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REVIEW

Toshiaki Kudo · Moriya Ohkuma · Shigeharu Moriya
Satoko Noda · Kuniyo Ohtoko

Molecular phylogenetic identification of the intestinal anaerobic microbial community in the hindgut of the termite, *Reticulitermes speratus*, without cultivation

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Abstract A termite maintains an anaerobic microbial community in its hindgut, which seems to be the minimum size of an anaerobic habitat. This microbial community consists of bacteria and various anaerobic flagellates, and it is established that termites are totally dependent on the microbes for the utilization of their food. The molecular phylogenetic diversity of the intestinal microflora of a lower termite, *Reticulitermes speratus*, was examined by a strategy that does not rely on cultivation of the resident microorganisms. Small subunit ribosomal RNA (ssrRNA) genes were directly amplified from the mixed-population DNA of the termite gut by polymerase chain reaction (PCR) and clonally isolated. Most sequenced clones were phylogenetically affiliated with the four major groups of the domain Bacteria: the *Proteobacteria* group, the *Spirochete* group, the *Bacteroides* group, and the Low G + C gram-positive bacteria. The 16S rRNA sequence data show that the majority of the intestinal microflora of the termite consists of new species that are yet to be cultured. The phylogeny of a symbiotic methanogen inhabiting the gut of a lower termite (*R. speratus*) was analyzed without cultivation. The nucleotide sequence of the ssrDNA and the predicted amino acid sequence of the *mcrA* product were compared with those of the known methanogens. Both comparisons indicated that the termite symbiotic methanogen belonged to the order *Methanobacteriales* but was distinct from the known members of this order. The diversity of nitrogen-fixing organisms was also investigated without culturing the resident microorganisms. Fragments of the *nifH* gene, which encodes the dinitrogenase reductase, were directly amplified from the mixed-population DNA of the termite gut and were clonally isolated. The phylogenetic analysis of the *nifH* amino acid sequences showed that there was a remarkable diversity of nitrogenase genes in the termite gut. The molecular phylogeny of a symbiotic hypermastigote *Trichonympha agilis* (class Parabasalia; order Hypermastigida) in the hindgut of *R. speratus* was also examined by the same strategy. The whole-cell hybridization experiments indicated that the sequence originated from a large hypermastigote in the termite hindgut, *Trichonympha agilis*. According to the phylogenetic trees constructed, the hypermastigote represented one of the deepest branches of eukaryotes. The hypermastigote along with members of the order Trichomonadida formed a monophyletic lineage, indicating that the hypermastigote and trichomonads shared a recent common ancestry.

Key words Minimum size of anoxic habitat · Termite hindgut · Molecular phylogeny

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T. Kudo (✉) · M. Ohkuma · S. Moriya · S. Noda · K. Ohtoko
The Institute of Physical and Chemical Research (RIKEN),
Hirosawa 2-1, Wako-shi, Saitama, 351-01, Japan
Tel. +81-48-467-9544; Fax +81-48-462-4672
e-mail: tkudo@postman.riken.go.jp

Introduction

Life originated in the pre-oxic worlds, and anoxic environments still persist in many places on Earth such as lake sediments, the gut of ruminants, and the deep waters of some marine basins. A termite maintains an anaerobic microbial community in its hindgut, which seems the minimum size of an anaerobic habitat. This microbial community consists of bacteria and various anaerobic flagellates, and it is established that termites are totally dependent on the microbes for the utilization of their food (Breznak and Brune 1994) (Fig. 1).

Despite the isolation and cultivation of several bacteria and protists from within this community, 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.

The application of molecular phylogenetic analysis to ecological studies has enhanced our ability to assess natu-

rally occurring biodiversity in mixed microbial assemblages (Amann et al. 1995). In this approach, genes encoding small subunit ribosomal RNA (16S-like rRNA) derived from the extracted nucleic acids of mixed microbial populations are clonally isolated, sorted, and sequenced (Fig. 2). These sequences can then be compared with each other as well as against databases of rRNA sequences from well-characterized microorganisms to determine the identity and ecology of uncultured organisms present in natural microbial communities.

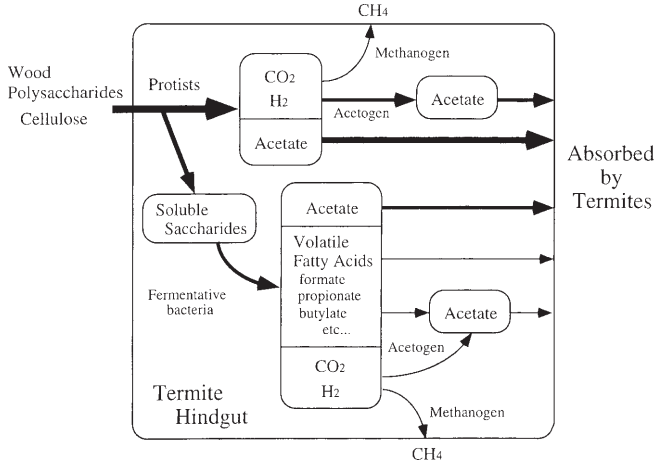

Fig. 1. Energy metabolism of termite hindgut

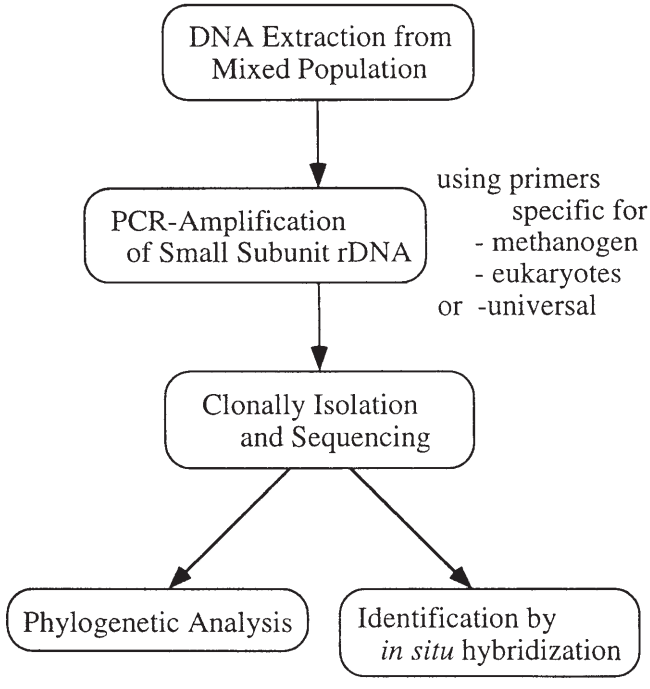

Fig. 2. Experimental strategy based on rRNA sequences for characterization of the termite symbiotic microbial community without cultivation

Here, we summarize the molecular phylogenetic diversity of the intestinal microbial community of the lower termite *Reticulitermes speratus* as determined by a comparison of partial sequences of cloned genes encoding small subunit ribosomal RNA directly amplified from extracted DNA by the polymerase chain reaction (PCR).

Bacterial diversity of the lower termite *Reticulitermes speratus*

The lower termite *Reticulitermes speratus* was reared for approximately 1 month on a sterile diet. DNA was directly extracted from the gut contents, and 16S rRNA genes (16S rDNAs) were amplified from universal primers (519FB and 1392RS). Although these primers only sample the 3′-proximal two-thirds of the gene, they allow unequivocal detection of rRNA genes for any type of organism. PCR products were electrophoresed, and bands of the expected size range for prokaryotic 16S rDNA (~0.9 kb) were recovered from the gel and cloned into *E. coli.* For ease on cloning, *Bam*HI and *Sal*I restriction sites, which occur rarely in 16S rRNA sequences, were introduced at the 5′- and 3′-ends of the amplified products of 16S rDNAs. Approximately 300 bases of each clonal segment corresponding to *E. coli* position 534–834 were sequenced. The sequence information obtained from this region of the 16S rRNAs has previously been shown to provide the level of resolution necessary to facilitate futher analysis (Fuhrman et al. 1993). 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 approximate estimation of phylogenetic similarity.

A total of 55 clones were sequenced and shown to belong within the domain Bacteria. Based on sequence similarity, these clones were classified into several clusters corresponding to the major divisions of the Bacteria. 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. 3). Most of the clones were phylogenetically affiliated with four of the major divisions of the Bacteria: the *Proteobacteria* group, the *Spirochete* group, the *Bacteroides* group, and the Low G + C gram-positive bacteria. More detailed information concerning phylogenetic relationships of the clones with known bacteria is mentioned by Ohkuma and Kudo (1996).

Phylogenetic analysis of the clonally isolated 16S rRNA genes shows that the intestinal microflora of the termite consists of many diverse microbial species, many of which have not been previously characterized. Surprisingly, approximately two-thirds of the analyzed clones share less than 90% sequence identity with the known 16S rRNA sequences of cultivated organisms. More significantly, ten clones bear no close sequence similarity to any recognized bacterial taxon in the database of rRNA. Among them, the presence of eight clonal isolates belonging to termite group I suggests that these unaffiliated microbes constitute

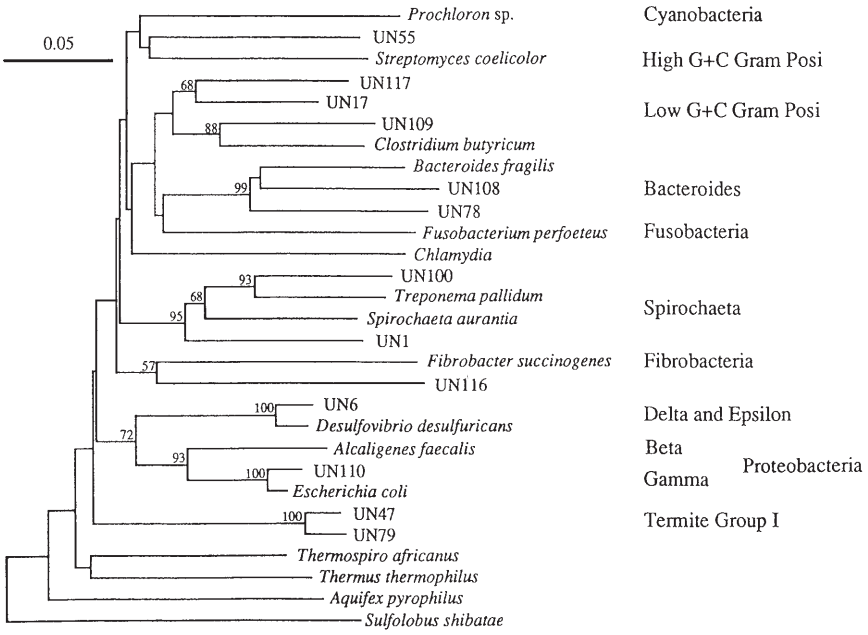

Fig. 3. A large phylogenetic tree including members of all groups found in the clone library, but not including all individual clones (other clones are in subsequent figures). The tree was constructed using the neighbor-joining method based on 271 homologous bases corresponding to *E. coli* 16S rRNA position 534–826. The 16S rRNA sequence of *Sulfolobus shibatae* was used as the outgroup. The *scale bar* represents 0.02 substitutions per nucleotide position. *Numbers at nodes* indicate bootstrap values for each node of 100 bootstrap resampling (values below 50 are not shown). The *parentheses* after the clone names indicate the database accession numbers of the termite clones

a significant quantity of the organisms in the termite intestine. The phylogeny of isolated clones indicates that the intestinal bacterial microflora of the termite consists of species related to the enteric bacteria *Desulfovibrio*, *Treponema*, *Bacteroides*, and *Clostridium*. All the known species among these groups are either strict or facultative anaerobes and have been frequently isolated from animal intestines. Because the termite gut, especially much the microbe-packed portion of the hindgut, is anoxic and has a low redox potential, the presence of strict and fucultatively anaerobic organisms in the termite gut is a reasonable expectation. In fact, several different bacterial species belonging to the *Enterobacter*, *Citrobacter*, *Desulfovibrio*, *Bacteroides*, and *Clostridium* genera have been isolated from the gut of many termite species (Eutick et al. 1978; Schultz and Breznak 1978; Trinkerl et al. 1990). Even when using strict anaerobic isolation techniques, however, the recovery of bacteria via culture from termite guts was only 0.7%.

The N_2 fixation mediated by gut bacteria is one of the crucial aspects of termite symbiosis because termites feed on nitrogen-poor wood. Nitrogen-fixing bacteria isolated from several kinds of termites have been identified as *Citrobacter freundii* and *Enterobacter agglomerans* (French et al. 1976; Potrikus and Breznak 1977). As the members of the termite gamma *Proteobacteria* cluster within our clone library are closely related to the *Citrobacter* and *Enterobacter*, these clones may play a role in N_2 fixation. Because several *Clostridium* and *Desulfovibrio* species are also known to be N_2-fixing bacteria, the organisms picked up in our clone library that cluster with them also represent possible N_2-fixing candidates in the termite hindgut. We discuss N_2 fixation in a later section.

The ability to reduce CO_2 to acetate also plays a crucial role in microbial fermentation in the termite gut. A few CO_2-reducing acetogens have been isolated from termite guts in pure culture and identified as species of *Sporomusa*, *Acetonema*, and *Clostridium* (Breznak et al. 1988; Kane and Breznak 1991; Kane et al. 1991). 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 them.

As most of the *Bacteroides* species are known to be fermentative and acidogenic, the organisms assigned to the termite *Bacteroides* cluster may also function as such. Isolation of *Bacteroides* species from termite guts has also been reported (Schultz and Breznak 1978). In one reported case, a cross-feeding of lactate from a lactate producer to a *Bacteroides* sp. was demonstrated, while in another case, a uric acid-degrading bacterium isolated from the termite gut was identified as *Bacteroides termitidis* (Potrikus and Breznak 1980). Uric acid is a metabolic waste 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 with known *Bacteroides* species.

A sulfate-reducing bacterium has been reported to be isolated from the termite gut by enrichment culture. The existence of the termite *Desulfovibrio* cluster, consisting of five nearly identical clones, indicates that the sulfate-reducing bacteria are present in significant quantity in situ within the termite gut. As discussed by Breznak and Brune, the 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, sugar monomers) as oxidizable electron donors, rather than as H_2 acceptors oxidizing sulfur compounds, given the low sulfate concentration in the termite gut (Breznak and Brune 1994).

In contrast to the bacterial genera just discussed, very little is known about the role of *Spirochete* species in the termite gut because 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 gut contents of an Australian termite was reported, but the sequence reported shares less than 90% similarity with any of our *Spirochete* clones (Bermudes et al. 1988). The clone most similar to it in our library (i.e., UN114) showed only 89% identity.

Although *Streptococcus* species have been reported as a major isolate from several termites (Schultz and Breznak 1978), none of our clones were related to *Streptococcus*. We have, however, indeed isolated in pure culture a *Streptococcus*-related species, identified by 16S rRNA analysis. The termites used for analysis were reared for about 1 month before DNA extraction. Under these culture conditions, the termites were active, 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 independent of their surrounding environment, and we expect to obtain reproducible results.

The abundance of clones of high similarity within the library, especially the six nearly identical clones forming termite group I and the five in each of the *Bacteroides* and the *Desulfovibrio* clusters, respectively, may reflect their numerical abundance in the termite gut. However, because PCR may bias the representation of clones, a quantitative analysis on abundance would be 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 also allow us to identify the relevant strains in situ by whole-cell hybridization. As microorganisms in the termite gut show complex spatial interactions with each other and with the epithelium cells of the termite (e.g., there are endo- and ectosymbionts of protists as well as epithelium-attached microorganisms), such an in situ identification allows an informative assay into the structural and spatial heterogeneity of symbiosis in the termite gut (Ohkuma and Kudo 1996).

Phylogeny of symbiotic methanogens in the gut of the termite *Reticulitermes speratus*

Almost all termites harbor methanogenic bacteria (methanogens) in their gut, and these methanogens are thought to produce methane primarily from CO_2 and H_2, which are produced from intermediate metabolic steps of lignocellulose digestion (Brauman et al. 1992). Because their diet, wood, is a poor source of nitrogen, methanogens, as well as nitrogen fixation bacteria, play an important role for balancing the C:N ratio in termite symbiotic system by means of releasing carbon atoms as methane. Emission of methane from termites has often been claimed to be a major contribution to global atmospheric methane, but it now appears that termites account for less than 5% of global methane emissions. Nevertheless, because the population of termites on earth is large, especially in tropical and subtropical areas, the study of methanogenesis from termites is important for understanding global ecology.

Methanogens in the hindgut contents of a lower termite *Zootermopsis angusticollis*, which was visualized by epifluorescence microscopy of methanogen-specific autofluorescence, have been reported (Lee et al. 1987). Small, rod-shaped methanogens were found associated with some kinds of flagellated protozoa. In axenic cultures of cellulolytic protozoa (*Trichomitopsis termopsidis*) from the same termite, methanogens appeared to be endosymbionts harbored within the protozoa cells (Odelson and Breznak 1985). The metabolic functions and roles of symbiotic methanogens in termite gut are not yet clear because neither their cultivation and characterization nor their phylogeny have been reported.

Although the bacterial clone library does not contain any methanogen-related sequences, PCR amplification of the methanogen 16S rDNA was successfully obtained from the same DNA pool using methanogen-specific primers. The small subunit ribosomal RNA gene (ssrDNA) and a 640-bp portion of the gene encoding subunit A of methyl coenzyme M reductase (*mcrA*) were amplified from a mixed-population DNA of the termite gut by PCR and cloned. The nucleotide sequence of the ssrDNA and the predicted amino acid sequence of the *mcrA* product were compared with those of the known methanogens. Both comparisons indicated that the termite symbiotic methanogen belonged to the order *Methanobacteriales* but was distinct from the known members of this order. In the gut of the termite *R. sperutas*, rod-shaped methanogens were found by epifluorescence microscopy, not only inside the cells of the some kinds of protozoa but also in association with the epithelium surface of the gut. Free-living methanogens were rarely observed. The PCR-amplified ssrDNA suggests that a single or set of closely related methanogen species inhabits the termite gut (Ohkuma et al. 1995).

Diversity of nitrogen fixation genes in the symbiotic intestinal community of the termite *R. speratus*

Although their diet is usually low in nitrogen sources, these termites thrive in great abundance, particularly in tropical regions. Nitrogen fixation in termites has been demonstrated by using the acetylene reduction assay (Benemann 1973; Breznak et al. 1973). The activity was shown to be associated with termite gut bacteria. A few nitrogen-fixing bacteria, however, have been isolated from termite guts (French et al. 1976; Potrikus and Breznak 1977). Identification that depends on culturing microorganisms may provide limited information on the diversity or types of organisms that fix nitrogen in termites because many yet uncultured bacteria are present in termite guts, based on analysis of rDNA sequences directly amplified and isolated from the mixed population of the termite intestinal community. The

gene *nifH* encodes dinitrogenase reductase and is conserved among diverse nitrogen-fixing microorganisms. The *nifH* gene is often used to detect nitrogen fixation genes in natural microbial communities, and analysis of *nifH* sequences provides information about the phylogenetic diversity of nitrogen-fixing microorganisms present.

The diversity of nitrogen-fixing organisms in the symbiotic intestinal microflora of the lower termite *R. speratus* was investigated without culturing the resident microorganisms. Fragments of the *nifH* gene were directly amplified from the mixed-population DNA of the termite gut and clonally isolated. Phylogenetic analysis of the *nifH* amino acid sequences showed that there was a remarkable diversity of nitrogenase genes in the termite gut. A large number of the termite *nifH* sequences were most closely related to those of a firmicute, *Clostridium pasteurianum*, with a few being most closely related to either the cluster of γ-proteobacteria or a sequence of *Desulfovibrio gigas*. Some of the others were distantly related to the bacteria and were seemingly derived from Archaea. The phylogenetic positions of these *nifH* sequences corresponded to those of genera found during a previous determination of rRNA-based phylogeny of the termite intestinal microbial community, of which a majority consisted of new, as yet uncultivated species. These results revealed that we have little knowledge of the organisms responsible for nitrogen fixation in termites.

The termite *R. speratus* can live on a diet of pure cellulose, suggesting N_2 fixation in this termite as in many other termites. In fact, N_2 fixation in *R. speratus* after the culturing condition described here was demonstrated by the reduction of acetylene to ethylene using live workers. It must be emphasized that the existence of *nifH* sequences does not always mean that N_2-fixing activity is being expressed by the respective organisms, since nitrogenase is regulated at the transcriptional and posttranslational levels. It must be also noted that the distribution of *nifH* sequences as final clones may not reflect the real distribution of *nifH* genes in the original microbial community as there are some differences in the efficiency of DNA extraction, PCR amplification, and cloning. Hybridization experiments using specific probes will be profitable for the ecological study of the natural community, and the sequences described here will be useful in the design of specific probes. The analyses with *nifH* together with rRNA will give us useful information concerning nitrogen economy and ecology within the symbiotic community in the termite gut (Ohkuma et al. 1996).

Phylogenetic identification of the symbiotic hypermastigote *Trichonympha agilis* in the hindgut of the termite *R. speratus*

The symbiotic protists may have a role in the digestion of cellulose. Morphological and ultrastructural studies have shown that these symbiotic protists belong to the orders Trichomonadida, Hypermastigida, and Oxymonadida. The former two orders together are classified into the class Parabasalia, and a common apomorphic character of the members of this class are so-called parabasal bodies that represent aggregates of apparently very large Golgi dictyosomes with kinetosome-associated fibrillar systems.

Based on ssrDNA sequences, the phylogeny of a variety of trichomonad species including species symbiotic in termites has been reported (Berchtold and König 1995; Berchtold et al. 1995; Gunderson et al. 1995). However, none of the members of the order Hypermastigida or Oxymonadida has been characterized on the basis of molecular sequences. Because they lack mitochondria and peroxisomes like the microsporidia and the diplomonads, they are believed to be some of the earliest emerging groups of eukaryotes. Despite their well-known morphological and ultrastructural characteristics, their exact phylogenetic position is not certain, and little is known about their physiology and function in the hindgut. The main problem is that only a few of them have been cultivated so far (Odelson and Breznak 1985; Yamin 1978, 1981).

Using DNA directly extracted from the whole gut of the termites as a template, with the eukaryote-specific primers, the ssrDNA gene was amplified by PCR. The determined sequences of the 5′-region of the five clones from the 1.6-kb product were almost identical (only one nucleotide difference in one clone) and showed a high degree of similarity to the ssrDNA sequences of trichomonads. The small sequence differences among the individual clones may result from amplification errors or intraspecific sequence variability.

The 1.6-kb PCR product from one representative clone, EH11, was sequenced completely. Comparison of the EH11 sequence with ssrDNA sequences available in the databases revealed that it showed significant similarity to the ssrDNA sequences of trichomonads. The EH11 sequence showed the highest similarity to the unknown trichomonad sequence 2, found in symbiont DNA from the related termite *Reticulitermes flavipes*. A model of the secondary structure of the EH11 sequence was con-structed. The overall structure was closely similar to that proposed for the trichomonad *Tritrichomonas foetus*, with slight differences in the variable regions, and shared complementarity with those of other eukaryotic organisms. This complementarity indicates that the sequence encodes a functional rRNA. As the DNA employed as the starting material for PCR was extracted from a mixed population in the contents of the termite gut, the origin of the resulting sequence was unclear. To assign the EH11 sequence to the corresponding species, we applied the whole-cell hybridization technique (Amann et al. 1995). The phase-contrast image showed the several flagellated protist species in the contents of the termite gut. This preparation was simultaneously probed with a eukaryote-targeted oligonucleotide labeled with Texas-Red (Euk1190) and an FITC-conjugated EH11-specific oligonucleotide (EH821). Hybridization with Euk1190 resulted in a fluorescence signal for each protist species, indicating that the technique is applicable in this case. Using the EH11-specific probe EH821, only a large hypermastigote, *Trichonympha agilis*, gave a strong fluorescence signal.

None of the cells of oxymonads or trichomonads in the termite hindgut gave a fluorescence signal with the EH821 probe in spite of the presence of fluorescence signals with the Euk1190 probe. The other hypermastigotes in the hindgut, such as *Teranympha mirabilis*, gave a strong signal with the Euk1190 probe but not with the EH821 probe. Thus, the EH11 sequence was assigned to *T. agilis*.

The hypermastigotes are unique in nature insofar as they occur only in termites and related insects. They are characterized by the large number of flagella inserted at the anterior end or at the anterior periphery of the cell. Members of the genus *Trichonympha* are one of the most common and abundant protists symbiotic in lower termites and are thought to play important roles in digestion of ingested cellulose and wood.

Phylogenetic analysis indicated that the hypermastigote shares a recent common ancestry with the trichomonads. Because the orders Hypermastigida and Trichomonadida constitute the class Parabasalia, based on morphological and ultrastructural features, the results concerning molecular phylogeny were consistent with morphological classification. The shallow branching pattern among the members of Parabasalia suggests a recent diversification within this class, consistent with their evolution as metazoan symbionts as discussed previously (Gunderson et al. 1995).

The lower termite, *R. speratus*, has been reported to harbor at least 12 flagellated protists. Characterization of more clones is necessary for the phylogenetic identification of the other symbiotic protists. This experimental strategy, which does not rely on cultivation, is advantageous because most of the termite symbiotic protists are difficult to cultivate in vitro.

Conclusion

Molecular phylogenetic analysis of the clonally isolated rRNA genes shows that the intestinal microflora of the termite consists of many diverse microbial species, many of which have not been previously characterized. Most bacterial sequenced clones were phylogenetically affiliated with the four major groups of the domain Bacteria: the *Proteobacteria* group, the *Spirochete* group, the *Bacteroides* group, and the Low G + C gram-positive bacteria. The nucleotide sequence of the ssrDNA and the predicted amino acid sequence of the *mcrA* product were compared with those of the known methanogens. Both comparisons indicated that the termite symbiotic methanogen belonged to the order *Methanobacteriales* but was distinct from the known members of this order. The phylogenetic analysis of the *nifH* amino acid sequences showed that there was a remarkable diversity of nitrogenase genes in the termite gut. The molecular phylogeny of a symbiotic hypermastigote, *Trichonympha agilis* (class Parabasalia; order Hypermastigida), in the hindgut of *R. speratus* was also examined by the same strategy. The whole-cell hybridization experiments indicated that the sequence originated from a large hypermastigote in the termite hindgut, *Trichonympha agilis.*

The rRNA approach, together with other molecular methodologies, bears great potential for an analysis of microbial diversity that is not biased by the limits of pure culture techniques. As microorganisms in the termite gut show complex spatial interactions with each other and with the epithelium cells of the termite (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 into the structural and spatial heterogeneity of symbiosis in the termite gut.

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