Effect of Temporal Sample Preservation on the Molecular Study of a Complex Microbial Community in the Gut of the Termite Microcerotermes sp.

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We investigated the effect of preserving samples with ethanol and acetone on molecular analyses of a complex microbial community in the gut of the termite Microcerotermes sp. Although the yield of DNA extracted from the guts decreased as the period of preservation increased (three days, one week, and one month), terminal-restriction fragment length polymorphism (T-RFLP) profiles of bacterial 16S rRNA genes amplified from the extracted DNA were conserved with respect to the occurrence of major taxa. Bacterial diversity detected in the analyses decreased only slightly after the preservation, and an analysis of clones demonstrated that the difference in terms of coverage was statistically insignificant before and one month after preservation with acetone, the treatment which resulted in the greatest dissimilarity in T-RFLP. However, the relative abundance of some taxonomic groups was remarkably changed after one month of preservation, especially in acetone. The results suggest that both ethanol and acetone well preserve bacterial DNA from a complex gut community, but that the DNA should be extracted as soon as possible. If the period of preservation exceeds one week, the molecular community profiles should be interpreted with caution, and it is better to avoid abundance-based comparisons.

Key words: DNA, preservation, termite, symbiosis, microbial community structure

It goes without saying that the collection and storage of natural samples are of primary importance in ecological and environmental studies. DNA extracted from biological materials is essential for molecular analyses, the significance of which has been growing for the evaluation of biological diversity and for evolutionary and ecological investigations. Thus, proper preservation for the maintenance of DNA is critical when DNA cannot be extracted at the time of sampling. Although freezing is usually used to preserve samples, it is not always applicable in the field. For the convenient preservation of DNA in biological materials, a high concentration of ethanol is widely used, while acetone has been recommendable in analyses of various insects and their intracellular bacteria2. Comparisons of various methods of preserving DNA in different biological materials are reviewed in reference 2). However, these methods have only been applied to studies on single organisms or symbiotic bacteria comprised of just one or a few species. The effect of these preservatives on a complex microbial community has never been investigated.

In this study, we investigated the effect of temporal pres-
ervation on microbial community structure detected by molecular analyses. For this purpose, we preserved the guts of the termite Microcerotermes sp. with ethanol and acetone. Termites harbor a complex microbial community which aids digestion\(^1\). Microbial diversity in the termite gut has been investigated based on molecular sequences, and a great majority of the community represent hitherto-undescribed species including novel candidate bacterial phyla\(^5\). Microcerotermes is one of the most extensively investigated genera in the gut bacterial community\(^5,16,17\), allowing us valid comparisons. Before and after preservation, PCR-amplified bacterial 16S rRNA genes from extracted DNA of the gut community were compared by T-RFLP and clone-based analyses.

**Materials and Methods**

**Termite and sample preservation**

A wood-feeding termite, Microcerotermes sp. (order Isoptera, family Termitidae), was collected from Prachinburi in Thailand. Based on morphological and molecular analyses, the termite sample (designated colony M2PB4) has been identified as the species M2, though its specific epithet has not yet been given\(^9\). Part of a nest containing termites was sampled and transported carefully without heating, drying, or exposure to sunlight. Only the worker caste was used. The collection and preservation were done the same day.

Pure-grade preservatives, acetone (BDH Laboratory Supplies, Poole, UK) and ethanol (Merck KGaA, Darmstadt, Germany), and 80% ethanol prepared with MilliQ water were used. The termite gut was carefully dissected with a pair of sterile, fine-tipped forceps, and 20 dissected guts were directly put into 1 mL of each preservative in a 1.5-mL microcentrifuge tube. The samples were kept at room temperature, and examined after three days, one week, and one month. Twenty entire bodies were also kept in 3 mL of each preservative in a screw-capped 10-mL glass vial.

**DNA extraction**

The samples were placed on clean paper towel to remove the preservatives. DNA was extracted from the gut homogenate using a Nippon Gene Isoplant II kit and further purified using a QIAGEN DNeasy kit as described previously\(^7\). The same method has been used to extract DNA from samples immediately after their collection (fresh guts) to study the gut bacterial community\(^3\), and this DNA was also used here for comparison. The yield of the extracted DNA was determined spectrophotometrically by measuring the absorbance at 260 nm.

**T-RFLP analysis**

Bacterial 16S rRNA genes were amplified from the extracted DNA (1.5 ng each) with Takara ExTaq polymerase using the universal primers 27F and 1389R\(^7\). The 27F primer was labeled with 6-carboxyfluorescien (FAM) at 5' terminus. The reaction was run with an initial denaturation at 95°C for 2 min, 25 cycles of 95°C for 30 sec, 50°C for 1 min, and 72°C for 4 min, and a final elongation step of 72°C for 10 min. The PCR products were size-fractionated with agarose gel electrophoresis and the products of expected size (1.3–1.5 kbp) were purified using a QIAGEN MinElute gel extraction kit. The purified products were digested with either the HaeIII or Hhal restriction enzyme. T-RFLP was analyzed using an ABI377 genetic analyzer in the GeneScan v.3.1.2 mode with a GS-1000ROX internal size standard. The entire procedure starting with the PCR was replicated in order to confirm its reproducibility.

The peak height in the T-RFLP profiles was standardized as described previously\(^6\), using 100 fluorescence units as the threshold of the baseline noise. The standardized data were analyzed with BioCLUST v.1.0 software\(^16\) using a dissimilarity index, calculated as follows: \(D = 1/2 \times (\sum_{ij} x_i - x_j)\), where \(x_i\) and \(x_j\) are the ratios (\%) of the \(k\)th peak height in samples \(i\) and \(j\), respectively\(^1\). \(D = 0\) when the two samples are identical and \(D = 100\) when they are absolutely different. A dendrogram showing a relationship among the T-RFLP profiles was constructed using the neighbor-joining algorithm followed by the tree-bisection-reconnection procedure in PAUP* (version 4.0b10; Swofford, D.L. 2003. Sinauer Associates, Sunderland, MA, USA). Diversity was evaluated using the divergence index \(MDt=(\sum x_i x_j)^2\), where \(x_i\) is the ratio (0<\(x_i<1\)) of the \(k\)th peak height in a single profile\(^6\). \(MDt\) increases as the sample is more diverse.

**Clone-based analysis**

A clone library of the amplified 16S rRNA genes from the DNA extracted from guts preserved in acetone for one month was constructed. In order to reduce the effects of bias and drift during the amplification, the PCR was performed using 12 cycles, and the products of four independent PCRs were mixed and used\(^1\). The PCR products were purified using a QIAGEN MinElute PCR purification kit, and used for the construction of the clone library with an Invitrogen TOPO TA cloning kit. One hundred clones of the library were randomly picked for DNA sequencing. The sequencing was performed using the ABI377 with primers T7 and T3. Chimeric sequences were eliminated as described
previously\textsuperscript{9). The sequences were aligned with ARB software\textsuperscript{10}, and 527 unambiguously aligned nucleotide positions corresponding to 563–1117 in \textit{Escherichia coli} (J01695) were used for the subsequent analyses.

The results of an analysis of 96 clones in a library constructed from fresh guts using the same method has already been published\textsuperscript{10} and these sequences were used for comparison. The sequences were sorted into phylotypes using the DOTUR program\textsuperscript{14} and the furthest neighbor clustering algorithm with the criterion of 97.0\% sequence identity. Analyses of rarefaction curves and Chaoy richness estimators were conducted with the DOTUR program. The difference between clone libraries was examined with the program j-LIBSHUFF v1.3\textsuperscript{19}, permuting the clones of the libraries 10,000 times. The distance matrix analyzed by j-LIBSHUFF was generated using the ARB software with the Jukes-Cantor model. The entire DNA sequence of the clone representing each phylotype was determined and used for construction of a phylogenetic tree by the maximum likelihood method using the PHYML v.2.4.4 program\textsuperscript{3} with a 100-bootstrap resampling test. The 16S rRNA gene sequences reported in this study have been deposited in DDBJ under accession numbers AB243251–AB243294.

\section*{Results}

\section*{Effect on DNA extraction}

Dissected guts of the termite \textit{Microcerotermes} sp. were preserved in absolute acetone, absolute ethanol, or 80\% ethanol. After three days, one week, and one month, DNA was extracted from guts containing a symbiotic microbial community. Figure 1 shows the yields of DNA extracted. The yield decreased as the period of preservation increased. The best yield was obtained when dissected guts were preserved in absolute ethanol. Bacterial 16S rRNA genes were successfully amplified from DNA of every preserved sample. No difference in amplification efficiency was observed among the samples because almost the same amount of PCR product was obtained from a definite amount of DNA. Thus, these preservation conditions allow the extraction of DNA suitable for PCR-based molecular studies.

When entire bodies were preserved, DNA yields of the gut after three days were lower than those from directly preserved samples regardless of the preservative used. Since dissection of the gut from the body became difficult after one week due to coherence of termite tissues, we did not investigate further the preservation of entire bodies.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Yield of DNA extracted from the guts after preservation. The yield of DNA extracted immediately after the collection (the fresh guts sample) is indicated on day zero. The samples were preserved in absolute ethanol (squares), 80\% ethanol (circles), and absolute acetone (triangles) for different periods. Dissected guts were preserved for one month (closed symbols). Entire bodies were preserved only three days (open symbols) due to difficulty in dissecting the guts afterwards.}
\end{figure}

\section*{Effect on bacterial community structure}

Bacterial communities in the gut were compared among samples by conducting T-RFLP analyses of the amplified 16S rRNA genes. Figure 2 shows representative T-RFLP profiles before and after each preservation. The profiles appeared to be conserved even after one month of preservation, particularly with respect to the occurrence of major taxa. The presence of T-RFs with a peak height of more than 5\% in the fresh guts was completely conserved in all the preserved samples. The profiles of the preserved samples retained significant T-RF diversity with divergence indices (\textit{MDt}) of \textit{HaeIII} profiles in the range of 8.8–10.7, while the index for the fresh samples was 11.2. The indices of the samples preserved for three days were quite high (\textit{MDt}>10.1). The dendrogram shown in Fig. 3 demonstrates the relationships among T-RFLP profiles based on comparisons of dissimilarity indices (\textit{D}). Each of the samples preserved for three days in absolute ethanol and acetone showed a close relationship with the fresh guts. The dissimilarity index of their \textit{HaeIII} profiles compared with that of the fresh samples was 9.8 and 11.5, respectively, and the dissimilarity was considered to be insignificant since the average index between duplicated T-RFLP profiles in a single sample was 7.5±3.5 (mean±SD; range, 4.8–15.5). Therefore, the preservation did not greatly affect microbial community structure in the termite guts. Above all, three days of preservation had the slightest effect.
The profile for one month of preservation in acetone was the most distantly related to that of the fresh guts (Fig. 3). The dissimilarity index of the two HaeIII profiles was 28.4, which was considerably higher than the indices between the fresh samples and the samples preserved in absolute or 80% ethanol (D<18.0). The one-week acetone treatment also gave a higher index, 21.5. In fact, the T-RF of 177 bases in the HaeIII profile (Fig. 2) was remarkable in the one-month acetone-preserved samples, amounting to 19% of total peak height. The peak height of the corresponding T-RF after one week in acetone was also high, 12% of the total, whereas that in the fresh guts and the guts preserved in absolute and 80% ethanol was 3–9%. The T-RF of 73 bases in the HhaI profiles also showed similar features. Thus, in spite of the apparent similarity, differences in the T-RFLP profiles were not negligible for the longer period of preservation in acetone, particularly with respect to the relative abundance of some taxa.

**Bacterial diversity in the sample preserved in acetone**

Since the T-RFLP analysis revealed significant similarity in the bacterial community structure in the gut after preservation, we then investigated bacterial taxonomic diversity in the sample most affected, that preserved in acetone for one month, and compared the result with that reported previously for fresh guts. One hundred clones in the library of 16S rRNA genes amplified from the gut microbial community in the one-month acetone-preserved sample were sorted into 44 phylotypes according to a criterion of 97.0% sequence identity, whereas 96 clones from the fresh guts were sorted into 50 phylotypes. A comparison of rarefaction curves (Fig. 4) revealed that the preserved sample contained slightly less bacterial diversity than the fresh guts. Indeed, expected numbers of phylotypes with the Chao1 richness estimator were 86 (95% confidence interval, 60–154) for the acetone-preserved guts and 106 (73–186) for the fresh guts.
However, the differences in rarefaction curves and Chaol estimators were not significant, respectively, owing to overlaps of the confidence intervals.

The two clone libraries were compared statistically with the J-LIBSHUFF test, demonstrating that the difference in their diversity was insignificant (P=0.8662 for X vs. Y and P=0.7415 for Y vs. X, where X and Y are libraries of the fresh guts and the acetone preserved guts, respectively). This test is based on a comparison of coverage within and between libraries[5]. In fact, as shown in the phylogenetic tree (Fig. 5), 15 phylotypes overlapped before and after the preservation, and they represented abundant phylotypes in terms of clone numbers, comprising 43 and 64 clones of the fresh and the preserved guts, respectively. Great bacterial diversity with up to 1,200 phylotypes is estimated in the gut of Microcerotermes sp.[3], and when compared with the phylotypes analyzed so far in this termite genus, a total of 26 phylotypes in the clone library of the preserved guts were shared among Microcerotermes spp. An additional 10 phylotypes were closely related to phylotypes from this termite genus, forming a number of phylogenetic clusters (see Fig. 5).

The taxonomic composition of the two clone libraries was different. When the major bacterial taxonomic groups were compared, clone abundance was found to be significantly different between the libraries (χ²=18.437, df=4, P=0.001). Also, the difference in clone numbers of abundant phylotypes between the libraries was significant (χ²=33.922, df=7, P<0.0001). After the preservation, members of Firmicutes accounted for 28% of the clones, versus 17% in the fresh guts (see Fig. 5). The phylotype M2PB4a-091 in Firmicutes showed the most remarkable change in clone numbers, from 3 to 17 after the preservation, and this phylotype corresponded to the marked peaks of 177 bases in HaeIII and of 73 bases in Hhal profiles of T-RFLP (see Fig. 2). The proportion of Fibrobacteres also increased to 29% from 12%, and some phylotypes in Fibrobacteres increased in clone number (e.g. M2PB4a-033, from 2 to 10; M2PB4a-004, from 1 to 7). In contrast, the proportion of Spirochaetes dominant in the fresh guts (52%) decreased to 29% after the preservation. In Spirochaetes, phylotypes M2PB4a-015 and M2PB4a-025 decreased in clone number after the preservation (from 10 to 1 and from 8 to 2, respectively), while M2PB4a-043 increased in number from 1 to 7. Although the number of clones analyzed here (100) represented a mere fraction of the great diversity in the termite gut, these results indicated that bacterial diversity detected in the analysis of clones was insignificantly different as a whole before and after the preservation, but the abundance in clone numbers changed considerably in several taxa.

Discussion

Preserving samples with either ethanol or acetone was found to be useful for PCR-based molecular studies of a complex microbial community in the termite gut, particularly in terms of the detection of major taxa. The bacterial diversity in the preserved samples decreased only slightly. However, the abundance of some taxa changed considerably in the acetone-preserved samples, as typified by the phylotype M2PB4a-091 in Firmicutes. Therefore, the data obtained after preservation should be carefully considered in this respect. Nevertheless, these convenient methods are advantageous for the temporal preservation of samples in the field. The direct preservation of dissected guts is recommended, and dissection is not problematic in the field because it is simple and easy using only forceps. The shorter preservation period (e.g. three days) has the least effect even on the abundance data, suggesting that temporally preserved samples ought to be used for DNA extraction as soon as possible. Preservation in absolute ethanol is suitable, since the DNA yield was the best and the abundance of taxa detected after the preservation was not affected significantly.

Acetone was recommended previously for the preservation of insects and their symbiotic bacteria[3], but acetone had the greatest effect on molecular analyses of the complex microbial community structure in the termite gut. The ap-
Fig. 5
parent changes in abundance of some taxa before and after preservation are probably due to different effects of acetone on individual community members. Because the bacterial taxa that changed greatly, such as those represented by phylotype M2PB4a-091, have not yet been cultivated, the reason for this change is unclear. Since the recovery of DNA decreased during the preservation, taxa that apparently increased after the preservation in acetone are thought to be resistant to DNA degradation, or to be unaffected in terms of extraction efficiency, while the other taxa are affected greatly. It is conceivable that the ability of acetone to penetrate cells may differ among the gut community members, and/or that some unknown interaction with acetone at the cell surface may occur with specific bacterial groups. Acetone-based preservation is reported to protect against contamination by water. However, the use of acetone resulted in a lower yield of extracted DNA than the use of absolute ethanol, although the presence of water indeed affected the yield when compared with the preservation in absolute and 80% ethanol. The dehydrating activity of acetone may not be significant in the case of the termite gut community.

Comparisons of intra- and interspecific termite hosts have revealed that the gut microbial community is remarkably conserved within some termite genera, with small but significant differences among species and sampling sites.

But bacterial species in the gut differ almost completely between termite genera. Hence, studies of a variety of genera and species in various locations are anticipated in order to understand the true diversity and coevolution of termite gut symbionts, most of which are novel microorganisms. A great diversity of termites, nearly 280 genera and more than 2600 species, exist on Earth, particularly in tropical regions which are often far removed from laboratories. Although only one termite species is investigated here and more termites should be analyzed, the temporal preservation described in this study will be useful for studies of the complex microbial communities in the gut of a variety of termites.

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References

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