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Phylogenetic Diversity of the Intestinal Bacterial Community in the Termite *Reticulitermes speratus*

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The phylogenetic diversity of the intestinal microflora of a lower termite, *Reticulitermes speratus*, was examined by a strategy which does not rely on cultivation of the resident microorganisms. Small-subunit rRNA genes (16S rDNAs) were directly amplified from the mixed-population DNA of the termite gut by the PCR and were clonally isolated. Analysis of partial 16S rDNA sequences showed the existence of well-characterized genera as well as the presence of bacterial species for which no 16S rDNA sequence data are available. Of 55 clones sequenced, 45 were phylogenetically affiliated with four of the major groups of the domain *Bacteria*: the *Proteobacteria*, the spirochete group, the *Bacteroides* group, and the low-G+C-content gram-positive bacteria. Within the *Proteobacteria*, the 16S rDNA clones showed a close relationship to those of cultivated species of enteric bacteria and sulfate-reducing bacteria, while the 16S rDNA clones in the remaining three groups showed only distant relationships to those of known organisms in these groups. Of the remaining 10 clones, among which 8 clones formed a cluster, there was only very low sequence similarity to known 16S rRNA sequences. None of these clones were affiliated with any of the major groups within the domain *Bacteria*. The 16S rDNA gene sequence data show that the majority of the intestinal microflora of *R. speratus* consists of new, uncultured species previously unknown to microbiologists.

One of the most fascinating examples of symbiosis is that displayed between the xylophagous termites and their intestinal microbiota, which includes both protist and prokaryotic communities (8). The mutualistic relationship between them enables termites to live by xylophagy. Several possible beneficial roles of the microbiota in the termite gut ecosystem have been discussed: carbon and energy nutrition derived from lignocellulose digestion (10), methanogenesis and acetogenesis from H_2 and CO_2 (7), nitrogen fixation (4, 9), recycling of uric acid nitrogen (35), and maintenance of a low redox potential and prevention of entry of foreign bacteria (43). Despite the isolation and cultivation of several bacteria and protists from within this community (15, 31, 40, 47, 48), our understanding of the biology and the physiology of intestinal microbiota is poor because many of the predominant species within the community, such as the spirochete-like bacteria and flagellated protists, have proven difficult or even impossible to culture in laboratories. They have been characterized only on the basis of their morphology (6, 24).

The application of molecular phylogenetic analysis to ecological studies has enhanced our ability to assess naturally occurring biodiversity in mixed microbial assemblages (reviewed in reference 2). In this approach, genes encoding small-subunit rRNA (16S-like rRNA) derived from the extracted nucleic acids of mixed microbial populations are clonally isolated, sorted, and sequenced. These sequences can then be compared with each other as well as with databases of rRNA sequences from well-characterized microorganisms to determine the identities and ecological roles of uncultured organisms present in natural microbial communities. Biodiversity studies based on rRNA sequences cloned directly from natural biomass have been performed with microbial communities from marine (12, 14, 18–20, 39), hot spring (3, 36, 44), soil (27), and other communities. These studies have shown that natural ecosystems in general often include species which are unknown to microbiologists because of our inability to successfully culture all the members of the community in the laboratory.

Here, we report the phylogenetic diversity of the intestinal bacterial community of the lower termite *Reticulitermes speratus* as determined by a comparison of partial sequences of cloned 16S rRNA genes (16S rDNAs) directly amplified from extracted DNA by the PCR.

MATERIALS AND METHODS

Collection and culture of termites. Wood-eating termites [*R. speratus* (Isoptera: Rhinotermitidae)] were collected in the vicinity of Ogose, Saitama Prefecture, Japan, in July 1994. Termite-infested wood moistened with distilled water was kept in plastic boxes at 25°C for rearing. Termites were reared in disposable polystyrene petri dishes (6 cm in diameter; Falcon catalog no. 1007) packed with an autoclaved mixture containing 3 g of dry pine chips, 3 g of cellulose powder (Nakarai), and 9 ml of distilled water (45). Approximately 100 termites collected from the infested wood were reared in each petri dish, and after 3 to 4 weeks, pseudergates (worker-like larvae) were removed from the petri dishes for DNA extraction.

DNA extraction. Approximately 300 termites were collected, and, after their exterior surfaces were washed with distilled water, their entire guts were removed with forceps. The intestinal contents were gently squeezed into a 0.4% NaCl solution, divided among 10 microcentrifuge tubes, harvested by centrifugation, washed with the same solution, and then resuspended in 0.4 ml of buffer (10 mM Tris-HCl [pH 7.5], 50 mM EDTA) in the presence of lysozyme (5 mg/ml). After incubation at 37°C for 20 min, proteinase K was added at 2 mg/ml, and the mixture was incubated for a further 40 min. At the end of incubation, sodium dodecyl sulfate was added at 1% (wt/vol), and the solution was mixed gently by inversion. Nucleic acids were released by three cycles of freezing in a liquid nitrogen bath followed by thawing in a 65°C water bath. The mixture was then extracted with an equal volume of phenol (saturated with 10 mM Tris-HCl, pH 8.0) and then with phenol-chloroform-isoamyl alcohol (24:24:1 [vol/vol/vol]). Bulk nucleic acids were precipitated from solution with isopropyl alcohol and by centrifugation, rinsed with 70% ethanol, and resuspended in 50 μl of TE (10 mM Tris-HCl [pH 7.6], 1 mM EDTA). Lithium chloride was added at a final concentration of 2.5 M, and the suspension was mixed, incubated on ice for 30 min, and centrifuged. Nucleic acids were precipitated from the supernatant with ethanol and resuspended in TE in the presence of 50 μg of RNase A per ml. After incubation at 37°C for 30 min, high-molecular-weight DNA was isolated by electrophoretic size fractionation on 1% low-melting-point agarose (SeaPlaque GTG; FMC) by the standard protocol (38).

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PCR amplification and cloning. rDNAs were amplified from the purified DNA by PCR with *Taq* DNA polymerase (Takara) according to the manufacturer's directions. The PCR primers contained 5′ restriction site linkers and corresponded to nucleotide positions 519 to 533 of *Escherichia coli* 16S rRNA for the forward primer (519FB; 5′-ATTGGATCCCAGCMGCCGCGGTAA-3′) and to *E. coli* 16S rRNA positions 1392 to 1405 for the reverse primer (1392RS; 5′-TGAGTCGACACGGGCGGTGTGTRC-3′), where R represents A or G and M represents A or C, with restriction site (underlined)-containing linkers at the 5′ ends. These primers were based on the two most widely spaced of the three universal primers for direct small-subunit rRNA sequencing (26). The reaction conditions were as follows: 30 cycles at 94°C for 30 s, 45°C for 45 s, and 72°C for 2 min. PCR products corresponding to the expected size of the bacterial rDNA (0.9 kb) were purified on an agarose gel, digested with *Bam*HI and *Sal*I, and cloned into pUC119. The isolated clones were designated UN1 through UN119.

Nucleotide sequencing and phylogenetic analysis. Single-stranded clonal DNAs were prepared from randomly picked recombinants and used as templates in sequencing with the universal M13 primer contained in a PRISM Ready Reaction Dye Primer UniCycle Sequencing Kit (Applied Biosystems). Sequencing reactions were performed with an automatic sequence analyzer (Applied Biosystems model 373). The previously determined rRNA sequences used for comparisons in this study were retrieved from the GenBank, EMBL, and DDBJ nucleotide sequence databases. Sequences were checked by the CHECK_CHIMERA program of the Ribosomal Database Project (29) to detect the presence of possible chimeric artifacts. Sequence data were aligned by using the CLUSTAL V package (21) and then corrected by manual inspection, and nucleotide positions of ambiguous alignment were omitted from subsequent phylogenetic analyses. Phylogenetic trees were constructed by the neighbor-joining method (37) with the PHYLIP package (version 3.5c, from J. Felsenstein, University of Washington). Bootstrap analyses for 100 replicates (16) were performed to provide confidence estimates for tree topologies.

Nucleotide sequence accession numbers. The sequence data determined in this study will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers D63587 to D63641.

RESULTS

Lower termites of the species *R. speratus* were reared for approximately 1 month on a sterile diet. DNA was directly extracted from the gut contents, and 16S rDNAs were amplified from universal primers (519FB and 1392RS). Although these primers sample only the 3′-proximal two-thirds of the 16S rDNA, they allow unequivocal detection of rDNAs for any type of organism (26). PCR products were electrophoresed, and bands of the expected size range for prokaryotic 16S rDNA (ca. 0.9 kb) were recovered from the gel and cloned into *E. coli*. For ease of cloning, *Bam*HI and *Sal*I restriction sites, which occur rarely in 16S rDNA sequences (27), were introduced at the 5′ and 3′ ends of the amplified products of 16S rDNAs. Although most of the DNA inserts in the clones were the expected full-length amplification products (0.9 kb), some clones contained short fragments of about 0.3 kb that were probably due to internal restriction sites.

Since our primary aims were to characterize the breadth of biodiversity in the termite gut microflora and to estimate the phylogenetic relationships among clones, we designed our analysis to screen relatively short sequences from multiple clones rather than to screen long sequences from a few clones. We sequenced approximately 300 bases of each clonal segment corresponding to *E. coli* positions 534 to 834. The sequence information obtained from this region of the 16S rDNAs has previously been shown to provide the level of resolution necessary to facilitate our analysis (19). However, the branching orders for many of the phylogenetic trees reported in this study, particularly those deep branches for distantly related taxa, represent only an estimation of phylogenetic similarity.

A total of 55 clones were sequenced and shown to belong to the domain *Bacteria*. On the basis of sequence similarity, these clones were classified into several clusters corresponding to the major divisions of the *Bacteria* (46). A large-scale phylogenetic tree containing representative members of each of the identified clusters, but not all members of each cluster, shows that the isolated clones correspond to many diverse groups of bacteria (Fig. 1). Most of the clones were phylogenetically affiliated with four of the major divisions of the *Bacteria*: the group *Proteobacteria*, the spirochete group, the *Bacteroides* group, and the low-G+C-content gram-positive bacteria. Table 1 shows the numbers and identities of the clones which were assigned to these groups. More-detailed information concerning the phylogenetic relationships of the clones and known bacteria is shown in subsequent figures (Fig. 2; also see Fig. 4 to Fig. 7).

The cluster containing clones UN47 and UN79 is deeply branched but fails to branch within any of the major groups of the *Bacteria* (Fig. 1). We designated this cluster termite group I. Figure 2 shows the phylogenetic relationships of the eight members in this group. The clones in termite group I showed more than 95% nucleotide identity to each other: the sequences of four clones, UN47, UN64, UN75, and UN104, are identical; clone UN81 has only one different nucleotide; and clone UN30 is different from the four identical clones only by the insertion of a single nucleotide. These clones had less than 83% sequence identity to previously sequenced organisms in the database, indicating that the members of termite group I are evolutionarily divergent within the domain *Bacteria*. Since all members of termite group I have short fragment inserts (ca. 0.3 kb) due to the internal *Sal*I site (*E. coli* position 856), it was impossible to determine the full-length sequences for these clones. Figure 3 shows the predicted secondary structure for one of the termite group I clones, UN47. The predicted secondary structure of clone UN47 shares complementarity with that of other bacteria (30), indicating that the sequence encodes a functional rRNA. Figure 3 also shows that sequence variations among the members of termite group I are located primarily in a stem region corresponding to variable region 4 of *E. coli.*

Figure 1 also includes the two clones, UN55 and UN116, that did not cluster with more than two identified sequences and could not be assigned to any of the known major groups of the *Bacteria*. These clones were deeply branched in the tree, indicating that they, like the members of termite group I, are evolutionarily divergent species within the domain *Bacteria*. Of the sequences in the database, clone UN55 showed the highest level of sequence identity (86.5%) to a rumen bacterium, *Synergistes jonesii* (1). When the sequence of *S. jonesii* was added to the phylogenetic analysis shown in the large tree (Fig. 1), UN55 and *S. jonesii* formed a cluster with a 100% bootstrap value for clustering. None of the 16S rDNA sequences within the database had more than 80% identity to UN116. Clone UN116 does form a monophyletic branch with *Fibrobacter succinogenes*, but the bootstrap value for this node was only 57% (Fig. 1).

Eleven clones were assigned to the group *Proteobacteria* (Fig. 4), with most of the members of this group sharing a high degree of similarity to previously sequenced organisms in the databases. Five clones of this group (UN6, UN59, UN93, UN50, and UN40) were clustered and closely related to sulfur and sulfate reducers (designated the termite *Desulfovibrio* cluster). Less than 3% nucleotide divergence separated the sequence of *Desulfovibrio desulfuricans* from this cluster, and this low level of divergence was reflected in the 100% bootstrap value for the node between the members of this cluster and the *Desulfovibrio* species. Five other clones formed clusters belonging to the gamma subdivision of the *Proteobacteria*, which includes the enteric bacteria. This cluster was most closely related to the enteric bacteria; thus, we have designated them the termite enteric cluster. Three clones of this cluster (UN34, UN92, and UN106) were closely related to *Citrobacter freundii*, while the sequences of another two clones (UN110 and

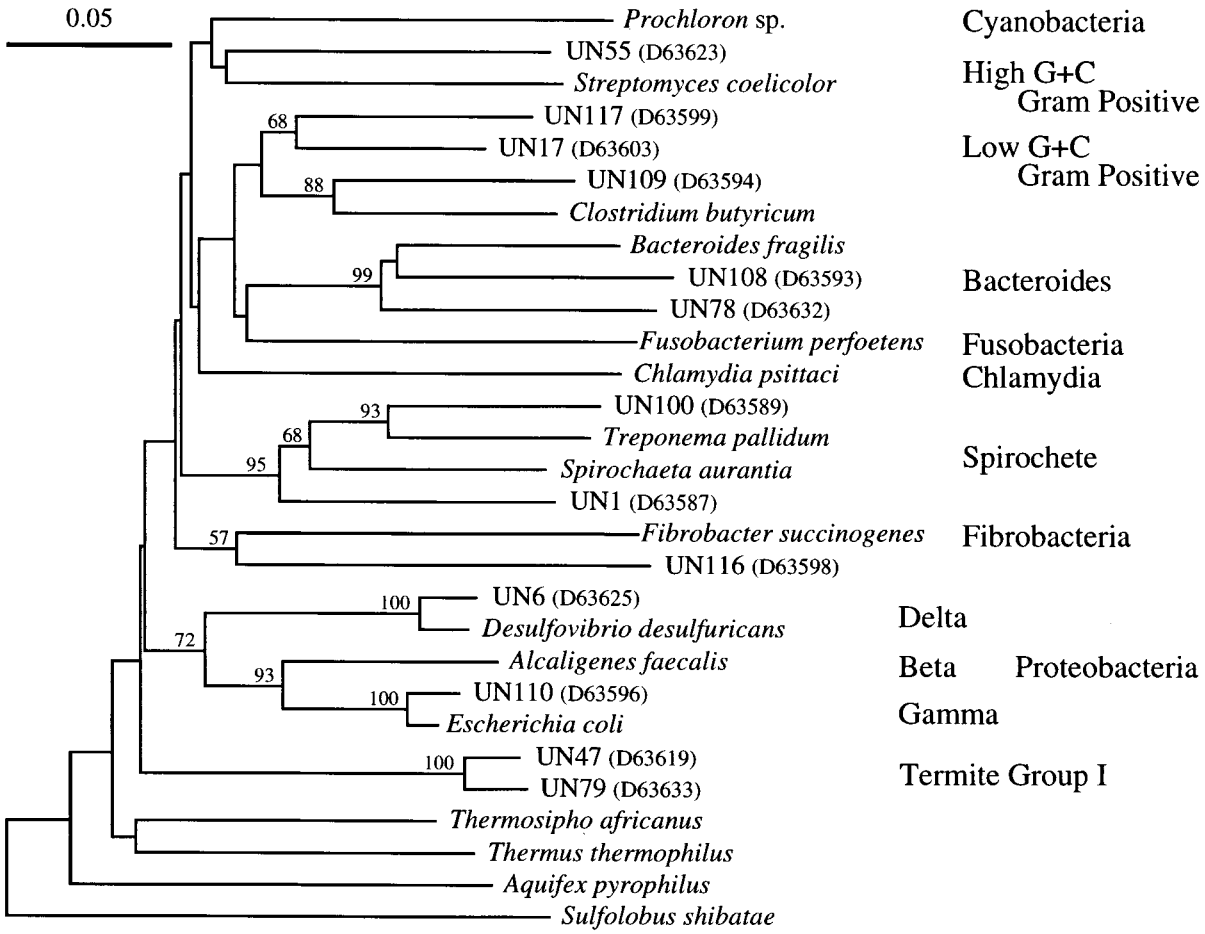

FIG. 1. Large phylogenetic tree including members of all groups found in the clone library but not including all individual clones (some clones are in subsequent figures). The tree was constructed by the neighbor-joining method on the basis of 271 unambiguously aligned bases corresponding to *E. coli* 16S rRNA positions 534 to 826. The 16S rDNA sequence of *Sulfolobus shibatae* was used as the outgroup. The scale bar represents 0.05 substitutions per nucleotide position. Numbers at nodes indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown). The database accession numbers of the termite bacterial clones are in parentheses.

UN119) differed at only two bases from the sequences of *Plesiomonas shigelloides* and *Enterobacter agglomerans* (now renamed *Rahnella aquatilis*). Clone UN45 did not fall within any clusters containing more than two sequences but clearly branches within the beta subdivision of the *Proteobacteria*. It does not, however, show close similarity to any known species.

Figure 5 shows the phylogenetic relationships of the 10 clones which were assigned to the spirochete group. Although none of the clones of this group showed more than 90% nucleotide identity to the database sequences, the bootstrap value of 95% for the node containing UN100, UN1, *Treponema pallidum*, and *Spirochaeta aurantia* in the large tree (Fig. 1) supports the assignment of these clones to the spirochete group. Among them, the three clones UN39, UN100, and UN10 were almost identical to each other, and clones UN1 and UN28 were almost identical to each other. All 10 termite clones were related to the members of the genus *Treponema*. The members of this group were divided into two clusters, one related to *Treponema pectinovorum* and *Treponema bryantii* (designated termite *Treponema* cluster I) and the other related to *Spirochaeta stenostrepta* (designated termite *Treponema* cluster II). It has been reported that *S. stenostrepta* is phylogenetically more closely related to members of the genus *Treponema* than to other members of the genus *Spirochaeta* (32). The bootstrap values for the node distinguishing each *Treponema* cluster were only 58% for termite *Treponema* cluster I and 74% for termite *Treponema* cluster II.

Figure 6 shows the phylogeny of nine clones which form a cluster and have been assigned to the *Bacteroides-Flexibacter-Cytophaga* group; we designated this cluster the termite *Bacteroides* cluster. This assignment was supported by a bootstrap

TABLE 1. Number of isolated clones in each group

Group or cluster	No. of clones
Proteobacteria	11
Desulfovibrio cluster	5
Beta subdivision	1
Gamma subdivision	5
Spirochetes	10
Treponema cluster I	4
Treponema cluster II	6
Bacteroides	9
Gram-positive bacteria	15
Termite group I	8
Unaffiliated	2
Total	55

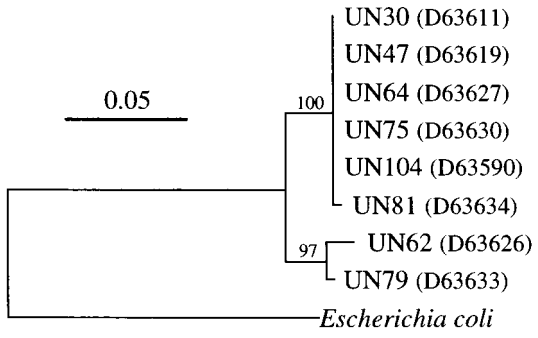

FIG. 2. Phylogenetic tree of the clones in termite group I, with *E. coli* as the outgroup. The tree is based on 326 aligned bases. The scale bar represents 0.05 substitutions per base position. Bootstrap values above 50 from 100 resampled data sets are shown for each node. The vertical line connecting six clones indicates that they are identical clones for the aligned bases (gaps were excluded from analysis). The database accession numbers of the termite bacterial clones are in parentheses.

value of 99% at the node for the cluster containing *Bacteroides fragilis*, UN108, and UN78 in the large tree (Fig. 1). No sequences in the database were found to have more than 87% nucleotide identity to the members of this cluster. Five clones (UN19, UN108, UN26, UN27, and UN5) were almost identical to each other (fewer than two nucleotide differences), as were two other clones (UN77 and UN85). The termite *Bacteroides* cluster may be divided into two subclusters consisting of seven and two clone members. The bootstrap values supporting this division were 97 and 98% for nodes in the two subclusters, respectively.

The last group, containing 15 clones, was the largest and most diverse group and was assigned to the low-G+C-content gram-positive bacteria (Fig. 7). Among them, four clones (UN11, UN117, UN68, and UN16) were somewhat related to each other and showed less than 90% nucleotide identity to other clones and the sequences in the databases. The bootstrap value of 76% at the node tenuously supports the grouping of these four clones. The other 11 clones are clearly related to the genus *Clostridium*, while two sets of clones each contained three almost identical clones. One of the three-clone clusters, containing clones UN17, UN24, and UN38, showed 95% nucleotide identity to *Clostridium celerecrescens* and a bootstrap value of 98%. The other set (UN36, UN84, and UN109) showed 95% nucleotide identity to *Clostridium bifermentans*, 94% identity to *Clostridium sordellii* and *Eubacterium tenue*, and a bootstrap value of 83%. The sequences of the other five clones showed less than 93% nucleotide identity to the sequences of the known organisms in the databases. Clone UN15 and *Clostridium piliforme*, however, appear to be related, as suggested by the bootstrap value of 99% at their node.

Chimeric rDNA clones, composed of rDNAs from different organisms, can arise during PCR amplification of mixed-population DNAs (28). Inspection of the predicted secondary structures of our cloned segments (as shown in Fig. 3), phylogenetic analyses of the 5′- and 3′-half portions of the sequences (25), and evaluation by the CHECK_CHIMERA program of the Ribosomal Database Project (29) indicated that the sequences studied in this paper showed no obvious evidence of chimeric artifacts. Only one clone, UN116, which deeply branched in the large tree (Fig. 1), looked suspicious when analyzed by the CHECK_CHIMERA program because the highest similarity values were obtained from different phylogenetic groups in the 5′-terminal two-thirds and the 3′-terminal one-third of the UN116 sequence. However, since the similarity values obtained are very low (the highest similarity values are 0.431 and 0.455), it is difficult to prove that the UN116 sequence is chimeric, as discussed for novel 16S rDNA sequences which have no close relatives in the databases (25).

DISCUSSION

Phylogenetic analysis of clonally isolated 16S rDNAs shows that the intestinal microflora of termites consists of many diverse microbial species, many of which have not been previously characterized. Surprisingly, approximately two-thirds of the analyzed clones have less than 90% sequence identity to

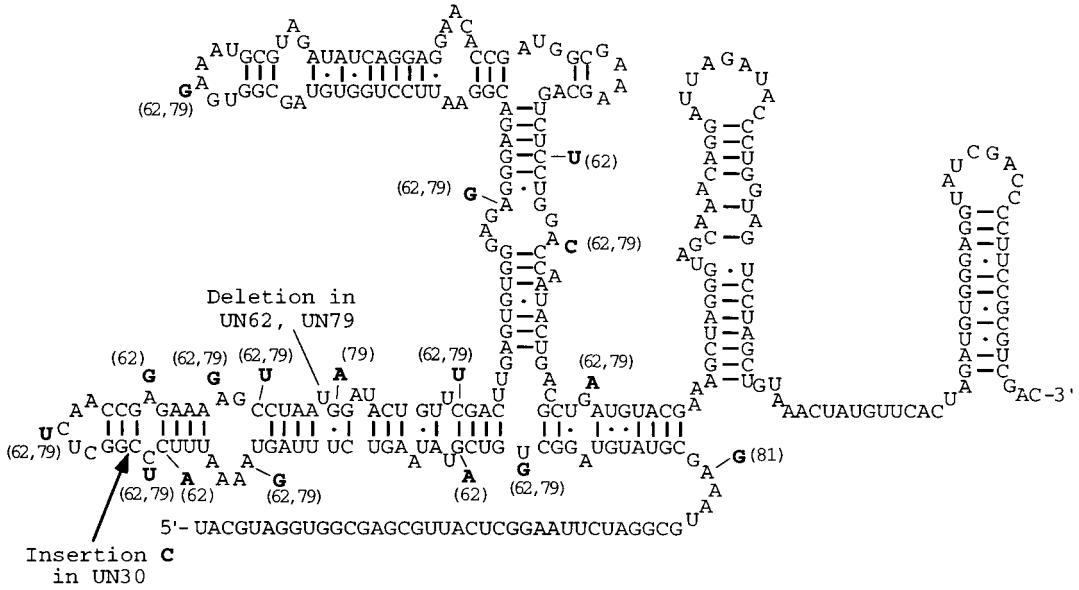

FIG. 3. Secondary structure model of the 16S rRNA of clone UN47, which belongs to termite group I. The corresponding positions in the *E. coli* 16S rRNA are positions 534 to 863. Clones UN64, UN75, and UN104 have sequences identical to those of clone UN47. The variations in clones UN62, UN79, and UN81 are noted in boldface type, with the clone numbers in parentheses.

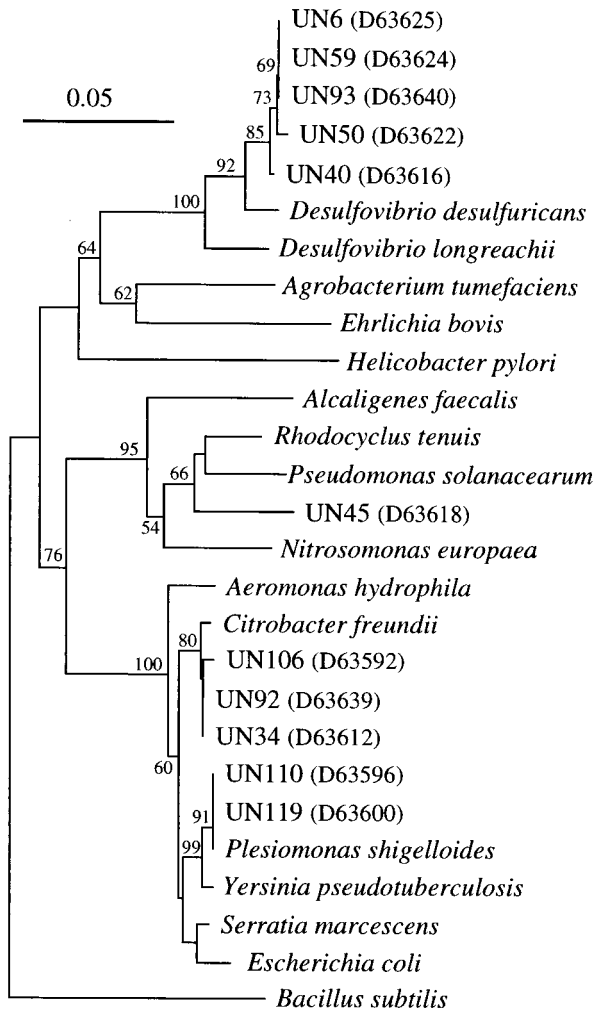

FIG. 4. Phylogenetic tree of the clones in the group *Proteobacteria*, with *B. subtilis* as the outgroup. The tree is based on 261 aligned bases. The scale bar represents 0.05 substitutions per base position. Bootstrap values above 50 from 100 resamplings are shown for each node. The database accession numbers of the termite bacterial clones are in parentheses.

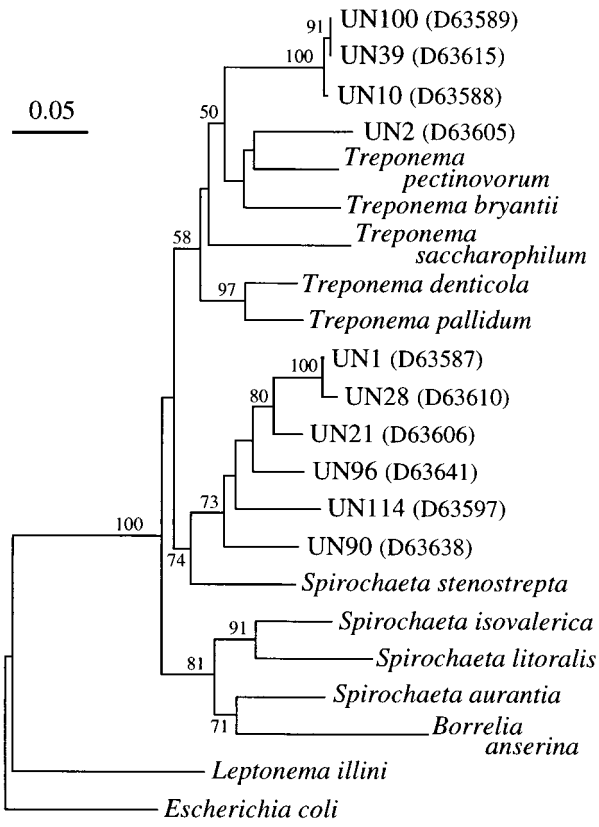

FIG. 5. Phylogenetic tree of the clones in the spirochete group, with *E. coli* as the outgroup. The tree is based on 277 aligned bases. The scale bar represents 0.05 substitutions per base position. Bootstrap values above 50 from 100 resamplings are shown for each node. The database accession numbers of the termite bacterial clones are in parentheses.

the known 16S rDNA sequences of cultivated organisms. More significantly, 10 clones bear no close sequence similarity to any recognized bacterial phylum in the rRNA database. Among them, the presence of eight clonal isolates belonging to termite group I suggests that these unaffiliated microbes constitute a significant quantity of the organisms in termite intestines. The phylogeny of isolated clones indicates that the intestinal bacterial microflora of *R. speratus* termites consists of species related to the enteric bacteria and the genera *Desulfovibrio*, *Treponema*, *Bacteroides*, and *Clostridium*. All of the known species among these groups are either strict or facultative anaerobes and have been frequently isolated from animal intestines. Since the termite gut, especially the hindgut, contains a high concentration of microbes, is anoxic, and has a low redox potential, the presence of strict and facultatively anaerobic organisms in the termite gut is a reasonable expectation. In fact, several different bacterial species belonging to the genera *Enterobacter*, *Citrobacter*, *Desulfovibrio*, *Bacteroides*, and *Clostridium* have been isolated from the guts of many termite species (15, 17, 22, 33, 34, 40, 42). Even with strict anaerobic isolation techniques, however, the level of recovery of bacteria via culture from termite guts was only 13% (40). To our knowledge, this is the first report of a comprehensive phylogenetic analysis of the intestinal bacterial diversity of a termite species in which formerly uncultivated bacteria have been found.

N_2 fixation mediated by gut bacteria is one of the crucial aspects of termite symbiosis, since termites feed on nitrogen-poor wood. Nitrogen-fixing bacteria isolated from several kinds of termites have been identified as *Citrobacter freundii* and *Enterobacter agglomerans* (17, 33). As the members of the termite gamma proteobacterial cluster within our clone library are closely related to the genera *Citrobacter* and *Enterobacter*, these clones may play a role in N_2 fixation. Since several *Clostridium* and *Desulfovibrio* species are also known to be N_2-fixing bacteria, the organisms in our clone library which cluster with them also represent possible N_2-fixing candidates in the termite hindgut.

The ability to reduce CO_2 to acetate also plays a crucial role in microbial fermentation in termite guts (7). A few CO_2-reducing acetogens have been isolated from termite guts in pure culture and identified as species of *Sporomusa*, *Acetonema*, and *Clostridium* (11, 22, 23). None of the clones in our library show high similarity to either the *Sporomusa* or *Acetonema* species (less than 83% identity), but a number of *Clostridium*-related clones were identified, indicating that the role of CO_2-reducing acetogen may be fulfilled by these clones.

Since most of the *Bacteroides* species are known to be fermentative and acidogenic, the organisms assigned to the ter-

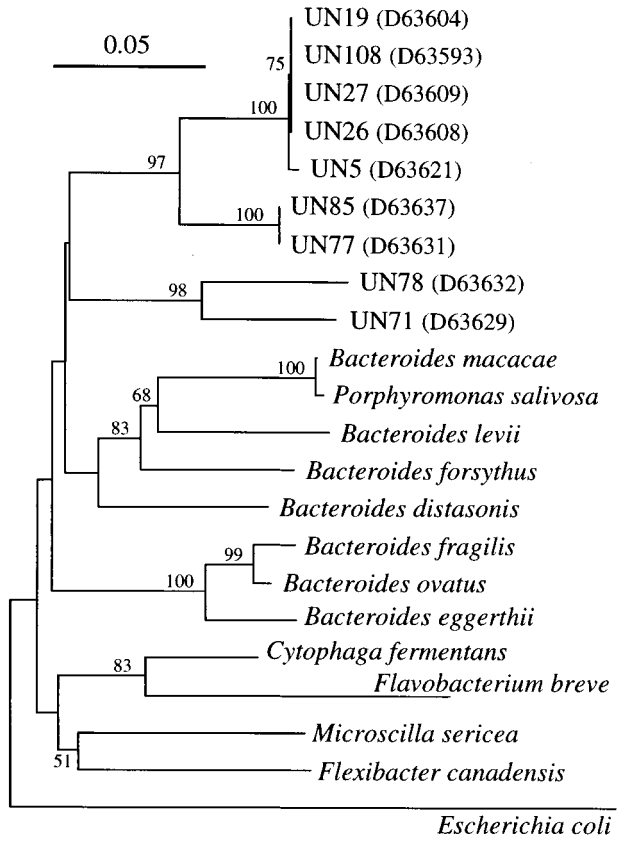

FIG. 6. Phylogenetic tree of the clones in the *Bacteroides* group, with *E. coli* as the outgroup. The tree is based on 304 aligned bases. The scale bar represents 0.05 substitutions per base position. Bootstrap values above 50 from 100 resamplings are shown for each node. The database accession numbers of the termite bacterial clones are in parentheses.

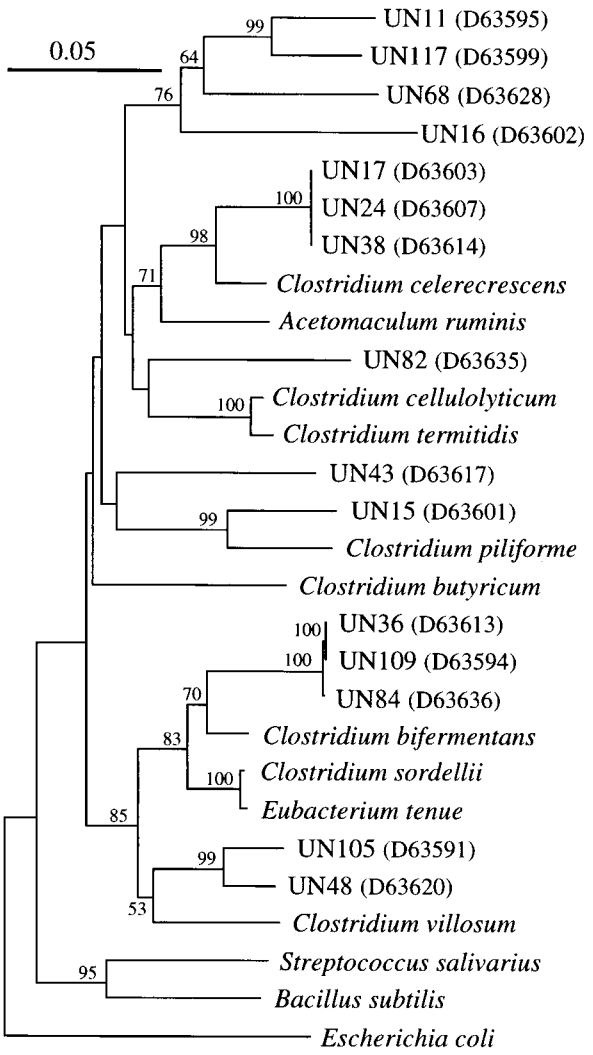

FIG. 7. Phylogenetic tree of the clones in the low-G+C-content gram-positive group of bacteria, with *E. coli* as the outgroup. The tree is based on 268 aligned bases. The scale bar represents 0.05 substitutions per base position. Bootstrap values above 50 from 100 resamplings are shown for each node. The database accession numbers of the termite bacterial clones are in parentheses.

mite *Bacteroides* cluster may also function as such. Isolation of *Bacteroides* species from termite guts has also been reported. In one reported case, cross-feeding of lactate from a lactate producer to a *Bacteroides* sp. was demonstrated (41), while in another case, a uric acid-degrading bacterium isolated from the termite gut was identified as *Bacteroides termitidis* (34). Uric acid is a metabolic waste product of termites, and the recycling of uric acid nitrogen by symbiotic bacteria is an important mechanism of nitrogen conservation in termites. However, none of the sequences for our isolated clones show a close relationship to known *Bacteroides* species.

A sulfate-reducing bacterium has been reported to be isolated from the termite gut by enrichment culture (42). The existence of the termite *Desulfovibrio* cluster, consisting of five nearly identical clones, indicates that sulfate-reducing bacteria are present in significant quantity in situ within the termite gut. As discussed by Breznak and Brune (10), sulfate-reducing bacteria would be more likely to function in interspecies hydrogen (H_2) transfer as H_2 donors, using small organic compounds (e.g., pyruvate, lactate, and sugar monomers) as oxidizable electron donors, than as H_2 acceptors oxidizing sulfur compounds, given the low sulfate concentration in the termite gut.

In contrast to the bacterial genera discussed above, very little is known about the role of spirochete species in the termite gut, as none have been isolated in pure culture. Nevertheless, they are believed to be nonpathogenic and to play an important role in the termite gut, as termites harboring them appear to be both vigorous and healthy. Recently, the 16S rDNA sequence of an uncultivated spirochete species from the gut contents of an Australian termite was reported (5), but the reported sequence has less than 90% similarity to the sequences of our spirochete clones. The most similar clone in our library (i.e., UN114) showed only 89% identity to the uncultivated spirochete.

Although *Streptococcus* species have been reported as major isolates from several termites (15, 40), none of our clones were related to the genus *Streptococcus*. We have, however, isolated in pure culture a *Streptococcus*-related species identified by 16S rRNA analysis. The presence of methanogenic bacteria in the termite gut has been suggested by means of fluorescence microscopy, since methanogens have a specific autofluorescence. Although our clone library does not contain any methanogen-related sequences, we have succeeded in PCR amplification of the methanogen 16S rDNA from the same DNA pool, using methanogen-specific primers (data not shown). It is possible that some biases of DNA extraction, PCR amplification, or cloning precluded their isolation.

Other than bacteria, protists are major constituents of the termite gut microflora and are present at concentrations of about 10^4 cells per gut (in comparison with 10^5 to 10^6 bacterial cells per gut). Although the primers used in our study are universal primers and are thought to amplify 16S rDNAs from all types of organisms, we could not isolate any 16S-like rDNA clones belonging to protists. In the experiment reported here, we fractionated the PCR products by gel electrophoresis and used only the 0.9-kb products expected for prokaryotic 16S rDNA. The 1.3-kb products expected for the eukaryotic small-subunit rDNA were also present in significant amounts, but analysis of a few clones derived from the 1.3-kb products revealed that they were clones of sequences from the termites themselves (unpublished data). Our analysis of one protist clone amplified with the PCR primers specific for eukaryotic small-subunit rDNAs indicates that the protist's sequence does not match that of 519FB (unpublished data), suggesting that the protists in the termite gut may be evolutionarily divergent. In fact, they represent one of the surviving relics in the very early stages of eukaryotic evolution, according to a recent reclassification of the kingdom Protozoa (13).

The termites used for analysis were reared for about 1 month prior to DNA extraction. Under our culture conditions, the termites were active and vigorous and survived for more than half a year without significant mortality. We have attempted to evaluate pure and stable symbiotic relationships within the termite gut that are independent of their surrounding environment, and we expect to obtain reproducible results. Our focus in this initial report in our study is to characterize the microbial diversity within the microcosm of the termite intestine rather than to describe the overall termite ecosystem.

The abundance of clones of high similarity within the library, especially the six nearly identical clones forming termite group I and the five each in the *Bacteroides* and *Desulfovibrio* clusters, may reflect their numerical abundance in the termite gut. However, since the PCR may bias the representation of clones, a quantitative analysis of abundance (2) is necessary. The data described here will allow us to investigate the relative abundance of relevant strains and to assay population changes with nucleic acid probes. Nucleic acid probes will also allow us to identify the relevant strains in situ by whole-cell hybridization (2). As microorganisms in the termite gut show complex spatial interactions with each other and with the epithelial cells of the termites (e.g., there are endo- and ectosymbionts of protists as well as epithelium-attached microorganisms), such an in situ identification will allow an informative assay of the structural and spatial heterogeneity of symbiosis in the termite gut.

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