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Matsuka, Akira
Laboratory of Soil Microbiology,Division of Soil Science and Plant Production,Department of Plant Resoruces,Graduate School of Bioresource and Bioenvironmental Sciences,Kyushu University

Sakai, Masao
Laboratory of Soil Microbiology,Division of Soil Science and Plant Production,Department of Plant Resources,Faculty of Agriculture,Kyushu University

Kanawaza, Shinjiro
Laboratory of Soil Microbiology,Division of Soil Science and Plant Production,Department of Plant Resources,Faculty of Agriculture,Kyushu University

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Application of T-RFLP Analysis for Bacterial Community Structure of Colonies Grown on Agar Plates

Akira MATSUKA*, Masao SAKAI†, and Shinjiro KANAZAWA

Laboratory of Soil Microbiology, Division of Soil Science and Plant Production, Department of Plant Resources, Faculty of Agriculture,
Kyushu University, Fukuoka 812–8581, Japan
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As a prompt and efficient method for analysis of community structure of bacterial colonies grown on agar plates, we investigated the application of terminal restriction fragment length polymorphism (T-RFLP) analysis. To deduce the community structure of colonies grown on agar plates, analysis of a number of colonies is necessary. In this analytical method, many colonies were collected using a Pasteur pipette to easily collect a constant amount of cells, and the colonies were collectively subjected to T-RFLP analysis to estimate the community structure of colonies. To analyze dominant bacterial species in test soils, 100 colonies were sufficient. Using this analytical method, we investigated the influence of plating method for colony count (pour-plate method vs. spread-plate method) on the constitution of bacterial species grown on the medium. Difference in plating method did not markedly change the bacterial species grown, but it did affect the constitution ratio of some bacterial species.

INTRODUCTION

A variety of bacterial species with various functions inhabits soils. These bacterial groups are involved in the plant production and clean-up functions of soils. To analyze the dynamics and functions of individual bacterial groups with specific functions, various selective media have been designed and used for studies of soil microorganisms. For example, selective media have been used to investigate the numbers of nitrogen-fixing bacteria and cellulose-assimilating bacteria and isolate bacteria having these functions. However, to evaluate the usefulness of these selective media and determine the application ranges, colonies grown on the medium had to be investigated one by one, which was an obstacle to development of selective media. If community structure (bacterial species and their constitution ratios) of bacterial species grown on a selective medium can be rapidly analyzed, it may be useful for design and improvement of a new selective medium.

In recent years, various methods as shown below have been developed as methods for analysis of bacterial community structure: 1) Methods using nucleic acids (gene): DNA reassociation analysis, DNA (G+C%) density fraction analysis, cross DNA hybridization analysis, PCR-amplified DNA clone library method, and various genetic fingerprint analyses (DGGE, TGGE, ARDRA, T-RFLP, SSCP, RISA, and RAPD) (Ranjard *et al.*,

* Laboratory of Soil Microbiology, Division of Soil Science and Plant Production, Department of Plant Resources, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University
† Corresponding author (E-mail: msakai@agr.kyushu-u.ac.jp)

2000), 2) methods using cellular components (biomarkers): phospholipid fatty acid (PLFA) analysis (Arao *et al.*, 1998), quinone profile analysis (Fujie *et al.*, 1998), and 3) methods using carbon source–assimilating property: diversity analysis based on the carbon source utilization patterns of isolates (Yokoyama, 1996), and community–level physiological profile (CLPP) analysis (Konopka *et al.*, 1998). These methods allowed rapid progress of studies of bacterial community structure. Particularly, terminal restriction fragment length polymorphism (T–RFLP) analysis is superior in simplicity, sensitivity, and reproducibility (Liu *et al.*, 1997; Marsh, 1999; Moeseneder *et al.*, 1999).

In this paper, we investigated the application of T–RFLP analysis as a prompt and efficient method for analysis of community structure of colonies grown on agar plates. Furthermore, using this method, changes in community structure of colonies due to differences in the plating method for colony count (pour–plate method vs. spread–plate method) were investigated.

MATERIALS AND METHODS

Soil sample and plate dilution culture method

A soil sample collected from a strawberry–cropping field located in Yunoura, Kumamoto Prefecture was passed through a 2 mm–sieve and used as the test soil. According to the dilution plate method described in Experimental Methods of Soil Microorganism (1992), suspensions of soil dilutions were prepared and plated by the pour method or spread method on four plates at each dilution step. Plates were incubated at 28 °C for 10 days. As medium for aerobic heterotrophic bacteria, 0.1 TSA medium (peptone, 1.7 gL^{-1}; soytone, 0.3 gL^{-1}; glucose, 0.25 gL^{-1}; K_2HPO_4, 0.25 gL^{-1}; NaCl, 0.5 gL^{-1}; agar, 15 gL^{-1}; pH 7.2) was used. As medium for crystal violet (CV)–resistant bacteria, 5 mgL^{-1} of CV was added to 0.1 TSA medium, 0.1 TSA+CV medium, was used.

Colony collection and DNA extraction

From four plates at a dilution step at which about 100 colonies were grown per plate, 100 colonies were randomly collected. To obtain a constant amount of cells from colonies with a diameter greater than 1 mm, colonies were sucked with medium one by one using a Pasteur pipette (1 mm inner diameter at the tip). These 100 colonies were placed in a 2–ml microtube with a screw cap. To the microtube, glass beads (2.5 mm ϕ, 0.06 g; 1 mm ϕ, 0.3 g; 0.5 mm ϕ, 0.4 g; 0.1 mm ϕ, 0.4 g; BIOSPEC Co.), 400 μL of SDS lysis mixture (100 mM NaCl, 500 mM Tris–HCl (pH 8.0), 10% SDS), 300 μL of 120 mM sodium phosphate buffer (pH 8.0), and 400 μL of phenol: chloroform: isoamylalcohol (25:24:1, pH 8.0) were added. Using a MINI BEADBEATER (BIOSPEC Co.), cells were destroyed at 5,000 rpm for 20 seconds. After centrifuge at 12,000 rpm for 30 seconds at room temperature, the supernatant (aqueous phase) was transferred to a new 1.5–mL microtube. To this tube, 1/10 volume of 3M CH_3COONa (pH 4.8) and 2.5 volumes of 100% ethanol were added and mixed, and kept standing at room temperature for 10 minutes. The mixture was then centrifuged at 12,000 rpm for three minutes at room temperature to separate DNA. The supernatant was removed, and 1 mL of 70% ethanol was added to the pellet, mixed slightly, and centrifuged at 12,000 rpm for three minutes at room temperature. The supernatant was removed, and the pellet was air–dried for five

minutes. Each DNA pellet was dissolved in 50 μL of ultrapure water.

PCR and restriction digests for T-RFLP

Purified DNA (10 ng) was amplified by PCR using the forward primer 63f-Cy5 (5'-[Cy5]CAGGCCTAACACATGCAAGTC-3' [Marchesi *et al.*, 1998]) labeled with a fluorescent dye (Cy5) and the unlabeled reverse primer 907r (5'-CCGTCAATTCMTTTRAGTTT-3' [Lane, 1991]). Both primers are considered to be specific for eubacteria. The reactions were performed in 50 μL (final volume) mixtures containing 1 × PCR buffer, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, each primer at a concentration of 1.2 μM, and 1.25 U of *Taq* DNA polymerase (Takara Shuzo). Samples were amplified in a thermocycler (ASTEC PC-707) by using the following protocol: an initial denaturation step at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. Cycling was completed by a final extension at 72 °C for 10 min. Amplification of DNA was confirmed by electrophoresis of aliquots of PCR mixtures (2 μL) in 1.5% agarose in 1 × TAE buffer.

The PCR products labeled with a fluorescent dye were purified by using the Qiaquick PCR Purification Kit (Qiagen) according to the instructions of the manufacturer. Aliquots of the purified 16S rDNA were digested by restriction enzymes. Digestion reaction mixtures (20 μL) contained 0.1 μg of the 16S rDNA and 10 U of either *Hha*I (TOYOBO, Japan) in the manufacturer's recommended reaction buffers, and were incubated for 6 h at 37 °C.

T-RFLP analysis

The lengths of the labeled TRFs from the amplified rDNA products were determined by electrophoresis with an automated DNA sequencer (ALFexpress; Amersham Pharmacia Biotech), as follows. Electrophoresis samples containing 5 μL of digestion reaction, 3 μL of Loading Dye (Amersham Pharmacia Biotech), and 2 fmol Sizer50 size standard (Amersham Pharmacia Biotech) were denatured at 95 °C for 5 min and then immediately stored on ice until loading onto the gel. Approximately 9 μL of each sample was applied to a 5% polyacrylamide gel and run on the Sequencer for 10 h under the following conditions: 1,500 V, 55 mA, and 25 W. The laser scanning system of this DNA sequencer detected only the labeled 5'-terminal fragments. The TRF size was determined in comparison to the pattern of the external lane standard (Sizer50-500 size standard; Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Analysis of colony community structure by T-RFLP

To investigate reliability of the method for analysis of colony community structure using T-RFLP, quadruplicate analyses were compared. The test soil was divided into four samples (A, B, C, and D) and each sample was subjected to T-RFLP analysis of colony community plated by the pour method and grown on 0.1 TSA medium. As shown in Fig. 1, 7 T-RF peaks were detected in each sample, suggesting the presence of 7 dominant species. There were no marked differences in the T-RF peak positions (T-RF sizes) or

relative fluorescence intensities (amounts of T–RF) among the samples, and the T–RF patterns were similar. Based on this highly reproducible T–RFLP analysis, the points below are discussed.

Since soils are generally not homogenous, soil bacteria are unlikely to homogenously distribute. Accordingly, to deduce the bacterial community structure in soils, the influence of uniformity of soils is great, and different results among the repeated analyses were predicted. However, high reproducibility was obtained in this study, indicating that sufficiently homogenous dispersion of bacteria was obtained by the preparation of the test soil sample.

Next, the number of colonies selected also affects the estimation of the bacterial community structure. Torsvik *et al.* (1990a,b) investigated diversity of colonies of soil samples cultured on agar plates by DNA reassociation analysis and estimated that investigation of at least 90 colonies included dominant bacterial species. Accordingly, considering the work efficiency, we predicted that investigation of 100 colonies grown on an agar plate is sufficient for analysis of community structure of culturable bacteria. If 100 colonies were insufficient, i.e., if more than 100 bacterial species were present, the results of repeated analyses would not have been consistent and various patterns would have been seen. The results of the quadruplicate T–RFLP analyses shown in Fig. 1 exhibited similar patterns, confirming that in this method of analyzing bacterial community in the culture of the test soils by this method, selection of 100 colonies including dominant bacterial species was sufficient. Furthermore, use of a Pasteur pipette to collect a constant amount of cells may have lead to the good reproducibility of this analytical method.

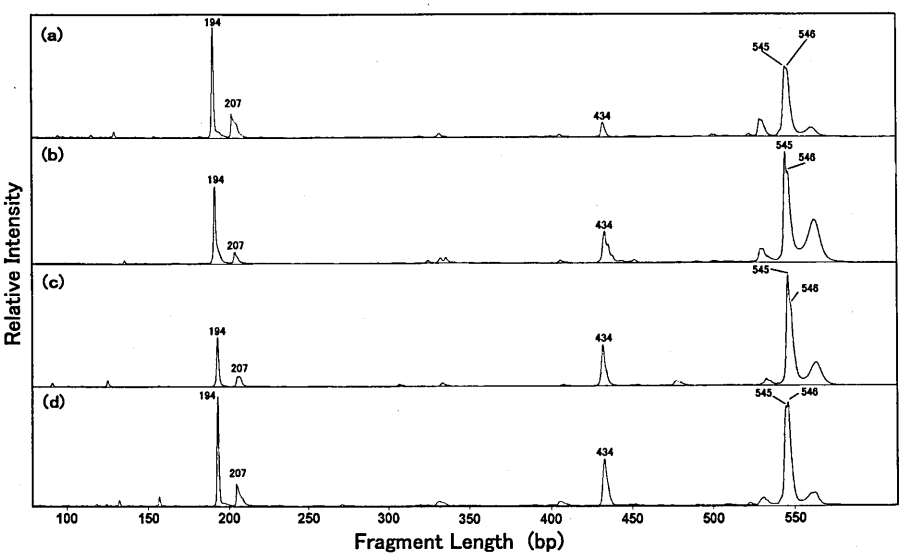

Fig. 1. T–RFLP patterns of colony community plated by the pour method and grown on 0.1 TSA medium. (a), (b), (c), and (d) represent T–RFLP patterns of colony communities derived from four soil samples A, B, C, and D. The values shown in Figure are fragment lengths of major T–RF peaks.

Changes in bacterial community structure due to plating method

As an example of applying the colony community structure analysis using T-RFLP, the influence of the plating method (pour-plate vs. spread-plate method) in the dilution plate method on the colony community structure was investigated. Suspensions of diluted soils A and B prepared in the above experiment were plated by the spread method. The results of T-RFLP analysis are shown in Fig. 2. On comparison of the results of analyses using the pour-plate method shown in Fig. 1 (a),(b) and spread-plate method shown in Fig. 2 (a),(b), the positions of T-RF were not different and the T-RF patterns were similar. However, the fluorescence intensity of 207-bp peak was the highest in the analysis using the spread method, but low in the analysis using the pour method, showing that although the constituent bacterial species were the same in the analyses using the spread and pour methods, the constitution ratios of some bacterial species were different.

Next, using 0.1 TSA+CV medium, the soil sample was plated by the spread and pour methods and compared by the analysis of colony community structure using T-RFLP. The suspension of diluted soil A prepared in the above experiment was plated by the pour and spread methods. The T-RFLP patterns of the colonies grown on 0.1 TSA+CV medium plated by the pour and spread methods are shown in Fig. 3. On comparison of T-RFLP patterns of colonies grown on 0.1 TSA medium (Figs. 1 and 2) and those on 0.1 TSA+CV medium (Fig. 3), the T-RFLP patterns were completely different and no T-RF was consistent, showing that the selectivity of crystal violet allowed different bacterial species to dominate on 0.1 TSA+CV medium.

Regarding the influence of the plating method on culture on 0.1 TSA+CV medium, the positions of T-RF were similar, but changes in the T-RF peak fluorescence intensity were greater than those on 0.1 TSA medium, showing that difference in the plating method does not greatly affect the types of CV-resistant bacteria grown on the medium, but the constitution ratios of some bacterial species were affected.

As described above, the simple and rapid method for analysis of colony community structure using T-RFLP proposed in this study allowed identification of the bacterial community structure with high reproducibility. Furthermore, this method sufficiently

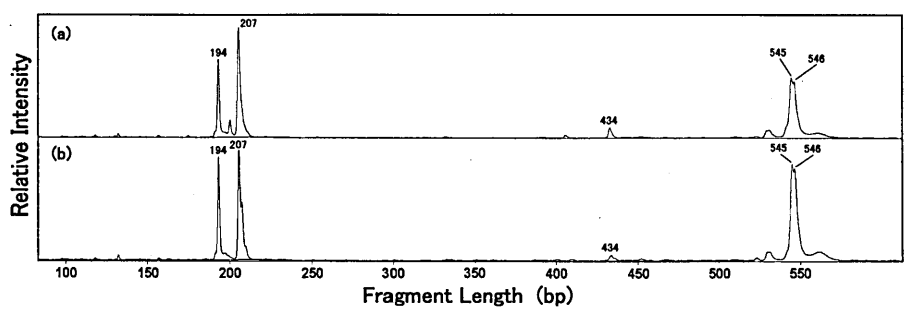

Fig. 2. T-RFLP patterns of colony community plated by the spread method and grown on 0.1 TSA medium. (a) and (b) represent T-RFLP patterns of colony communities derived from two soil samples A and B. The values shown in Figure are fragment lengths of major T-RF peaks.

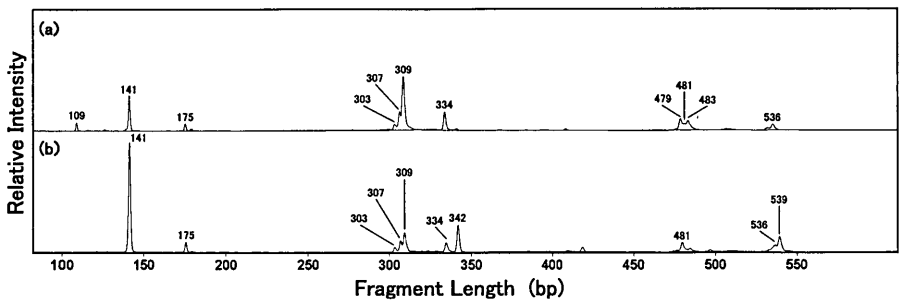

Fig. 3. T-RFLP patterns of colony community grown on 0.1 TSA+CV medium. T-RFLP patterns of soil sample A-derived colony communities plated by (a) pour method and (b) spread method. The values shown in Figure are fragment lengths of major T-RF peaks.

detected differences in the bacterial community structure caused by the different plating methods.

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