Studies of Diversity and Functions of Soil Microbes and Nematodes in NIAES Using Nucleic Acids Extracted from Soil

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Abstract: Since we succeeded in establishment of an improved DNA extraction method using skim milk from soils that strongly adsorb DNA, we have studied the structure and functions of soil microorganisms using nucleic acids extracted from soil. By analyzing DNA extracted from 3-chlorobenzoate (3CB) amended soil using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), we succeeded in isolating effective 3CB-degraders and full length of 3-CB degradative genes from the soil and the metagenome of the soil, respectively. We could show the possibility to use DGGE bands as indicators of soils biological properties for soil management: we detected PCR-DGGE bands derived from ammonia-oxidizing bacteria specific in a field under long-term application of chemical N-fertilizer or organic manure. Based on these achievements, since 2006, National Institute for Agro-Environmental Sciences (NIAES) play a central role in a national project sponsored by Ministry of Agriculture, Forestry and Fisheries of Japan addressing development of analytical methods for soil biological properties using environmental DNA (eDNA) which is directly extracted from soil. In the project, we have optimized and standardized the conditions for preparation of DNA from soil and PCR-DGGE using soil eDNA to compare the microbial communities in different soil samples. We determined the most suitable primer sets and "DGGE markers" for each of soil bacterial, fungal and nematode analysis and optimized conditions in PCR amplification, electrophoresis and other experimental steps. In parallel, we have been collecting soil samples under various types of agricultural management at different localities from the northern to the southern Japan, and analyzing the eDNA extracted from each soil sample by the standardized PCR-DGGE method. The obtained data have been used to construct a database (eDDASs: eDNA database for agricultural soils) collecting the results of soil eDNA analysis accompanying the physical and chemical soil properties and information about crop productivity in each agricultural fields. On the other hand, we constructed an RNA extraction method from soil with high purity. Using RNA purified from 3-CB amended soil, we could detect 3-CB-induced gene expression in soil environment. Our final goal is to determine the relationships between the biological properties evaluated by eDNA or RNA analysis and the soil physicochemical properties as affected by cultivation practice.

Keywords: Environmental DNA, PCR-DGGE, eDDASs: eDNA database for agricultural soils,

1. Introduction

To achieve sustainable production of high quality crops and vegetables, it is essential to maintain soil fertility and to overcome soil disease such as sickness due to continuous cropping. For this purpose, we have to evaluate not only physical and chemical properties of soils but also biological ones and to utilize their information for soil management. Especially, it is important to elucidate how soil microbes and nematodes are involved in the phenomena in agricultural fields, such as crop growth, pest infestation and suppressiveness. We need to collect the information of soil biota, and compare it across soil samples. Soil microorganisms are small but important component of soil organic matter, which are thought to exert a key controlling influence on the rate at which N, C and other nutrients cycle through agricultural and other ecosystems [1]. Soil microbial biomass and activities are frequently used as an early indicator of changes in soil chemical and physical properties resulting from soil management and environmental stresses in agricultural ecosystems [2]. However, our knowledge on the soil biological properties has been still far from enough to evaluate the soil biological properties for soil management. This is mainly because most of soil microorganisms cannot be cultivated with conventional methods. Useful general methods for examining soil microbial population and functions have been desired for long time. Recent methodological developments in molecular microbial ecology have made it possible to analyze the microbial communities in environmental samples without any culture step. Today, one of such methods, denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified genes from environmental samples, is a useful tool in environmental microbiology [3, 4]. For example, the PCR-DGGE method targeting the 16S rRNA gene (16S rDNA) [5] is most widely applied for the studies of bacterial community structure in the environment, because this gene is essential to all living procaryotes and helpful in tracing phylogenetic relationships. The PCR-DGGE targeting appropriate sequences can also be used for studying the community structures of other microorganisms in the environment, such
as fungi [6-8] or nematodes [9-12]. The method, like PCR-DGGE, analyzing nucleic acids directly extracted from the environment will bring us more wide range of information about communities and functions of microorganisms existing in soil environment.

Since we succeeded in establishment of improved DNA extraction methods using skim milk from soils that strongly adsorb DNA [13], we have developed new methods using nucleic acids for analyzing the structure and functions of soil microorganisms. Our several research achievements are reviewed.

2. Establishment of improved DNA extraction methods using skim milk from soils

Direct extraction of DNA from soil is essential for the study of microbial ecology. This method facilitates the analysis of whole microbial communities, including unculturable microbes. Many protocols have been developed to yield DNA for molecular analysis. However, these methods are not satisfactory for some types of soil, especially for Andisols, which are volcanic ash soils widely distributed all over the world and especially in the circum-Pacific Ring of Fire, including Japan, East Africa, and the Mediterranean. We devised an improved method of extracting DNA from Andisols [13]. Seven Andisol samples from 6 agricultural fields and a forest in Japan were tested with a commercially available kit that uses bead-beating. Soil DNA could be extracted from only 2 of the samples (Fig. 1). Pure DNA added to the soil was adsorbed to the clay particles, revealing why DNA extraction is difficult in Andisols. However, when we added skim milk to the extraction buffer at a rate of 40 mg g soil−1, DNA could be detected by electrophoresis from all the soil samples (Fig. 1). Certain molecules, such as RNA and skim milk, are known to be adsorbed to soil colloids in a similar manner to that of DNA. Soil extracts proved to be suitable for PCR when skim milk was added, rather than RNA. Addition of skim milk did not affect PCR-DGGE profiles. PCR-DGGE is a complementary tool for determining the genetic diversity of natural microbial communities in a culture-independent fashion. Our improved method is applicable to the molecular analysis of microbial communities in soils that strongly adsorb DNA.

3. Isolation of Effective 3-Chlorobenzoate-Degraders in Soil using Community Analyses by PCR-DGGE

Screening of pollutant degraders by relying only on cultivation techniques such as liquid enrichment often fail to isolate the actual degraders in the environment. In fact, we found that PCR-DGGE patterns targeting benzoate 1,2-dioxygenase alpha subunit genes (benA) of soil culture amended with 3-chlorobenzoate (3CB), substrate for ben operon, were completely different from those of liquid culture (Fig. 2). We succeeded in demonstrating that community analyses by PCR-DGGE were successfully used to detect and isolate bacteria that can degrade 3CB effectively in soil [14, 15]. A forest soil sample was repeatedly dosed with 3CB (500 mg Kg−1) to enrich the indigenous 3CB-degraders, and changes in the bacterial community were monitored by PCR-DGGE of 16S rRNA genes and benA. Initially, it required about 3 weeks to degrade 3CB in the soil, whereas it took only 3 days after the third dose. With this accelerated degradation, several intensified bands appeared in the DGGE profiles of both 16S rRNA and benA genes. We succeeded in isolating five 3CB-degrading Burkholderia strains corresponding to these bands from the 3CB-dosed soil by direct plating, while most of them fail to proliferate (or grow) in liquid enrichment [15]. Inoculation of the strains into the soil demonstrated that the five strains could degrade 3CB effectively in the soil [15]. This study clearly shows significant bias during the liquid enrichment process and the advantage of using PCR-DGGE in screening effective degraders under environmental conditions.

4. Retrieve Full Lengths of Functional Genes from Soil by PCR-DGGE and Metagenome walking
In the previous study, PCR-DGGE analysis succeeded in showing that several types of benA became dominant in 3CB-dosed soil, and isolating 3CB-degradable bacteria that had corresponding benA sequences. However, some of the 3CB-inducible benA sequences in soil samples could not be found in the isolated bacteria. In addition, the sequence analysis clearly indicated that the deduced amino acid sequences of them were phylogenetically different from those of the cultured strains. Therefore, we thought that the benA sequences were most likely to be derived from unculturable strains which played an important (some) role in biodegradation of 3CB in soil environment. We tried a new approach to retrieve full lengths of functional genes from soil DNA using PCR-DGGE followed by metagenome walking [16]. Partial fragments of benA were detected from a 3CB-dosed soil by PCR-DGGE, and one DGGE band induced by 3CB was used as a target fragment for metagenome walking (Fig. 3). The walking succeeded in retrieving the flanking regions of the target fragment from the soil DNA, resulting in recovery of the full length of benA and also downstream gene (benB). To demonstrate the generality of our approach, the same approach was also applied to another gene, tfdC encoding chlorocatechol 1,2-dioxygenase. As a result of metagenome walking, a complete tfdC and two downstream genes were obtained from the same soil [16]. PCR-DGGE allows screening for target genes based on their potential for degrading contaminants in the environment. This feature provides an advantage over other existing metagenomic approaches.

5. Community Structure of Ammonia-oxidizing Bacteria Revealed by PCR-DGGE Analysis

PCR-DGGE analysis also reveal the effects of mineral fertilizer (NPK) and organic manure on the community structure of soil ammonia-oxidizing bacteria (AOB) in a long-term fertilizer experiment, which has been carried out for 16 years in agricultural fields in China [17]. The experiment included seven treatments: organic manure, half-organic manure N plus half-fertilizer N, fertilizer NPK, fertilizer NP, fertilizer NK, fertilizer PK and the control (without fertilization). N fertilization greatly increased soil nitrification potential, and mineral N fertilizer had a greater impact than organic manure, while N-deficiency treatment (PK) had no significant effect [17]. AOB community structure was analyzed by PCR-DGGE targeting the amoA gene, which encodes the α subunit of ammonia monoxygenase. DGGE profiles showed that AOB community was more diverse in N-fertilized treatments than those in the PK treatment and the control, while one dominant band observed in the control could not be detected in all fertilized treatments (Fig. 4). Phylogenetic analysis showed that the DGGE bands derived from N-fertilized treatments, belonged to Nitrosospira cluster 3, indicating that N fertilization resulted in the dominance of Nitrosospira cluster 3 in soil [17]. These results demonstrate that long-term application of N fertilizers could result in the increased soil nitrification potential, and the AOB community shifts in soil. On the other hand, PCR-DGGE analysis targeting the 16S rDNA gene revealed that mineral fertilization did not affect General bacterial community structure, while specific DGGE band was observed in organic manure-fertilized soils [18].

Similar approach using PCR-DGGE analysis was done to reveal the effect of former upland conversion (upland rice cultivation or soybean/wheat cultivation) on AOB community in a paddy rice field in NIAES [19]. Rotation of paddy rice and upland crop cultivation is a major type of land use in eastern Asia, and temporary upland conversion of paddy field also occurs frequently in this region. Little is known about the affects of these agricultural practices on soil microbial communities. Community structures of AOB were
determined by PCR-DGGE, while its population size was quantified by real-time PCR. Soil AOB communities were more diverse and had significantly larger population size in the plots with former soybean/wheat cultivation than that in consecutive paddy rice plots, while former upland rice cultivation had much less effect on the community structure and population size of AOB. The results demonstrate a significant and lasting effect of upland conversion history on soil AOB communities in paddy field, which depend strongly on the type of upland crop while upland conversion practice itself seems to have less effect.

These DGGE bands specifically observed in soil could be candidates for indicators of soils biological properties for soil management.

6. eDNA Project

In 2006, Ministry of Agriculture, Forestry and Fisheries of Japan started a project addressing development of analysis methods for soil biological properties using environmental DNA (eDNA) which is extracted from soil. It united researchers of national institutes, universities and prefectural agricultural experiment stations in Japan. The project consists of three subthemes. In the subtheme 1, we have optimized PCR-DGGE procedure for each of bacterial, fungal and nematode communities, and make up a manual. With the standardized and optimized DGGE procedures we developed, clear and sharp band patterns can be produced (Fig. 5).

Fig. 5 Soil biological analysis by PCR-DGGE. Soil samples were prepared from various agricultural fields and DNA was extracted from them according to the methods in the manual. The extracted DNA was used to amplify bacterial 16S rDNA, fungal 18S rDNA or nematodes 18S rDNA by PCR with the specific primer sets that we had chosen. The PCR fragments were analyzed by DGGE according to the standardized method in the manual. Community structures of bacteria, fungi or nematodes in each agricultural field can be evaluated as band patterns. The PCR fragments obtained from each soil are aligned in the same order in each gel. “M” indicates DGGE marker we developed to compare band patterns across gels.

The subtheme 2 aims to develop analytical methods for elucidation of relationship between soil biodiversity and crop productivity. We have been collecting soil samples under various types of agricultural management at different localities from the northern to the southern Japan (Fig. 6), and analyzing the eDNA extracted from each soil sample by the standardized PCR-DGGE method. The subtheme 3 has been constructing a database (eDDASs, eDNA database for agricultural soils) collecting the results of soil eDNA analysis accompanying the physical and chemical soil properties with information about crop productivity in each agricultural fields. This database also provides some tools for data retrieval and analysis developed by this project and others. Applications of new analytical methods using microarray or metagenomic approach are also considered in this project. We expect to elucidate the mechanisms in which soil biota affect agricultural production, by analyzing the biological information revealed in our project. Our final goal is to determine the relationships between the biological properties evaluated by eDNA or RNA analysis and the soil physicochemical properties as affected by cultivation practice.

7. Improvement of the Method of RNA Preparation from Soil

To fully understand the physiological response of microorganisms to various factors in the soil environment, it is necessary to examine gene expression in the soil. As the first step of such a study, a method of RNA extraction from soil microorganisms has to be established. Although there are some reports describing RNA extraction from microorganisms in soil, the documented methods handled small amounts of soil only or resulted in the co-extraction of humic substances together with RNA. Humic substances interfere with many enzyme reactions, nucleic acid
detection and measurement, and RNA hybridization. So, we developed a new method of extracting RNA with high purity from soil reproducibly [20, 21]. A soil RNA extraction method was improved by optimization of lysis conditions and further purification by a spin column, in terms of removing humic substances efficiently which hinder enzymatic reactions of the obtained RNA. Fluorescence spectrophotometry demonstrated that the improved method removed both humic and fulvic acids efficiently (Fig. 7). By the improvement, the signal of gene expression detected by real-time RT-PCR increased 10-fold [21]. Using the method, we extracted RNA from a sterilized field soil, which was inoculated with Pseudomonas putida KT2440 transformed with a chloroaromatic degrading plasmid, in the presence or absence of 3-CB. Real-time RT-PCR performed with the extracted RNA confirmed the induction of chloroaromatic degrading genes in 3-CB amended soil [21].

8. Conclusions

The improved soil-DNA extraction method enabled us to isolate effective 3CB-degraders and full length of 3-CB degrading genes from soil using PCR-DGGE. We found DGGE bands which could be candidates for indicators of soils biological properties that can be used for soil management. Based on these achievements, NIAES play a central role in a national project addressing development of analytical methods for soil biological properties using eDNA (eDNA project). In the project, we have been collecting soil samples under various types of agricultural management at different localities from the northern to the southern Japan, and analyzing the eDNA extracted from each soil sample by the standardized PCR-DGGE method. The obtained data have been used to construct a database (eDDASs) collecting the results of soil eDNA analysis accompanying the physical and chemical soil properties and information about crop productivity in each agricultural fields. The data will be used to analyze the relationships between the biological properties evaluated by eDNA or RNA analysis and the soil physicochemical properties as affected by cultivation practice. We could also detect 3-CB-induced gene expression in soil environment using a soil-RNA extracted by the method we developed.

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References


