Analysis of the Bacterial Diversity in Soil Using 16S Ribosomal DNA

Kiminori Itoh[1,2], Teruhisa Amano[1] and Masayuki Murabayashi[1,2]

[1] Institute of Environmental Science and Technology, Yokohama National University, 79-7 Tokiwadai, Hodogaya-ku, Yokohama 240-0067, JAPAN

[2] CREST, Japan Science and Technology Corporation.

Key Words: bacteria, diversity, soil, 16S ribosomal DNA, PCR, electrophoresis, temperature gradient gel electrophoresis

Abstract

A method is proposed to monitor complicated bacterial communities in soil, and preliminary results are given. An appropriate section of 16S rRNA gene DNA (16S rDNA) was amplified with polymerase chain reaction (PCR) and the resultant fragments were analyzed in terms of GC (guanine + cytosine) content with temparature gradinent gel electrophoresis (TGGE). Soil samples taken from a forest and a lawn field gave different patterns of GC content distribution. Thus, the TGGE analysis proved effective for monitoring the bacterial community in soil. To incease the efficiency of the method, primers were designed to separate the bacteria into several taxonomical groups.

1. Introduction

The diversity of bacteria in soil is a good measure of environmental quality as well as the different stresses applied to soil (Atlas 1984), but conventional methods based on culturing bacteria extracted from the soil in question inevitably highly modify the original distribution of bacteria in soil because of the limited number of culturable species. In fact, more than 10,000 species of bacteria are considered to exist in 10 g of soil on the basis of DNA-DNA hybridization measurements, while only several hundred are culturable (Hattori & Miyashita 1996). However, recent progress in the polymerase chain reaction (PCR) method (e.g. Cullen et al. 1998) and those in the database of 16S ribosomal RNA gene DNA (16S rDNA) may make it possible to carry out the reasonably accurate profiling of bacterial community in soil. As we have recently demostrated this principle (Itoh 1998), the GC content and chain length of 16S rDNA fragments, together with the data of The Ribosomal Database Project (RDP) (Maidak et al. 1997), can be used to analyze PCR-amplified fragments consisting of a large number of different DNA chains corresponding to different bacterial species. In this report, we show preliminary experimental results of the analysis of the bacterial community in soil. In particular, thermal gradient gel electrophoresis (TGGE) proved to be effective in analyzing the GC content of the DNA fragments of the soil bacteria.

2. Method

2.1 16S rDNA as a tool of bacterial community analyses

As a result of evolution, different bacteria species have different 16S rDNA base sequences. Thus, on the basis of the database established by Woese 's group (Woese 1987; Maidak et al. 1997), 16S rDNA can be used to distinguish or identify different bacteria. Figure 1 is an example of chain length vs GC content relations for DNA fragments corresponding to the first ca. 350 bp part of eubacteria 16S rDNA. About 200 species were selected from the RDP data so as to represent each taxonomically numbered group which approximately corresponds to the genus level. The figure shows that GC content changes about 30% and bp length about 20% among the bacteria examined. W suggest that this diagram may give information about the diversity of bacteria mixtures semiquantitatively; the complexity of the diagram will change depending on the complexity of the mixtures (Itoh 1998). Although we cannot directly identify each bacteria on the diagram, each spot denotes approximately a single species if the resolution is sufficiently high. As a first step, one dimentional analyses were carried out in this study.

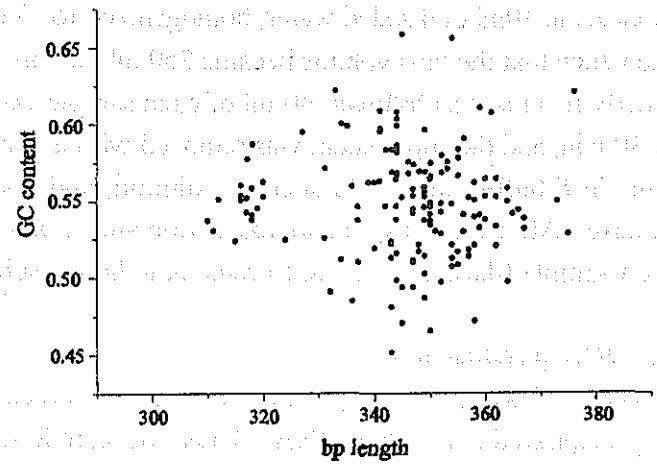

Fig.1. GC content vs bp length for the first ca. 300 bp part of 16S rRNA for 200 selected species of eubacteria.

2.2 Electrophoresis

To obtain the map shown in Fig. 1 experimentally, denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman 1989), or temperature gradient gel electrophoresis (TGGE) (Abrams and Stanton 1992) can be used in principle. In DGGE, the gradient of DNA denaturant in the gel separates DNA fragments according to their GC content while temperature gradient works in TGGE. Their principles are almost the same; DNA fragments are suddenly slowed down at the point corresponding to their respective melting point because the denaturing from double strands to single strands takes place. For instance, Muyzer et al. have reported an analysis of sulfur reducing bacteria in biofilm using DGGE (1993). We employ here TGGE because this method appears to be more facile than DGGE when applied to two-dimensional electrophoresis to obtain the GC content - bp length maps. Although our final goal is two-dimensional electrophoresis to obtain two-dimensional maps, we start with one-dimensional TGGE electrophoresis to obtain GC content profiles.

3. Experimental

3.1 Extraction of DNA from soil.

Two kinds of soil were used in this experiment; one from a lawn field and the other from a forest. The soil samples were put through a sieve (2 mesh), then 25 g of each sample was dispersed in 10ml of distilled water, homogenized for 5 min at 1800rpm, and diluted with distilled water such that the final volume became 250 ml. In this experiment, bacterial DNA was extracted directly from soil as follows: 40 ml of each sample was centrifuged in a 50 ml tube at 4500 rpm for 30 min, and the supernatant was removed with a micropipette. The sample pellet was washed with TNT buffer and 25 % sucrose solution, and lysis was performed by using lysozyme and pronase. After the lysis, phenol extraction and ethanol precipitation were performed. The final DNA sample (dark brown due to remaining humic substances) was dried over a desiccator.

3.2 DNA purification.

The DNA samples were purified with ethanol precipitation. The humic substances seemed to be precipitated faster than DNA. Thus, the sample solution was centrifuged at 6000 rpm after adding salt, and the supernatant was centrifuged further at 15000 rpm for 20 min. In addition, several column chromatography methods and ozone treatments were examined.

3.3 PCR.

The PCR primers used are those reported by Lane (27f and 342r, synthesized by Lifetech Oriental Co., Ltd.), which amplify regions corresponding to positions from 8 to 357 in the *E. coli* number. For PCR reactions, 22.5μl of Supermix^{TM} (Lifetech Oriental Co., Ltd.), 0.5μl of the DNA solution and 1μl of each primer solution (0.5μM) were added to a 200μl PCR tube. If necessary, the DNA samples were diluted with doubly distilled water, or T4 gene 32 protein (Tebbe and Vahjen, 1993) was added to the tube to reduce the inhibition effect of the humic material. PCR was performed with a thermal cycler (Corbett Research, Co., Ltd.) at the following thermal cycles: 1) 94° C, 3min; 57° C, 30s; 72° C, 30s; 2) - 35) 94° C, 30s; 57° C, 30s; 72° C, 30s; 36) 94° C, 30s; 57° C, 30s; 72° C, 5min.

3.4 Electrophoresis.

The PCR products were first analyzed with submarine-type elctrophoresis using agarose gels. When sufficiently intense amplification was observed, the sample was then analyzed by TGGE as follows: 1) 50 μl of the sample was applied to the wells of 1mm-thick polyacrylamide gels containing urea and formamide as denaturants in a vertical-type bath, and a voltage of 200 V was applied for 5 min. This procedure served as a pre-run. 2) The gel was removed from the cell glass plates, transferred onto a plastic sheet, and placed on the temperature-control stage of the TGGE equipment (TAITEC Co., Ltd.). A voltage of 230 V was applied to the sample for 1-2h. 3) The gel was dyed with SYBR^{TM} Green I fluorescent dye (PanVera Corporation) for 20 min, observed with a transilluminator, and photographs were taken. The electrophoresis patterns were analyzed with densitometry (ATTO Co., Ltd).

4. Results and Discussion

4.1 DNA extraction and purification.

The direct lysis of the microbial cells in the soil led to a large amount of remaining impurities (mainly humic substances) after the conventional ethanol precipitation as proved by the dark brown color of the precipitate. Most of the impurities were eliminated by centrifuging at relatively low rotation speeds after addition of the salt solution. The resultant DNA samples were white so that they seemed to be sufficiently pure. However, PCR amplification of these samples was unsuccessful, showing that a trace of inhibitor still remained in the samples. Thus, T4 gene 32 protein was added to the PCR solution to suppress the inhibition effect of the humic substances; PCR amplification was observed only for samples containing the protein.

Several DNA purification methods we have tested here were unsuccessful in that no PCR amplification was observed without T4 gene 32 protein.

4.2 PCR.

Figure 2 shows a typical agarose gel electrophoresis pattern for DNA amplified with primers 27f and 342r. As expected, a single band was observed at the length of about 300 bp for all the samples although a dim band attributable to primer dimers was observed at shorter regions. DNA extracted from cultured bacteria gave strong bands while those from soil were weak even with the addition of T4 gene 32 protein. This is likely due to the inhibition effect of humic substances. For quantitative PCR experiments, which will be needed for further analyses of bacterial communities, more efficient PCR amplification is required. Thus, establishing DNA purification techniques is necessary in the near future. Fortunately, recent papers have reported promising methods to purify DNA extracted from soil (e.g., Cullen and Hirsch 1998), and in fact, we are examining these purification methods now in our laboratory.

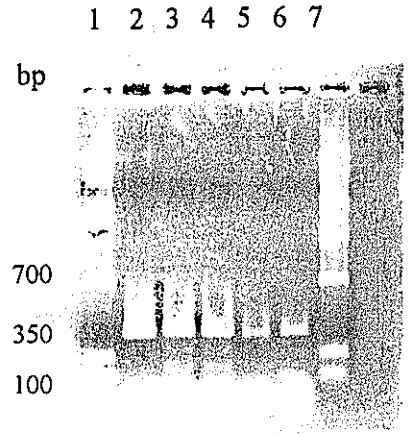

Fig.2. An agarose gel electroporresis pattern for 16S rDNA amplified with primers 27f and 342r. Lane 1 and 7, marker; lane 2, Lactobacillus; lane 3 and 4, commercial bacterial mixture; lane5, bacteria from a forest soil; lane 6, bacteria from a lawn field.

4.3 Thermal Gradient Gel Electrophoresis (TGGE).

Figure 3 shows an example of the vertical TGGE analysis where temperature gradient is vertical to the direction of the electric field, and hence, to the direction of the DNA movement. A sharp bend was observed at 65° C for DNA of Lactobacillus, showing that double-strand DNA was denatured to give slower mobility at this temperature. Thus it was confirmed that TGGE works for bacterial DNAs.

Figure 4 shows parallel TGGE patterns analyzed with densitometry for DNA fragments amplified from bacterial DNA taken from different soil samples (Fig. 4a, forest soil; Fig.4b, lawn field soil). Two main lobes and a number of small peaks are found in the figure. Considering the resolution of the gel, i.e.

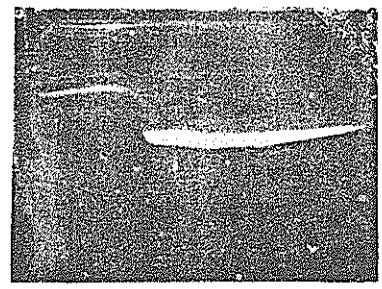

Fig.3. Vertical temperature gradient gel electrophoresis for 16S rDNA amplified with primers 27f and 342r for Lacto-bacillus. Arrows show the position of the DNA fragment.

about 1 mm, the small peaks appear to be noise due to densitometry. The main lobes consist of several broad peaks which represent different DNA fragments with different GC contents. The lobe at right side in Fig. 4b is much broader than that in Fig. 4a. This likely shows that the forest soil sample has a more complicated GC content profile, although detailed analyses are not yet completed. This may show the larger diversity of the bacteria community in the forest soil. Thus, we conclude that TGGE is effective in analyzing the diversity of the bacterial community in soil. For the detailed analyses we have to employ GC content markers which enable us to give accurate GC values on the gels.

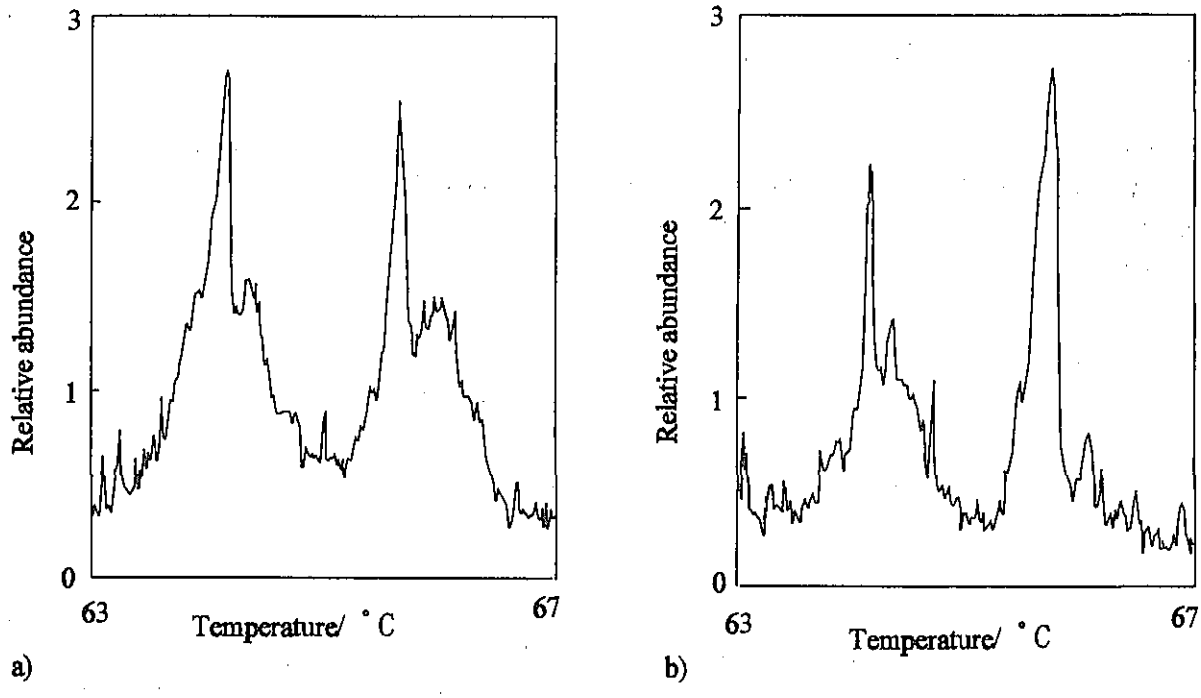

Fig.4. GC content distributions of PCR-amplified bacterial 16S rDNA taken from different soils: a) forest soil, b) lawn field soil.

4.4 Primers specific to different bacteria groups.

In Figs. 1, 4, and 5, PCR primers used are designed as to fit most eubacteria, and thus the results obtained tend to become complicated because of a large number of bacterial species. We can overcome this disadvantage by using PCR primers specific to different bacterial groups. For instance, Lane (1991) proposed a set of primers to separate bacteria into three groups: primer 685r1 (5'TCTACGRATTTCACCYCTAC) for alpha and delta proteobacteria and fusobacteria: 685r2 (5'TCTACGCATTTCACYGCTAC) for all beta and gamma proteo-bacteria, and 685r3 (5'TCTRCGCATTYCACCGCTAC) for most gram-positive bacteria, cyanobacteria, and some miscellaneous bacteria. Here, R denotes G or A, Y is C or T, and hence, 685r1 consists of four components, 685r2 of two components, and 685r3 of four components. In fact, different TGGE patterns were obtained in our measurements by using these primers (data not shown), but it was found that one of the components of 685r2 is completely the same as that of 685r3 although Lane did not mention it. Thus, this set of primers are inadequate for the precise taxonomical separation

of eubacteria in soil. Hence, we have designed a set of new primers which work in a manner similar to 685r (1 to 3) but more accurately.

We modified primers reported so far to obtain new primers. In Table 1, two bases next to primer 27f are compared for the selected bacterial species. Thus, all bacteria can be separated into four groups by using modified 27f primers. Each group can then be analyzed with TGGE to give a diagram shown in Fig.1. In this manner, analyzing the complicated bacterial community will become more facile than with the whole bacterial DNA. In a similar manner, we can design different useful primers. For instance, using two bases next to primer 342r can divide gram-positive bacteria into two groups: one containing high-G+C subdivision, Clostridia, and anaerobic halophiles, and the other containing Mycoplasmas and Bacillus-Lactobacillus-Streptococcus subdivision.

Table 1. Comparison of bases next to 27f primer between different bacterial groups (approximately corresponding to phyla).

Bases next to 27f	Bacterial classification
CG	Thermophilic oxygen reducers
AR (AA or AG)	Fibrobacter, Spirochetes, Proteobacteria alpha, delta, epsilon
AT	Proteobacteria beta and gamma
GR (GA or GG)	Flexibacter, Fusobacteria, Gram-positive bacteria, and minor bacteria

5. References

Abrams, E. S. and Stanton, V. P. Jr., *Meth. Enzymol.*, 212, 71-104 (1992)

Atlas, R. N., *Amer. Soc. Microbiol.*, 540-545 (1984)

Cullen, D. W. and Hirsch, P. R., *Soil Biol. Biochem.*, **30**, 983-993 (1998).

Fischer, S. G. and Lerman, L. S., Proc. Natl. Acad. Sci. USA, 80, 1579-1583 (1989)

Hattori, T. and Miyashita, K., Microbiology of Soil (Yokendo, Tokyo, 1996) p.141.

Itoh, K., Proceedings of the 1st Internatinal Workshop on Ist Evaluation and Management of Chemicals (Yokohama, January 22-23, 1998) p.139-142.

Lane, D. J., In Nucleic Acid Techniques in Bacterial Systematics, (eds. Stackebrandt, E. and Goodfellow, M., John Wiley & Sons Ltd., New York, 1991) pp. 115-175.

Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J., and Woese, C. R., *Nucleic Acids Res.* **25**, 109-111 (1997)

Muyzer, G., De Waal, E. C., and Uitterlinden, A. G., *Appl. Environ. Microbiol*, **59**, 695-700 (1993)

Tebbe, C. C. and Vahjen, W, *Appl. Env. Microbiol*, **59**, 2657-2665 (1993)

Woese, C. R., *Microbiological Reviews*, **51**, 221-271 (1987).