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Estimation of Distribution of a Commensal Thermophile in Soil by Competitive Quantitative PCR and Terminal Restriction Fragment Length Polymorphism Analysis

RHEE, SUNG-KEUN, SEUNG-PYO HONG[1], JIN-WOO BAE, CHE OK JEON, SEUNG-GOO LEE, JAE JUN SONG, HARYOUNG POO, AND MOON-HEE SUNG*

Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yusong, Daejeon 305-343, Korea
[1]BioLeaders Corp. Bio-Venture Center, KRIBB, Yusong, Daejeon 305-343, Korea

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Abstract *Symbiobacterium toebii* has been previously reported as a novel commensal thermophile exhibiting a commensal interaction with thermophilic *Geobacillus* sp. SK-1. We investigated the distribution of this commensal thermophile in various soils using molecular methods, such as quantitative PCR and terminal restriction fragment polymorphism analysis. Based on a nested competitive quantitative PCR, the 16S rDNA of the commensal thermophile was only detected in compost soils at about 1.0×10^4 copies per gram of soil, corresponding to 0.25×10^4 cells per gram of soil. However, in an enrichment experiment at 60°C, about 1.0×10^8 copies of 16S rDNA molecules were detected per ml of enriched culture broth for all the soils, and more than 0.1 mM indole accumulated as the product of commensal bacterial growth. When incubated at 30°C, neither the 16S rDNA of the commensal bacterium nor any indole accumulation was detected. Accordingly, even though the 16S rDNA of the bacterium was only detected in the compost soils by a nested PCR, the presence of the 16S rDNA molecules of commensal thermophile and accumulation of indole in all the enriched cultures appeared to indicate that the commensal thermophile is widely distributed in various soils.

Key words: Commensal, distribution, soil, *Symbiobacterium*, thermophile

Until recently, the estimation of bacterial diversity has been restricted due to limitations associated with the cultivation of bacteria from natural environments. Due to inability to understand and to reproduce real microenvironmental niches, only a small fraction of bacterial communities can be cultivated using current techniques. Recent molecular techniques have also highlighted that uncultured fractions are a major component in all bacterial communities [2, 7, 15].

It is difficult to isolate and cultivate certain microorganisms, because of their interactions with other organisms. For example, *Dehalococcoides ethenogens*, which reductively dechlorinates tetrachloroethene, requires a mixed culture extract for its growth [8]. The growth of the bacterial parasite, *Bdellovibrio*, requires a Gram-negative bacteria host [13], and syntrophic bacteria grow only when other bacteria remove hydrogen, the metabolic product of syntrophic bacteria [9]. Therefore, understanding of these bacterial interactions can help unravel the microbial diversity in the natural ecosystem and also contribute to the discovery of new unknown microbial groups.

We previously detected a new thermophilic Gram-negative bacterium, *Symbiobacterium toebii*, and reported on its novel interaction with *Geobacillus* sp. SK-1 [12], which appears to be an example of a microbe-microbe interaction that prevents such microorganism from being cultivated in general nutrient media. Suzuki *et al.* [14] previously reported that *Symbiobacterium thermophilum* also exhibits this type of bacterial interaction. Recently, we showed that *S. toebii* has a 98.5% 16S rRNA homology, yet less than 30% DNA-DNA hybridization homology, with *S. thermophilum*. The factors involved in a commensal interaction are still unclear [10, 12].

Understanding of the potential importance of this interaction requires information on the distribution and diversity of the microorganism. Accordingly, we estimated the abundance of the commensal thermophile *Symbiobacterium toebii* in soils, employing a competitive quantitative PCR (CQ-PCR) and terminal restriction fragment length polymorphism (TRFLP) analysis. The results presented show evidence of unknown novel microbial interactions that

*Corresponding author
Phone: 82-42-860-4372; Fax: 82-42-860-4595;
E-mail: smoonhee@mail.kribb.re.kr

affect the growth of previously uncultivated microorganisms in the ecosystem.

MATERIALS AND METHODS

Soil Sampling and Isolation of Chromosomal DNA

Different soils from the Gongjoo region in Korea were selected for analysis, including compost soils obtained from compost sites for cow manure and rice straw, agricultural soils obtained from farm fields used in soybean cultivation, and playground soils with no previous agricultural use.

The total soil DNA was extracted and purified using a modified version of the procedure described by Zhu *et al.* [18]. 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, 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. This was 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 a TE buffer. The purity and quantity of DNA were optically verified by a spectrum of 220 nm to 320 nm.

Enriched Culture of Commensal Thermophile and Isolation of Chromosomal DNA

The commensal thermophile in the soil samples was enriched in a nutrient medium (PEP) at 60°C with a low agitation speed (about 80 rpm) to maintain a low oxygen tension. Five grams of the soil samples were added to 250 ml Erlenmyer flasks containing 100 ml of the PEP medium, which contained 3 g of K_2HPO_4, 1 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 5 g of polypeptone, 0.5 g of L-tryptophan, 0.5 g of L-tyrosine, and 1 g of yeast extract per liter of deionized water. The $MgSO_4 \cdot 7H_2O$ was sterilized separately before being mixed into the PEP. During incubation, the tryptophan indole-lyase activity was determined using Ehrlich's reagent [16]. To detect the commensal bacterium under mesophilic culture conditions, the incubation temperature was changed to 30°C.

The total DNAs were extracted from 0.5 ml of the initial enriched cultures. The cells were harvested by centrifugation at 10,000 ×g for 2 min. The cell pellets were then resuspended in 0.5 ml of a TE buffer containing 4 mg per ml of lysozyme. The samples were incubated for 30 min at 37°C. After adding 0.1 ml of 10% SDS (wt/vol), the mixture was vigorously vortexed and incubated at 50°C for 30 min. This was followed by extraction with an equal volume of $CHCl_3$-isoamylalcohol (24:1, vol/vol). Thereafter, the DNA was precipitated with isopropanol and the resulting pellet was dried and dissolved in 0.1 ml of TE. As a standard, the total DNA of *Symbiobacterium toebii* was isolated from a pure culture using the same method.

Primers and Competitors

The PCR primers were specifically designed to probe the commensal thermophile *Symbiobacterium*. These PCR primers were based on the 16S rDNA region, which is not homologous with any other related bacteria. Based on a sequence similarity analysis, the 16S rDNA sequence of *S. toebii* (GenBank accession no. AF190460) exhibited less than 87% similarity with all other known sequences. Primer sc16s62F (5'-GGGTTTCGGGGCTTCGGCCCT-GAGA-3') and primer sc16s1037R (5'-AGAGGCACTC-CCGCATCTCTGCAGG-3') were designed to amplify a 976-bp region encompassing nucleotides 62 to 1037 of *Symbiobacterium toebii* 16S rDNA. The specificities of these primers were checked by sequencing clones obtained from the amplified DNA of the soil samples. The sequencing of the amplification products confirmed that the PCR products had higher than 96% similarity with *S. toebii*. The primer specificities were also checked using Ribosomal Database Project II (http://rdp.cme.msu.edu/html/analyses.html). Eubacterial universal primers 8F (5'-AGAGTTTG-ATCCTGGCTCAG-3'), corresponding to positions 8 to 27 of *E. coli* 16S rDNA, and 1542R (5'-AGAAAGGAGGT-GATCCAGCC-3'), corresponding to positions 1542 to 1525 of *E. coli* 16S rDNA, were also used to amplify the total eubacterial 16S rDNA.

For CQ-PCR of the commensal thermophile, competitor DNA was constructed as follows. The 16S rDNA of *S. toebii* was cloned into the TA cloning vector pT7Blue (Novagen) and the cloned plasmid designated as pSC. A 269-bp fragment was then deleted from the 16S rDNA gene of pSC by digestion with *Stu*I (Boehringer Mannheim, Germany), thereby yielding the competitor, pSC-C. The resulting inserts in pSC and pSC-C were 1,561 and 1,292 bp, respectively. As such, the resulting amplification products from pSC and pSC-C as templates with the *Symbiobacterium toebii*-specific primers were 976 bp and 707 bp, respectively.

CQ-PCR Protocol

The competitive PCR was conducted using the method developed by Zachar *et al.* [17]. To quantify the commensal thermophile in each sample, a single set of samples containing a known amount of target DNA was prepared. Each reaction mixture (50 µl) contained 1 pg of pSC-C as a competitor, and all samples were amplified by a PCR using the commensal thermophile-specific primer

set (sc16s62F and primer sc16s1012R). The PCR was performed using a thermal cycler (BioRad Lab., Hercules, CA, U.S.A.). All cycles were programmed to perform 35 cycles consisting of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, followed by a final extension step of 10 min at 72°C. The PCR products were visualized by agarose gel electrophoresis.

In order to construct a calibration curve for each amplification, the ratio of the intensity of target DNA to that of competitor band was plotted against mass of the input target DNA. The mass of target DNA in a soil sample can be determined by relating the ratio of the amplification product of the unknown to that of the target DNA in the calibration curve [17]. To quantify the commensal thermophile based on the copy numbers of 16S rDNA, the DNA extracted from the pure culture of *Symbiobacterium toebii* was used as the standard. When the DNA from the pure culture of *Symbiobacterium toebii* was used, a cell contained about 4 copies of 16S rDNA.

A nested PCR method was used for the direct quantification of the commensal thermophile present in the soils [1]. In the first PCR, 0.1 fg of the competitor (pSC-C) was added to reaction mixtures of 1 g soil samples. The samples were first amplified for 30 cycles using the outer universal primer set (27F and 1525R) under the same PCR conditions as described above. The PCR product was purified using a 30 kDa cut-off membrane, and 1 μl was then reamplified in a second PCR for 35 cycles using the inner commensal thermophile-specific primer set.

Fingerprinting Using TRFLP Analysis

A direct fingerprinting method was used to assess the commensal thermophile composition in the bacterial communities in the various samples. This method was based on amplification of the 16S rRNA target genes using the universal eubacterial primers 27F and 1525R, with 27F fluorescently labeled with 6-carboxyfluorescein (96-FAM; Perkin-Elmer, Norwalk, CT, U.S.A.) on the 5' end. The amplification conditions were the same as those described above for the CQ-PCR method. The amplified 16S rDNA was purified using a 30 kDa cut-off membrane, then a 10 μl sample was digested with *Alu*I (Boehringer Mannheim), precipitated with n-butanol, and dried. The pellet was dissolved in 10 μl of a loading buffer containing 1 μl of ROX GS500 (Perkin-Elmer) as the internal size standard and loaded in an ABI 373 DNA automated sequencer (Perkin-Elmer). The 16S rDNA of *Symbiobacterium toebii* and thermophilic *Geobacillus* strain SK-1 (GenBank accession no. AF326278) were used as the standards for this experiment. The size, in base pairs, of the terminal restriction fragments (TRFs) was estimated with reference to the internal standard.

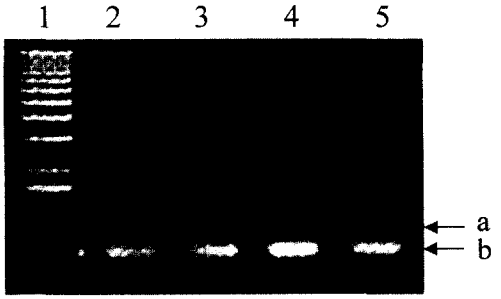

Fig. 1. Nested CQ-PCR of 16S rDNA of commensal thermophile in DNA extracted from 1 g sample soils.
A competitor (0.1 fg) was used as the internal standard. Lanes: 1, 1 kb DNA ladder; 2, manure compost soil (A1); 3, agricultural soil (B1); 4, playground soil (C); 5, straw compost soil (A2). The bands indicated by the solid arrows a and b (between 1,018 bp and 517 bp fragments of 1 kb ladder) show the positions of the 16S rDNA of the commensal thermophile and the competitor, respectively.

RESULTS

Extraction of DNA from Soils and CQ-PCR of Commensal Thermophile

The DNA recovered from the representative soils, cow-manure compost soil (A1), agricultural soil (B1), playground soil (C), and rice-straw compost soil (A2), was 1.2, 4.2, 2.2, and 2.8 μg of DNA per g of soil, respectively. The purity of the extracted DNA was above 80%.

No 16S rDNA from the commensal thermophile was detected in any of the DNA samples extracted from the soils by the first PCR using the specific primer set (sc16s62F and primer sc16s1012R) (data not shown). However, the nested PCR did detect the 16S rDNA of the commensal thermophile (Fig. 1). In the first PCR, pSC-C was added as a competitor and the full length (about 1.5 kb) of the eubacterial 16S rDNA was amplified (data not shown). In the second PCR using the first PCR products as templates, an internal 976 bp fragment of the commensal thermophile was amplified from two soil samples, A1 and A2, (cow-manure compost soil and rice-straw soil, respectively). The copy numbers of the 16S rDNA of the commensal thermophile in the two DNA samples extracted from 1 gram of soil were 1.0×10^{4} and 1.2×10^{4}, respectively. The detection limit of the nested PCR in this experiment was about 1.0×10^{3} copies per g soil.

CQ-PCR of Commensal Thermophile in Enriched Cultures

When the various soil samples were incubated in a PEP medium at 60°C, indole was detected in all cultures as the product of L-tryptophan indole-lyase (Table 1). In two cultures with the A1 and A2 soils, the indole rapidly accumulated up to 0.2 mM after 16 h of incubation. At the same time, the commensal thermophile 16S rDNA

Table 1. Indole concentration accumulated during enrichment cultures of various soils[a].

Soil	Indole concentration (mM)	
	16 h	72 h
Agricultural soil (B1)	0.029	0.12
Agricultural soil (B2)	0.035	0.15
Cow manure compost soil (A1)	0.20	0.19
Playground soil (C)	0.004	0.12
Straw compost soil (A2)	0.16	0.12

[a]Incubation conditions are described in Materials and Methods.

detected in the A1 and A2 samples was about 3.9×10^8 and 2.5×10^8 copies per ml, respectively (Fig. 2A). The results of calibration experiments using various amounts of *S. toebii* DNA as standards also revealed a statistically meaningful evaluation of the competitive PCR (data not shown). In contrast, less than 1.0×10^7 copies per ml of the commensal thermophile 16S rDNA were detected in the B1 and B2 soils after 16 h of incubation. However, after 72 h of incubation, more than 1.0×10^8 copies of the commensal thermophile 16S rDNA per ml were detected in all samples (Fig. 2B). In addition, after 72 h of incubation, more than 0.12 mM of indole was detected in all the enriched samples. Based on microscopic observations, the cell numbers were about 3×10^9 cells per ml, and cells exhibiting a slim morphology and Gram-negativity made up the majority of the population. Although these phenotypes are typical characteristics of *Symbiobacterium toebii*, when considering the 16S rDNA results of the CQ-PCR, not all of these bacteria were the commensal

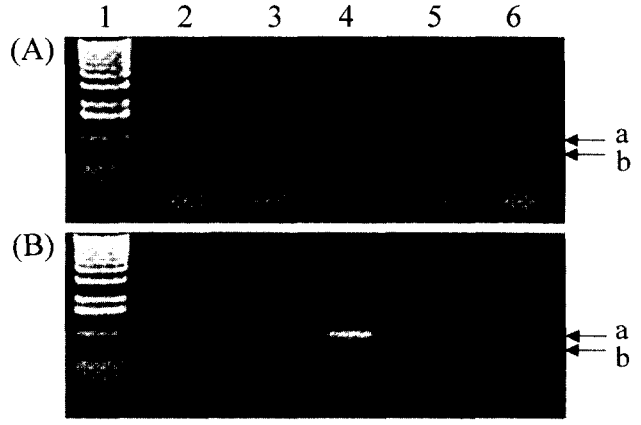

Fig. 2. CQ-PCR of 16S rDNA of commensal thermophile in DNA extracted from cultures incubated with soils for (A) 16 h and (B) 72 h.

A competitor (1 pg) was used as the internal standard. Lanes: 1, 1 kb DNA ladder; 2, agricultural soil (B1); 3, agricultural soil (B2); 4, manure compost soil (A1); 5, playground soil (C); 6, straw compost soil (A2). The bands indicated by the solid arrows a and b (between 1,018 bp and 517 bp fragments of 1 kb ladder) show the positions of the 16S rDNA of the commensal thermophile and the competitor, respectively.

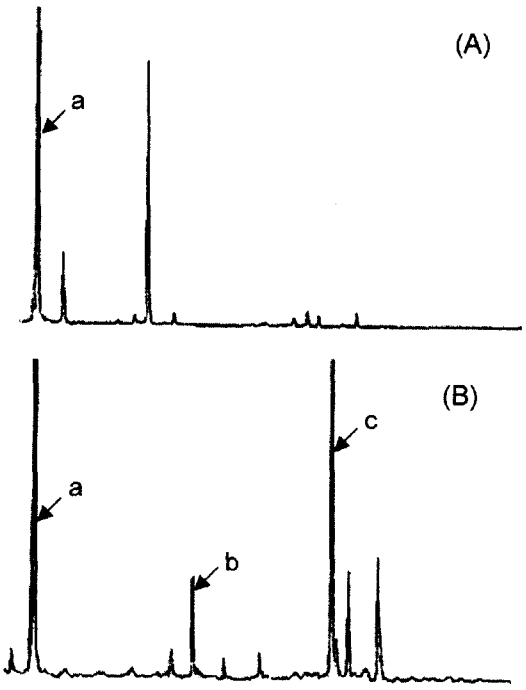

Fig. 3. TRFLP fingerprints of 16S rDNA amplified from DNA extracted from (A) soil A2 and (B) after 72 h of enrichment with soil A2.

TRF: a, 74 bp (*Geobacillus*); b, 172 bp (commensal thermophile); c, 257 bp (previously uncultivated bacteria).

bacterium. When 1% of the A1 and A2 cultures were transferred to a fresh PEP medium, the accumulation of indole and enrichment of the commensal thermophile reoccurred after 16 h.

Under mesophilic incubation conditions, only a slight amount of indole was accumulated (less than 0.003 mM) and no commensal thermophile 16S rDNA was detected. In addition, when the cultures were transferred, no indole or commensal thermophile 16S rDNA was detected.

Commensal Thermophile in Soil Bacterial Community (TRFLP Analysis)

To analyze the commensal thermophile in the soil bacterial communities using the TRFLP technique, the 16S rDNA in the compost soils was amplified using a fluorescein-labeled eubacterial primer, and the 5'-labeled amplification product was digested with a restriction enzyme. When the 5' fragment of the 16S rDNA from the soil samples was examined, the TRF of the commensal thermophile 16S rDNA (172 bp) was not detected in any of the soil samples. *Bacillus* and *Geobacillus* were the main components in all the samples. As shown in Fig. 3, a typical *Geobacillus* TRF (74 bp) and unidentified TRF (152 bp) were the main components (about 90%) in the PCR-amplified samples of soil A2. All other components appeared only as minor fractions in the soils.

Commensal Thermophile in Bacterial Communities of Enriched Cultures (TRFLP Analysis)

After incubating the soil samples in a PEP medium, the 16S rDNA fragment pattern changed significantly, as shown in Fig. 3B. A TRF (172 bp) corresponding to the *Alu*I fragment of the 16S rDNA of *Symbiobacterium toebii* was detected, comprising about 5% of the total 16S rDNA. A thermophilic *Geobacillus* TRF (about 50% of the total 16S rDNA) remained as the major fraction after enrichment. The most significant change was the appearance of a new main TRF of 257 bp (about 40% of the amplified 16S rDNA) in all the samples. When the amplified 16S rDNA was cloned and sequenced, sequences with a TRF of 257 bp were found. Based on a sequence similarity analysis, the 16S rDNA sequence exhibited a less than 90% similarity with any other known sequence, including *Symbiobacterium toebii* (data not shown). Since the most similar 16S rDNA sequences were those of *Clostridium hastiforme* [4], this result would seem to indicate the presence of a new thermophile member of the *Bacillus-Clostridium* subphylum [3].

DISCUSSION

It has been suggested that there are large numbers of uncultivated microorganisms in natural environments, which at present can only be seen under a microscope or detected using molecular ecological methods [2]. It is also assumed that biological interaction is one growth factor for certain microorganisms, such as commensal eubacteria [8, 9, 13] and psychrophilic crenarchaeon on marine sponges [11]. A novel commensal thermophile was previously reported to require essential growth factors from its partner thermophilic *Geobacillus* sp. SK-1 [11]. Since understanding of this interaction requires knowledge about the ecology of this microorganism, we investigated in the current study the abundance of the commensal thermophile in soils using a CQ-PCR and terminal restriction fragment length polymorphism (TRFLP) analysis. The results obtained the presence of unknown novel microbial interactions among uncultivated microorganisms in the ecosystem.

An attempt was made to quantify the 16S rDNA of the commensal thermophile in soils using a CQ-PCR. The commensal thermophile 16S rDNA was only detected in the DNA samples from manure and rice straw compost soils when the nested CQ-PCR method was used. 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. However, when considered the low recovery of DNA from soils, as estimated by Lee *et al.* [7], the commensal thermophile count was more likely to be about 2 orders of magnitude higher in reality. Accordingly, these results indicate that compost would be a growth environment for the commensal bacterium.

In the enrichment culture experiment, the commensal thermophile 16S rDNA was detected in all the soils. Furthermore, indole, the enzymatic reaction product of tryptophan-indole lyase, was also accumulated in all the samples. Since tryptophan-indole lyase activity has not been reported in any other thermophile, except for commensal bacteria such as *S. toebii* [12] and *S. thermophilum* [14], the indole produced would appear to have resulted from the enzyme reaction of the commensal thermophile. This is also supported by the proportional relationship between the commensal thermophile 16S rDNA content and the indole concentration in the culture medium. Based on the presence of the commensal thermophile 16S rDNA and indole accumulation in all the culture samples, it was concluded that the commensal thermophile was widely distributed in all the soil environments, even though the nested CQ-PCR detected only the commensal thermophile 16S rDNA in certain soils. In fact, when more than 20 kinds of soils were incubated under enrichment conditions, indole was accumulated in all the samples within one week of incubation.

The indole accumulation was faster in the culture experiment using compost soils than in an earlier commensal co-culture study of *Symbiobacterium toebii* with thermophilic *Geobacillus* sp. SK-1 [12]. Although no indole was detected within 24 h of incubation in the commensal co-culture study, 0.20 mM of indole was accumulated within 16 h of incubation in the compost soil samples. This rapid accumulation of indole was maintained despite successive transfers to fresh PEP media. The possibility of the presence of free-living relatives of the commensal thermophile was excluded, based on the failure to detect any free-living commensal thermophiles. Furthermore, no other indole-producing thermophile was detected during screening of the bacteria from the culture samples. Therefore, it would seem that unknown interactions made the commensal thermophile grow faster in the enrichment culture experiment than in the defined co-culture study.

Since no accumulation of indole or the commensal thermophile 16S rDNA was detected in the samples incubated at 30°C, the presence of a mesophilic relative of the commensal thermophile in nature is not evident. This means that the 16S rDNA of the commensal bacterium detected in the soil samples did not originate from a commensal mesophile.

The TRFLP analysis confirmed the results of the CQ-PCR. No TRF corresponding to the commensal thermophile was detected in the 16S rDNA products directly amplified from the soils. Excluding *Geobacillus*, all other eubacteria appeared to constitute small fractions in the soils. However, during the culture experiment, even though the microbial community structure changed, a TRF of the commensal thermophile 16S rDNA was still detected in all the soils. Furthermore, the presence of a novel 16S rDNA

showing a TRF of 257 bp was highly enriched. When the bacteria containing the novel 16S rDNA sequence were isolated under a microaerobic atmosphere, the strains exhibited no tryptophan indole-lyase activity, although the novel group of bacteria showed a similar morphology to *Symbiobacterium toebii* (data not shown).

The enriched samples from the compost soils that exhibited rapid accumulation of indole (within 16 h) contained a large amount of the commensal thermophile 16S rDNA. This indicates that the commensal thermophile may be released in mesophilic soil environments after proliferation in thermophilic composts. Since composts have been traditionally used in agriculture, this may be one reason for the wide distribution of the commensal thermophile in the various soils.

According to most molecular phylogenetic surveys, a good deal of microbial diversity remains to be discovered and identified. With regard to interactions among bacteria in the natural ecosystem, the current knowledge in this area is only scratching the surface. Thus, more studies are needed to elucidate the diversity of the bacteria affected by these microbial interactions since commensal thermophiles are surely not the only examples in the ecosystem.

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