12 Diversity and Molecular Analyses of Yet-Uncultivated Microorganisms

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12.1 Introduction

Termites harbor a dense and diverse population of microorganisms consisting of both eukaryotes and prokaryotes in their gut. The relationship between termites and the gut microorganisms is a well-known example of symbiosis, and it is established that termites are totally dependent on the microbes for the utilization of their food. The metabolism in the gut and the roles of the symbionts in host nutrition have been extensively studied (see other chapters in this volume and recent reviews: Bignell 2000; Breznak 2000; Brune and Friedrich 2000; Inoue et al. 2000; König et al. 2002; Ohkuma 2003). A large number of studies involving pure cultures of symbionts have contributed to the remarkable progress. However, our understanding of biology of the gut microbiota is still poor because many of dominant species within the community are yet-uncultivated in laboratories. An estimation of culturability of the gut microbiota is at most 10% of the direct microscopic count. Such a situation is not unusual in microbial ecology study in which culture-based methods enable to sample only a limited fraction of inhabitants in natural environments.

The application of molecular methodology to ecological studies in the past decade has enhanced our ability to assess naturally occurring biodiversity. Culture-independent molecular approaches are advantageous to estimate real microbial assemblages. In those approaches, small-subunit rRNA genes in nucleic acid extracted from natural microbial communities are usually analyzed. Such approaches have been applied to the analysis of the gut community of termites, to demonstrate that a majority of the gut community consists of a diverse array of yet-uncultivated and novel species (Ohkuma 2002). In this chapter, we outline the recent advances in the culture-independent molecular studies of the gut microbial community, particularly that of lower termites which harbor flagellated protists (single cell eukaryotes) in the gut.

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12.2 Phylogenetic Identification of Symbiotic Protists

Molecular phylogeny of the symbiotic flagellate protists in the termite gut has been studied in most cases based on the 16S-like rRNA genes. These genes are obtained from a mixed-population of gut protists by PCR-based methods, and the origins of the sequences are identified by whole-cell in situ hybridization using sequence-specific probes (Berchtold and König 1995; Ohkuma et al. 1998, 2000; Dacks et al. 2001; Gerbod et al. 2002; Keeling and Leander 2003; Moriya et al. 2003; Stringl and Brune 2003). Alternatively, a careful collection of specific cells is subjected to PCR (Dacks and Redfield 1998; Keeling et al. 1998; Fröhlich and König 1999; Keeling 2002). The genes encoding phylogenetic indicators such as elongation factor-1α, and α-tubulin are also studied (Moriya et al. 1998, 2001).

The gut flagellates have been classified by their morphology and they belong to the orders Hypermastigida, Trichomonadida, and Oxymonadida. The former two are classified into the phylum Parabasalia. Hypermastigotes and oxymonads are unique in nature in that their occurrence has been documented only in termites and wood-eating cockroaches. A sister group relationship between trichomonads and hypermastigotes is evident, supporting the classification of Parabasalia. Parabasalids together with diplomonads represent earlier extant lineages of eukaryotes. Parabasalids, diplomonads, and oxymonads lack mitochondria, while parabasalids contain instead an anaerobic metabolic organelle called a hydrogenosome. Oxymonads and diplomonads seem to have neither mitochondria nor hydrogenosomes, and thus a close evolutionary relationship between them was proposed. However, a specific sister relationship between oxymonads and diplomonads is ruled out. The 16S-like rDNA studies reveal that oxymonads are related to an excavating group of protists (the genus *Trimastix*) which have small membrane-bound organelles resembling hydrogenosomes in the cell, suggesting that oxymonads are not primitively amitochondriate but that they lost mitochondria secondarily (Dacks et al. 2001). Although oxymonads and parabasalids share a similar cytoskeletal structure of huge bundled microtubules, called an axostyle, they are distantly related, which is also evident by the analysis of microtubule using α-tubulin sequences (Moriya et al. 2001).

16S-like rDNA sequences of a number of parabasalids including yet-unidentified taxa have been investigated (Berchtold and König 1995; Gunderson et al. 1995; Dacks and Redfield 1998; Keeling et al. 1998; Ohkuma et al. 1998, 2000; Fröhlich and König 1999; Gerbod et al. 2000, 2002; Keeling 2002; Keeling and Leander 2003). Trichomonads seem to form a monophyletic group while hypermastigotes are paraphyletic, and trichomonads have emerged within a lineage of the hypermastigotes. Hypermastigotes are

distinguished from trichomonads by having a large number of flagella and they show a more complex cellular morphology than trichomonads. The molecular studies imply that loss of cytoskeletal structures and reduction of the number of flagella may have occurred during parabasalian evolution (Viscogliosi et al. 1999; Ohkuma et al. 2000). A number of oxymonad taxa have also been studied with 16S-like rDNA sequences and they are found to be monophyletic (Keeling and Leander 2003; Moriya et al. 2003; Stringl and Brune 2003). One of the outstanding cytoskeletal features of oxymonads is the holdfast or attachment apparatus used to adhere to the gut wall of the host. It is postulated that this apparatus may have occurred once during their evolution and then lost in some lineages (Moriya et al. 2003).

12.3 Methanogenic Archaea

Methane emission from termites has often been claimed as a significant contribution to global atmospheric methane. Methanogenic archaea in the gut of termites have been characterized by culture-independent analyses of archaeal 16S rDNA (Ohkuma et al. 1995, 1999b; Fröhlich and König 1999; Shinzato et al. 1999, 2001; Tokura et al. 2000; Brauman et al. 2001; Friedrich et al. 2001). Methanogens in the gut of lower termites are mostly related to the genus *Methanobrevibacter* in the family Methanobacteriaceae, whereas those in the gut of higher termites belong to the family Methanosarcinaceae or the order Methanomicrobiales in addition to *Methanobrevibacter*. The former is related to the genus *Methanomicrococcus* and the latter represents a not-yet-cultivated genus. Archaeal 16S rDNAs affiliated with the Termoplasmales and with the Crenoarchaeota were also identified from the gut of some termites (Shinzato et al. 1999; Friedrich et al. 2001). In general, soil-feeding higher termites emit more methane than wood-feeders, and a correlation of relative archaeal abundance with the methane emission rates is observed (Brauman et al. 2001).

12.4 Diversity of Eubacteria

The gut eubacterial population is extensively investigated in the termite *Reticulitermes speratus* by the 16S rDNA sequences. In order to avoid some biases in PCR, several combinations of different primers and several PCR conditions are examined. A total of 1923 clones in the 16 clone libraries of different PCR conditions are analyzed (Hongoh et al. 2003a,b). The clones are sorted into phylotypes using the criterion of 97% identity of nearly

entire sequences of 16S rDNA region, to obtain up to 312 phylotypes. There are few numerically dominant and many very rare phylotypes, which is different from a species abundance deduced theoretically to approximate a log-normal distribution, namely, few dominant and few rare species but many species of intermediate abundance. This difference implies that the number of sequenced clones is still insufficient to reflect the diversity of all bacteria in the termite gut, though the coverage of eubacterial population by the clone analysis is expected higher than 90%. The eubacterial diversity is roughly estimated as about 6000 phylotypes per ml and 700 phylotypes per gut. It is noted that some dominant phylotypes represent the bacteria associated physically with the gut protists (see below), thus the accurate estimation of the microbial diversity in the gut must take much their localization into account.

Table 12.1 shows phylogenetic affiliation of the eubacterial 16S rDNA phylotypes found in the gut community of *R. speratus*. Spirochetes are the most abundant eubacterial population in the gut accounting for about a half of the analyzed clones and sorted to 61 phylotypes. Most of the spirochete phylotypes are affiliated to the genus *Treponema* (57 phylotypes) and divided into two phylogenetic clusters (Fig. 12.1). The cluster I contains diverse phylotypes of gut spirochetes and includes strains isolated recently as pure cultures from the termite gut (Leadbetter et al. 1999), whereas cluster II is also abundant in clone numbers but the fewer phylotypes. Similar diversity in spirochete population is shown in *Reticulitermes flavipes* and *Neotermes koshunensis* (Lilburn et al. 1999; Noda et al. 2003) by using a spirochete-specific PCR primer.

The clones affiliated with the low G+C Gram-positive bacteria particularly those with clostridia were very diverse and sorted into 134 phylotypes, though the clone abundance of about 10% was less than that of spirochetes. Other predominant groups of 5–15% clone abundance were *Bacteroides*-related and Termite group I clones. The termite group I of bacteria are distantly related to any known eubacterial division (Ohkuma and Kudo 1996), and only five phylotypes within the range of more than 95% sequence similarity are found from the termite so far. The rest are comprised of Proterobacteria, Actinobacteria, *Mycoplasma*, and so on (see Table 12.1).

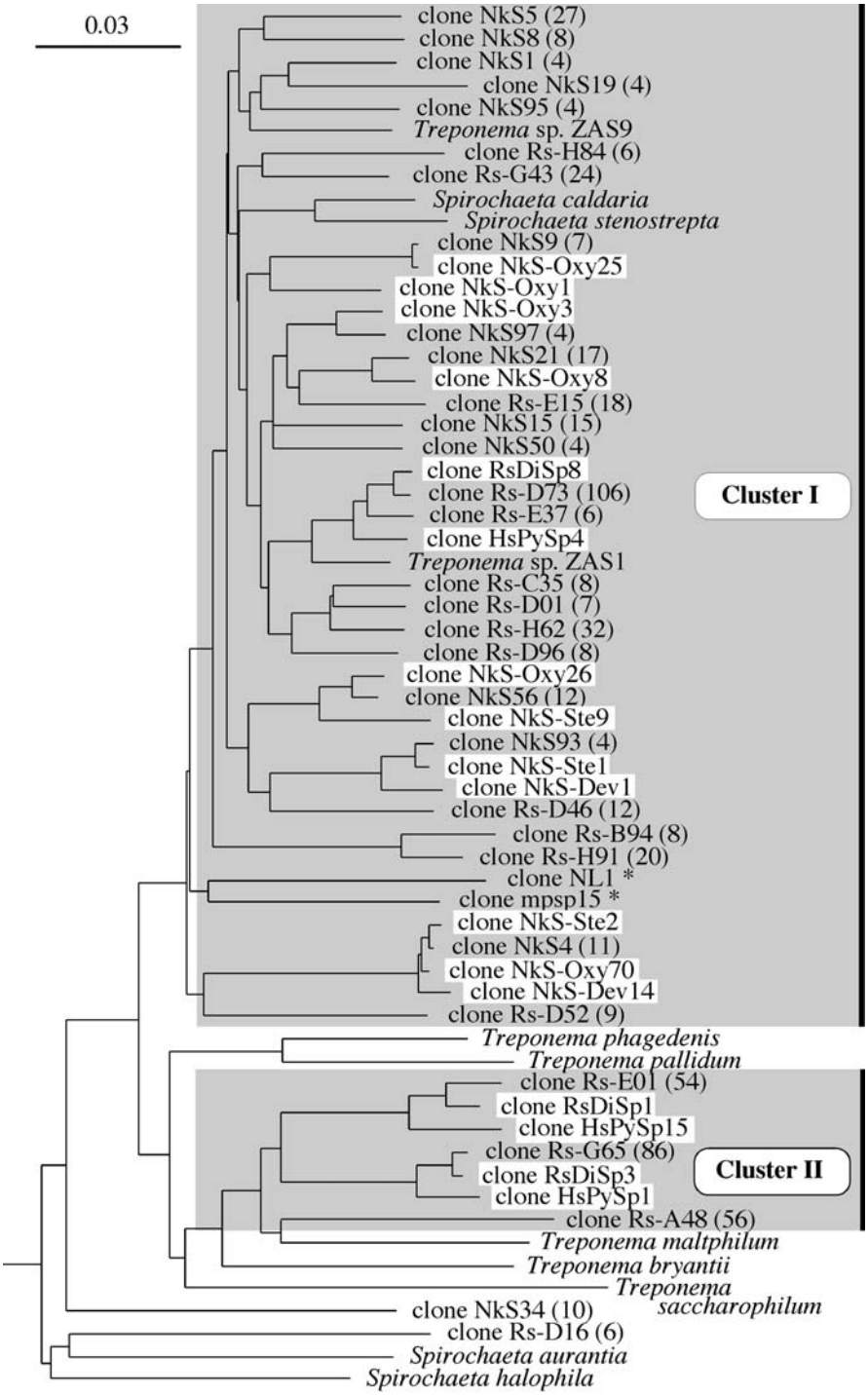
Comparisons with the database sequences reveal that almost all phylotypes found in the gut are new sequences at the phylotype-level criterion (97% nucleotide identity). Some phylotypes are clustered with each other and/or with sequences derived from other termite species, implying the existence of bacterial lineages unique in the termite guts. These results indicate that the termite gut community is a rich reservoir of novel and yet-uncultivated bacterial diversity. When spirochetes are compared between two *Reticulitermes* species, several phylotypes are closely

Table 12.1. Classification and number of eubacterial 16S rDNA phylotypes found in the gut community of *Reticulitermes speratus*

Taxon	RDP-code	Number of phylotypes
Spirochaetes		
Treponema	[2.27.3.2]	57
Spirochaeta	[2.27.3.1, 3]	3
Undescribed cluster	None	1
CFB group		
Bacteroides and relatives	[2.15.1]	36
Low G+C Gram-positives		
Clostridium/Eubacterium	[2.30.4, 5, 9]	134
Mycoplasma	[2.30.8.4]	7
Lacto-, *Enterococcus*	[2.30.7.20, 21]	4
Desulfosporosinus	[2.30.3.2]	1
Termite group I	[2.19]	5
Actinobacteria	[2.30.1]	14
Proteobacteria		
Alpha-	[2.28.1]	17
Beta-	[2.28.2]	4
Delta-	[2.28.4.1, 10]	6
Epsilon-	[2.28.5]	3
Gamma-	[2.28.3]	2
Planctomycetes	[2.20]	4
Anaerobaculum	[2.11]	3
Verrucomicrobia	[2.10]	2
Cyanobacteria	[2.21]	1
Acidobacteria	[2.25.3.6]	1
OP11	[2.28.4.2]	2
TM7	[2.21.5]	1
Unidentified cluster	None	3
Unidentified bacterium	None	1
Total number of phylotypes		312

Table is modified from Hongoh et al. (2003a). See the reference for the taxa not defined by RDP code. The data include the results obtained in Hongoh et al. (2003b)

related to each other. However, phylotypes of distinct phylogenetic positions are usually obtained from different termite genera when comparing both spirochetes and *Bacteroides*-related clones (Ohkuma and Kudo 1998; Ohkuma et al. 1999a, 2002). In terms of co-evolution with the host termites, the gut symbionts shows complex evolutionary history due to their occurrence in numerous phylogenetic positions. Probably, phylogenetically distinct species have been acquired as symbionts at many times by diverse termite species and then evolved within the gut to the present diversity.


0.03
clone NkS5 (27)
clone NkS8 (8)
clone NkS1 (4)
clone NkS19 (4)
clone NkS95 (4)
Treponema sp. ZAS9
clone Rs-H84 (6)
clone Rs-G43 (24)
Spirochaeta caldaria
Spirochaeta stenostrepta
clone NkS9 (7)
clone NkS-Oxy25
clone NkS-Oxy1
clone NkS-Oxy3
clone NkS97 (4)
clone NkS21 (17)
clone NkS-Oxy8
clone Rs-E15 (18)
clone NkS15 (15)
clone NkS50 (4)
clone RsDiSp8
clone Rs-D73 (106)
clone Rs-E37 (6)
clone HsPySp4
Treponema sp. ZAS1
clone Rs-C35 (8)
clone Rs-D01 (7)
clone Rs-H62 (32)
clone Rs-D96 (8)
clone NkS-Oxy26
clone NkS56 (12)
clone NkS-Ste9
clone NkS93 (4)
clone NkS-Ste1
clone NkS-Dev1
clone Rs-D46 (12)
clone Rs-B94 (8)
clone Rs-H91 (20)
clone NL1 *
clone mpsp15 *
clone NkS-Ste2
clone NkS4 (11)
clone NkS-Oxy70
clone NkS-Dev14
clone Rs-D52 (9)
Cluster I
Treponema phagedenis
Treponema pallidum
clone Rs-E01 (54)
clone RsDiSp1
clone HsPySp15
clone Rs-G65 (86)
clone RsDiSp3
clone HsPySp1
clone Rs-A48 (56)
Cluster II
Treponema maltphilum
Treponema bryantii
Treponema saccharophilum
clone NkS34 (10)
clone Rs-D16 (6)
Spirochaeta aurantia
Spirochaeta halophila

◀ **Fig. 12.1.** Phylogenetic relationship of gut spirochetes of the termites *Reticulitermes speratus*, *Neotermes koshunensis*, and *Hodotermopsis sjoestedti*. Clone names are tagged with the initials of the host termites (*Rs*, *Nk* and *Hs*, respectively). The number of clones belonging to each phylotype is indicated in parentheses. Only the phylotypes that represent more than six clones from *R. speratus* and four from *N. koshunensis* are shown. The two clusters are *highlighted on the gray background*. The clones *on the white background* are obtained from ectosymbiotic spirochetes of gut protists. The clones tagged with *RsDiSp* and *HsPySp* are from the ectosymbionts of the oxymonads *Dinenympha* and *Pyrsonympha*, respectively (Iida et al. 2000). The clones tagged with *NkS-Dev*, *NkS-Ste*, and *NkS-Oxy* are from the ectosymbionts of the trichomonads, *Devescovina* and *Stephanonympha*, and the oxymonad *Oxymonas*, respectively (Noda et al. 2003). The clones marked with *asterisks* denote that origins of the sequences are identified as free-swimming spirochetes by in situ hybridizations (Berchtold and König 1996; Paster et al. 1996). Tree was constructed by the neighbor-joining method and rooted with other spirochete genera (not shown). The *scale bar* represents 0.03 substitutions per nucleotide position

12.5 Spatial Organization of Gut Community

The gut microbial community is not evenly dispersed. A variety of associations of prokaryotes with gut protists are observed (Radek 1999; Dolan 2001; Dyer 2002; Ohkuma 2002, 2003; Wenzel et al. 2003). Dense populations of endosymbiotic bacteria are frequently occurred inside the cells of protists. On the cell surfaces of the protists, the attachment of prokaryotes (ectosymbiosis) is also frequently observed. A typical case for the ectosymbiosis is the attachment of spirochete-like bacteria on the protist cells. In addition, there are significant amount of both free-swimming populations and populations associated directly or indirectly with gut epithelium. In order to investigate the distribution of respective microbial population in the gut, several approaches have been applied. In situ hybridization targeting rRNA of individual cells is a powerful approach to visualize localization and morphology of the cells (Paster et al. 1996; Berchtold et al. 1999; Berchtold and König 1996; Iida et al. 2000; Tokuda et al. 2000; Noda et al. 2003). Micro-scale fractionation is also advantageous. A micromanipulator-aided microcapillary has been successfully applied to collect a single cell or a pool of the cells under microscopy (Fröhlich and König 1999; Iida et al. 2000; Tokura et al. 2000; Noda et al. 2003).

Methanogens, which are easily detected by their autofluorescense, often occur on and within the cells of the gut protists. Associations of methanogens with the gut wall are also known. Methanogens free in the gut fluid are rarely observed at least in lower termites. After the micro-scale fractionation of the gut protist species, the endosymbiotic methanogens are phylogenetically identified as yet-uncultivated species of

Methanobrevibacter (Fröhlich and König 1999; Tokura et al. 2000). Some closely related methanogens are shared between different protist species. The phylogenetic relationships of the endosymbiotic methanogens tend to be closer among those harbored by different protists within a single termite species than among those harbored by the respective protists in different termite species. The methanogens may have the nature to infect or to be transferred to the protists and it is possible that the protists have acquired their endosymbionts within the gut of each termite species. After careful fractionation of the gut epithelium, the resident methanogens are also investigated phylogenetically (Tokura et al. 2000). The sequences identified from the gut wall are related to the *Methanobrevibacter* species isolated from the termite gut (Leadbetter and Breznak 1996; Leadbetter et al. 1998). These cultivated species are morphologically similar to the cells residing on or near the gut epithelium. The protist-associated methanogens are phylogenetically distinct from those attached to the gut epithelium. Thus, a single termite species harbors several methanogen species and they show a distinct spatial distribution within the gut.

The ectosymbiotic spirochetes attached onto the cell surfaces of the gut protist species have been identified phylogenetically by the clone analyses after micro-fractionations and in situ hybridizations (Iida et al. 2000; Noda et al. 2003). Spirochetes belonging to both clusters I and II are identified as the ectosymbionts in the termites *R. speratus* and *Hodotermopsis sjoestedti* (Fig. 12.1). A group-specific probe for cluster II enables to detect a large population of ectosymbionts.Two 16S rDNA phylotypes in cluster II are identified in each protist species. A probe for cluster I also detects a population of the ectosymbionts in each protist. Thus, at least three spirochete species attach to a single protist cell, though the composition of each spirochete species is different among the protist species. The cluster I probe recognizes free-swimming spirochetes as well as the ectosymbionts whereas the cluster II probe rarely detects free-swimming ones.

In the case of the termite *N. koshunensis*, the cluster II probe gives no positive signal in the gut community while the cluster I probe detects both ectosymbiotic and free-swimming spirochetes. The absence of the cluster II spirochete population is supported by the analyses of 16S rDNA clones from the whole gut community (Noda et al. 2003). In the gut of *N. koshunensis*, three protist species harbor ectosymbiotic spirochetes, and at least two distinct phylotypes of the cluster I spirochetes are detected as ectosymbionts in each protist. The results indicate that attachment of multiple spirochete species to a single protist cell is a general feature. Common phylotypes are often shared among the protist species. Since the ectosymbiotic spirochetes are dispersed in the phylogenetic tree (Fig. 12.1), there seem to be multiple independent origins of spirochete attachment to gut protists. Some spirochetes distribute randomly over the surface of the host protist cells

whereas others locates on a restricted portion of the protist cells, which is especially the case with *Mixotricha paradoxa*, a rare example of moving symbiosis between protists and spirochetes (Wenzel et al. 2003). The latter observation evokes a specific relation between the cellular organization of the protists and the role of the spirochetes. Most of the ectosymbiotic spirochete species correspond to the abundant phylotypes in the 16S rDNA clone libraries from the whole gut community, implying the ectosymbionts represent dominant populations in the gut.

The studies of methanogens and spirochetes reveal that gut microbes are not evenly dispersed but occupy distinct micro-niches within the termite guts. The various associations involving diverse microbial species are observed with gut protists and with gut wall. Not only the micro-scale fractionation and in situ hybridization but also sectioning of gut is advantageous as the structural integrity of gut is retained (Berchtold et al. 1999; Tokuda et al. 2000). It is noted that the relationships between the gut protists and their associated prokaryotes are attractive to investigate in terms of the symbiosis-accelerated evolution of eukaryotic cells, since the gut protists represent early emerging groups of eukaryotes. Meanwhile, studies using microelectrodes have shown the presence of steep gradients of oxygen and hydrogen within the gut, and these physicochemical conditions have a significant impact on the microbial activities (Brune and Friedrich 2000). Since the gut community is highly structured, a link between microbial activities and spatial organization of respective populations is important in order to understand real nature of the gut symbiotic system.

12.6 Toward the Function of Gut Symbionts

Analysis of rRNA sequences has opened a window to investigate the diversity and composition of natural microbial communities, avoiding the largely unrepresentative nature of microbial cultivation. In some instances, metabolic functions of microbes enable to be inferred within specific rRNA phylogenetic groups such as methanogens. However, in general, physiological properties of individual microbial populations in a community cannot be predicted on the basis of only the rRNA sequences. Particularly, yet-uncultivated groups of microbes are hard to predict their functions (cultured microorganisms from the termite gut: cf. Chap. 14) Under such circumstances, genes encoding metabolically important enzymes can be useful to investigate the microbial functions in the community.

The gut protists are essential for decomposition of the ingested cellulose as well as hemicellulose in lower termites. It is considered that the ingested cellulose can be partially degraded by the cellulases of termite origin, which

are excreted from the salivary glands or the mid-gut, and the cellulose not hydrolyzed in the anterior portion of the gut then travels to the hindgut, where it can be endocytosed and fermented by the symbiotic protists. The existence of this dual system explains the capacity of termites to assimilate cellulose almost completely. Cellulases (endo-β-1,4-glucanases) of termite origin have been identified and characterized (Watanabe and Tokuda 2001). However, phylogenetic studies tell nothing about the cellulolytic system of the gut protists. Through the analyses of cDNA library constructed with the mixed-population of gut protists, their cellulase genes have been identified (Nakashima et al. 2002; Ohtoko et al. 2000; Watanabe et al. 2002; Li et al. 2003). The cellulases of the gut protists identified so far are classified into glycosyl hydrolase families (GHF) 7 and 45, whereas those of the termite origin to GHF9. A diverse array of cellulase genes are detected, suggesting a cooperation of multiple cellulases within a single protist cell to attain their efficient decomposition.

Nitrogen fixation by the gut symbionts is critical since termites thrive on a nitrogen-poor diet. Identification based on the cultivation as well as by the 16S rDNA analysis, however, provides limited information on the diversity and types of organisms that fix nitrogen in termites. Indeed, culture-independent analyses of nitrogen fixation gene *nifH*, which is PCR-amplified and characterized from the gut microbial community as in the case of 16S rDNA clones, have provided evidence for a previously unexpected diversity of nitrogen fixing microbes in diverse termites (Ohkuma et al. 1996, 1999c; cultured nitrogen fixing bacteria: cf. Chap. 14). The gene *nifH* encoding dinitrogenase reductase is evolutionarily conserved and has often been used to detect nitrogen-fixing microorganisms in natural samples. Most of the *nifH* clones analyzed from the gut communities so far are affiliated to the anaerobic *nif*, the alternative *nif*, and pseudo-*nif* groups, the latter of which probably functions in some process other than nitrogen fixation. The anaerobic *nif* group includes *nifH* from anaerobic microorganisms such as clostridia and sulfur reducers. The alternative *nif* group consists of nitrogenases carrying no molybdenum (Mo) as a cofactor and those from some Archaea. The detected *nifH* groups are similar within each termite family but different among termite families, suggesting an evolutionary trend of diazotrophic inhabitants of the gut community. Most of the sequences from the termites form lineages distinct from those previously recognized in studies of cultivated nitrogen fixers, although some lineages are related to the *nifH* sequences identified from spirochetes that includes isolates from the termite gut (Lilburn et al. 2001). The results indicate the presence of diverse potentially nitrogen-fixing microbial assemblages in the guts of termites, and the majority of them are as yet uncharacterized.

Since the expression of nitrogenase genes is strictly regulated, it must be carefully addressed that the existence of *nifH* sequences does not always

mean that nitrogen fixing activity is being expressed by the corresponding microbes. In fact, the analysis of the *nifH* mRNA constituents of the gut microbial community in *N. koshunensis* reveals that only a few among the diverse *nifH* sequences found in the gut community are preferentially expressed in the gut (Noda et al. 1999). The preferentially expressed gene encodes a member of the alternative *nif* group. The quantitative analysis of its mRNA under several feeding conditions of the termite indicates that the expression level of this gene is critical for nitrogen fixation activity of the termite. The expression is completely repressed with the presence of nitrogen sources in the termite food, whereas no significant decrease in the expression level is observed when the diet contains Mo, which ordinarily represses alternative *nif* genes. In contrast, the *nifH* mRNA analysis of the gut community in *Coptotermes formosanus*, in which no alternative *nif* member is found, reveals that members of the anaerobe group of *nif* are preferentially detected (Noda et al. 2002). The factors involved in the choice of diazotrophic symbionts by termites need to be clarified in order to understand the nature of symbiotic nitrogen fixation in termites.

As shown in the analyses of nitrogen fixation genes, the marker genes directly linked to the microbial activities are useful to characterize the responsible populations. It is emphasized that not only the presence of the genes but their expression should be studied to know real contribution of the respective microbial populations. This points to an important concept in microbial ecology in general. Even if the cultivation in laboratory reveals a microbial function, the pure cultures in any artificial condition could not represent their natural habitats. Therefore, it is very important to know the real contribution of respective microbial populations to a certain activity in natural environments. Studies on diverse microbial functions with more genes are anticipated, and for this purpose, the development of PCR primers or probes for targeting functions is important. As shown in the studies of the protist cellulases, genome or cDNA surveys may also be advantageous, though exhaustive, to search for a novel function of microbes, which is particularly attractive because the gut community consists of a numerous number of novel and yet-uncharacterized microbes.

12.7 Conclusions

The application of culture-independent molecular approaches provides a new way to characterize the microbial populations in the gut community of termites. Beyond the mere description of phylogenetic diversity, the future studies will be directed to the characterization of the in situ localization of individual populations, and to the direct link of the identity

of individual cells to their functions. We must make much account of the various interactions among the symbionts and with the host termites. Cultivation and characterization of some specific microbes is greatly anticipated, which may be helpful if aided by molecular probes. However, now that the gut symbiotic community of termites has been shown to consist of yet-uncharacterized novel microbes, culture-independent approaches will be of more and more significance.

References

Berchtold M, König H (1995) Phylogenetic position of the two uncultivated trichomonads *Pentatrichomonoides scora* Kirby and *Metadevescovin a extranea* Kirby from the hindgut of the termite *Mastotermes darwiniensis* Froggatt. System Appl Microbiol 18:567–573

Berchtold M, König H (1996) Phylogenetic analysis and in situ identification of uncultivated spirochetes from the hindgut of the termite *Mastotermes darwiniensis*. System Appl Microbiol 19:66–73

Berchtold M, Chatzinotas A, Schönhuber W, Brune A, Amann R, Hahn D, König H (1999) Differential enumeration and in situ localization of microorganisms in the hindgut of the lower termite *Mastotermes darwiniensis* by hybridization with rRNA-targeted probes. Arch Microbiol 172:407–416

Bignell DE (2000) Introduction to symbiosis. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer Academic Publishers, Dordrecht, pp 189–208

Brauman A, Dore J, Eggleton P, Bignell DE, Breznak JA, Kane MD (2001) Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. FEMS Microbiol Ecol 35:27–36

Breznak JA (2000) Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. In: Abe T, Bignell DE, Higashi M (eds) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers, Dordrecht, pp 209–231

Brune A, Friedrich M (2000) Microecology of the termite gut: structure and function on a microscale. Curr Opin Microbiol 3:263–269

Dacks JB, Redfield RJ (1998) Phylogenetic placement of *Trichonympha*. J Eukaryot Microbiol 45:445–447

Dacks JB, Silberman JD, Simpson AGB, Moriya S, Kudo T, Ohkuma M, Redfield RJ (2001) Oxymonads are closely related to the excavate taxon *Trimastix*. Mol Biol Evol 18: 1034–1044

Dolan MF (2001) Speciation of termite gut protists: the role of bacterial symbionts. Int Microbiol 4:203–208

Dyer BD (2002) Termite hindgut symbionts. In: Seckbach J (ed) Symbiosis: Mechanisms and Model Systems. Kluwer Academic Publishers, Netherlands, pp 703–713

Friedrich MW, Schmitt-Wagner D, Lueders T, Brune A (2001) Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil- feeding termite *Cubitermes orthognathus*. Appl Environ Microbiol 67: 4880–4890

Fröhlich J, König H (1999) Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. System Appl Microbiol 22:249–157

Gerbod D, Edgcomb VP, Nöel C, Delgado-Viscogliosi P, Viscogliosi E (2000) Phylogenetic position of parabasalid symbionts from the termite *Calotermes flavicollis* based on small subunit rRNA sequences. Int Microbiol 3:165–172

Gerbod D, Noël C, Dolan MF, Edgcomb VP, Kitade O, Noda S, Dufernez F, Ohkuma M, Kuda T, Capron M, Sogin ML, Viscogliosi E (2002) Molecular phylogeny of parabasalids inferred from small subunit rRNA sequences, with emphasis on the Devescovinidae and Calonymphidae (Trichomonadea). Mol Phylogenet Evol 25: 545–556

Gunderson J, Hinkle G, Leipe D, Morrison HG, Stickel SK, Odelson DA, Breznak JA, Nerad TA, Müller M, Sogin M (1995) Phylogeny of trichomonads inferred from small-subunit rRNA sequences. J Eukaryot Microbiol 42:411–415

Hongoh Y, Ohkuma M, Kudo T (2003a) Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol Ecol 44, 231–242

Hongoh Y, Yuzawa H, Ohkuma M, Kudo T (2003b) Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. FEMS Microbiol Lett 221:299–304

Iida T, Ohkuma M, Ohtoko K, Kudo T (2000) Symbiotic spirochetes in the termite hindgut: phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol Ecol 34:17–26

Inoue T, Kitade O, Yoshimura T, Yamaoka I (2000) Symbiotic associations with protists. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer Academic Publishers, Dordrecht, pp 275–288

Keeling PJ (2002) Molecular phylogenetic position of *Trichomitopsis termopsidis* (Parabasalia) and evidence for the Trichomitopsiinae. Eur J Protistol 38:279–286

Keeling PJ, Leander BS (2003) Characterization of a non-canonical genetic code in the oxymonad *Streblomastix strix*. J Mol Biol 326:1337–1349

Keeling PJ, Poulsen N, McFadden GI (1998) Phylogenetic diversity of parabasalian symbionts from termites, including the phylogenetic position of *Pseudotrypanosoma* and *Trichonympha*. J Eukaryot Microbiol 45:643–650

König H, Fröhlich J, Berchtold M, Wenzel M (2002) Diversity and microhabitats of the hindgut flora of termites. Recent Res Devel Microbiol 6:125–156

Leadbetter JR, Breznak JA (1996) Physiological ecology of *Methanobrevibacter cuticularis* sp. nov and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl Environ Microbiol 62:3620–3631

Leadbetter JR, Crosby LD, Breznak A (1998) *Methanobrevibacter filiformis* sp. nov., a filamentous methanogen from termite hindguts. Arch Microbiol 169:287–292

Leadbetter JR, Schmidt TM, Graber JR, Breznak JA (1999) Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283:686–689

Li L, Fröhlich J, Pfeiffer P, König H (2003) Termite's symbiotic gut Archaezoa are becoming living metabolic fossils. Eukaryotic Cell. 2:1091–1098

Lilburn TG, Schmidt TM, Breznak JA (1999) Phylogenetic diversity of termite gut spirochaetes. Environ Microbiol 1:331–345

Lilburn TG, Kim KS, Ostrom NE, Byzek KR, Leadbetter JR, Breznak JA (2001) Nitrogen fixation by symbiotic and free-living spirochetes. Science 292:2495–2498

Moriya S, Ohkuma M, Kudo T (1998) Phylogenetic position of symbiotic protist *Dinenympha exilis* in the hindgut of the termite *Reticulitermes speratus* inferred from the protien phylogeny of elongation factor 1α. Gene 210:221–227

Moriya S, Tanaka K, Ohkuma M, Sugano S, Kudo T (2001) Diversification of the microtubule system in the early stage of eukaryote evolution: elongation factor 1α and α-tubulin protein phylogeny of termite symbiotic oxymonad and hypermastigote protists. J Mol Evol 51:6–16

Moriya S, Dacks JB, Takagi A, Noda S, Ohkuma M, Doolittle WF, Kudo T (2003) Molecular evolution of three oxymonad genera: *Pyrsonympha*, *Dinenympha* and *Oxymonas*. J Eukaryot Microbiol 50:190–197

Nakashima K, Watanae H, Azuma J-I (2002) Cellulase genes from the parabasalian symbiont *Pseudotrichonympha grassii* in the hindgut of the wood-feeding termite *Coptotermes formosanus*. Cell Mol Life Sci 59: 1554–1560

Noda S, Ohkuma M, Usami R, Horikoshi K, Kudo T (1999) Culture-independent characterization of a gene responsible for nitrogen fixation in the symbiotic microbial community in the gut of the termite *Neotermes koshunensis*. Appl Environ Microbiol 65:4935–4942

Noda S, Ohkuma M, Kudo T (2002) Nitrogen fixation genes expressed in the symbiotic microbial community in the gut of the termite *Coptotermes formosanus*. Microb Environ 17:139–143

Noda S, Ohkuma M, Yamada A, Hongoh Y, Kudo T (2003) Phylogenetic position and in situ identification of ectosymbiotic spirochetes on protists in the termite gut. Appl Environ Microbiol 69:625–633

Ohkuma M (2002) Symbiosis in the termite gut – culture-independent molecular approaches. In: Seckbach J (ed) Symbiosis: mechanisms and model systems. Kluwer Academic Publishers, Netherlands, pp 717–730

Ohkuma M (2003) Termite symbiotic systems: efficient bio-recycling of lignocellulose. Appl Microbiol Biotechnol 61:1–9

Ohkuma M, Kudo T (1996) Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. Appl Environ Microbiol 62:461–468

Ohkuma M, Kudo T (1998) Phylogenetic analysis of the symbiotic intestinal microflora of the termite *Cryptotermes domesticus* 164:389–395

Ohkuma M, Noda S, Horikoshi K, Kudo T (1995) Phylogeny of symbiotic methanogenes in the gut of the termite *Reticulitermes speratus*. 134:45–50

Ohkuma M, Noda S, Usami R, Horikoshi K, Kudo T (1996) Diversity of nitrogen fixation genes in the smbiotic intestinal microflora of the terrmite *Reticulitermes speratus*. Appl Environ Microbiol 62:2747-2752

Ohkuma M, Ohtoko K, Grunau C, Moriya S, Kudo T (1998) Phylogenetic identification of the symbiotic hypermastigote *Trichonympha agilis* in the hindgut of the termite *Reticulitermes speratus* based on small-subunit rRNA sequence. J Eukaryot Microbiol 45:439–444

Ohkuma M, Iida T, Kudo T (1999a) Phylogenetic relationships of symbiotic spirochetes in the gut of diverse termites. FEMS Microbiol Lett 181:123–129

Ohkuma M, Noda S, Kudo T (1999b) Phylogeny of symbiotic methanogenes in diverse termites. FEMS Microbiol Lett 171:147–153

Ohkuma M, Noda S, Kudo T (1999c) Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. Appl Environ Microbiol 65:4926–4934

Ohkuma M, Ohtoko K, Iida T, Tokura M, Moriya S, Usami R, Horikoshi K, Kudo T (2000) Phylogenetic identification of hypermastigotes, *Pseudotrichonympha*, *Spirotrichonympha*, *Holomastigotes*, and parabasalian symbionts in the hindgut of termites. J Euk Microbiol 47:249–259

Ohkuma M, Noda S, Hongoh Y, Kudo T (2002) Diverse bacteria related to the bacteroides subgroup of the CFB phylum within the gut symbiotic communities of various termites. Biosci Biotechnol Biochem 66:78–84

Ohtoko K, Ohkuma M, Moriya S, Inoue T, Usami R, Kudo T (2000) Diverse genes of cellulase homologues of glycosyl hydrolase family 45 from the symbiotic protists in the hindgut of the termite *Reticulitermes speratus*. Extremophiles 4:343–349

Paster BJ, Dewhirst FE, Cooke SM, Fussing V, Poulsen LK, Breznak JA (1996) Phylogeny of not-yet-cultured spirochetes from termite guts. Appl Environ Microbiol 62:347–352

Radek R (1999) Flagellates, bacteria and fungi associated with termites: diversity and function in nutrition. Ecotropica 5:183–196

Shinzato N, Matsumoto T, Yamaoka I, Oshima T, Yamagishi A (1999) Phylogenetic diversity of symbiotic methanogens living in the hindgut of the lower termite *Reticulitermes speratus* analyzed by PCR and in situ hybridization. Appl Environ Microbiol 65:837–840

Shinzato N, Matsumoto T, Yamaoka I, Oshima T, Yamagishi A (2001) Methanogenic symbionts and the locality of their host lower termites. Microb Environ 16:43–47

Stingl U, Brune A (2003) Phylogenetic diversity and whole-cell hybridization of oxymonad flagellates from the hindgut of the wood-feeding lower termite *Reticulitermes flavipes*. Protist 154:147–155

Tokuda, G, Yamaoka, I, Noda, H (2000) Localization of symbiotic clostridia in the mixed segment of the termite *Nasutitermes takasagoensis* (Shiraki). Appl Environ Microbiol 66:2199–2207

Tokura M, Ohkuma M, Kudo T (2000) Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol Ecol 33:233–240

Viscogliosi E, Edgcomb VP, Gerbod D, Noël C, Delgado-Viscogliosi P (1999) Molecular evolution inferred from small subunit rRNA sequences: what does it tell us about phylogenetic relationships and taxonomy of the parabasalids? Parasite 6:279–291

Watanabe H, Tokuda G (2001) Animal cellulases. Cell Mol Life Sci 58:1167–1178

Watanabe H, Nakashima K. Saito H, Slaytor M (2002) New endo- β-1,4-glucanases from the parabasalian symbionts, *Pseudotrichonympha grassii* and *Holomastigotoides mirabile* of *Coptotermes* termites. Cell Mol Life Sci 59:1983–1992

Wenzel M, Radek R, Brugerolle G, König H (2003) Identification of the ectosymbiotic bacteria of *Mixotricha paradoxa* involved in movement symbiosis. Eur J Protistol 39:11–23