Intra- and Interspecific Comparisons of Bacterial Diversity and Community Structure Support Coevolution of Gut Microbiota and Termite Host†

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Received 17 December 2004/Accepted 1 June 2005

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† Supplemental material for this article may be found at http://aem.asm.org/.

We investigated the bacterial gut microbiota from 32 colonies of wood-feeding termites, comprising four Microcerotermes species (Termitidae) and four Reticulitermes species (Rhinotermitidae), using terminal restriction fragment length polymorphism analysis and clonal analysis of 16S rRNA. The obtained molecular community profiles were compared statistically between individuals, colonies, locations, and species of termites. Both analyses revealed that the bacterial community structure was remarkably similar within each termite genus, with small but significant differences between sampling sites and/or termite species. In contrast, considerable differences were found between the two termite genera. Only one bacterial phylotype (defined with 97% sequence identity) was shared between the two termite genera, while 18% and 50% of the phylotypes were shared between two congenic species in the genera Microcerotermes and Reticulitermes, respectively. Nevertheless, a phylogenetic analysis of 228 phylotypes from Microcerotermes spp. and 367 phylotypes from Reticulitermes spp. with other termite gut clones available in public databases demonstrated the monophyly of many phylotypes from distantly related termites. The monophyletic “termite clusters” comprised of phylotypes from more than one termite species were distributed among 15 bacterial phyla, including the novel candidate phyla TG2 and TG3. These termite clusters accounted for 95% of the 960 clones analyzed in this study. Moreover, the clusters in 12 phyla comprised phylotypes from more than one termite (sub)family, accounting for 75% of the analyzed clones. Our results suggest that the majority of gut bacteria are not allochthonous but are specific symbionts that have coevolved with termites and that their community structure is basically consistent within a genus of termites.

Termites harbor an abundance and diversity of gut bacteria, which are thought to play essential roles in the carbon and nitrogen metabolism of their host termites. While these characteristics of gut bacteria have been extensively studied by tracing the flow of carbon and nitrogen or characterizing isolated strains of bacteria (5, 25), the bacterial microbiota has remained a black box due to difficulties in cultivation of most of these bacteria. This has been an obstacle to a comprehensive understanding of symbiosis between gut bacteria and their host termites. Recently, we conducted a detailed census of the bacterial community in the gut of the termite Reticulitermes speratus by analyzing clones of 16S rRNA (18, 19). We found 314 phylotypes (defined with 97.0% sequence identity) of 16S rRNA from 1,923 analyzed clones. The majority of the clones were affiliated with groups of anaerobic bacteria such as the genus Treponema and the orders Clostridiales and Bacteroidales, and most of the phylotypes were found for the first time. Many of them constituted novel lineages in several bacterial phyla, including the candidate phylum termite group I (TG1), which was one of the dominant groups in R. speratus (18, 34). For the African soil-feeding termite Cubitermes orthognathus, a culture-independent analysis of gut bacteria was also conducted. It was revealed that distinct bacteria inhabited each of the distinct gut compartments, and most of the bacteria were considered novel species (42). Although the novelty of these bacterial lineages prompts us to assume that they are not allochthonous but rather autochthonous symbionts that are specifically associated with termites, only a few studies have examined the phylogenetic relationship of gut bacteria among different termite samples. Besides, since these studies focused only on the genus Treponema (27, 33) and the order Bacteroidales (35), they were insufficient to evaluate the overall relationship of the diverse termite gut bacteria, which are affiliated with more than 10 phyla (18). In addition, little is known about the variation of bacterial gut microbiota between individuals, colonies, locations, and species of termites.
For this study, we collected 32 termite colonies from various locations to investigate and compare their bacterial gut microbiota, using a combination of terminal restriction fragment length polymorphism (T-RFLP) analysis and clonal analysis of 16S rRNA. We used four *Reticulitermes* species as representatives of the lower termites, which have a simply structured gut and harbor gut protistan symbionts, and four *Microcerotermes* species as representatives of the higher termites, which have a highly compartmentalized gut and lack protistan symbionts. The phylogenetic relationship of gut bacteria and the variation of their diversity and community structure within and between host termite species are discussed.

### MATERIALS AND METHODS

**Collection of termites.** We collected 25 colonies comprising four species of the wood-feeding higher termites belonging to the genus *Microcerotermes* (order Isoptera, family Termitidae, subfamily Termitinae) and 7 colonies comprising four species of the lower termites belonging to the genus *Reticulitermes* (family Rhinotermitidae). The locations of the sampling sites and the abbreviations of the samples used in this study are shown in Fig. 1. The termites were identified based on their morphology and the DNA sequence of mitochondrial cytochrome oxidase II. Detailed information on the classification of the genus *Microcerotermes*, including two undescribed species, *Microcerotermes* species M1 and M2, has been described elsewhere, using the same colony sample names (22). Another undescribed species, a *Reticulitermes* sp., from Nan province in Thailand, is designated species R1 in this study. The termites with their nest were carefully transported to our laboratories in Thailand or Japan without heating or sunlight exposure.

**DNA extraction and PCR amplification.** The collected termites were immediately subjected to DNA extraction, except for *Reticulitermes* samples collected in Tanzawa and Amami Island in Japan, which were kept in the laboratory with their nest log for 7 months before being processed. Whole guts were isolated from 20 randomly chosen workers per colony by using sterilized forceps. DNAs were extracted from the gut homogenates using an Isoplant II kit (Nippon Gene), which chemically lyzes bacterial cell walls and membranes with benzyl chloride. The extracts were further purified using a DNeasy tissue kit (QIAGEN) as described previously (48). To test for differences in gut bacterial community structure between individual termites, DNAs were also extracted from the guts of five randomly chosen workers from colony M1NP1, and the samples were named M1NP1a to e. The DNA sample from *R. speratus* collected at Ogose in Japan (RsOg, Fig. 1) was prepared for our previous study using a QIAGEN RNA/DNA kit (18).

PCR was performed with the *Bacteria*-specific primer pair 27F and 1389R (18) to amplify the near-full-length 16S rRNA gene. For T-RFLP analysis, the 27F primer was labeled with a 6-carboxyfluorescein (FAM) dye. To minimize PCR bias (38), the PCR mixture contained 1.0 μM of the primer set, 0.025 U/μl of ExTaq polymerase (Takara), its attached buffer, a deoxynucleoside triphosphate mixture, and the DNA template adjusted to 0.1 ng/μl. The concentration of the DNA template was further adjusted to produce approximately the same amount of final PCR product between samples in order to diminish the effect of PCR bias. The reaction tubes were placed into the preheated (95°C) block of a PTC-200 thermal cycler (MJ Research), and the reaction was run with the following program: an initial 2 min of denaturation at 95°C, 12 or 20 cycles of denaturation (30 s at 95°C), annealing (1 min at 50°C), and extension (4 min at 72°C), and a final 10-min extension at 72°C.

**T-RFLP analysis.** The PCR products (50 μl) were loaded into a 1% agarose gel (Scakem GTG; BMA) and electrophoresed to separate the products. The products at the target size (1.3 to 1.5 kb) were excised from the gel and purified using a MinElute gel extraction kit (QIAGEN). A 5-μl aliquot of the purified sample was digested with the restriction enzyme HhaI or HaeIII (Takara). From this reaction mix, 2 μl was mixed with formamide and a GS-1000ROX internal size standard (Applied Biosystems) and loaded into an ABI377 genetic analyzer. Electrophoresis and data extraction, using the software GeneScan Analysis 3.1.2 (Applied Biosystems), were conducted according to the manufacturer's instructions. The whole procedure was replicated in order to check the reproducibility, using freshly diluted DNA templates for PCRs.

Although peaks were basically identifiable in increments of one base, only a larger peak height was taken into account for all the samples when adjacent peaks were indistinguishable. The peak height was standardized before comparison, basically according to the method of Dunbar et al. (10), using 50 fluorescence units as the threshold of the baseline noise. After the fourth iteration procedure, the standardized data were analyzed with the software BioCLUST v. 1.0 (24), using a dissimilarity index, calculated as follows: $D = 1/2 \times \sum x_i - \bar{x}_i$, where $x_i$ and $\bar{x}_i$ are the ratios (%) of the $k$th peak heights in the samples $i$ and $j$, respectively. When the two samples were identical, $D = 0$, and when they were absolutely different, $D = 100$. (16) dendrograms were constructed based on this dissimilarity index, using the neighbor-joining (NJ) algorithm followed by the tree bisection-reconnection procedure in PAUP* (version 4.0b10; D. Swofford, Sinauer Associates, Sunderland, MA). To test for a differentiation of clusters, $P$ values for a fixation index, $F_{ST}$ (see below), were calculated by permuting samples 10,000 times, using the software ARLEQUIN (version 2.001; S. Schneider, D. Roessli, and L. Excoffier, University of Geneva, Switzerland [http://www.unige.ch/arlequin/]).

**Clone libraries and sequencing.** Nine colony samples (M1NP1, M1NP2, M1PT1, M1PT2, M2PT2, RsTz, RsTu, and RPK) were also subjected to clonal analysis. Four 50-μl reaction mixtures containing DNA templates from the same colony sample were combined after PCR to decrease the effects of PCR.

![FIG. 1. Sampling sites of termites and abbreviations of colony samples used for this study. Four *Reticulitermes* species (*R. speratus*, *R. okinawanus*, *R. amanianus*, and *Reticulitermes* species R1) were collected in Japan (top) or Thailand (bottom). Four *Microcerotermes* species (*M. crassa*, *M. minutus*, and *Microcerotermes* species M1 and M2) were collected in Thailand. One (M1BK, M2BK, MmPP, RsOg, RsTz, Rama, Roki, and RPK), two (M1PT, M2PT, and RsTu), or three (M1NP, M1PL, M2PB, McCM, McMS, and McPP) colonies were collected from each sampling area for one species. These colony samples were named, e.g., M1NP1, M1NP2, and M1NP3. The three species *Reticulitermes* species R1 and *Microcerotermes* species M1 and M2 are undescribed species. Both species M1 (colonies M1PT1 and M1PT4) and species M2 (colonies M2PT2 and M2PT3) were collected at the same orchard in Pathum Thani, and colonies M1BK1 and M2BK2 were collected at the same university campus in Bangkok. In *M. minutus* (colony MmPP5) and *M. crassa* (colonies McPP2 to 4) were collected at Phu Phan in Sakon Nakhon province, but from distinct forests.](image-url)
The 16S rRNA sequences generated in this study have been deposited with DDBJ under accession numbers AB191790 to AB192133 for bacterial sequences from Microcerotermes spp., AB192134 to AB192291 for those from Reticulitermes spp., and AB193191 to AB193243 for mitochondrial cytochrome oxidase II gene sequences of termites.

RESULTS

T-RFLP analysis. T-RFLP analysis of the bacterial gut microbiota was performed for the 32 termite colonies and five individual termites. Representative electropherograms are shown in Fig. 2. The T-RFLP patterns were highly concordant with the prediction from the analysis of each corresponding clone library, and the taxonomic origins of approximately 60 and 70% of the detected peaks, including almost all of the prominent peaks, in the HhaI and HaeIII restriction profiles, were assignable (see Fig. S1 in the supplemental material). As predicted by the clonal analysis, Hhal digestion produced only one T-RF peak from the Treponema clones and also one or a few peaks from other dominant groups, while HaeIII digestion generated many T-RF peaks from clones of Treponema and other dominant groups.

Both the Hhal and HaeIII profiles exhibited surprisingly high similarity within each termite genus, irrespective of the individual, colony, sampling site, and termite species. In the HaeIII analyses, small but significant differences were also further demonstrated among colony samples within each termite genus. Figure 3 shows the relationship of the HaeIII profiles based on the dissimilarity index (D). The profiles robustly clustered for each termite genus; the average dissimilarity indices were 22.4 ± 6.2, 24.4 ± 5.9, and 81.0 ± 2.7, within genus Microcerotermes, within genus Reticulitermes, and between the two genera, respectively. This differentiation was statistically supported (Fp < 0.0001). Within each termite genus, the profiles were further clustered by termite species and/or sampling site. In the genus Reticulitermes, four profiles from R. speratus were clustered and differentiated from the other Reticulitermes samples (Fp = 0.3200; P = 0.0289;
In the genus Microcerotermes, all of the profiles for Microcerotermes crassus clustered together, except for that of colony McCM2. The differentiation of the M. crassus profiles from those for Microcerotermes species M1 and M2, respectively, was statistically supported (for $H_0$ of $M. crassus = M1$, $F_{ST} = 0.3012$ and $P < 0.0001$; for $H_0$ of $M. crassus = M2$, $F_{ST} = 0.2801$ and $P = 0.0002$). However, the profiles for species M1 and M2 intermingled and formed clusters by sampling site rather than termite species. The four colony samples M1PT3, M1PT4, M2PT1, and M2PT2 from the same sampling site, Pathum Thani, clustered together, and the two colony samples M1BK2 and M2BK1 from Bangkok also showed a close relationship. This was consistent with the acceptance of the null hypothesis that profiles for species M1 are not significantly different from those for species M2 ($F_{ST} = 0.01268; P = 0.2859$). The other Microcerotermes samples from the same sampling areas also tended to cluster together, as shown by the circles in Fig. 3. For colony M1NP1, a similarity in the gut bacterial communities of the individual termites within a single colony was also demonstrated (Fig. 3).

**Estimation of gut bacterial diversity.** For the genus Microcerotermes, 228 bacterial phylotypes were found from 576 sequenced clones. For the genus Reticulitermes, 144 phylotypes were found from 384 clones analyzed, including 53 novel phylotypes not found among the 314 phylotypes obtained for R. speratus in our previous studies (18, 19). The rarefaction analysis of the 10 clone libraries (M1NP1, M1NP2, M1PL1, M1PT4, M2PT2, and M2PB4 from genus Microcerotermes and RsOg, RsTz, RsTu1, and RPK from genus Reticulitermes) showed similar curves within each termite genus and suggested that our sequencing effort was not enough to represent the diversity of bacteria in the gut (data not shown). Therefore, the number of phylotypes was estimated using both parametric and nonparametric analyses (Table 1). The data from the clone libraries that were not significantly different (see below) were combined and used for estimation, in order to improve the reliability of the estimations, by increasing the number of sampled clones. Thus, we estimated the bacterial diversity in each termite species from one or more colonies. To calculate Curtis’s parametric estimator, the number of bacterial cells per gut of Microcerotermes species M1 ($6.24 \times 10^6 \pm 2.40 \times 10^6$) or Reticulitermes species R1 ($1.24 \times 10^6 \pm 0.22 \times 10^6$) was used as the total number of individuals ($N_F$) in each bacterial community for the Microcerotermes and Reticulitermes samples, respectively. The most abundant phylotypes in the sequenced clones were Treponema phylotypes M2PT2-56 (8.3%), M2PT2-87 (8.3%), Rs-G65 (8.0%), and Rs-A48 (10.4%), for termite species M1, M2, R. speratus, and R1, respectively. These values were used as the reciprocal of the ratio $N_F/N_{max}$, where $N_{max}$ is the number of individuals in the most abundant species (8). The estimated numbers were approximately five to six times larger by Curtis’s parametric method than by the Chao1 method, except for R. speratus, for which the difference was relatively small (Table 1). Since these methods estimate the maximum and minimum possible diversity, respectively (4, 7, 8), the diversity of gut bacteria is thought to be within this range.

**Phylogenetic affiliation and abundance of gut bacteria.** Most of the 16S rRNA phylotypes, 227 of 228 from genus Microcerotermes and 362 of 367 from genus Reticulitermes, showed <97.0% sequence similarity to any clones from other environments and any cultured strains in the public databases. Since only one phylotype, Rs-A44, was shared between these termite genera, it is implied that, in total, 99% of the 594 phylotypes represent novel and as yet uncultured bacteria. These phylotypes were affiliated with 17 phyla (15 from genus Microcerotermes and 15 from genus Reticulitermes), including the novel
candidate phyla TG2 and TG3, according to the phylogenetic analysis (Fig. 4). In both Microcerotermes and Reticulitermes termites, phylotypes affiliated with the genus Treponema (phylum Spirochaetes) were predominant, and those affiliated with the orders Clostridiales (phylum Firmicutes) and Bacteroidales (phylum Bacteroidetes) were also dominant in the analyzed clones (Fig. 5). The predominance of the genus Treponema in the guts of these termites was supported by the result that 50.8%/H11006 2.8% and 40.6%/H11006 3.2% of bacterial cells were spirochete-like in the genera Microcerotermes and Reticulitermes, respectively, upon microscopic observation. Conversely, clones affiliated with the phylum Fibrobacteres and the novel candidate phylum TG3 were dominant specifically in the genus Microcerotermes, and TG1 clones were dominant specifically in the genus Reticulitermes.

The novel candidate phylum TG3 was comprised of six phylotypes from Microcerotermes species M1 and M2 and clustered with five uncultured clones from other environments (Fig. 6). This phylum can be divided into subphyla I and II. Subphylum I includes three phylotypes that were dominantly and consistently found in Microcerotermes colony samples and related to three short sequences from other environments. These three phylotypes, M1PT4-09, M1PT4-86, and M1PT4-23, accounted for approximately 6% of the sequenced clones from Microcerotermes spp. The other three phylotypes from Microcerotermes spp. in subphylum II were rarely found and formed a monophyletic cluster with clone R76-150B, amplified from the vent worm Riftia pachyptila (28). The sequence similarity between the termite gut clones and R76-150B was only 80.2 to 82.6%, and the sequence similarity within this phylum was 77.1 to 96.5%. Another novel candidate phylum, TG2, was rarely found in R. speratus and Microcerotermes species M2 (Fig. 6). Five phylotypes from termite guts were clustered with five uncultured clones from other environments. The sequence similarity within this phylum was 80.1 to 96.4% and to any other sequences was <78%.

**Specificity and monophyly of gut bacteria.** Within each termite genus, some phylotypes were shared among distinct colony samples and species of termites. For the genus Microcerotermes, 68 of 228 phylotypes (29.8%) were shared by more than one termite colony, and 42 of 228 (18.4%) were shared by species M1 and M2. The latter corresponds to 49.7 and 53.6% of the sequenced clones from species M1 and M2, respectively. For the genus Reticulitermes, 25 of 50 phylotypes (50.0%) from species R1 were shared by R. speratus, corresponding to 72.9% of the sequenced clones from species R1. For R. speratus, 39 of 55 (70.9%) and 40 of 53 (75.5%) phylotypes from colonies RsTz and RsTu1, respectively, had already been found in our previous studies (18, 19). These correspond to 77.0 and 80.2%
of the sequenced clones from colonies RsTz and RsTu1, respectively. These data indicate that a substantial portion of bacterial phylotypes are shared among congeneric termites, while only one phylotype was shared between the two termite genera. Nevertheless, many phylotypes from these termites constituted monophyletic clusters (termite clusters) together with clones from other termites, as shown in Fig. S2 to S9 in the supplemental material. Of the 960 clones analyzed in this study, 95% were contained in the termite clusters constituted by those from more than one termite species. These clusters were distributed among 15 phyla. Moreover, the termite clusters constituted by phylotypes from even more diverse termite species, i.e., more than one termite (sub)family, occupied 75% of the clones analyzed in this study (see Table S1 in the supplemental material). These termite clusters were distributed among 12 phyla (Fig. 4).

Among the termite clusters, the largest was *Treponema* termite cluster I, which was designated previously (21, 27). This cluster contains phylotypes from 14 termite species in five families (2, 27, 32, 33), including 120 and 68 phylotypes from the genera *Microcerotermes* and *Reticulitermes*, respectively. Phylogenetic analysis of this cluster revealed that the phylotypes from congeneric termites tend to form subclusters, although these genus-specific subclusters are paraphyletic and some phylotypes from distinct termite genera together formed subclusters outside the genus-specific subclusters, as shown in Fig. 7.

**Statistical comparison of clone libraries.** As shown in Fig. 5, the taxonomic composition of clones was remarkably similar within each genus of the termites, whereas great differences existed between the two termite genera. Pairwise statistical comparisons using the Libshuff-test, *F*<sub>ST</sub> test, and *P* test were conducted, and the results are published online as Table S2 in the supplemental material; the results of multiple comparisons based on these pairwise comparisons are summarized in Table 2. For multiple comparison, the null hypothesis that the samples were drawn from the same population was rejected when compared within each termite genus, although the *P* values for pairwise comparisons between congeneric termites were generally much higher than those between the two termite genera. For example, the two clone libraries were not significantly different in any of the three statistical tests. These results indicate that (i) there was a considerable difference in clone libraries between *Microcerotermes* and *Reticulitermes* samples; (ii) only samples collected from the same sampling site were not significantly different in any of the three statistical tests. These were the sets M1NP1 and M1NP2; M2PT2, M2PB4, and M1PT4; and RsOg, RsTz, and RsTu1. These results indicate (18, 19) were removed from the phylogeny. The phylum-level clusters, comprising 228 phylotypes from the genus *Microcerotermes* and 367 phylotypes from the genus *Reticulitermes*, are shown in a compressed form, depicted using ARB software. All of the sequences used are shown in the trees for each phylum (Fig. 6; see Fig. 7. **Taxonomic composition of 16S rRNA clones in libraries prepared from the guts of *Microcerotermes* and *Reticulitermes* termites.** See Fig. 1 for the abbreviations of samples.

**FIG. 5.** Taxonomic composition of 16S rRNA clones in libraries prepared from the guts of *Microcerotermes* and *Reticulitermes* termites. See Fig. 1 for the abbreviations of samples.
were not significantly different from each other but were different from the R1 sample (colony RPK). These results were exactly concordant with the results of the T-RFLP analysis (Fig. 3).

**DISCUSSION**

In this study, we aimed to examine whether gut bacteria of termites are allochthonous or autochthonous and also whether they have coevolved with their termite hosts by evaluating the consistency of bacterial gut microbiota with the phylogeny of host termites. Our results clearly demonstrated that congeneric termites harbored very similar bacterial gut microbiota, irrespective of the individual, colony, location, and host species. The similarity in bacterial gut microbiota among congeneric termites was also recently demonstrated with the African soil-feeding termites *Cubitermes* spp. (family Termitidae; subfamily Termitinae), where moderately or considerably high similarity was shown by T-RFLP analysis in a comparison of each gut compartment between three *Cubitermes* species (41). These data suggest that the high similarity of bacterial gut microbiota within a termite genus may be a general trait for termites and that the symbiotic relationship between gut bacteria and their host termites is very stable and strong. Since the majority of gut bacteria from distantly related termites, including the genera *Microcerotermes*, *Reticulitermes*, and *Cubitermes*, constituted

**FIG. 6.** Phylogenetic tree showing the relationships of 16S rRNA phylotypes affiliated with the novel candidate phyla TG2 and TG3. The sequences of related phyla were used as references to demonstrate the monophyly and distinctness of each novel candidate phylum. A maximum likelihood (ML) tree as the framework tree was reconstructed with the Olsen model using ARB software, based on unambiguously aligned regions (1,227 nucleotide sites) of the alignment of near-full-length sequences (≥1,300 bp). The clones connected by dotted lines were short (362 to 841 bp) and were added later to the framework tree by means of the ARB parsimony tool, without changing the overall topology. Novel phylotypes obtained in this study are shown in bold. Termite clusters are shown by thick lines. When a phylotype was found from multiple colony samples, the names of the colony samples are shown in parentheses. When bootstrap values obtained using the PHYML program to construct ML trees under the HKY model are ≥95% or 70 to 94%, they are shown with filled or open circles, respectively. The “Rs-” clones were obtained from *Reticulitermes speratus* collected at Ogose in our previous studies (18, 19). See Fig. 1 for the abbreviations of samples.
FIG. 7. Phylogenetic tree showing the relationships of 16S rRNA phylotypes affiliated with Treponema termite cluster 1 obtained from three termite genera, i.e., Microcerotermes, Reticulitermes, and Neotermes. A maximum likelihood (ML) tree was reconstructed based on 1,288 unambiguously aligned nucleotides (positions 64 to 1388 in E. coli) of 111 sequences, comprising 49 phylotypes from Microcerotermes species M1 and M2, 38 from Reticulitermes speratus (Rs) (18) and Reticulitermes species R1 (RPK), 18 from Neotermes koshunensis (Nks) (31), and the outgroup sequences Spirochaeta halophila (M34262), Spirochaeta asiatica (X93926), Spirochaeta marmaduqueae (U05897), Treponema pallidum (M88726), Treponema bryantii (M57737), and Treponema brennaborense (Y16568). Only phylotypes representing more than one clone were used. Novel phylotypes obtained in this study are shown in bold. The tree was inferred with the HKY + G (gamma distribution) model, using PHYML v2.4.4. The parameters for each of 100 bootstrap resamplings. Bootstrap values of >75% (filled circles) and 50 to 74% (open circles) are shown. The monophyletic clusters consisting of phylotypes from one termite genus were compressed. Clusters containing phylotypes from different termite genera are shown with brackets. The full tree is published online as Fig. S10 in the supplemental material.

TABLE 2. P values for multiple comparisons of 16S rRNA clone libraries prepared from the guts of Microcerotermes and Reticulitermes termites

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a If P < 0.05 (shown in bold), there was at least one pair of significantly different clone libraries within each group.

b M, Microcerotermes spp.; M1, Microcerotermes species M1; M2, Microcerotermes species M2; R, Reticulitermes spp.; Rs, R. speratus.

c Number of compared libraries.

the extent of cospeciation of gut bacteria in termites here due to the lack of ample sequence data from various termite species, it appears that the phylotypes from one termite genus tend to cluster together, as shown in Treponema termite cluster I (Fig. 7). Nevertheless, it also seems obvious that the cospeciation is not as strict as that of intracellular symbionts such as Buchnera aphidicola in aphids (13) considering that the complex phylogeny of gut bacteria is not readily comparable to the phylogeny of the host termites (see Fig. S2 to S9 in the supplemental material), as exemplified by the paralogy of the genus-specific subclusters in Treponema termite cluster I (Fig. 7).

Although termite gut bacteria are considered to be transmitted vertically from generation to generation basically via protodel trophallaxis, as known for the gut symbiotic protists in lower termites (23), the horizontal transfer of gut bacteria among at least congeneric termites is also inferred from the clustering pattern of the bacterial community profiles from Microcerotermes species M1 and M2, which were clustered by sampling site rather than by host termite species. Considering that species M1 and M2 are sympatric in some localities, it is conceivable for the transmission of gut bacteria to occur between these congeners in the same niche via feces excreted into the immediate environment. However, there are other possible factors affecting the variation in bacterial gut microbiota within congeneric termites, such as ambient temperature, food quality, and humidity. In any case, this clustering pattern suggests a great impact of environmental factors comparable to the host species barrier within a termite genus. Unfortunately, it was impossible to confirm this trait for other pairs of congeneric termites, because other species inhabit different localities. Therefore, the significant differentiation in bacterial community profiles for M. crassus and R. speratus from those of other congeneric termites might also be due to the difference in sampling sites.

The difference in gut bacterial lineages between the genera Microcerotermes and Reticulitermes may be partly related to the difference in gut structure and the presence or absence of gut protists. For example, seven phylotypes from Microcerotermes species M1 and M2 were affiliated with a termite P1 cluster in the family Eubacteriaceae found in our recent study (47), while no phylotypes from lower termites were affiliated with this
FIG. 8. Phylogenetic tree showing the relationships of 16S rRNA phylotypes affiliated with the family Eubacteriaceae in the phylum Firmicutes. A maximum likelihood tree with bootstrap values was constructed as described in the legend to Fig. 6, using 1,232 unambiguously aligned nucleotides (positions 64 to 1388 in E. coli). A subset of the sequences shown in Fig. S5 in the supplemental material were used. Novel phylotypes obtained in this study are shown in bold. M1- and M2- clones were obtained from Microcorterines species M1 and M2, respectively. Several phylotypes from species M1 and M2 were clustered with clones obtained from the highly alkaline gut regions of higher termites. NT-1 and NT-2 were obtained from the mixed gut segment of Nasutitermes takasagoensis (49). Clones M-P1-, Pl-P1-, Tc-P1-, and S-P1- were obtained from the P1 gut segment of Microcorterines sp., Pericapritermes latagnathus, Termes comis, and Speculitermes sp., respectively (47). Bootstrap values of >95% (filled circles) and 70 to 94% (open circles) are shown. The outgroup sequences were Acidaminobacter hydrogenoformans (L04168), Clostridium purinolyticum (M60491), and Peptostreptococcus anaerobius (L04168).

cluster (Fig. 8). This cluster comprises phylotypes from the highly alkaline gut compartments (pH 10 to 12), called the mixed segment and the P1 section, both of which are unique to higher termites (3, 6). Since several “P1 clusters” like this have been found in higher termites (42, 47) and since it was revealed that distinct gut compartments harbor distinct bacterial communities (42), some part of the gut bacteria may have differentiated as symbionts specifically associated with gut structures and physiological conditions unique to higher termites. Conversely, the absence of TG1 clones from Microcorterines spp. may be due to the absence of gut flagellates found only in lower termites, which are thought to be the sole habitats of the termite-associated TG1 bacteria (36, 44).

The coevolution of gut microbiota with the termite host has also been suggested for protistan symbionts in lower termites and some archaeal symbionts. The majority of the protistan gut microbiota is unique to lower termites and wood-feeding cockroaches belonging to the genus Cryptocercus (23). These symbiotic protists are basically species specific to their host termites and are stably harbored, although the coevolutionary process cannot be explained by a purely vertical transmission (23). Some archaeal lineages, e.g., a cluster in the order Methanomicrobiales and clusters in the genus Methanobrevibacter, are also considered unique to termites (9, 12, 50). Combined with the results from the present study, it seems most likely that the majority of gut microbiota, comprising bacteria, archaea, and protists, are autochothonous symbionts that have coevolved with their host termites.

ACKNOWLEDGMENTS

We thank T. Takematsu and O. Kitade for helpful advice on the identification of termites. We also thank C. Dissen, K. Sirihongsuwan, L. Ekponnprasit, C. Boontong, and H. Yuzawa for assisting with experiments. We thank A. Hiraishi for kindly providing the software BioCLUST.

Y. H. is a recipient of a special postdoctoral research fellowship from RIKEN. This work was partially supported by grants for the Bioarchitect Research Program and the Eco Molecular Science Research Program from RIKEN.

REFERENCES


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References:


