J. Microbiol. Biotechnol. (2003), **13**(6), 1008–1012

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Application of Denaturing Gradient Gel Electrophoresis to Estimate the Diversity of Commensal Thermophiles

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Received: April 14, 2003
Accepted: June 4, 2003

Abstract *Symbiobacterium toebii* has been reported as a thermophile exhibiting a commensal interaction with *Geobacillus toebii*. The distribution of the commensal thermophiles in various soils was investigated using a denaturing gradient gel electrophoresis (DGGE). Based on the DGGE analysis, the enrichment condition for the growth of *Symbiobacterium* sp. was found to also enrich populations of several other microbial spp. as well as *Symbiobacterium* sp. In the enrichment experiment, several different 16S rDNA sequences of commensal thermophiles were detected in all of the soil samples tested, indicating that commensal thermophiles are widely distributed in various soils.

Key words: DGGE, diversity, *Symbiobacterium toebii*

It is estimated that there are large numbers of unculturable microorganisms in natural environments, which can only be detected by molecular ecological methods [1, 4, 8, 9, 19]. Among the reasons that many microorganisms can not be cultured in laboratory conditions is the fact that a biological interaction is an essential factor in the growth of these unculturable microorganisms [2]. *Symbiobacterium* sp. is a thermophile exhibiting a commensal interaction with *Geobacillus* sp. [14, 15, 16, 17]. The commensal interaction is a unique example of microbe-microbe interaction. Previous studies have revealed the presence of novel lineages of heterotrophic commensal thermophilic organisms, but only two strains of commensal thermophiles, i.e. *S. toebii* SC-1 and *S. thermophilum* strain T, have been grown in culture and the factors involved in the commensal interaction are still unclear [11, 12, 18, 20]. Among the molecular ecological methods, DGGE has been the most widely used to investigate distribution patterns of soil bacterial assemblages [3, 6, 7, 21]. However, this technique has not been applied to the estimation of commensal bacterial diversity, because of lack of enough information to understand and reproduce real micro-environmental niches.

Among recent molecular ecological studies, there are reports on competitive quantitative PCR (CQ-PCR) and terminal restriction fragment length polymorphism (TRFLP) which focused on the whole *Symbiobacterium* assemblage by using *Symbiobacterium*-specific primers [16, 20] and these studies analyzed the diversity of *Symbiobacterium* from various environment sources, but did not find any populations that existed together with the commensal thermophiles in the natural environmental niche. In the present study, we examined the appropriateness of the enrichment condition of commensal thermophiles with DGGE by comparing enriched samples with non-enriched soil samples. We optimized the conditions for universal primer sets and tested their performance with enriched cultures and environmental samples. The relative effectiveness of each set was evaluated by comparing community profiles obtained from different compost samples from Korea. Finally, the DGGE results were compared with the results obtained by using two other molecular analyses, namely, CQ-PCR and TRFLP analysis. Since knowledge about the ecology of the microorganism is required to understand the microbial interaction, we also investigated possible abundance of the commensal thermophiles in soils using a PCR-DGGE analysis. The results obtained may provide evidence for the presence of unknown novel microbial interactions among uncultivated microorganisms in the ecosystem.

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Different soils from the vicinity of Gongju in Korea were collected for analysis, including manure compost and straw compost obtained from compost sites with no previous history of agricultural use. For the enrichment of the soil samples, 5 g of the soil samples were added to 250-ml Erlenmyer flasks containing 100 ml of a basal medium (BM), which contained, per liter of deionized water, 6 g of K_2HPO_4, 2 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 5 g of polypeptone, 3 g of KNO_3, and 10 g of yeast extract. The $MgSO_4 \cdot 7H_2O$ was sterilized separately before mixing into the BM. The enrichment of the soil samples was performed at 60°C under anaerobic conditions. For cultivation under anaerobic conditions (CO_2/N_2, 10:90), BM containing 30 mM nitrate as an electron acceptor was used in an anaerobic jar (Difco) with Anaerobic system and palladium catalysts (Difco). The growth of the commensal thermophiles was monitored by determining the nitrite accumulated with a colorimetric method described by Hanson and Phillips [5]. The shapes and sizes of living and stained cells were determined by light microscopy.

All the soil DNA was extracted and purified using a modified version of the procedure described by Zhou *et al*. [22]. One gram of each soil sample was mixed with 3 ml of benzyl chloride, 1 ml of 10% sodium dodecylsulfate (SDS), and 5 ml of an extraction buffer (100 mM Tris-HCl, 40 mM EDTA, pH 9.0). The mixture was vortexed and incubated at 50°C for 30 min with shaking, and then 3 ml of 3 M sodium acetate (pH 5.0) was added. After centrifugation at 6,000 ×g for 15 min, the DNA in the supernatant was precipitated with an equal volume of isopropanol. The pellet was dried and dissolved in 0.5 ml of TE (pH 8.0) buffer, followed by extraction with an equal volume of $CHCl_3$-isoamylalcohol (24:1, vol/vol). The sample was treated with 0.2% polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, MO, U.S.A.) and filtered with a 30 kDa cut-off membrane. The DNA was finally recovered with 0.1 ml of TE (pH 8.0) buffer. The purity and quantity of DNA were optically verified at an absorbance spectrum between 220 nm and 320 nm. As a standard, the 16S rDNA of the *Symbiobacterium toebii* strain SC-1^T and the *Geobacillus toebii* strain SK-1^T was isolated from a pure culture. PCR amplification of 16S rRNA genes for cloning and sequence analysis was carried out with primers 27f and 1492r, as described by McCaig *et al.* [10]. Products for DGGE analysis were amplified with primers GC338f and 518r [11], which amplify a 180-bp fragment of the 16S rRNA gene, including the variable V3 region, and include a 40-bp GC clamp at the 5' end of p3. Amplification reactions were performed as follows: 95°C for 5 min, followed by 10 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, 25 cycles of 92°C for 30 s (95°C is not necessary to denature ~200-bp products and the lower temperature preserves enzyme activity), 55°C for 30 s, and 72°C for 45 s, and a final incubation at 72°C for 10 min.

DGGE was performed with 8% (wt/vol) acrylamide gels in a linear 40 to 65% denaturant agent gradient (100% denaturant agent was defined as 7 M urea and 40% deionized formamide). Gels were run for 3 h at 200 V with a Dcode Universal Mutation System (Bio-Rad) at 60°C. DNA was visualized after CYBR Green 1 staining by UV transillumination. The major bands were excised for the identification of the bacterial species. The bands were placed into sterilized vials with 20 µl of sterilized distilled water and stored overnight at 4°C to allow the DNA to passively diffuse out of the gel strips. Sequence identification was performed by using the BLAST database (National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the Sequence Match Facility of the Ribosomal Database Project (www.cme.msu.edu/RDP).

DGGE analysis of 16S rDNA fragments was used to examine the diversity of the commensal thermophiles and their neighborhood in the enriched and un-enriched soil samples. Figure 1 shows the DGGE patterns of the 16S rDNA fragments (primers P338f and P518r) amplified from two kinds of compost soils and control soils after 3 days of enrichment. No dominant bands could be observed in the lanes loaded with DNA purified directly from soils. However, the drastic effect of enrichment was observed after 3 days of enrichment, as shown in the DGGE banding pattern in Fig. 1. After 3 days of enrichment, there was a significant shift in the microbial community structure. The 16S rDNA bands from the commensal thermophiles could

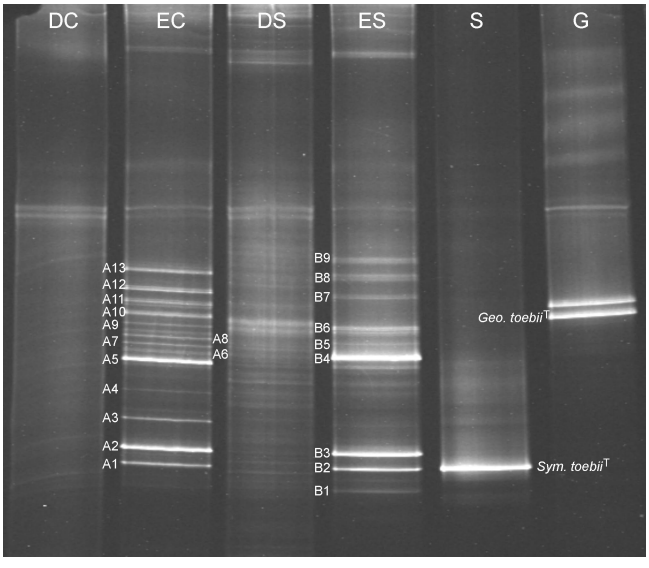

Fig. 1. DGGE analysis of PCR-amplified 16S ribosomal DNA fragments from soil bacterial communities.
DNA was derived from the direct compost soil and direct straw soil (lane DC and DS, respectively), the same soil enriched for 3 days at 60°C in anaerobic condition (lane EC, ES, respectively), and axenically cultivated *Symbiobacterium toebii* (lane S) and *Geobacillus toebii* (lane G). The bands discussed in the text and Fig. 2 are indicated with positions and numbering.

not be recovered in compost soil lanes with universal primer sets (primers GC338f and 518r). However, in the lanes loaded with amplified DNA from the enrichment samples, more than twenty 16s rDNA bands could be detected. The results indicated that the enrichment condition for the growth of *Symbiobacterium* sp., i.e., anaerobic condition at 60°C for 3 days, could also enrich the several other commensal microbial populations. In the soil samples, the maximum number of different rDNA fragments separated by DGGE may be vastly underestimated. Torsvik [19] found that there might be as many as 10^4 different genomes present in 1 gram of soil samples, suggesting that DGGE cannot separate all of the 16S rDNA fragments obtained from soil microorganisms, but only the dominant species. Therefore, the banding patterns obtained in this study reflect the most abundant rDNA types in the community after 3 days of enrichment.

The analysis of the enriched bacterial species was carried out with two enriched soil samples as shown in Fig. 2. The bands selected for analysis are shown in Fig. 1. As expected, the enrichment condition applied revealed the microorganisms belonging to the genus *Symbiobacterium* predominant. The derived sequences from these bands confirmed that B2 was 100% identical to *S. toebii* SC-1^T and that A1 had 99% similarity to *S. thermophilum.* Almost all the enriched microorganisms were Gram-positive thermophiles. Several bands such as A5, A6, A7, A9, B4, and B5 did not belong to any phylogenetic groups, indicating that certain groups of uncultivated microorganisms as well as *Symbiobacterium* could be enriched in this enrichment condition. The isolation of the uncultivated population which did not belong to any phylogenetic group is being undertaken.

When the various soil samples were incubated in the basal medium at 60°C, nitrite was detected as the product of the initial nitrate reduction in all the cultures. In addition, after 72 h of incubation, more than 20 mM nitrite was detected in all the enriched samples. Based on microscopic observations on enriched cultures, the cell numbers were about 3×10^9 cells per ml, and cells exhibiting a slim morphology and Gram-negativity were found to comprise the majority of the population. Although these phenotypes are typical characteristics of the *Symbiobacterium toebii* strain SC-1^T, when considering the 16S rDNA results of the DGGE, these bacteria could not all be composed of the commensal bacterium. When 1% of the enriched cultures was transferred to a fresh basal medium, the accumulation of nitrite and enrichment of the commensal thermophiles recurred after 16 h.

Previously, an attempt was made to quantify the 16S rDNA of the commensal thermophiles in soils using CQ-PCR and TRFLP [16]. From the CQ-PCR method, the

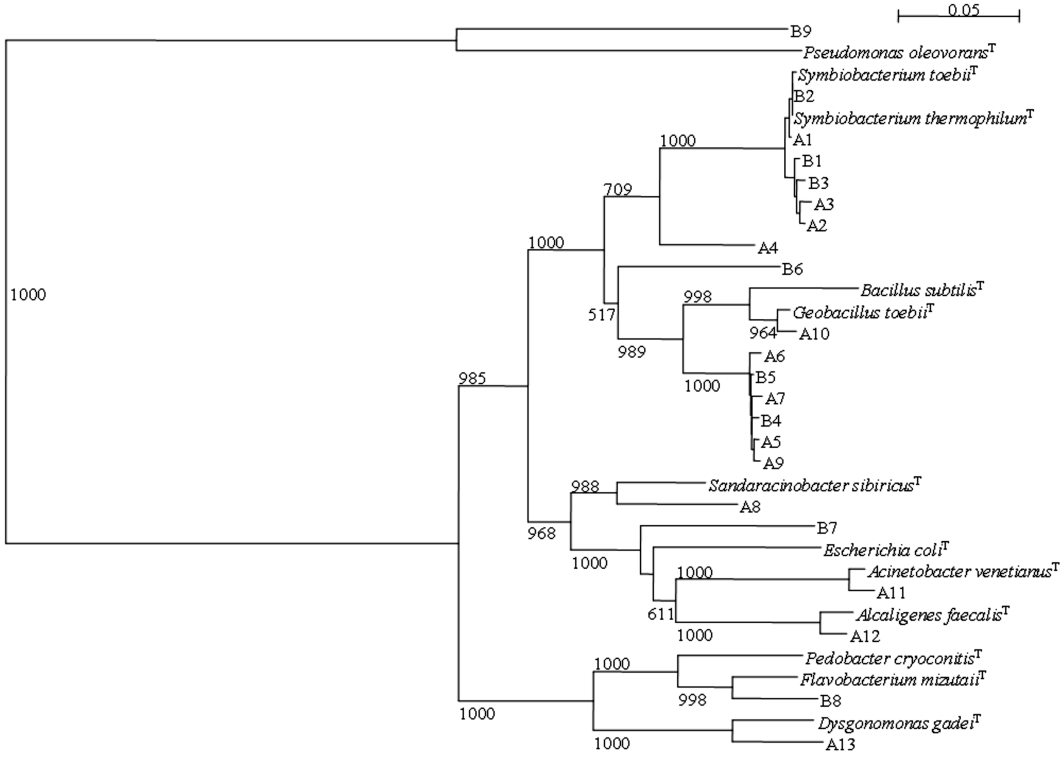

Fig. 2. Phylogenetic tree representing the genetic similarity of the microbial community profiles obtained by PCR-DGGE.
Bootstrap values are shown at branching points.

commensal thermophile 16S rDNA was only detected in the DNA samples from manure and rice straw compost soils. The estimated 16S rDNA copy number was about 1.0×10^4 copies per g of soil, corresponding to 0.25×10^4 cells per g of soil, thus indicating that the compost is a productive growth environment for the commensal bacterium. The TRFLP analysis also confirmed the results of the CQ-PCR. No TRF corresponding to the commensal thermophile was detected in the 16S rDNA products, when amplified directly from the soils. Although numerous kinds of other eubacteria in small fractions appeared in the soils during the culture experiment, the microbial community structure changed and a TRF of the commensal thermophile 16S rDNA was consequently detected in all the soils. Furthermore, a novel 16S rDNA showing a TRF of 257 bp was present in a highly enriched state. However, the analyses by CQ-PCR and TRFLP did not show a population enriched under the same condition. Therefore, a neighborhood that could become a commensal host to *Symbiobacterium* was detected only by DGGE analysis among the molecular ecological methods. According to the molecular phylogenetic surveys, a great deal of microbial diversity remains to be discovered and identified. With regard to interactions among bacteria in the natural ecosystem, our current knowledge in this area is extremely superficial. Thus, further studies are needed to elucidate the diversity of the bacteria affected by these microbial interactions, because commensal thermophiles are certainly not the only examples in the ecosystem. Understanding the potential importance of this interaction requires detailed in-depth information on the distribution and diversity of the microorganism. Thus, our present study estimated the abundance of the commensal thermophiles in soils based on DGGE analysis. The results obtained may provide information on hither-to-unknown novel microbial interactions that affect the growth of previously uncultivated microorganisms in the ecosystem.

Acknowledgment

This work was supported by National Research Laboratory (NRL) program grant NLM0020224 from the Ministry of Science and Technology of Korea.

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