, the microbial population and its succession in a circulation flush toilet were monitored by T-RFLP and cloning analyses. Clone libraries of 16S rRNA gene on day 3 and day 127 were constructed. The experimental results are summarized as follows.

1. On day 3, 102 clones were sequenced and *Proteobacteria* and *Bacteroidetes* accounted for 27% and 45%, respectively. On the other hand, *Proteobacteria* increased to 43% and *Bacteroidetes* decreased to 26% of a total of 100 clones on day 127.
2. In T-RFLP analysis, the relative abundances of *Nitrosomonas* increased from 1% to 6% with commencement of nitrification and denitrification. Similarly, relative abundance of T-RFs generated from *Xanthomonas* increased from 3% to 10%. Therefore, these bacteria could play a prominent role in this process.
3. The microbial community structure changed markedly, particularly during the start-up period of the system. The plots then became stable after the start of nitrification and denitrification by multidimensional scaling (MDS) analysis. This result suggests that the succession of microbial community structure has a correlation with performance of the system.

Information on the microbial population is important to establish and enhance the reliability of this system for long-term operation without maintenance. It is anticipated that further monitoring and accumulation of T-RFLP data would enable us to predict the deterioration of water quality. Further work is needed to determine the active portion of the bacteria concerned with nitrification and denitrification. A combination of T-RFLP analysis and stable isotope probing methods (1) will clarify these points.

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Chapter 5

Molecular diversity of bacterial chitinases in arable soils and effects of environmental factors on chitinolytic bacterial community

SUMMARY

The molecular diversity of bacterial chitinases in the bulk soils of arable lands was investigated using culture-independent methods. The results suggest that the bacterial chitinase genes in arable soils are highly diverse and contain the unique groups of bacterial chitinases when compared to sequences presently in the public databases. Secondly, the compositions of bacterial chitinase genes were evaluated based on the polyphasic analyses including the conventional phylogenetic analysis, the application of UniFrac analysis and MDS analysis to T-RFLP profiles of bacterial chitinase genes for elucidating the relationship between the diversity of bacterial chitinases and soil characteristics. The results indicate that the environmental factors such as soil types and pH could be responsible for shaping the compositions of bacterial chitinases. Moreover, the potential usefulness of the combination with UniFrac analysis and MDS analysis to T-RFLP profiles are shown in the assessment of bacterial chitinase compositions, indicating the importance of polyphasic analyses.

5.1 INTRODUCTION

Chitin is the second most abundant polysaccharides in nature and is widely distributed across diverse environments as a constituent of several organisms, including fungal cell walls and the exoskeletons of insects (12). Chitin degradation is one of the key steps in the recycling of carbon and nitrogen resources in soil ecosystems. Chitin degrading enzymes, the chitinases, are also found in a wide variety of organisms, including fungi, plants, insects, crustaceans and bacteria (6). In bacteria, the primary role of the chitinases is thought to be the digestion and utilization of chitin as a carbon and energy source (2). Bacterial chitinases are often associated with the outer membrane or are secreted as extracellular enzymes (19, 20).

Chitinases are classified as either family 18 or 19 of the glycosyl hydrolases based on amino acid sequence similarity (13). The vast majority of known bacterial chitinases is grouped into family 18 (37). Previous studies have proposed to subdivide family 18 bacterial chitinases into groups A, B, and C, based on their amino acid sequence similarities in the catalytic domains (36). So far, the most information on the diversity and distribution of the bacterial chitinases is obtainable for the group A enzymes, and it has been speculated that the group A chitinase genes are more abundant than the group B or C enzymes in nature (28).

To date, only a few studies have investigated chitinase diversity within terrestrial ecosystems such as the chitin bags (28), and rhizosphere (18), whereas bacterial chitinases have been widely retrieved from aquatic ecosystems by culture-independent methods (3, 4, 32). These studies were evaluated on the analyses of one or a few environmental samples. While these studies revealed the high diversity and the presence of novel groups of bacterial chitinases in various environments, little is known about the relationships between the compositions of bacterial

chitinases and environmental factors. This is because of that most of previous studies investigated the diversity of bacterial chitinases in each sample not multiple samples by the application of conventional phylogenetic analysis. In addition, it would be difficult to visualize the relationships among multiple environmental samples based on the large set of sequence data. However, comparisons of bacterial chitinase sequences retrieved from multiple soil samples with distinct chemical and physical characteristics may yield insights into how soil environmental conditions affect the structure of chitinolytic bacterial communities.

Recently, new phylogenetic methods, such as UniFrac (25) and TreeClimber (34), have been introduced in order to measure the distance of molecular diversities between microbial communities. While these methods so far have been used on the basis of 16S rDNA sequence data (26, 41), no attempt has been made on the basis of functional genes such as bacterial chitinases with these new approaches. Meanwhile, terminal restriction fragment length polymorphism (T-RFLP) analysis has been shown to be a reliable fingerprint method for analyzing complex bacterial communities in nature (15, 16, 24, 31). In previous studies, the potential usefulness of T-RFLP analysis was demonstrated for analyzing the molecular diversity of bacterial chitinases in soils (16). Furthermore, statistical analyses, such as principal component analysis (PCA) and multidimensional scaling (MDS) analysis, of T-RFLP fingerprinting profiles are preferred methods for visualizing microbial diversity to facilitate the comparisons of complex microbial communities (5, 14, 42).

The aim of this chapter is to investigate the molecular diversity of bacterial chitinases in the bulk soils of multiple arable lands and effects of environmental factors on the compositions of bacterial chitinases. The compositions of bacterial chitinases were evaluated based on the polyphasic analyses including the conventional phylogenetic analysis, the application of UniFrac analysis, and MDS analysis to T-RFLP profiles of bacterial chitinases for elucidating the relationships between the diversity of bacterial chitinases and soil characteristics.

5.2 MATERIALS AND METHODS

5.2.1 Soil samples and DNA extraction

The soil collection sites and soil properties are shown in Table 1 as previously described (17). The soil samples were collected from diverse geological locations in Japan (Table 1). Briefly, about 1 kg of soil was sampled in each location from a 2~20 cm depth, without the surface soil (0~2 cm depth), and immediately transported to our laboratory and stored at 4˚C.

Table 1. Sampling locations and soil characteristics.

Sample name	Sampling location Prefecture/City	FAO soil grouping	pH	EC[c]	PAC[d]	%C[e]	%N[f]	Organic Content (%)	Humic acid Content (%)
EH	Hokkaido/Eniwa	Andosol	5.4	10.2	1930	3.8	0.7	11	8.5
GG	Gifu/Gifu	Andosol	6	7.5	544	1.3	0.5	4.1	2.7
HA	Aomori/Hirosaki	Andosol	5.2	81.7	1880	6.5	0.9	16.6	13.1
HO	Osaka/Habikino	Andosol	5.2	33.3	470	1.9	0.5	4.6	3.9
IN	Nagasaki/Isahaya	Acrisol	4.2	24.8	1200	1	0.6	11.6	2.5
KH	Hokkaido/Kasai	Andosol	5.6	12.6	1750	2.8	0.6	9.8	7
KK	Kagawa/Takamatsu	Andosol	6	10.7	516	1.6	0.5	3.9	3.9
KN	Nara/Kasihara	Gleysol	5.4	13.9	826	2	0.8	5.4	8.9
MH	Hokkaido/Bibai	Histosol	7.1	16.4	976	3.8	0.6	8.6	6.1
MH2	Hokkaido/Bibai	Histosol	5.6	73.6	1590	17.2	1.4	33	39.8
MI	Iwate/Morioka	Andosol	5.8	11.7	2370	9.3	1	23.6	21.1
SA	Akita/Daisen	Andosol	6.5	6.2	1550	3.2	0.5	11.3	7
ST	Tochigi/Sakura	Andosol	7.6	12.2	1980	3	0.5	15.1	7.2
UT	Ibaraki/Tsukuba	Andosol	6.1	6.6	1880	4.3	0.6	15.9	10.2
YH	Hyogo/Kato	Cambisol	5.5	16.7	802	2.8	0.6	6.7	10.3

[a]The east longitude. [b]The north latitude. [c]Electrical conductivity. [d]Phosphate adsorption coefficient. [e]Total carbon level. [f]Total nitrogen level.

After sieving the soils (2 mm, diameter), 0.5 g aliquots were collected into 2 ml microtubes and stored at -80˚C. All samples were collected between the end of October and the beginning

of November, 2004. The quantified soil characteristics that we previously reported (17) were subjected to principal component analysis using the software 'Excel statistics 2002' (Social Survey Research Information Co., Ltd, Tokyo, Japan). DNA extraction was carried out by a soil DNA extraction method previously reported by our group (17).

5.2.2 PCR amplification of bacterial chitinase gene

PCR amplifications of bacterial chitinase gene were carried out based on a protocol as previously described (18). Briefly, the PCR primers used were GA1F (cgt cga cat cga ctg gga rtd bcc) and GA1R (acg ccg gtc cag ccn ckn ccr ta), as previously reported (43). The PCR reaction mixture (total 25 μl) contained 2.5 μl of 10 x buffer, 5 μg of BSA, 0.5 μM of primers, 200 μM of each dNTP and 2 U of Ex *Taq* HS DNA polymerase (TaKaRa Bio, Shiga, Japan). One microliter (5 ng of DNA) of soil DNA was used in each amplification reaction as a template. The PCR amplification program consisted of 2 min at 94˚C, 35 cycles of 30 sec at 94˚C and 30 sec at 68˚C, and a final extension time of 7 min at 72˚C in a GeneAmp PCR System 9700 (Applied Biosystems, Tokyo, Japan). PCR products were verified by electrophoresis on 2% (w/v) agarose gels, followed by 20 min of staining with ethidium bromide (10 $\mu l\ l^{-1}$).

5.2.3 Cloning and sequencing of PCR products

The resulting PCR products (10 μl) were then purified with Microcon YM-100 (Millipore, Bedford, Mass.) and eluted in 20 μl of TE buffer. These purified PCR products were subsequently ligated to a pCR4-TOPO Vector and transformed into *ECOS* Competent *E. coli* DH5α Cells (NIPPON GENE, Tokyo, Japan) using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA). Transformants were plated on LB-agar containing 50 μg/ml ampicillin, and were incubated overnight at 37˚C. Colonies of transformed cells were picked at random,

and were suspended in PCR reaction mixtures for insert check. The PCR reaction mixture (total 20 μl) contained 2 μl of 10 x buffer, 0.4 μM of M13 primers, 200 μM of each dNTP and 0.5 U of Blend *Taq* -Plus- DNA polymerase (TOYOBO, Osaka, Japan). The PCR amplification program used for insert check consisted of 2 min at 94˚C, 30 cycles of 30 sec at 94˚C, 20 sec at 55˚C and 1 min at 72˚C, and a final extension time of 7 min at 72˚C. The amplified inserts were verified by electrophoresis on 2% (w/v) agarose gels as described above. The amplified inserts (10 μl) were then purified with Montage PCR kit (Millipore) and eluted in 15 μl of TE buffer. DNA sequencing reactions were performed by using M13 reverse and forward primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol, and the reaction products were analyzed on ABI3130 Genetic Analyzer. Sequence data were assembled using ATGC software (GENETYX corporation, Tokyo, Japan).

5.2.4 Sequence analyses

Sequence data were compared to public database entries using BLASTX and sequence matches were considered to be significant for scores >50 (29). For phylogenetic analysis, alignments of deduced amino acid sequences were performed using the CLUSTAL W program (39) and the neighbor-joining method was used for building the phylogenetic trees (33). The Phylip format tree output was applied using the bootstrapping procedure (7) (the number of bootstrap trials used was 1000).

UniFrac (25) was applied to a test for examining statistical differences of bacterial chitinase compositions among soil samples. First, phylogenetic trees were constructed for the chitinase gene sequences using the neighbor-joining method as implemented in PAUP 4.0 beta Win. Testing was then carried out to detect differences of molecular diversity of bacterial chitinases among soil samples using the UniFrac statistics (http://bmf.colorado.edu/unifrac/index.psp).

5.2.5 T-RFLP analysis

Terminal restriction fragment length polymorphism (T-RFLP) analysis was carried out with the minor modifications previously described (38). Briefly, PCR amplifications were performed essentially as described above, except that the 5' end of the reverse primer was labeled with 6-carboxyrhodamine (Sigma Genosys, Hokkaido, Japan) for fluorescent detection. PCR products were verified by electrophoresis on 2% (w/v) agarose gels as described above. Fluorescently labeled PCR products (10 μl) were purified with a Microcon YM-100 (Millipore) and eluted in 20 μl of TE buffer. Aliquots (5 μl) were then digested in a total volume of 20μl for 4 h at 37˚C with AfaI and HhaI (TaKaRa Bio), respectively. Two restriction enzymes were used in the present study, because the results with one restriction enzyme may be insufficient to evaluate the microbial diversity using T-RFLP due to the possible bias of restriction sites (23). The digests (10 μl) were purified by ethanol precipitation. The precipitate was mixed with 0.5 μl of GeneScan 500-LIZ size standard (Applied Biosystems) and 30 μl of deionized formamide. After DNA denaturation at 94˚C for 5 min and immediate chilling on ice, the fluorescently labeled terminal fragments were analyzed by electrophoresis on an ABI PRISM 3100 Genetic Analyser automated sequence analyser (Applied Biosystems) in the GeneMapper mode. The injection time was 15 sec and the run time was 30 min. Reproducibility of T-RFLP profiles was confirmed by conducting duplicate T-RFLP analyses using the same DNA extract.

5.2.6 MDS Analysis of T-RFLP profiles for bacterial chitinase genes

Signals with a peak area contribution below 1% (27) were regarded as background noise and excluded from analysis. Moreover, in order to avoid detection of primers and uncertainties of size determination, terminal fragments smaller than 30 bp were excluded from further analysis. The relative abundance of terminal fragments within the sections was determined by calculating the ratio between the height of each peak and the total height of all peaks within one sample.

The Euclidean distance between each sample was calculated based on the data of peak size and relative abundance, and then, differences among communities were assessed graphically using the ordination method of nonmetric multidimensional scaling (MDS) (21). All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL).

5.2.7 Nucleic sequence accession numbers

Bacterial chitinase genes sequenced in the present study have been deposited in the DDBJ database under the accession numbers AB361658-AB362158.

5.3 RESULTS

5.3.1 Soil analysis

The similarities of soil characteristics among the samples were examined by PCA. The first principal component, PC1, and the second principal component, PC2, explained 64.7 and 19.2% of the variance of the data, respectively (Fig. 1). The results of PCA showed that the ordering on PC1 mostly corresponded to organic and humic acid content, since high-content samples and low-content samples were located at opposite ends on the PC1 axis, respectively. Likewise, the ordering on PC2 mostly corresponded to pH of soil samples (Fig. 1).

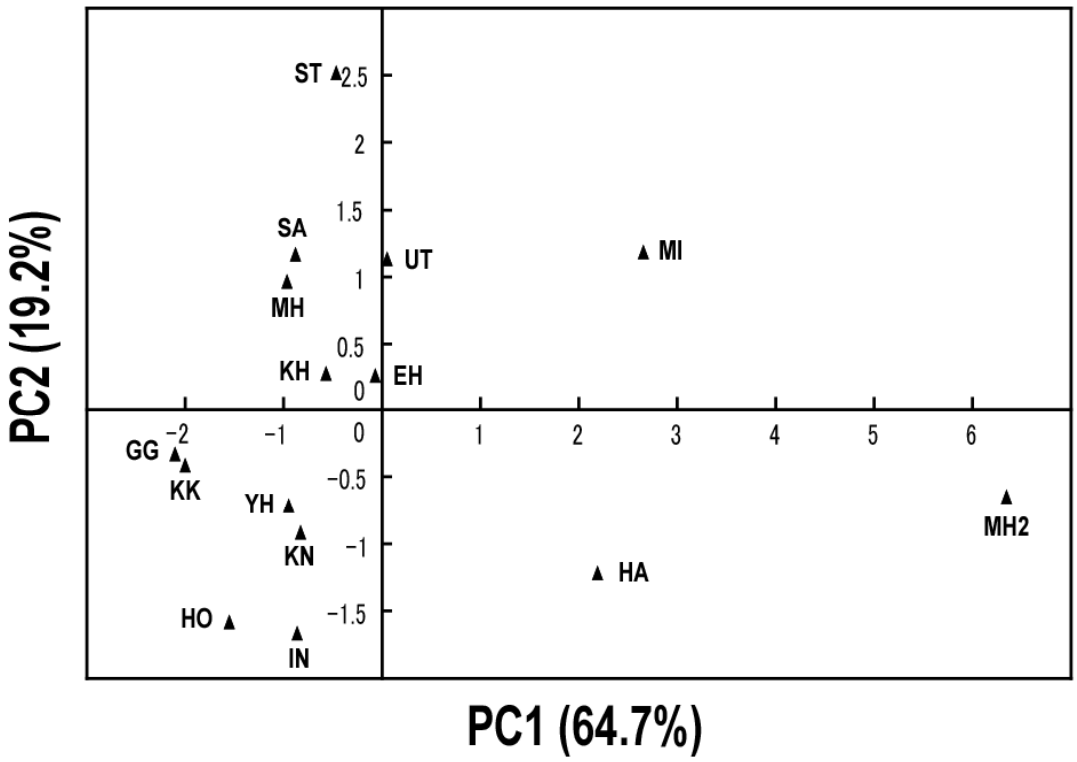

Fig. 1 PCA analysis of soil characteristics

5.3.2 Phylogeny inference of the bacterial chitinases cloned from bulk soils

In order to investigate the diversity of bacterial chitinases in bulk soils, 15 arable soil samples were selected based on the results of PCA for the soil characteristics. Bacterial chitinase genes were amplified, cloned, and then sequenced. A total of 501 clones were obtained from 15 arable soil samples. BLASTX searches of these clones revealed that these sequences had 40 to 70% similarities to chitinases listed in the current public databases at the amino acid level (August 2007). About 100 sequences of bacterial chitinases were appended to the phylogenetic analysis, whose sequences are composed of the representative chitinase genes and the most closely related genes of each of clones with respect to BLASTX searches. Phylogenetic analysis placed the deduced amino acid sequences of chitinases cloned in the present study into nine clusters, designated clusters A to I (Fig. 2).

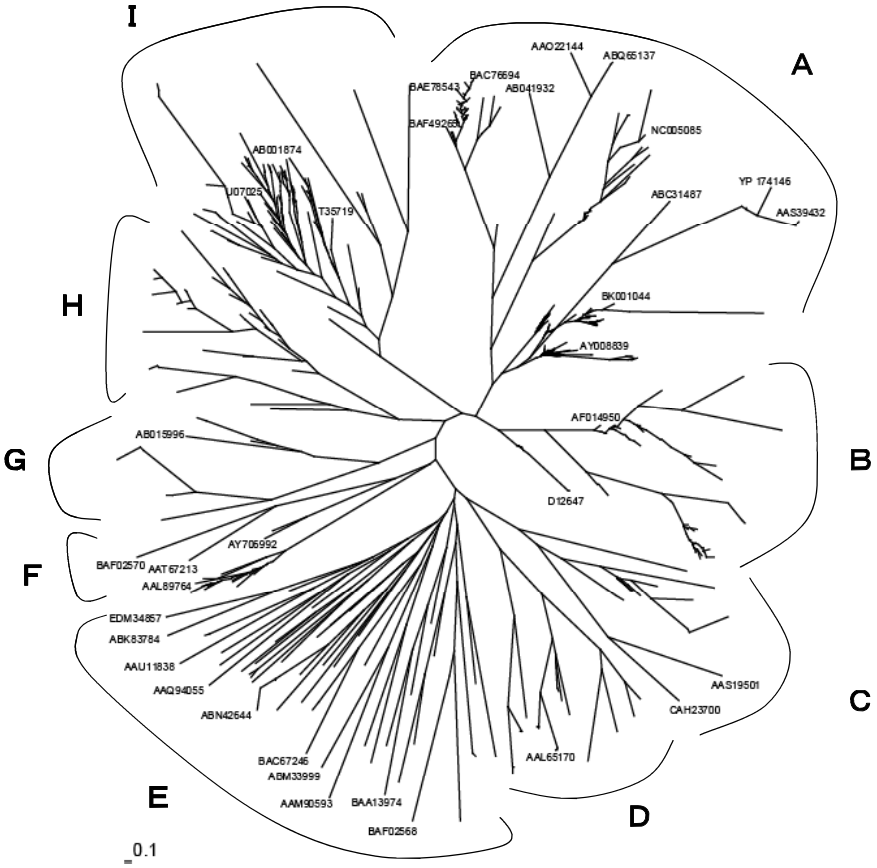

Fig. 2 Phylogenetic analysis of 501 clones and about 100 sequences of bacterial chitinases, Accession number indicate several sequences of public database

Clusters A and E contained a number of known chitinase genes. Cluster A contained the chitinases of *Bacillus* and *Streptomyces* sp. such as Chi C of *Bacillus circulans* (D89568), Chi B of *Bacillus cereus* (AAS39432), Chi C of *Streptomyces avermitilis* (NC003155) and Chi D of *Streptomyces coelicolor* (AB017011). Cluster E contained the chitinases from a wide range of known species such as *Burkholderia cepacia* (ABI86986), Chi A of *Alteromonas* sp. (D13762), Chi II of *Saccharopolyspora erythraea* (CAM05726) and so on. The other clusters contained only a few chitinases retrieved from culturable bacteria in each cluster. Especially cluster H contained only the sequences cloned in the present study.

5.3.3 The polyphasic analyses of the diversity of bacterial chitinase genes

UniFrac analysis and MDS analysis to T-RFLP profiles were employed in order to compare the diversity of bacterial chitinases among soil samples. Firstly, the diversity of bacterial chitinases was evaluated by UniFrac analysis using sequence data as shown in Fig. 3 (25). The andosol samples converged in a large cluster (cluster A in Fig. 3). The other samples with different soil types such as acrisol, cambisol, histosol and gleysol were branched in deep-rooted positions of the dendrogram (cluster B in Fig. 3).

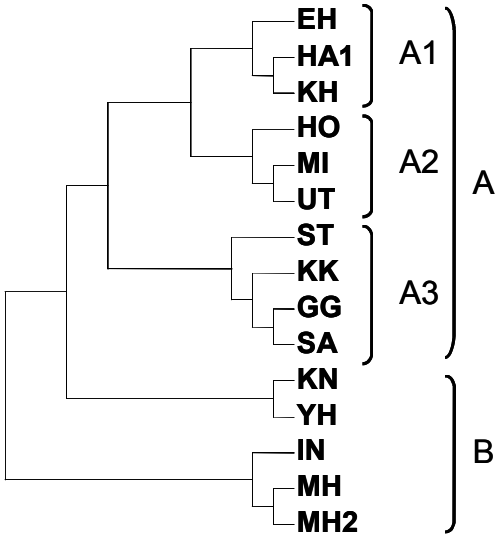

Fig. 3 Dendrogram of fifteen soil samples using UniFrac analysis

Secondly, the similarity of compositions of bacterial chitinases was visualized by MDS analysis to T-RFLP profiles. Amplification of bacterial chitinase genes with a fluorescently labeled primer set yielded a single band of the expected size (approximately 400 bp). T-RFLP profiles were generated using PCR products digested with *Afa*I and *Hha*I (data not shown). In the present study, duplicate experiments of T-RFLP analyses with *Afa*I and *Hha*I showed the identical MDS patterns, respectively, indicating high reproducibility of T-RFLP profiles. MDS analysis to T-RFLP profiles with *Afa*I digestions placed fifteen soil samples into three clusters (Fig. 4-A). On the other hand, as for *Hha*I digestions, MDS analysis placed fifteen soil samples into two clusters and several scattered points (Fig. 4-B). One cluster with both *Afa*I and *Hha*I digestions consisted of same soil samples (GG, KH, KK, MH2, MI, SA1, and UT). The other cluster with both *Afa*I and *Hha*I digestions consisted of almost all same soil samples (EH, HA, and KN) except for the samples (IN and MH). These results indicated that the diversity of chitinases was successfully visualized using MDS analysis to T-RFLP profiles.

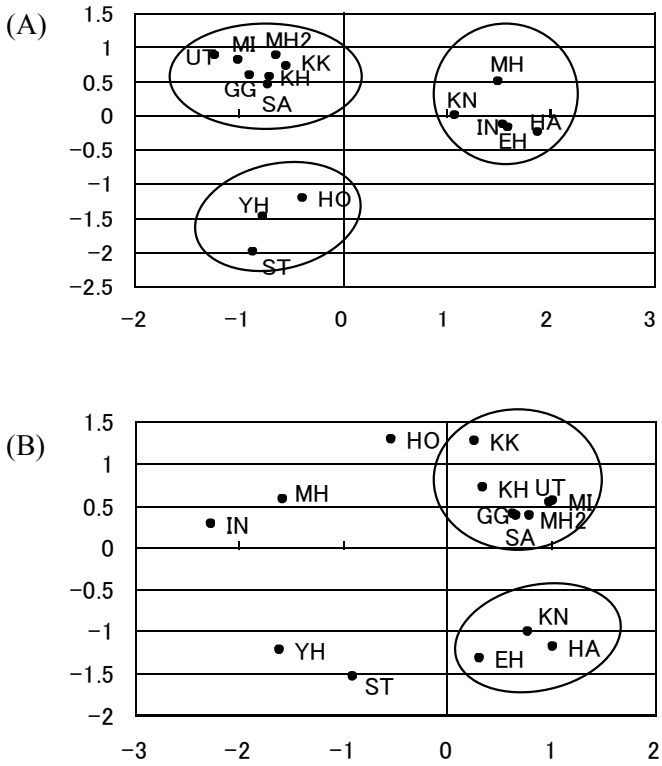

Fig. 4 Plots of MDS analysis to terminal restriction fragment length results. (A) *Afa*I, (B) *Hha*I

5.4 DISCUSSION

5.4.1 Phylogeny inference of the bacterial chitinases cloned from bulk soils

Phylogenetic analysis indicate the cloned chitinases are evenly distributed among each of clusters regardless of the types of soil samples, suggesting that there is no clear relationship between the molecular diversity of bacterial chitinases and soil characteristics. About 40% of sequences cloned in the present study are included in the cluster A (Fig. 2). Cluster A contained a number of chitinases of *Bacillus* and *Streptomyces* species, both of which are well known as important chitin degraders in soils (10, 11, 30, 40, 43). Thus, it is reasonable to assume that about 40% of sequences cloned in the present study are shown to be closely related to the chitinases of *Bacillus* and *Streptomyces* species. In contrast, the other clusters contained only a few chitinases retrieved from culturable bacteria in each cluster, except for cluster E that contained chitinases from a wide range of bacterial species. These clusters suggest the presence of novel groups of bacterial chitinases in bulk soils of arable lands. Especially, the phylogenetic analysis clearly indicate that clusters G and H are quite unique groups because a few sequences presently in the public databases are included in the cluster G and H, according to phylogenetic analysis appended to 2,690 sequences of bacterial chitinases. A total of 2,690 sequences of bacterial chitinases were collected from GenBank as of 4 January 2008 [We estimated the total number of sequences of bacterial chitinases published in GenBank with an Entrez search with chitinase AND bacteria.] in order to compare with bacterial chitinase sequences currently deposited in the public databases.

Then, the chitinases sequenced in the present study were compared with the chitinases retrieved from the chitin bags, since this report described the first molecular diversity of

chitinase in terrestrial ecosystems (28). Although the same primers were employed, the chitinases of our results were noticeably different. All the chitinases retrieved from the chitin bags were contained in the cluster E (data not shown). This difference might be explained by several reasons such as the biased diversity of microbial communities within chitin bags, the difference of soil types, or the use of different conditions for PCR amplifications. Secondly, the chitinases sequenced in the present study were compared with the chitinases of a maize field (18), since this report described the first molecular diversity of bacterial chitinases retrieved from DNA extracted from soil samples. The chitinases retrieved from the maize field were also included into the clusters A to I, except for cluster G (data not shown). According to phylogenetic analysis appended to 2,690 sequences of bacterial chitinases, cluster G contained bacterial chitinases retrieved from aquatic ecosystems such as marine sediment and intertidal hot spring. Thus, the chitinases in the cluster G were first shown to be present in terrestrial ecosystems in the present study. As for the cluster H, the chitinases retrieved from rhizosphere and ground water were merged (data not shown). Thus the chitinases in the cluster H were first shown to be present in bulk soils of arable lands in the present study. These results clearly indicate that the compositons of bacterial chitinases in bulk soils of arable lands are diverse and unique than those of previous studies based on both cultured and uncultured based methodologies.

5.4.2 The polyphasic analyses of the diversity of bacterial chitinase genes

UniFrac analysis and MDS analysis of T-RFLP profiles were employed in order to compare the diversity of bacterial chitinases among soil samples with the distinct characteristics. To date, a few studies have compared the molecular diversity of chitinases across a broad range of distinct environments (22). UniFrac analysis is a suit of tools for the comparison of microbial communities using phylogenetic information, and has been used on the basis of sequence data

of 16S rRNA (26, 41). In the present study, UniFrac analysis was adapted on the basis of sequence data of bacterial chitinases. The results indicate that the compositions of the bacterial chitinases are mostly affected by soil types as shown by clusters A and B in Fig. 4. Similar results were reported in the previous studies showing that soil type was the key factor determining bacterial community composition in soil environments such as the rhizosphere (1) and three separate arable farm soils (9). Moreover another previous study described that the bacterial community were significantly affected by particle size (35). Thus it is reasonable to assume that the bacterial chitinases are affected by soil types. Meanwhile three sub-clusters (A1, A2 and A3 in Fig. 4) formed within cluster A ranged in pH 5.2-5.6, pH 5.2-6.1 and pH 6.0-7.6, respectively. This result further suggests that the soil pH may be partially responsible for shaping the compositions of bacterial chitinases. Previous study have shown that the diversity and richness of soil bacterial communities could largely be explained by soil pH (8).

As contrasted with the sequence-based analyses, T-RFLP is a simple and powerful fingerprinting method for evaluating the diversity of a microbial community. However, a few studies have analyzed the diversity of functional genes using a T-RFLP profiles (44). MDS analysis was used to visualize the relationships among the samples based on the results of T-RFLP analysis. The distance among plots in MDS analysis indicates the similarity among the samples (Fig. 5). We focused our attention on the cluster contained same number of the samples both *Afa*I and *Hha*I (GG, KH, KK, MH2, MI, SA1, and UT in Fig. 4). The pH of these soil samples ranged 5.6-6.5, whereas pH of other scattered samples ranged below 5.5 or above 7.1. This result indicates that compositions of bacterial chitnases around pH 6 are broad range of chitinolytic bacterial communities and suggests that soil pH could be partially responsible for shaping the compositions of bacterial chitinases, similar to the results of UniFrac analysis.

With regard to usefulness of polyphasic analyses, it is assumed to be inappropriate for phylogenetic analysis to visualize the relationships among soil samples because of the large set

of sequence data. Compared to phylogenetic analysis, UniFrac analysis and MDS analysis to T-RFLP profiles were available for visualizing the relationships among multiple environmental samples. However, there were significant differences in existence or nonexistence of sequence data between UniFrac analysis and MDS analysis to T-RFLP profiles. A bias of cloning experiment via *E. coli* cells should be considered in UniFrac analysis, since UniFrac analysis must be based on the phylogenetic information on sequenced chitinases whereas MDS analysis to T-RFLP profiles might be evaluated all amplified chitinase genes. On the other hand, MDS analysis to T-RFLP profiles should consider a bias of restriction sites, although two restriction enzymes were used in the present study. In the present study, the result of UniFrac analysis clearly suggests that soil characteristics such as soil types and pH are important factors for affecting the compositions of the bacterial chitinases in bulk soils of arable lands. Meanwhile, the result of MDS analysis to T-RFLP profiles suggests that the soil pH may be partially responsible for shaping the compositions of bacterial chitinases. Thus these results indicates that soil pH may have a stronger influence on the compositions of bacterial chitinases than the soil types. Moreover, MDS analysis to T-RFLP profiles is effective in reducing a bias of cloning experiment via *E. coli* cells for the assessments of molecular diversity of functional genes.

5.5 CONCLUSION

In this chapter, we investigated the molecular diversity of bacterial chitinases in the bulk soils of multiple arable lands and effects of environmental factors on the compositions of bacterial chitinases. The compositions of bacterial chitinases were evaluated based on the polyphasic analyses including the conventional phylogenetic analysis, the application of UniFrac analysis, and MDS analysis to T-RFLP profiles of bacterial chitinases for elucidating the relationships between the diversity of bacterial chitinases and soil characteristics. The experimental results are summarized as follows.

1. The results in the present study suggest the high diversity of bacterial chitinases in bulk soils of arable lands and imply the presence of unique groups of bacterial chitinases.
2. The results also indicate that the environmental factors such as soil types and pH could be responsible for shaping the compositions of bacterial chitinases. Especially, the influence of the pH of soil samples is suggested by the both results of UniFrac analysis and MDS analysis to T-RFLP profiles.
3. These results indicate the potential usefulness of UniFrac analysis and MDS analysis to T-RFLP profiles in addition to a conventional phylogenetic analysis based on a sequence data set.

Thus the polyphasic analyses in the combinations of these molecular methods would facilitate the assessments of molecular diversity of functional genes in nature. Moreover, MDS analysis to T-RFLP profiles is effective in profiling the molecular diversity of functional genes and reducing a bias of cloning experiment via *E. coli* cells for the assessments of molecular diversity of functional genes.

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Chapter 6

The effectiveness of screening of environmental samples in metagenomic analysis using T-RFLP analysis

SUMMARY

The target genes represent a small proportion of the total nucleic acid fraction in the metagenomic analysis. It is important for suggesting the existence of the target genes, especially in the genes that include novel base sequence and/or characteristics. In this chapter, several environmental samples were profiled with T-RFLP and MDS analysis to assess the effectiveness of screening of environmental samples in metagenomic analysis. First, the molecular diversity of esterases among the environmental samples was investigated using T-RFLP and MDS analysis. MDS pattern indicate that there are different types of esterases among three clusters, while there are similar types of esterases among the same kinds of samples. Secondly, novel esterase genes were retrieved from environmental samples, and then examined the relationship between molecular diversity of esterases through MDS pattern and characteristics of novel esterase genes through phylogenetic analysis. As compared with MDS pattern, phylogenetic analysis of novel esterase genes shows a little relationship of MDS pattern. The potential usefulness of MDS analysis to T-RFLP profiles are shown in the assessment of the effectiveness of screening of environmental samples in metagenomic analysis. Thus MDS analyses to T-RFLP profiles make it possible to place the types of novel esterases based on the sequence similarity into each cluster corresponding to MDS pattern among environmental samples.

6.1 INTRODUCTION

Esterases (EC 3.1.1.x) are defined as hydrolases catalyzing the cleavage and formation of ester bonds and are considered to be some of the most useful biocatalysts in the synthetic organic chemistry (3). Two major classes of hydrolases are of utmost importance: carboxylesterases (EC 3.1.1.1) and lipases (EC 3.1.1.3). For example, lipases (EC 3.1.1.3) are defined as carboxylesterases which catalyze the hydrolysis of long-chain acylglycerols and .play an important role in a variety of biotechnological applications such as the processing of oils and fats, detergent manufacturing (8). Esterases are widely distributed in animals, plants and microorganisms. Especially, microorganisms have been found to be a major source of a variety of esterases.

However, current research indicates that more than 99% of the microorganisms in the environment can not be easily cultivated (1, 10). The genomes of as yet uncultured microorganisms represent rich repositories of genetic resources for novel genes and chemical compounds (9, 13). To overcome the difficulties and limitations associated with cultivation techniques, a new technology of the culture-independent genomic analysis of environmental DNA, metagenomics, has been developed over the past decade and has led to the discovery and characterization of a wide range of novel genes or biocatalysts (5, 12). In the metagenomics, the target genes represent a small proportion of the total nucleic acid fraction. Thus, pre-enrichment or screening of the sample thus provides an attractive means of enhancing the positive hit rate (4).

On the other hand, terminal restriction fragment length polymorphism (T-RFLP) is highly effective in fingerprinting the microbial community structure. Moreover, a combination of

T-RFLP with statistical analysis such as principal component analysis and multidimensional scaling (MDS) analysis provides useful information about visualizing the structure of microbial communities (6). However, there are very few studies on screening of the environmental samples in metagenomic analysis by applying for T-RFLP combined with statistical analysis. Hence, it is significant that T-RFLP combined with cloning and/or statistical analysis is applied to obtain the microbial community fingerprints for screening environmental samples.

The objective of this chapter is to evaluate the effectiveness of screening of environmental samples in metagenomic analysis through T-RFLP combined with cloning and/or statistical analysis. We have chosen the novel esterase as our target enzyme. We examined the relationship between molecular diversity of bacterial esterases through T-RFLP combined with statistical analysis and characteristics of novel esterase genes retrieved from environmental samples.

6.2 MATERIAL AND METHODS

6.2.1 DNA extraction from environmental samples

Environmental samples were collected from activated sludges, soils, stools, composts and appendices. DNA was extracted from approximately 5 g of each sample using ISOIL (NIPPONGENE, Toyama, Japan) according to the manufacturer's instructions. The yield and quality of the DNA were checked by measuring absorbance at 260 nm and 280 nm using a BECKMAN COULTER DU®640 spectrophotometer (Palo Alto, Calif).

6.2.2 PCR conditions

PCR amplifications were carried out using the minor modification as previously described by Bell *et al* (2). Briefly, the PCR primers used for the amplification of putative esterases were OXF1 (ccy gtk gts ytn gtn cay gg) and ACR1 (agg ccn ccc akn gar tgn sc). For fluorescence detection, the 5' end of the forward primer was labeled with BODIPY®FL (J-Bio21, Ibaraki, Japan). The PCR reaction mixture (total 25 µl) contained 2.5 µl of 10 x buffer, 4 µM of primers, 200 µM of each dNTP and 1 U of Blend *Taq* –Plus- DNA polymerase (TOYOBO, Osaka, Japan). Three microliter (10 ng/µl of DNA) of environmental DNA was used in each amplification reaction as the template. The PCR amplification program consisted of 2 min at 94˚C, 50 cycles of 30 sec at 94˚C, 30 sec at 50˚C and 90 sec at 72˚C, and a final extension time of 7 min at 72˚C in a GeneAmp PCR System 9700 (Applied Biosystems, Tokyo, Japan). PCR products were verified by electrophoresis on 2% (w/v) agarose gels, followed by 20 min of staining with ethidium bromide (10 µl l^{-1}).

6.2.3 T-RFLP and MDS analysis

T-RFLP analysis was carried out according to the method previously described (14). Briefly, fluorescently labeled PCR products (10 μl) were purified with a Microcon YM-100 (Millipore) and eluted in 20 μl of TE buffer. Aliquots (5 μl) were then digested in a total volume of 20μl for 4 h at 37˚C with *Hha*I (TaKaRa Bio). The digests (10 μl) were purified by ethanol precipitation. The precipitate was mixed with 0.5 μl of GeneScan 500-LIZ size standard (Applied Biosystems) and 30 μl of deionized formamide. After DNA denaturation at 94˚C for 5 min and immediate chilling on ice, the fluorescently labeled terminal fragments were analyzed by electrophoresis on an ABI PRISM 3100 Genetic Analyser automated sequence analyser (Applied Biosystems) in the GeneMapper mode. Reproducibility of T-RFLP profiles was confirmed by conducting duplicate T-RFLP analyses using the same DNA extract. Then, multidimensional scaling (MDS) analysis was also carried out according to the protocol previously described (14). All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL).

6.2.4 Retrieval of putative esterase genes from environmental samples

Putative esterase genes were retrieved from environmental samples according to the method, pre-amplified inverse PCR (PAI-PCR), previously described (18). Open reading flame (ORF) analysis was performed using GENETYX software (GENETYX, Tokyo, Japan). Putative ORFs were translated and used as queries for blastp searches against the NCBI protein database.

6.2.5 Phylogenetic analysis

For phylogenetic analysis, alignments of deduced amino acid sequences were performed using the CLUSTAL W program (15) and the neighbor-joining method was used for building the phylogenetic trees (11). The Phylip format tree output was applied using the bootstrapping procedure (7) (the number of bootstrap trials used was 1000).

6.3 RESULTS

6.3.1 T-RFLP and MDS analysis

Several environmental samples were profiled with T-RFLP and MDS analysis to assess the effectiveness of screening of environmental samples in metagenomic analysis. Environmental samples were collected from activated sludges, soils, stools, composts and appendices. PCR products from each environmental DNA were obtained using a degenerate esterase-targeted primer set labeled with BODIPY®FL to amplify appropriate 200-250 bp fragments (data not shown). T-RFLP electropherograms were generated using PCR products digested with *Hha*I. T-RFLP electropherograms differ significantly among environmental samples (Fig. 1). This result indicated that there were different types of esterases among environmental samples.

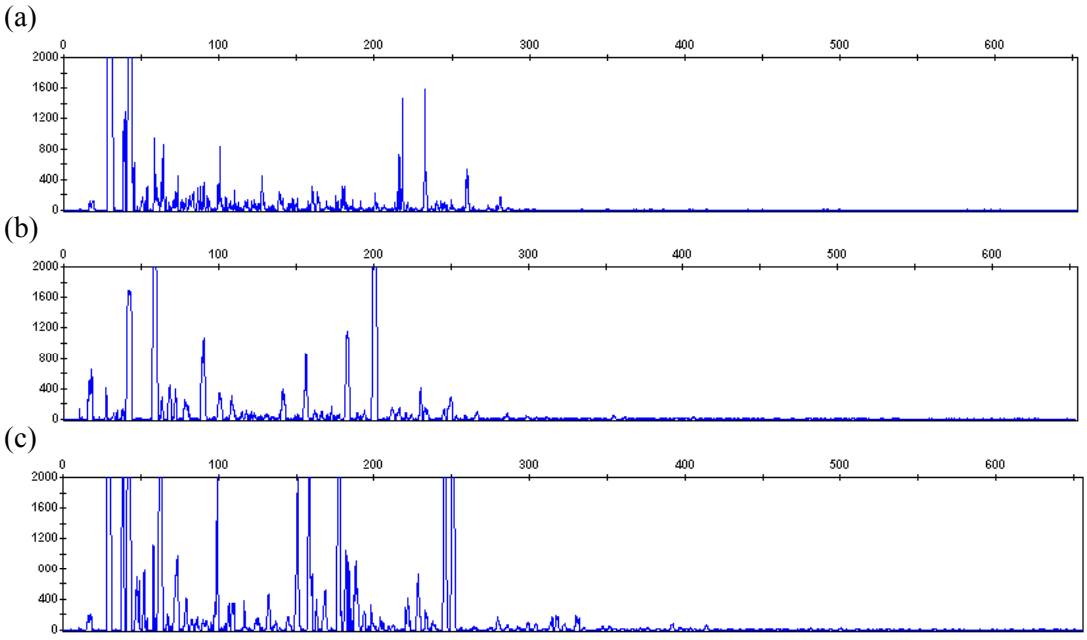

Fig. 1. T-RFLP electropherograms after digestion with *Hha*I

(a) activated sludge, (b) soil, (c)stool

Secondly, MDS analysis was then used to visualize the relationships among the samples based on the results of T-RFLP analysis. In this study, duplicate experiments of T-RFLP analyses with *Hha*I showed the MDS pattern. Points close to each other mean that the sequence similarity of esterases between samples is probably similar. The result indicated that MDS pattern of T-RFLP profiles with *Hha*I digestions placed nineteen environmental samples into three clusters (Fig. 2). The first cluster is composed of activated sludges and composts (Cluster I). The second cluster is composed of soils (Cluster II). The third cluster is composed of stools and appendix of horses (Cluster III). Moreover, points were close to each other among the same kinds of samples. For example, all points of activated sludges were included in cluster I, and all points of stools were included in cluster III, and so on. Thus, these results indicated that there were different types of esterases among three clusters, while there were similar types of esterases among the same kinds of samples.

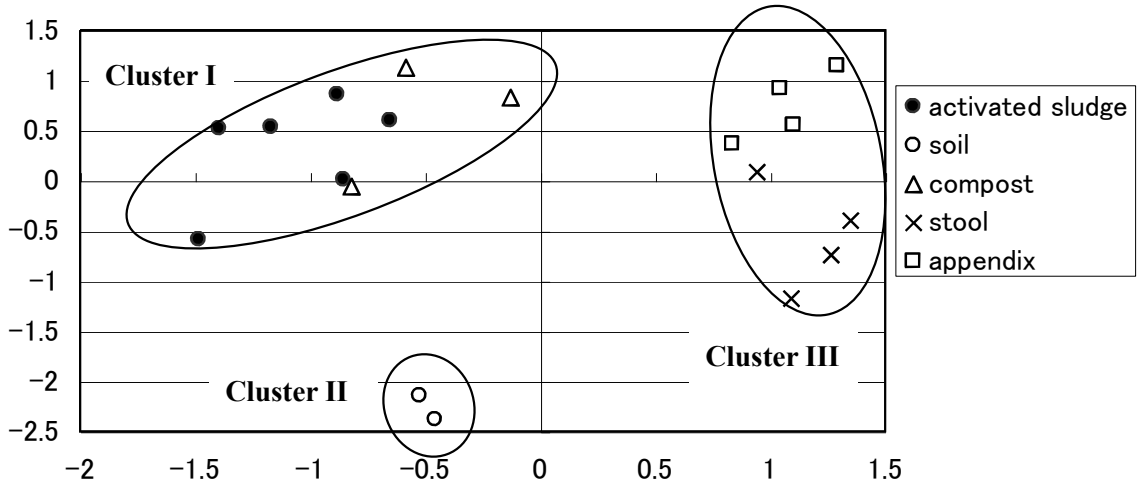

Fig. 2 Multidimensional scaling analysis of T-RFLP results. Symbols: closed circles; activated sludge, open circles; soil, open triangles; compost, crosses; stool, open square; appendix,

6.3.2 Retrieval of putative esterase genes from environmental samples

Putative esterase genes were retrieved from environmental samples according to PAI-PCR (18). PCR amplifications were performed essentially as described above, except that the 5' end of the forward primer was not labeled with BODIPY®FL. PCR products were ligated into a TA-cloning vector, and used to transform cells of *E. coli*. Transformants were randomly picked up and then the sequences were determined. The sequence similarity to the known esterases was used to select for the clones which should be amplified by PAI-PCR. Sequencing analysis of the amplification products by PAI-PCR demonstrated that thirteen putative novel esterase genes were retrieved from several environmental samples (Table 1).

Table 1 Characterization of putative esterase genes cloned from environmental samples

Gene	Sample	Size (aa)	Identities	E-value	Accession No.	Homologue
①	Sludge	304	37%	1.00E-38	ABD05759	Alpha/beta hydrolase [Rhodopseudomonas palustris HaA2]
②	Sludge	304	33%	7.00E-37	EAX43775	putative hydrolase protein [Ralstonia pickettii 12J]
③	Soil	293	76%	1.00E-95	ABF40304	hypothetical protein Acid345_1302 [Acidobacteria bacterium Ellin345]
④	Soil	291	41%	4.00E-57	AAG06337	probable lipase [Pseudomonas aeruginosa PAO1]
⑤	Soil	290	49%	3.00E-73	ABD25138	alpha/beta hydrolase [Novosphingobium aromaticivorans DSM 12444]
⑥	Compost	261	45%	8.00E-46	ABQ92942	alpha/beta hydrolase [Rseiflexus sp. RS-1]
⑦	Soil	315	36%	7.00E-47	AAM40072	conserved hypothetical protein [Xanthomonas campestris]
⑧	Stool	289	32%	1.00E-30	AAZ32719	lipase/esterase [uncultured bacterium]
⑨	Soil	260	72%	4.00E-66	ABC91194	putative hydrolase protein [Rhizobium etli CFN 42]
⑩	Stool	272	41%	1.00E-37	ABQ46908	phospholipase/Carboxylesterase [Thermotoga petrophila RKU-1]
⑪	Soil	231	46%	4.00E-50	EAP71282	esterase [Ralstonia solanacearum UW551]
⑫	Compost	315	48%	2.00E-75	CAK08822	putative lipase [Rhizobium leguminosarum bv. viciae 3841]
⑬	Soil	279	63%	4.00E-74	EAV06637	alpha/beta hydrolase fold [Burkholderia phytofirmans PsJN]

About thirty sequences of esterases were appended to the phylogenetic analysis, whose sequences are composed of the obtained putative esterases and the closely related genes of each of clones with respect to BLASTX searches. Phylogenetic analysis placed the deduced amino acid sequences of esterases cloned in the present study into four clusters, designated clusters A to D (Fig. 3).

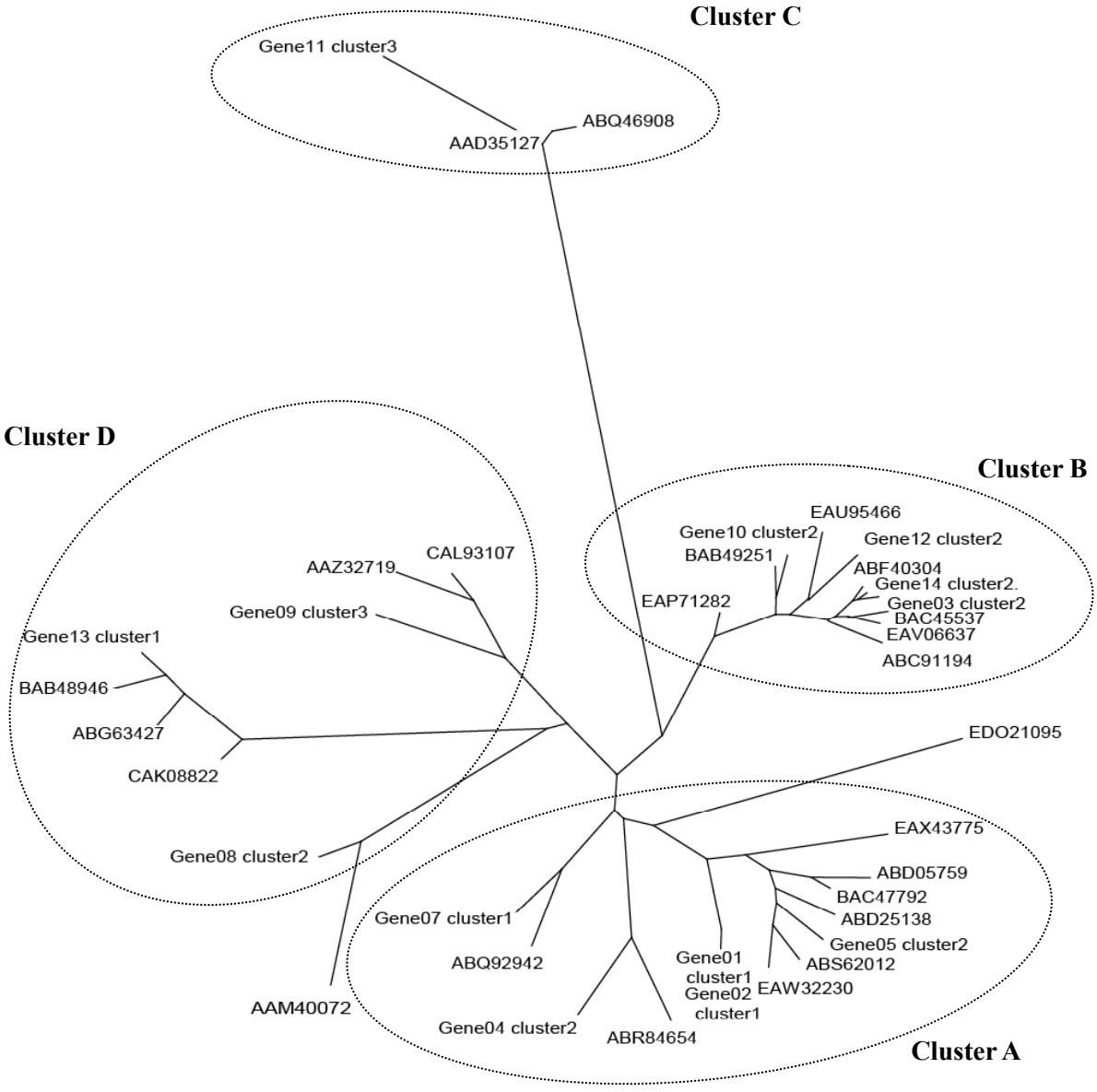

Fig. 3. Phylogenetic dendrogram based on the obtained esterases and the closely related genes

As compared with MDS analyses to T-RFLP profiles, phylogenetic analysis showed a little relationship of MDS pattern. Cluster A contained putative esterases retrieved from the sample of cluster I and II. Cluster B contained only putative esterases retrieved from the sample of cluster II. Cluster C contained only one putative esterase retrieved from the sample of cluster III. Cluster D contained putative esterases retrieved from the sample of all kinds of clusters. These results raised the high possibility that different types of esterases were retrieved from the samples shown different cluster by MDS pattern. Each cluster of MDS pattern roughly corresponded to the cluster in phylogenetic analysis. Thus, MDS analysis to T-RFLP profiles made it possible to place the types of novel esterases based on the sequence similarity into each cluster corresponding to MDS pattern among environmental samples.

6.4 DISCUSSIONS

Microbial communities are characterized by extraordinary numbers of cells and species richness (16). The target genes represent a small proportion of the total nucleic acid fraction in the metagenomic analysis. Thus it is important for suggesting the existence of the target genes, especially in the genes that include novel base sequence and/or characteristics. In this study, MDS analysis to T-RFLP profiles was adapted to esterases in order to elucidate the relationships between the diversity of esterases and characteristics of novel esterase genes among environmental samples. As contrasted with the sequence-based analyses, T-RFLP is a simple and powerful fingerprinting method for evaluating the diversity of a microbial community. On the other hand, T-RFLP profiles should consider the bias of PCR, degenerate primer set and restriction sites. However, a few studies have analyzed the diversity of functional genes using a T-RFLP profiles (17). MDS analysis was used to visualize the relationships among the samples based on the results of T-RFLP analysis. First, the molecular diversity of esterases among the environmental samples was investigated using T-RFLP and MDS analysis. The distance among plots in MDS analysis indicates the similarity among the samples (Fig. 2). We focused our attention on the samples forming a same cluster. Secondly, novel esterase genes were retrieved from environmental samples, and then examined the relationship between molecular diversity of esterases through MDS pattern and characteristics of novel esterase genes through phylogenetic analysis. The phylogenetic analysis raised the high possibility that different types of esterases were retrieved from the samples shown different cluster by MDS pattern. As compared with phylogenetic analysis, MDS analysis to T-RFLP profiles shows a little relationship of characteristics of novel esterase genes among environmental samples. Thus MDS analysis to

T-RFLP profiles makes it possible to place the types of novel esterases based on the sequence similarity into each cluster corresponding to MDS pattern among environmental samples.

6.5 CONCLUSIONS

In this chapter, several environmental samples were profiled with T-RFLP and MDS analysis to assess the effectiveness of screening of environmental samples in metagenomic analysis. First, the molecular diversity of esterases among the environmental samples was investigated using T-RFLP and MDS analysis. Secondly, novel esterase genes were retrieved from environmental samples, and then examined the relationship between molecular diversity of esterases through MDS pattern and characteristics of novel esterase genes. The experimental results are summarized as follows.

1. MDS pattern indicate that there are different types of esterases among three clusters, while there are similar types of esterases among the same kinds of samples. The first cluster is composed of activated sludges and composts (Cluster I). The second cluster is composed of soils (Cluster II). The third cluster is composed of stools and appendix of horses (Cluster III).
2. Thirteen putative novel esterase genes were retrieved from several environmental samples. According to phylogenetic analysis, phylogenetic analysis placed the deduced amino acid sequences of esterases cloned in the present study into four clusters. As compared with MDS pattern, phylogenetic analysis shows a little relationship of MDS pattern.

The potential usefulness of MDS analysis to T-RFLP profiles are shown in the assessment of the effectiveness of screening of environmental samples in metagenomic analysis. MDS analyses to T-RFLP profiles make it possible to place the types of novel esterases based on the sequence similarity into each cluster corresponding to MDS pattern among environmental samples.

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Chapter 7

Conclusion and perspectives

7.1 Summary of this study and conclusive remarks

The Molecular biology have been developed rapidly over the last few decades and shown that the diversity of environmental microorganism is greater than initially anticipated. Moreover, more than 99% of the environmental microorganisms are unculturable. These microorganisms have the potential to be utilized as two major aspects of biotic function and biological resources. In either case, to utilize the microorganisms more effectively, it is important for obtaining the microbial community fingerprints. Recently, some molecular methods have been developed for analysis of the microbial community structure of environmental samples. Especially, terminal restriction fragment length polymorphism (T-RFLP) is highly effective in fingerprinting the microbial community structure. Moreover, a combination of cloning and/or statistical analysis with T-RFLP is available for monitoring or screening the samples. However, sufficient studies combined with cloning and/or statistical analysis have not been conducted for monitoring microbial community transition or screening environmental samples. Hence, it is significant that T-RFLP combined with cloning and/or statistical analysis is applied to obtain the microbial community fingerprints for development of environmental biotechnology and utilization of environmental microorganisms.

Thus, the objective of this study is to demonstrate the efficacy of T-RFLP in applying for environmental biotechnology under the following points:

1. Evaluation of monitoring the microbial communities in the wastewater treatment processes through T-RFLP and MDS analysis,

2. Evaluation of screening the environmental samples through T-RFLP and MDS analysis.

Summary of each chapter is described as follows.

In chapter 1, the potential of the microorganisms for environmental biotechnology such as wastewater treatment processes and metagenomic analysis is described. Moreover, the principles, advantages, and disadvantages of fingerprinting techniques are described. And also, the meaning and objectives are indicated.

In detail, the efficacy of T-RFLP in monitoring the microbial communities in the wastewater treatment processes is described in chapter 2-4. Furthermore, the efficacy of T-RFLP in screening the environmental samples is described in chapter 5 and 6.

In chapter 2, the microbial population dynamics at the start-up stage of a wastewater treatment reactor was investigated using terminal restriction fragment length polymorphism (T-RFLP) analysis based on 16S rDNA and rRNA gene sequences. The experimental results are summarized as follows.

4. T-RFLP analyses of the 16S rDNA and rRNA genes demonstrate that the relative abundance and activities of nitrifying bacteria increase in association with the start of nitrification.
5. The relative ratios of the 16S rRNA of these nitrifying bacteria increase prior to those of 16S rDNA by calculating the ratio of peak height of selective peaks to total peak heights.
6. MDS analysis suggests that the diversity of 16S rRNA in the microbial community is strongly affected by environmental changes and that the composition of the microbial community changed in association with the start of nitrification.

Therefore, T-RFLP analysis can be useful to monitor microbial communities in wastewater treatment systems and to assess the condition of the reactor for control and improvement of such systems.

In chapter 3, the bacteria playing an important role in denitrification was investigated by monitoring the molecular dynamics accompanying the start of denitrification using T-RFLP. The experimental results are summarized as follows.

3. The bacteria belonging to the genera *Hydrogenophaga* and *Acidovorax* were increased in number after the start of denitrification
4. Combination of the results of T-RFLP analysis and 16S rRNA clone library indicate that the bacteria belonging to the genera *Hydrogenophaga* and *Acidovorax* play an important role in denitrification

The results of this study provide new insight to the 16S rRNA level of active denitrifying bacteria in wastewater treatment processes. Furthermore, the results obtained in this study show the potential of monitoring the transition of the microbial structure using T-RFLP targeting 16S rRNA for identification of the microbial population associated with denitrification. It is anticipated that genes and transcripts of nitrite reductase (nirS and nirK) will be analyzed by micro autoradiography (MAR)-FISH and/or stable isotope probing (SIP) methods, and the combination of the results based on 16S rRNA and functional genes could determine the bacteria that actually perform denitrification in wastewater treatment processes.

In chapter 4, the microbial population and its succession in a circulation flush toilet were monitored by T-RFLP and cloning analyses. Clone libraries of 16S rRNA gene on day 3 and day 127 were constructed. The experimental results are summarized as follows.

4. On day 3, 102 clones were sequenced and *Proteobacteria* and *Bacteroidetes* accounted for 27% and 45%, respectively. On the other hand, *Proteobacteria* increased to 43% and *Bacteroidetes* decreased to 26% of a total of 100 clones on day 127.
5. In T-RFLP analysis, the relative abundances of *Nitrosomonas* increased from 1% to 6% with commencement of nitrification and denitrification. Similarly, relative abundance of T-RFs

generated from *Xanthomonas* increased from 3% to 10%. Therefore, these bacteria could play a prominent role in this process.

6. The microbial community structure changed markedly, particularly during the start-up period of the system. The plots then became stable after the start of nitrification and denitrification by multidimensional scaling (MDS) analysis. This result suggests that the succession of microbial community structure has a correlation with performance of the system.

Information on the microbial population is important to establish and enhance the reliability of this system for long-term operation without maintenance. It is anticipated that further monitoring and accumulation of T-RFLP data would enable us to predict the deterioration of water quality. Further work is needed to determine the active portion of the bacteria concerned with nitrification and denitrification. A combination of T-RFLP analysis and stable isotope probing methods will clarify these points.

In chapter 5, we investigated the molecular diversity of bacterial chitinases in the bulk soils of multiple arable lands and effects of environmental factors on the compositions of bacterial chitinases. The compositions of bacterial chitinases were evaluated based on the polyphasic analyses including the conventional phylogenetic analysis, the application of UniFrac analysis, and MDS analysis to T-RFLP profiles of bacterial chitinases for elucidating the relationships between the diversity of bacterial chitinases and soil characteristics. The experimental results are summarized as follows.

4. The results in the present study suggest the high diversity of bacterial chitinases in bulk soils of arable lands and imply the presence of unique groups of bacterial chitinases.
5. The results also indicate that the environmental factors such as soil types and pH could be responsible for shaping the compositions of bacterial chitinases. Especially, the influence of

the pH of soil samples is suggested by the both results of UniFrac analysis and MDS analysis to T-RFLP profiles.

6. These results indicate the potential usefulness of UniFrac analysis and MDS analysis to T-RFLP profiles in addition to a conventional phylogenetic analysis based on a sequence data set.

Thus the polyphasic analyses in the combinations of these molecular methods would facilitate the assessments of molecular diversity of functional genes in nature. Moreover, MDS analysis to T-RFLP profiles is effective in profiling the molecular diversity of functional genes and reducing a bias of cloning experiment via *E. coli* cells for the assessments of molecular diversity of functional genes.

In chapter 6, several environmental samples were profiled with T-RFLP and MDS analysis to assess the effectiveness of screening of environmental samples in metagenomic analysis. First, the molecular diversity of esterases among the environmental samples was investigated using T-RFLP and MDS analysis. Secondly, novel esterase genes were retrieved from environmental samples, and then examined the relationship between molecular diversity of esterases through MDS pattern and characteristics of novel esterase genes. The experimental results are summarized as follows.

3. MDS pattern indicates that there are different types of esterases among three clusters, while there are similar types of esterases among the same kinds of samples. The first cluster is composed of activated sludges and composts (Cluster I). The second cluster is composed of soils (Cluster II). The third cluster is composed of stools and appendix of horses (Cluster III).
4. Thirteen putative novel esterase genes were retrieved from several environmental samples. According to phylogenetic analysis, phylogenetic analysis placed the deduced amino acid

sequences of esterases cloned in the present study into four clusters. As compared with MDS pattern, phylogenetic analysis shows a little relationship of MDS pattern.

The potential usefulness of MDS analysis to T-RFLP profiles are shown in the assessment of the effectiveness of screening of environmental samples in metagenomic analysis. MDS analyses to T-RFLP profiles make it possible to place the types of novel esterases based on the sequence similarity into each cluster corresponding to MDS pattern among environmental samples.

7.2 Perspective

T-RFLP was successfully applied to monitoring the microbial communities in the wastewater treatment processes and screening the environmental samples in combination with MDS analysis in this study. However, application of T-RFLP to environmental biotechnology remains some problems in analyzing microbial communities.

Monitoring the microbial communities is limited by a resolution of T-RFLP. The information of microbial community acquired by 16S rRNA gene analysis can not directly correlate with those acquired by functional gene analysis in the wastewater treatment processes. Thus we should investigate functional genes such as ammonia monooxygenase and nitrite reductase gene using T-RFLP, whereas sequence data of functional genes are very similar among different bacterial species. Therefore it seems to be difficult that T-RFLP is applied to analyze microbial communities by targeting functional genes because it is difficult to identify bacterial species from T-RFLP results. Recently, some techniques such as stable isotope probing and micro-autoradiography (MAR)-FISH have been developed to elucidate bacterial activity. Application of these techniques may bring some new information of microbial community.

On the other hand, screening the environmental samples is limited by numerous peaks obtained form T-RFLP. It seems to be difficult to narrow down samples that include abundant novel genes. Some hybridization techniques such as southern hybridization may be useful for screening the environmental samples when genome database is considered to be dramatically increased.

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Appendix

博士論文概要

従来の微生物学研究は環境中から採取した微生物の分離・培養を基盤に行われてきたが，近年，急速に発展してきた分子生物学的手法により，培養を伴わずに複合微生物生態系の解析が可能になってきた。分子生物学的手法によって環境微生物の分類学的な同定が行われた結果，微生物は複雑に相互作用しており，環境中での一連の物質代謝が複数の菌株の共代謝によって行われているケースが多いことなどが明らかになってきた。さらに，現在の技術で培養可能な微生物は実際に環境中に存在する微生物の 1%程度に過ぎないことも明らかになってきた。このような複合微生物生態系は多岐にわたって活用される可能性を秘めており，特に２つの観点で着目できる。一つは，生物機能として活用する場合であり，その機能を活かすためには複合微生物叢の理解が必要不可欠である。特に，反応を担う微生物群の挙動を把握すること，すなわちモニタリングすることが極めて重要である。もう一つは，培養可能な微生物は僅かであるため，生物資源として活用する場合である。最近，環境中から取得した DNA の解析，いわゆるメタゲノム解析が注目されているが，メタゲノム解析を網羅的に行うことはコストや時間を要するため，効率的なスクリーニング系を構築する必要がある。よって，両観点において複合微生物叢を簡易に把握，すなわちプロファイルし，モニタリングもしくはスクリーニングに適用できる手法が求められている。

本論文では，微生物叢プロファイリング手法として Terminal Restriction Fragment Length Polymorphism (T-RFLP)法に着目した。T-RFLP 法は蛍光標識したプライマーを用いて増幅した PCR 産物を制限酵素で断片化し，その断片を電気泳動によって分離する手法であり，簡便かつ迅速に微生物種とその存在比を解析できるために微生物叢のプロファイリングに有効である。そこで，本手法を用いて複合微生物叢を簡易に把握し，モ

ニタリングやスクリーニングに適用し，その有効性を検討した。具体的には，生物機能の観点からは生物学的排水処理に着目し，硝化工程，脱窒工程，および長期間連続運転時の排水処理微生物叢をそれぞれ T-RFLP 法によって解析し，その変遷をモニタリングした。また，排水処理能と微生物叢との関係および外部環境因子の変動に対する微生物叢の変遷を解析することにより，複合微生物叢のモニタリングに T-RFLP 法を適用することの有効性を示した。一方，生物資源の観点からは環境試料を未利用遺伝子資源と位置づけ，T-RFLP 法によって遺伝子の多様性を解析し，環境試料からの遺伝子スクリーニングへの適用について検討した。具体的には，T-RFLP 法とクローニング法を用いて土壌中のキチナーゼ遺伝子の多様性を解析・比較し，T-RFLP 法によって遺伝子の多様性を概ね把握できることを示した。また，T-RFLP 法によるリパーゼ遺伝子の多様性と環境試料から取得した新規リパーゼ遺伝子の多様性に基づき，環境試料をそれぞれグループ分けした結果に対応関係があることを見出し，環境試料からの遺伝子スクリーニングに T-RFLP 法を適用することの有効性を示した。

本論文は 7 章で構成されている。以下に各章の概要を述べる。

第 1 章では，分子生物学的手法の特徴やそれらを活用した微生物生態解析の現状について，既往の研究動向を整理して本論文の研究背景をまとめるとともに，意義および目的を述べた。

第 2 ～ 4 章では複合微生物叢のモニタリングへの T-RFLP 法の適用を検討した。第 2 章では，生物学的排水処理能と微生物叢との関係について調べるために，排水処理槽内の微生物叢を T-RFLP 法にてモニタリングすることが有効であることを示した。間欠曝気方式の排水処理槽を運転し，16S rDNA および rRNA 遺伝子に基づいて T-RFLP 法によって微生物叢の変遷をモニタリングした結果，硝化反応の開始時に特定の Terminal Restriction Fragments(T-RFs)が変化し，微生物叢が大きく変遷することがわかった。データベースを基に微生物種を同定したところ，大きく変化した T-RFs はアンモニア酸化細菌由来であることが推察され，硝化反応に関わるアンモニア酸化細菌が微生物叢内で優

占していたことを明らかにした。また，アンモニア酸化細菌の 16S rRNA は 16S rDNA に先んじて微生物叢内での割合が増加していたことが確認され，多次元尺度法にて解析した結果，微生物叢全体で 16S rRNA は 16S rDNA より環境変動に鋭敏に応答することが示された。以上の結果から，T-RFLP 法と多次元尺度法を組み合わせ，視覚化する方法が有効であることを示した。

第３章では，生物学的排水処理における脱窒工程に着目し，T-RFLP法によって排水処理槽内の微生物叢の変遷をモニタリングした。間欠曝気方式の排水処理槽を運転し，微生物叢を16S rRNA遺伝子に基づいてT-RFLP法およびクローニング法によって解析した。その結果，脱窒反応の開始時に増加したT-RFsはクローニング法により*Hydrogenophaga*と*Acidovorax*属細菌であることが示され，それらが微生物叢内で優占していたことを明らかにした。両細菌は既往の研究で脱窒細菌であると報告されていることから，本実験結果の妥当性が示された。また，主なT-RFsに相当する微生物の優占度をT-RFLP法とクローニング法からそれぞれ算出した。その結果，微生物の優占度は両手法でほぼ一致し，T-RFLP法がクローニング法と同程度に微生物叢を把握できる上，モニタリングに適していることが示された。

第４章では，第２，３章で示された成果に基づき，循環型水洗トイレの開発に向け，長期間にわたって循環型水洗トイレの活性汚泥の微生物叢をT-RFLP法によってモニタリングした。運転期間は200日で，1～2週間に一度サンプリングを行い，計21サンプルをT-RFLP法によって解析した。まず，100日間はアンモニアおよび硝酸態窒素が蓄積し硝化・脱窒反応は進行していなかったが，100日目に曝気条件を変更した後は速やかに硝化・脱窒反応が進行することを確認した。そして，3日目と127日目の活性汚泥サンプルを用い，クローニング法によって約100個のクローンをそれぞれ解析した。その結果，3日目の微生物叢は*Proteobacteria*と*Bacteroidetes*が各々27%と45%を占めていたが，127日目の微生物叢では*Proteobacteria*は43%に増加した一方で*Bacteroidetes*は26%に減少したことがわかった。つぎにT-RFLP法によって詳細なモニタリングを行った結果，硝化・

脱窒反応の進行に伴い*Proteobacteria*の*Nitrosomonas*属が1%から6%に，*Xanthomonas*属が3%から10%にそれぞれ増加しており，クローニング法のデータで*Proteobacteria*の割合が増加していたことが裏付けられた。*Nitrosomonas*属は代表的な硝化細菌，*Xanthomonas*属は脱窒細菌として報告されており，これらの微生物が循環型水洗トイレの主要な役割を担っていることが示唆された。さらに，循環型水洗トイレの性能と微生物叢との関連について調べるために，多次元尺度法によってT-RFLP法の結果を視覚化した。その結果，硝化・脱窒反応が進行していなかった100日後までの微生物叢は激しく変遷していたが，硝化・脱窒反応進行後は比較的安定していたことが示された。

第５，６章では環境試料からの遺伝子スクリーニングへの T-RFLP 法の適用を検討した。第５章では，15 種類の土壌試料中のキチナーゼ遺伝子の多様性について，T-RFLP 法とクローニング法による解析結果を比較・検討した。まず，T-RFLP 法のデータを多次元尺度法によって解析した結果, 15 種類の土壌試料は 3 つのグループに分けられた。一方，クローニング法では各土壌試料から 30～40 個ほどのクローンを取得し，合計 501 個を解析して，系統樹を作成した。さらに，ソフトウェア UniFrac によって統計解析した結果，土壌粒子の性状や pH によって，15 種類の土壌試料は 5 個のグループに分けられた。T-RFLP 法に比べ，クローニング法ではより詳細なグループに分けられたが，大局的には概ね一致しており，複数の環境試料中の遺伝子の多様性を T-RFLP 法と多次元尺度法による解析によって簡易に把握可能であることが示された。

第６章では，第５章で示された成果に基づき，環境試料から新規遺伝子を効率的に取得するため，環境試料のスクリーニングへの T-RFLP 法の適用を検討した。活性汚泥，土壌，堆肥などの環境試料中におけるリパーゼ遺伝子の多様性は，T-RFLP 法のデータを多次元尺度法にて解析した結果，3 つのグループに分けられた。また，各種環境試料から取得した新規リパーゼ遺伝子の塩基配列の多様性も同様に 3 つのグループに分けられ，対応関係があることを見出した。よって，T-RFLP 法を環境試料からの遺伝子スクリーニングに適用することは有効であり，環境試料から効率よく新規な有用遺伝子を

取得するための基盤技術になり得ることが示唆された。

第 7 章では，本論文の統括および展望を記述した。

以上，本論文では，T-RFLP 法を用いて複合微生物叢を簡易に把握することがモニタリングやスクリーニングへの適用に有効であることを示した。これらの研究成果は生物学的排水処理プロセスの向上のみならず環境浄化技術およびメタゲノム解析の効率化などの環境微生物を利用したバイオテクノロジーの発展に大いに寄与することが期待される。

研究業績

原著論文

(1)（報文）Kazutaka Yamada, Takeshi Terahara, Shinya Kurata, Toyokazu Yokomaku, Satoshi Tsuneda, and Shigeaki Harayama, Retrieval of Entire Genes from Environmental DNA by Inverse PCR with Pre-Amplification of Target Genes Using Primers Containing Locked Nucleic Acids, *Environ Microbiol*, (in press).

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(4)（報文）Takeshi Terahara, Tatsuhiko Hoshino, Satoshi Tsuneda, Akira Hirata, Yuhei Inamori, Monitoring the Microbial Population Dynamics at a Start-up Stage of Wastewater Treatment Reactor by Terminal Restriction Fragment Length Polymorphism Analysis Based on 16S rDNA and rRNA Gene Sequences, *J. Biosci. Bioeng.*, 98(6), 425-428 (2004).

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(1) Takeshi Terahara, Kazutaka Yamada, Shinya Kurata, Toyokazu Yokomaku, Satoshi Tsuneda, Shigeaki Harayama, Pre-amplification Technique of Flanking Region for Isolating Entire Gene from Environmental DNA by Inverse PCR, The 107th General Meeting on American Society for Microbiology, Toronto, 21-25, May, 2007

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