The influence of carbon sources on bacterial community structure in the gut of the wood-feeding higher termite *Nasutitermes takasagoensis* was investigated. 16S rRNA gene sequencing and terminal-restriction fragment length polymorphism (T-RFLP) analyses revealed that the bacterial community structure changed markedly depending on feed components at the phylum level. *Spirochaetes* was predominant in the clone libraries from wood- and wood powder-fed termites, whereas *Bacteroidetes* was the largest group in the libraries from xylan-, cellobiose-, and glucose-fed termites, and *Firmicutes* was predominant in the library from xylose-fed termites. In addition, clones belonging to the phylum Termite Group I (TG1) were found in the library from xylose-fed termites. Our results indicate that the symbiotic relationship between termite and gut microorganisms is not very strong or stable over a short time, and that termite gut microbial community structures vary depending on components of the feeds.

Key words: symbiosis; higher termite; *Nasutitermes takasagoensis*; carbon sources; 16S rRNA gene

Termites are important decomposers, particularly in tropical and subtropical ecosystems, and play important roles in the biorecycling of lignocellulose. They harbor abundant and diverse microorganisms (protozoa, fungi, bacteria, and archaea) in their gut. These intestinal microorganisms are considered to contribute to digestion and utilization of wood and plant litter as sources of nutrients. They establish symbiotic relationships not only with the host but also with other intestinal microorganisms. Therefore, the termite gut microbial ecosystem is one of the most interesting examples of a symbiotic relationship.

Termites comprise a complex assemblage of diverse species. They are roughly divided into six families of lower termites and a single family of higher termites. Lower termites harbor prokaryotes and flagellates in their guts, and in particular, the flagellates are essential for the digestion of the lignocellulose of wood. Higher termites lack flagellates and harbor only prokaryotes in their guts. It has been reported that higher termites produce their own cellulolytic enzyme, and that their gut is morphologically and physicochemically highly structured.

Most previous studies of the bacterial community in the gut of wood-feeding termites focused on lower termites. Recent culture-independent studies have provided many findings about the diversity of the bacterial community structure in the gut of various lower termites, but knowledge of the bacterial community structures in the gut of wood-feeding higher termites is still insufficient for a comprehensive understanding of the mechanisms underlying wood digestion in higher termites. Wood is chiefly composed of cellulose, hemicellulose, and lignin, and wood-feeding termites utilize these carbon sources in the wood for nourishment. To clarify the mechanisms underlying wood digestion in higher termites, it is important to determine the relationship between the carbon sources contained in wood and gut bacterial community structures. Moreover, such findings would be useful in elucidating the function and evolution of digestive symbiosis of higher termites.

In this study, we investigated the influence of car-
bohydrate contents in feeds on the bacterial community in the gut of the wood-feeding higher termite *Nasutitermes takasagoensis* fed with different feeds by culture-independent molecular approaches.

**Materials and Methods**

*Termites.* *N. takasagoensis* was collected from Iriomote Is. in Okinawa Prefecture (Japan). The collected termites were placed in containers with their nest materials and wood from the original collection site. They were maintained at 30°C and >90% relative humidity. For the experiment, only worker-caste termites were used.

**Artificial feeds and feeding experiments.** Artificial feeds were prepared as follows: The carbon source (40 g/l) and agar (15 g/l, Wako, Tokyo, Japan) were dissolved in distilled water, and the pH of the solution was adjusted to 6.8–7.0 before autoclaving at 121°C (40 g/l) and agar (15 g/l, Wako, Tokyo, Japan) were dissolved in distilled water, and the pH of the solution was adjusted to 6.8–7.0 before autoclaving at 121°C for 20 min. Powdered wood from the original collection site (wood powder), cellulose (CF31, Whatman Japan KK, Tokyo, Japan), cellobiose (Wako), glucose (Wako), xylan (from beechwood, Sigma-Aldrich, Tokyo, Japan), or xylose (Wako) was used as the carbon source. After autoclaving, the solution was solidified in sterilized glass petri dishes. Solidified agar gels were cut into blocks aseptically (one block = 25 × 25 × 5 mm). A group of 25 workers and one block of artificial feed were placed inside a sterilized polystyrene case (30 × 30 × 10 mm), and incubated at 30°C and 30% relative humidity for three weeks. During incubation, fresh feed was supplied every 3 d, and dead termites were removed. Viable termites were counted periodically and their survival ratios were calculated. All experiments were performed four times for each type of feed.

**DNA extraction.** Ten termites fed with wood as the natural feed or the various artificial feeds for three weeks were randomly collected. After their exterior surfaces were washed with 70% ethanol and sterilized distilled water, whole guts were removed with sterilized forceps. Total DNA in the guts of termites was extracted using a Fast DNA Kit (Qiogene, Carlsbad, CA, USA). The removed guts were added into 1 ml of CLS-TC buffer (Qiogene) in a Fast DNA tube containing a matrix designed for the lysis of most cell types. The mixture was processed in a Fast Prep 120 instrument (Qiogene) for 15 s at 4 m/s. The subsequent procedures were performed as described in the manufacturer’s instructions. DNA concentration was determined using a Pico green dsDNA Quantitation Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s instructions.

**Construction of 16S rRNA gene clone libraries.** Clone libraries of 16S rRNA genes were constructed from PCR products derived from the guts of termites fed with wood, wood powder, xylan, xylose, cellobiose, or glucose. The PCR primers used to amplify the bacterial 16S rRNA gene were 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1390R (5'-ACGGGCGGTGTGTRGAA-3'). The amplification reaction mixture (20 μl) consisted of 0.5 U of Taq DNA polymerase (Takara Bio, Shiga, Japan), 1.0 μl of 10× PCR buffer, 1.0 μM each of the primers, 200 μM of dNTP mixture, 3.0 ng of total DNA, and sterilized distilled water. The reaction was performed as follows: Initial denaturation at 95°C for 5 min, 18 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and then incubation of the reaction mixture at 72°C for 4 min. To minimize PCR bias, the number of PCR cycles was decreased to 18. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and cloned into pGEM-T Easy Vector system (Promega, Madison, WI, USA) in accordance with the manufacturer’s instructions. Clonal DNAs were amplified from randomly selected recombinants by direct PCR with T7 and SP6 primers (Promega). PCR products were purified using a MinElute 96 UF PCR Purification Kit (Qiagen) and used as templates for sequencing. Sequencing was performed with primer 907R (5'−CCGTCAGTTCTACCGAGTTG−3') using a DTCS Quick Start Kit (Beckman-Coulter, Fullerton, CA, USA), and a CEQ-8000 automated sequencer (Beckman). The sequences of all clones were compared with those in the GenBank database by BLAST search at the website of DDBJ (http://www.ddbj.nig.ac.jp/Welcome-j.html). Artifact chimeric sequences were detected using the CHECK-CHIMERA program of RDP project II. Phylogenetic analysis was performed using the ARB program package. All clonal sequences and the reference sequences from the GenBank database were imported into the database of the ARB program. After automatic and manual sequence alignment, phylogenetic trees were constructed by the neighbor-joining method, and bootstrap analysis for 1,000 replicates was performed using PAUP 4.0 b (Sinauer Associates, Sunderland, MA, USA). We defined the phyotypes as sequences showing >97% identity according to the clustering algorithm. Diversity coverage by each clone library was analyzed using the Analytic Rarefaction software program (Holland, SM, University of Georgia, Athens, version 1.3, http://www.uga.edu/~strata/software/).

**T-RFLP analysis.** Fragments of bacterial 16S rRNA genes were amplified using the BODIPY FL (D-6140, Molecular Probes)-labeled primer (5'−CCCTACGGGAGGCAGCAG−3') and primer 907R. Each PCR mixture (20 μl) consisted of 0.5 μl of 10× Taq DNA polymerase (Takara), 4.0 ng of total DNA, 2.0 μl of 10× PCR buffer, 0.2 μM of each of the primers, 200 μM of dNTP mixture, 0.25 mg/ml of bovine serum albumin, and sterilized distilled water. PCR was performed as follows: Initial denaturation at 95°C for 150 s, 30 cycles of 95°C for 20 s, 55°C for 10 s, and 72°C for 45 s, and
then incubation of the reaction mixture at 72°C for 2 min. PCR products were purified using a Microcon PCR filter (Millipore, Tokyo, Japan), digested with AluI, HaeIII, HinfI, HhaI, or MspI (New England Biolabs, Beverly, MA, USA), and analyzed using a ABI310 sequencer (Applied Biosystems, Tokyo, Japan) with a TAMRA size standard (Applied Biosystems). T-RFLP electropherograms were analyzed by GeneScan v.3.1 (Applied Biosystems).

Analysis of T-RFLP data. Signals with a peak height contribution lower than 3% were regarded as background noise and excluded from further analysis. Moreover, to avoid detection of primers and uncertainties in size determination, terminal restriction fragments (T-RFs) smaller than 17 bp and larger than 600 bp were excluded from further analysis. For statistical analysis, the Euclidean distance between the samples was calculated on the basis of peak size and relative abundance. Multidimensional scaling (MDS) analysis based on Euclidean distance(19) was performed using SPSS 14.0 software (SPSS, Chicago, IL, USA). MDS analysis was performed in this study solely to aid in visualization of the relationships among bacterial community structures (grouping and relative distances among structures).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of clones in the libraries from the gut of N. takasagoensis in this study have been deposited with DDBJ under accession nos. AB277868 to AB278069.

Results

Influence of artificial feeds on survival ratio of termites
A higher termite, N. takasagoensis, was fed with artificial feeds containing different carbon sources (wood powder, xylan, xylose, cellulose, cellobiose, and glucose) for three weeks. The survival ratios of termites fed with various artificial feeds after three weeks were higher than those fed with only agar without any carbon source, in the order wood powder, glucose, cellobiose, xylan, xylose, and cellulose (Fig. 1).

Clonal analysis of 16S rRNA gene
Bacterial community structures in the gut of termites fed with wood for three weeks as natural feed or various carbon sources (wood powder, xylan, xylose, cellulose, cellobiose, and glucose) as artificial feeds were analyzed on the basis of 16S rRNA gene sequences. A total of 388 clones of the six libraries were constructed. No cellulose library was constructed because of an insufficient amount of DNA collected, owing to the markedly low survival ratio. Table 1 shows the total number of clones, phylotype richness, Shannon–Weaver index, and evenness(21) of each clone library. Clonal analysis yielded between 58 and 71 clones in individual libraries. The phylotype richness among all libraries was lowest for the wood and wood powder libraries, and highest for the cellobiose library. The Shannon–Weaver index and evenness were calculated as measures of diversity, which were higher for the cellobiose library. Diversity coverage by each of the libraries was estimated from the rarefaction curve (Fig. 2). The steepness of the curves for the single-carbon-source libraries were larger than those for the wood or wood powder libraries. The highest diversity was found in the cellobiose library. These results indicate that bacterial diversity in the gut of termites was influenced by the feed components.

The relative frequencies of the clones in various bacterial phylogenetic groups at the phylum level are shown in Fig. 3. The relative clone frequencies of the major phylogenetic groups in the wood and wood powder libraries were very similar, but they were markedly different from those in the single-carbon-source libraries. In particular, the relative clone frequencies of the phyla Spirochaetes, Bacteroidetes, and
Firmicutes in the various libraries were markedly different. The clones with the highest relative frequencies in the wood and wood powder libraries were related to the phylum Spirochaetes. On the other hand, the relative clone frequencies affiliated with the phylum Bacteroidetes were high in the xylan, cellobiose, and glucose libraries. In the xylose library, the phylum Firmicutes was predominant. Moreover, the clone affiliated with the candidate phylum termite group I (TG1) was obtained only from the xylose library. This clone was related to the clones derived from the gut of the lower termite Reticuritermes santonensis (97% sequence identity) (data not shown).

Table 2 shows the number of phylotypes within the three major phyla (Spirochaetes, Bacteroidetes, and Firmicutes) and others in each libraries. The number of phylotypes affiliated with Spirochaetes and the Firmicutes showed only a few differences between the wood/wood powder libraries and the single-carbon-source libraries. Most of the phylotypes affiliated with the Firmicutes in the single-carbon-source libraries showed <97% sequence identity to any phylotype in the wood/wood powder libraries. Within the Bacteroidetes, the number of phylotypes in each single-carbon-source library was much larger than those in the wood/wood powder libraries, and many phylotypes within the

![Rarefaction Analysis of Bacterial 16S rRNA Gene Clones Recovered from the Whole Gut of N. takasagoensis Fed with Various Feeds.](image1)

The slope at the end of each curve indicates the rate at which new phylotypes (>97% sequence identity) were discovered at the point when we stopped sampling. The error bars show 95% confidence intervals.

![Relative Clone Frequencies of Major Phylogenetic Groups in Clone Libraries of 16S rRNA Genes of the Whole Gut of N. takasagoensis Fed with Various Feeds.](image2)
Bacteroidetes obtained from the single-carbon-source libraries were not obtained from the wood/wood powder libraries.

**T-RFLP analysis**

The bacterial community structures were compared among the termites fed with wood and artificial feeds on the basis of the T-RFLP fingerprinting of 16S rRNA gene fragments with AluI (Fig. 4). The T-RFLP profiles of the termites fed with wood and wood powder were very similar, but those of termites fed with artificial feeds were markedly different. The numbers of the peaks in the T-RFLP profiles of the termites fed with xylan, xylose, cellobiose, and glucose were larger than those of the termites fed with wood or wood powder. Using the predicted lengths for the T-RFs of the clonal sequences, the predominant peaks in the T-RFLP profiles were assigned to the clones in the corresponding libraries. The predominant peaks of the profiles of wood- and wood powder-fed termites matched those of the Spirochaetes-related clones (18 bp). A peak of 396 bp was assigned to the clones in our libraries affiliated with the Bacteroidetes, and that of 133 bp was assigned to the clones affiliated with the Clostridiales. These results corresponded to those for the abundant phylotypes in the clone libraries (Fig. 3), and this tendency was also observed for the fragments obtained using other restriction enzymes (HaeIII, Hinfl, HhaI, and MspI) (data not shown).

The relationships among the bacterial community structures of the various samples were visualized by MDS analysis of T-RFLP profiles obtained in experiments using five enzymes (AluI, HaeIII, Hinfl, HhaI, and MspI) (Fig. 5). The profiles of wood- and wood powder-fed termites were grouped more closely, indicating a similar T-RFLP pattern composition. Similarly, the profiles of xylan-, cellobiose-, and glucose-fed termites were grouped together, although they were distinct from those of wood- and wood powder-fed termites. The profile of xylose-fed termites was divergent from the other profiles. MDS plots showed that the bacterial community structures in the gut of termites fed with single carbon sources were markedly different from those of the termites fed with wood or wood powder.

**Table 2.** Phylotypes of Bacteria in Gut of Termites Fed with Various Feeds

<table>
<thead>
<tr>
<th>Taxon of bacteria</th>
<th>Wood</th>
<th>Wood powder</th>
<th>Xylan</th>
<th>Xylose</th>
<th>Cellobiose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spirochaetes</td>
<td>10</td>
<td>16</td>
<td>9</td>
<td>8</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>5</td>
<td>3</td>
<td>15</td>
<td>15</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>7</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Others</td>
<td>7</td>
<td>7</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
Discussion

In this study, we investigated the influence of carbon sources in feed components on the symbiotic bacterial community structure in the gut of the wood-feeding higher termite N. takasagoensis. Artificial feeds were developed for the feeding of N. takasagoensis, and the bacterial community structures in the termites fed with various artificial feeds were characterized by culture-independent methods. The results of cloning analysis and T-RFLP analysis based on 16S rRNA gene sequences revealed that the components in the feeds had a drastic influence on the bacterial community structure in the gut of the termites.

The survival ratios of the termites fed with artificial feeds depended on the carbon sources contained in the feeds. Although N. takasagoensis produces its own cellulase, surprisingly, the survival ratio of the termites fed with artificial feed containing cellulose powder was markedly low. Conversely, the survival ratios of the termites fed with xylan or xylose, which are hemicellulose components, were high. This finding suggests that N. takasagoensis has a high ability to metabolize hemicellulose.

The relative clone frequencies in wood and wood powder libraries were similar to those of wood-feeding higher termites Microcerotermes spp. and N. takasagoensis at the higher taxonomic level (data not shown), but the bacterial community structures in the gut of the termites fed with the various artificial feeds changed markedly at the phylum level, depending on the feed component. Moreover, there were many phyotypes within the Bacteroidetes and the Firmicutes that were obtained only from the single-carbon-source libraries. This indicates that the phyotypes that are not common to the wood/wood powder libraries and the single-carbon-source libraries contribute to the change in the relative clone frequencies in each library.

Thirty-eight phyotypes within the Spirochaetes, 50 phyotypes within the Bacteroidetes, and 34 phyotypes within the Firmicutes showed < 97% sequence identity to clones derived from the gut of wood-feeding higher termites Microcerotermes spp. and N. takasagoensis. In particular, most of the phyotypes affiliated with the Bacteroidetes showed phylogenetic distance from different clones derived from other environments and all cultured strains in public databases, suggesting that they represent novel and as yet uncultured bacteria. These results imply that feed components are one of the most important factors affecting the variation in bacterial community structures.

Spirochaetes is the major member of the bacterial community in the gut of wood-feeding termites. The first pure cultures of hindgut Spirochaetes were obtained from the lower termite Zootermopsis angusticollis (Treponema primitia strains ZAS-1 and ZAS-2, and Treponema azotonutricium strain ZAS-9). These strains show physiological features of acetogenesis from H\(_2\) plus CO\(_2\) or nitrogen fixation, but our results showed that Spirochaetes was not predominant in the gut of termites fed with low-molecular-weight single-carbon sources. It is likely that production of H\(_2\) and CO\(_2\) by the fermentation of artificial feeds is not sufficient for the growth of Spirochaetes. As another possibility, Spirochaetes in the gut of N. takasagoensis might play an important role in degrading wood to low-molecular-weight substances.

Bacteroidetes is also one of the most abundant groups of bacteria in the gut community of termites. Many bacteria affiliated with the Bacteroidetes specifically degrade polymers such as plant fibers, polysaccharides, and proteins. The remarkable dominance of Bacteroidetes members in the single-carbon-source libraries suggests that many of them utilize xylan, xylose, cellobiose, and glucose.

Firmicutes-related bacteria are the majority in the first proctodeal segment (P1), which has high alkalinity (pH 10–12), in the gut of higher termites. It has been found that clones NT-1 and NT-2, affiliated with the order Clostridiales, localize in the mixed segment, which also shows high alkalinity, of N. takasagoensis. The number of phyotypes related to these sequences from the single-carbon-source libraries were smaller than those from the wood and wood powder libraries. This implies that feed components also influence the bacterial community structure in the mixed segment. Many clones (28% of clones in the xylose library) related to Propionispora hippei (represented by Nt-O26, 98% sequence identity) were obtained specifically from the xylose library, suggesting that this species utilizes xylose in the termite gut. P. hippei, which ferments sugars and sugar alcohols to propionic and acetic acids, has recently been isolated from sewage sludge.

The candidate phylum TG1 from termites has been found only in lower termites, which harbor abundant flagellates. Some TG1 members have been found to localize in the cytoplasm of certain flagellates. In this study, however, clones belonging to the TG1 were obtained from the gut of N. takasagoensis, which harbors no protozoa. This is a novel finding, and it implies that the TG1 phylum is distributed widely in the gut of various termites, not only lower termites but also higher ones.

The results of this study indicate that the feed components markedly influenced the bacterial community structures in the termites. Each phylogenetic group may have its own specific role in the metabolic pathway of wood digestion, so that changes in the bacterial community indicate rapid functional adaptation to various components of feeds. Moreover, this finding suggests that the symbiotic relationship between termites and gut microorganisms is not very strong or stable, and that it can easily be changed by environmental factors such as feed components. Further studies are required to clarify the metabolic function and the transition of bacterial
community structures in each gut segment and to identify in situ individual populations.

Acknowledgments

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