

Termite Gut Flagellates and Their Methanogenic and Eubacterial Symbionts

Yuichi Hongoh and Moriya Ohkuma

Contents

1	Introduction	56
2	Methanogenic Endosymbionts of Termite Gut Flagellates	59
2.1	Phylogeny of Endosymbiotic Methanogens	59
2.2	Predicted Functions of Endosymbiotic Methanogens	61
3	Eubacterial Symbionts of Termite Gut Flagellates	65
3.1	Phylogeny of Ectosymbiotic Eubacteria	65
3.2	Phylogeny of Endosymbiotic Eubacteria	67
3.3	Predicted Functions of Eubacterial Symbionts	70
4	Genomics of Endosymbionts	71
5	Concluding Remarks and Future Perspectives	72
	References	74

Abstract Termites harbor an abundance and diversity of symbiotic microbes in their gut that comprise all the three domains of life: Eucarya, Bacteria, and Archaea. One of the most prominent features of this microbiota is the cellular association of the gut flagellates with eubacteria and/or methanogenic archaea. The eubacterial and methanogenic symbionts are observed both inside and on the surface of the host flagellate cells. Although molecular approaches have gradually revealed the phylogenetic and spatial structures of these as-yet-uncultivable symbiotic complexes, their functions remain largely unknown. Recently, a method to acquire the complete genome sequence of uncultured bacterial species from a small number of cells

Y. Hongoh (✉)

Department of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, O-okayama 2-12-1-W3-48, Meguro-ku, Tokyo 152-8550, Japan
Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Hirosawa 2-1, Wako, Saitama 351-0198, Japan
e-mail: yhongoh@bio.titech.ac.jp

M. Ohkuma

Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Hirosawa 2-1, Wako, Saitama 351-0198, Japan

has been developed; two complete genome sequences of endosymbiotic eubacteria of termite gut flagellates have been decoded. This novel genomic approach is expected to provide a great progress in the studies of this multilayered symbiotic system in termite gut.

1 Introduction

Termites are one of the most important decomposers in temperate to tropical regions (Sugimoto et al. 2000). Their ability to thrive on recalcitrant, nitrogen-poor lignocellulose is mostly attributable to the activities of the microbial community in the gut (Fig. 1) (Cleveland 1923; Eutick et al. 1978; Yoshimura 1995). In phylogenetically “lower” termites, the gut microbiota comprises both eukaryotes and prokaryotes, whereas most “higher” termites (family Termitidae) harbor only prokaryotic gut microbes. The majority of these gut microbes are as yet uncultivable; their phylogenetic and spatial distributions have been studied mainly by small subunit rRNA-based molecular analyses (Berchtold et al. 1994; Ohkuma and Kudo 1996; Ohkuma et al. 1998; Lilburn et al. 1999; Iida et al. 2000; Hongoh et al. 2003; Nakajima et al. 2005; Yang et al. 2005; Hongoh et al. 2006b). However, the detailed symbiotic mechanism remains unclear due to lack of effective methodologies for functional analysis of uncultivable microbes.

The eukaryotic gut symbionts comprise two distinct lineages of flagellated protists, belonging to either the phylum Parabasalia or the order Oxymonadida in the phylum Preaxostyla. They are unique to termites and the wood-feeding cockroach *Cryptocercus*, and each termite species possesses a specific set of flagellate species (Yamin 1979; Kitade 2004). Although the cultivation of these flagellates is very difficult, several studies on axenic or mixed cultures demonstrated that the

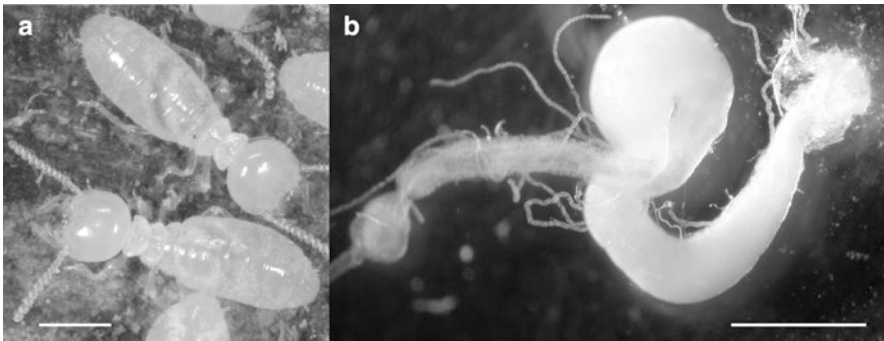


Fig. 1 Termites and a removed gut. (a) The lower termite *Reticulitermes speratus*. (b) A removed gut from *R. speratus*. Bars = 1 mm. Panel (b) was originally published in Hongoh et al. (2008a) as supporting information

flagellates are strictly anaerobic and ferment cellulose: $n(\text{C}_6\text{H}_{12}\text{O}_6) + n(2\text{H}_2\text{O}) \rightarrow n(2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2)$ (Yamin 1980, 1981; Odelson and Breznak 1985b).

In addition to the cellulolytic flagellates, lower termites harbor a diversity of eubacteria in their gut. Several hundred or more species of eubacteria inhabit the gut of a single termite species, and the community structure is basically consistent within a host species (Hongoh et al. 2005, 2006a). The eubacterial gut symbionts, distributed among 25 phyla, constitute one or more monophyletic clusters in each phylum, suggesting that they are not allochthonous, but autochthonous symbionts, inherited from parents to offspring through the proctodeal trophallaxis (i.e., transmission of gut contents from the anus of a donor to the mouth of a recipient) (Andrew 1930; Kitade et al. 1997; Hongoh et al. 2005).

Another domain of life, Archaea, is also found in termite gut. The majority are methanogens, which are less abundant and less diverse compared to the eubacterial gut symbionts (Ohkuma et al. 1999; Shinzato et al. 1999; Brauman et al. 2001; Friedrich et al. 2001; Donovan et al. 2004; Brune 2010). To date, three methanogenic strains have been isolated from a lower termite, *Reticulitermes flavipes*, and described as *Methanobrevibacter cuticularis*, *Methanobrevibacter curvatus* (Leadbetter and Breznak 1996), and *Methanobrevibacter filiformis* (Leadbetter et al. 1998). All these methanobrevibacters attach to the gut epithelium. The occurrence of methanobrevibacters and their attachment to the gut wall are observed in both lower and higher termites (Fig. 2a, b) (Tokura et al. 2000; Pester and Brune 2007). From a wood-feeding higher termite, a methanobrevibacter strain closely related to *Methanobrevibacter arboriphilus* has been isolated, and from higher termites of various feeding habits, three strains of the genus *Methanobacterium*, closely related to *Methanobacterium bryantii*, have also been isolated (Deevong et al. 2004).

In general, soil- and litter-feeding higher termites emit much more methane than wood-feeding higher and lower termites (Brauman et al. 1992; Sugimoto et al. 1998). In the gut of wood-feeding termites, unlike many other anoxic environments, H_2 -dependent acetogenesis outcompetes methanogenesis as “ H_2 -sink” (Odelson and Breznak 1983; Breznak and Switzer 1986; Pester and Brune 2007). Methanogens account for 0–10% of the gut prokaryotic population (Leadbetter and Breznak 1996; Brauman et al. 2001) and the rate of CH_4 emission is only 10% of that of CO_2 -reductive acetogenesis in the gut of lower termites (Pester and Brune 2007). Although acetogenesis from H_2 plus CO_2 appears nutritionally more beneficial to termites than methanogenesis because acetate is their chief energy and carbon source, the physiological and physicochemical basis for the outcompetition of methanogenesis by acetogenesis is unclear. The localization of methanogens in the gut might account in part for this outcompetition (Breznak 2000; Tholen and Brune 2000), but the question why the localization of methanogens is restricted to the gut wall and the cells of the relatively few species of flagellates in the gut of lower termites remains unanswered. Methane oxidation has never been observed in termite gut (Pester et al. 2007).

Whereas numerous eubacteria and methanogens reside as free or wall-attached forms in the termite gut, it is known that the majority of the prokaryotic members in

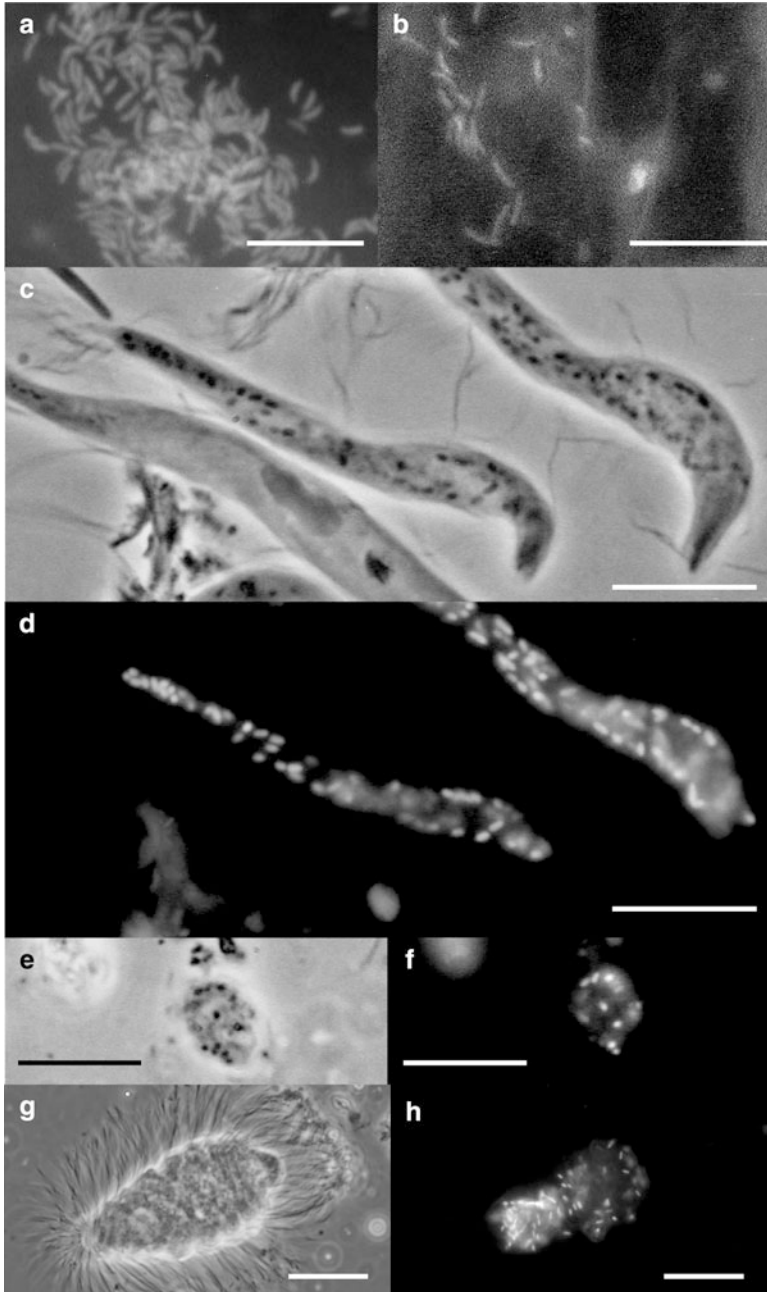


Fig. 2 Localization and morphology of methanogens detected by epifluorescence microscopy. (a) Epifluorescence image of methanogens on the gut epithelium of the lower termite *Reticulitermes speratus*. (b) Epifluorescence image of methanogens on the gut epithelium of the higher termite *Microcerotermes* sp. (c) Phase contrast image of the oxymonad protist *Dinenumpha parva*

the gut of lower termites exist as endo- or ectosymbionts of the gut flagellates (Berchtold et al. 1999). Indeed, the physical association of cells between the flagellates and prokaryotes is one of the most prominent features of the termite gut microbiota (Brune and Stingl 2006; Ohkuma 2008). In this chapter, the studies on the cellular association between the flagellates and methanogenic archaea, as well as between the flagellates and eubacteria, in termite gut are reviewed and future perspectives for the functional analysis of the endosymbiotic prokaryotes are presented.

2 Methanogenic Endosymbionts of Termite Gut Flagellates

This section describes the phylogeny and predicted roles of the methanogenic endosymbionts of flagellates in the gut of lower termites. Although there have been fewer reports on the endosymbiotic methanogens of termite gut flagellates compared to those on the eubacterial endosymbionts, the reports contain valuable data, which help us to capture a general tendency on the phylogeny and host specificity and to elucidate their functional roles in the symbiosis with the flagellate host.

2.1 Phylogeny of Endosymbiotic Methanogens

The presence of methanogens inside the cells of certain flagellate species in the termite gut was evidenced for the first time by Lee et al. (1987). *Methanobrevibacter*-like rod-shaped cells were detected on the basis of the autofluorescence from the cofactors F₄₂₀ and F₃₅₀, inside the cells of the parabasalid flagellates *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis* from the gut of the termite *Zootermopsis angusticollis*. The corresponding rod-shaped cells associated with these small parabasalids had been described by Kirby (1930). Endosymbiotic methanogens in these three flagellate species have also been reported in the congeneric termite *Zootermopsis nevadensis* (Pester and Brune 2007). No molecular data exist for these methanogens to date.

Tokura et al. (2000) discovered endosymbiotic methanogens inside the cells of the parabasalid flagellate *Microjoenia* sp. and the oxymonad flagellate *Dinenympha parva* in the gut of the termite *Reticulitermes speratus*, on the basis of the F₄₂₀ and

Fig. 2 (continued) from the gut of *R. speratus*. **(d)** Epifluorescence image of endosymbiotic methanogens in the *D. parva* cells. **(e)** Phase contrast image of the parabasalid flagellate *Microjoenia* sp. from the gut of *R. speratus*. **(f)** Epifluorescence image of endosymbiotic methanogens in the *Microjoenia* cell. **(g)** Phase contrast image of the parabasalid flagellate *Spirotrichonympha leidy* from the gut of the lower termite *Coptotermes formosanus*. **(h)** Epifluorescence image of endosymbiotic methanogens in the *S. leidy* cell. Bars = 10 μm. Panels **(a)** and **(c–f)** were originally published in Tokura et al. (2000) and slightly modified. Panels **(g)** and **(h)** were kindly provided by Jun-Ichi Inoue and a related study was published in Inoue et al. (2008)

F₃₅₀ autofluorescence (Fig. 2c–f). About 10–50 cells of methanogens were found constantly inside the cells of *Microjoenia* sp. and *D. parva*, respectively (Hara et al. 2004). The total numbers of methanogens associated with *Microjoenia* sp. and *D. parva* were 7.9×10^3 and 1.3×10^5 per gut, respectively. Methanogens were also observed in other species of *Dinenympha* and the oxymonad *Pyrronympha* sp., but the association was occasional. In total, approximately 4% of the flagellate cells in *R. speratus* guts were found to be associated with methanogens (Tokura et al. 2000).

In the termite *Hodotermopsis sjoestedti*, all the cells of *Dinenympha* and *Microjoenia* were found to be associated with methanogens. The population of the methanogen-associated flagellates was much larger than that in *R. speratus*; they accounted for 42% of the total flagellate cells. In both termite species, *R. speratus* and *H. sjoestedti*, methanogens free in the gut luminal fluid were rarely found (Tokura et al. 2000), while many were observed on the gut epithelium as in *R. flavipes*. *R. flavipes* and *Reticulitermes santonensis* [synonym of *R. flavipes*, found in European countries (Jenkins et al. 2001)] harbor no gut flagellates that are associated with methanogens (Leadbetter and Breznak 1996; Pester and Brune 2007).

To identify the phylogenetic position of these endosymbiotic methanogens in *R. speratus* and *H. sjoestedti*, clone analyses of archaeal 16S rRNA genes were performed. About 50 cells of *Dinenympha* and *Microjoenia*, respectively, were physically isolated by micromanipulation and directly used for PCR amplification. All of the sequenced clones were affiliated with the genus *Methanobrevibacter* (Fig. 3). In *R. speratus*, two phylotypes were obtained from *D. parva*, whereas a single phylotype was found from *Microjoenia* sp. Similarly, two phylotypes were from *Dinenympha* spp. and a single phylotype from *Microjoenia* sp. in *H. sjoestedti*. These phylotypes showed only 94.6–97.3% sequence identity to the closest cultured species, suggesting that they are novel *Methanobrevibacter* species (Tokura et al. 2000). It is unclear whether the two methanogen phylotypes from *D. parva* inhabit the cells of an identical host strain or of distinct strains of *D. parva*.

Interestingly, the methanogen phylotypes LRSd3 from *D. parva*, LRSm1 from *Microjoenia* sp., and RsW10 from the gut wall fraction of *R. speratus* showed >99.7% sequence similarity to one another (cluster A in Fig. 3) (Tokura et al. 2000). The localizations of cluster A methanogens inside the cells of *D. parva* and *Microjoenia* sp. were confirmed by fluorescent in situ hybridization (FISH) analysis (Hara et al. 2004). It remains unknown whether these phylotypes represent an identical species that can change its habitat or represent similar but distinct lineages that have adapted specifically to the respective habitats. No evidence of cospeciation between the endosymbiotic methanogens and the flagellate hosts has been found.

Another endosymbiotic methanogen is observed inside the cells of the parabasalid flagellate *Spirotrichonympha leidy* in the gut of the termite *Coptotermes formosanus* (Fig. 2g, h) (Tsunoda et al. 1993; Inoue et al. 2008). A single *S. leidy* cell harbors about 80 cells of methanogens, accounting, in total, for 0.3% of the prokaryotic community in *C. formosanus* guts. The archaeal 16S rRNA sequences obtained from *S. leidy* cells consisted of a single phylotype, SIMeN10, sharing >99% similarity with one another and 98% similarity with those of cluster A in Fig. 3 (Inoue et al. 2008).

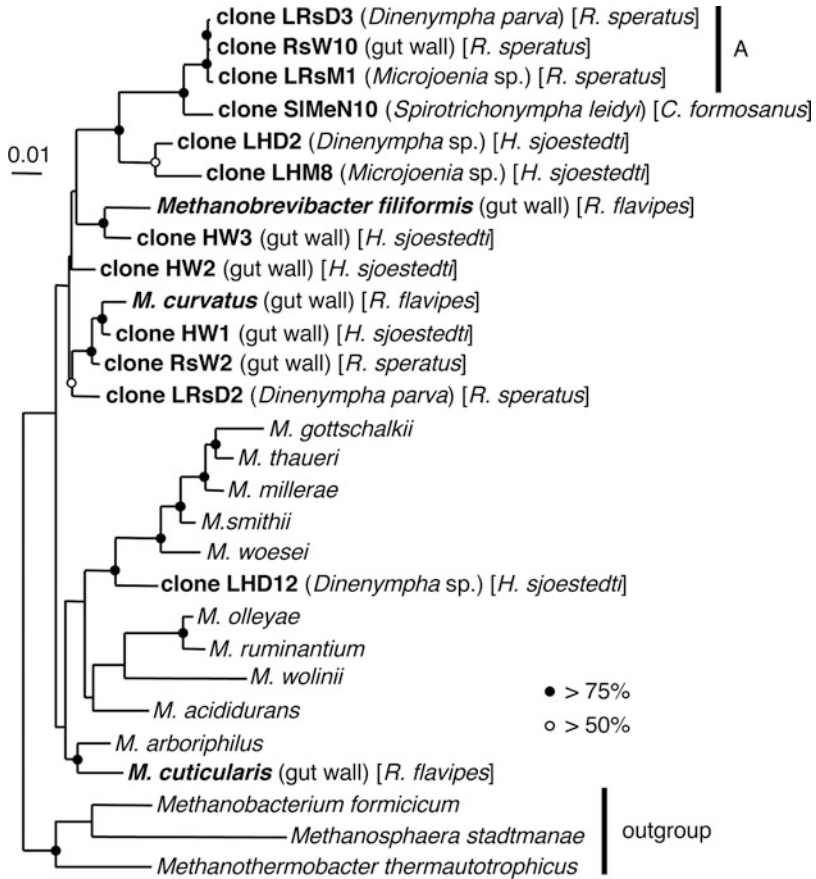


Fig. 3 Phylogenetic position of methanogens found from the gut of lower termites. Uncultured clones obtained by PCR from the gut of lower termites and type strains of described species belonging to the genus *Methanobrevibacter* were used to construct a phylogenetic tree. The localization or host protist species are shown with the host termite species in *parentheses*. Clones and isolates deriving from termite guts are shown in *bold*. The tree was constructed using PhyML v2.4.4 (Guindon and Gascuel 2003) with an HKY base substitution model. Bootstrap confidence values were calculated by 100 resamplings

2.2 Predicted Functions of Endosymbiotic Methanogens

Endosymbiotic methanogens inside flagellate cells in termite gut have never been cultured. In addition, no functional gene has been obtained from them. Therefore, there is no direct evidence for their functions. However, it is still possible to predict some of their functions from their taxonomic positions, localizations, and the data reported by Odelson and Breznak (1985a), who investigated the physiology of a

cultured flagellate, *Trichomitopsis termopsidis*, with and without “endogenous” methanogens.

The cultured strains closest to the endosymbiotic methanogens are *M. curvatus* and *M. filiformis*, which have been isolated from the gut of *R. flavipes*. The energy source of these isolates is restricted to H₂ plus CO₂, yielding CH₄ as the sole product: $4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$. They require one or more complex nutrients such as yeast extract and rumen fluid (Leadbetter and Breznak 1996; Leadbetter et al. 1998). Assuming that the endosymbiotic methanogens share basic physiologies with these isolates, a simple interpretation of the endosymbiosis is mutualism: the flagellate host provides H₂, CO₂, and other nutrients to the endosymbiotic methanogens, and in turn, the methanogens enhance the growth of the host flagellate by promoting the lignocellulose fermentation through the elimination of excess H₂.

The symbiosis mediated by interspecies transfer of H₂ between H₂-evolving fermentative anaerobes and H₂-consuming methanogens is not rare. In general, a high concentration of H₂ suppresses fermentation; the concentration of H₂ must be kept low (Worm et al. 2010). Actually, the growth rate of *Trichomitopsis termopsidis* 6057C, the sole axenic flagellate culture from the termite gut to date, was much enhanced when cocultured with an H₂-consuming methanogen, *Methanospirillum hungatii* (Odelson and Breznak 1985a). In the cocultivation, the produced gas was shifted from H₂ to CH₄, clearly suggesting the interspecies transfer of H₂. Heat-killed *M. hungatii* could not enhance the growth rate. Thus, it seems likely that the endosymbiotic methanogens benefit the flagellate host by lowering the H₂ concentration together with other H₂-consuming prokaryotes in the gut.

H₂ partial pressures, measured using agarose-embedded guts with microelectrodes, were very high in the dilated portion (paunch) of termite hindguts. In the paunch region, the values were 15–30 kPa in *R. santonensis*, 30–72 kPa in *Z. nevadensis* (Pester and Brune 2007), and 2–5 kPa in *R. flavipes* (Ebert and Brune 1997). However, since the hydrogen emission of the embedded guts was 30- to 50-fold higher than that in living termites (Pester and Brune 2007), the actual values in vivo might be much lower. This discrepancy was possibly caused by the damages of the H₂-consuming bacteria and a limited concentration of O₂ utilized to oxidize H₂ compared to living termites (Pester and Brune 2007). It has been demonstrated that the Fe-hydrogenases of a termite gut flagellate retain more than a half of their maximum H₂-evolving potential if the H₂ partial pressure can be kept lower than 20 kPa (Inoue et al. 2007).

Considering that the gut flagellates are the major source of H₂ and CO₂, produced during lignocellulose fermentation, it is reasonable that the methanogens have exploited the cytoplasm of the flagellates as their habitat. While the H₂ partial pressure is very high in the central region of a termite hindgut, it decreases toward the gut peripheral regions (Ebert and Brune 1997; Pester and Brune 2007). Messer and Lee (1989) demonstrated that exogenously supplied H₂ greatly enhanced the methanogenic activity of the termite *Z. angusticollis*, which harbors methanogen-associated flagellates as the main sites for CH₄ emission in the gut. In *Z. nevadensis*, a significant increase of CH₄ emission was also observed, even though the extent was smaller than that in *Z. angusticollis* (Pester and Brune

2007). These findings suggest that the H_2 concentration is a limiting factor for methanogenesis by the endosymbionts. In addition, the gut peripheral regions contain oxygen that suppresses methanogenesis, while the central region is almost completely anoxic (Ebert and Brune 1997). Therefore, the cytoplasm of the H_2 -evolving flagellates, a habitat in close proximity to the H_2 source and probably protected from penetrating oxygen, seems an ideal habitat for stable, highly active methanogenesis. Indeed, anaerobic flagellates in various environments occasionally harbor endosymbiotic methanogens as described elsewhere in this book (Fenchel and Finlay 2010). The endosymbiosis seems also beneficial to the methanogens in the termite gut for avoiding washout because methanobrevibacters are generally nonmotile.

Although these factors reasonably explain the merit of the endosymbiosis to the methanogens, the interspecies H_2 transfer cannot fully explain the benefit to the flagellate hosts. Since H_2 diffuses rapidly through the hindgut of termites, it is questionable that the endosymbiotic methanogens can create a boundary layer with a significantly lower H_2 partial pressure around the host flagellate cells within such a high concentration of H_2 (Breznak 2000). While the elimination of H_2 by H_2 -consuming prokaryotes including methanogens seems crucially important in the gut symbiotic system, the flagellates do not need to harbor them as intracellular symbionts that occupy large spaces in the cytoplasm. Actually, in *R. flavipes* and *R. santonensis*, the majority of H_2 -consuming methanogens were found on the gut epithelium and not associated with the flagellate cells (Leadbetter and Breznak 1996; Pester and Brune 2007; Brune 2010).

A clue to a factor that may benefit the flagellate hosts by the endosymbiosis with methanogens has been implied in the study of the cultured flagellate *Trichomitopsis termopsidis* derived from the gut of *Z. angusticollis*. The cultivation of *Trichomitopsis termopsidis* had been achieved by Yamin (1978). He treated a mixed culture comprising *Trichomitopsis termopsidis* and diverse gut bacteria with penicillin and streptomycin to acquire an axenic culture of *Trichomitopsis termopsidis*. Odelson and Breznak (1985a) used this *Trichomitopsis termopsidis* 6057 culture provided by Yamin, and they unexpectedly found that the “axenic” culture produced both CH_4 and H_2 . This clearly indicated that the *Trichomitopsis termopsidis* 6057 culture contained methanogenic archaea, which are insensitive to penicillin and streptomycin.

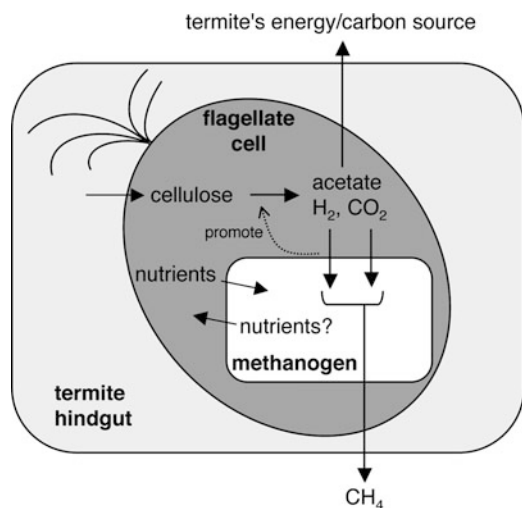
In their study, the coexistence of the methanogens persisted for a long time, and the emission of CH_4 resumed even after 1 year suppression of methanogenesis by changing nutrients in the culture medium. Their attempts to isolate methanogens from the *Trichomitopsis termopsidis* 6057 culture failed, although methanogens were readily isolated from another “axenic” culture of a parabasalid flagellate, *Trichonympha sphaerica*, which had also been established from a *Z. angusticollis* gut by Yamin (1981) using the same culture condition and medium. Epifluorescence microscopy of the *Trichomitopsis termopsidis* culture failed to detect F_{420} -fluorescent cells. From these results, Odelson and Breznak (1985a) speculated that the *Trichomitopsis termopsidis* culture 6057 contained a

small amount of “endogenous” methanogens inhabiting the cytoplasm of *Trichomitopsis termopsidis* as “energy parasites similar to chlamydiae”.

To eliminate the “endogenous” methanogens, Odelson and Breznak (1985a) treated *Trichomitopsis termopsidis* 6057 with bromoethanesulfonate (BES), an analog of a cofactor 2-mercaptoethanesulfonate (CoM), which inhibits methanogenesis, and they succeeded in establishing the putatively axenic culture *Trichomitopsis termopsidis* 6057C exhibiting no methanogenic activity. As described above, Lee et al. (1987) discovered that *Trichomitopsis termopsidis* in *Z. angusticollis* guts permanently harbors endosymbiotic methanogens. Hence, assuming that the “endogenous” methanogens were the intracellular symbionts that Lee and co-workers found later, the comparative experiments between *Trichomitopsis termopsidis* 6057 and 6057C conducted by Odelson and Breznak (1985a) should provide crucial information on the roles of the intracellular methanogens in the symbiosis with the flagellate host.

The elimination of methanogens with the BES treatment exhibited a drastic change in the growth of *Trichomitopsis termopsidis*. The growth rate of *Trichomitopsis termopsidis* 6057C decreased to one-eighth of that of *Trichomitopsis termopsidis* 6057. Interestingly, replacement of heat-killed rumen bacteria as a food source in the culture medium by a heat-killed eubacterial strain, *Bacteroides* sp. JW20, recovered the growth rate of *Trichomitopsis termopsidis* 6057C to a level comparable to that of *Trichomitopsis termopsidis* 6057. Various other strains of prokaryotes, including *M. hungatii*, could not replace *Bacteroides* sp. JW20. This implies that the endosymbiotic methanogens provide certain essential growth factors to the flagellate host, which could be replaced to some extent by a specific strain of heat-killed bacteria as a nutritional source. Thus, it is likely that endosymbiotic methanogens are nutritionally essential to the efficient growth of the flagellate host. A hypothesized symbiotic system between intracellular methanogens and their flagellate host is outlined in Fig. 4.

Fig. 4 Hypothesized roles of endosymbiotic methanogens. Endosymbiotic methanogens consume H_2 and CO_2 , which are abundantly produced during the lignocellulose fermentation by the flagellate host, and they emit CH_4 . The elimination of H_2 stimulates the fermentation process of the flagellate host. The host supplies certain nutrients to the methanogens and, in turn, the latter supply essential growth factors to the former



3 Eubacterial Symbionts of Termite Gut Flagellates

Numerous reports have been published on the eubacterial symbionts that are physically associated with the cells of the flagellates in the gut of lower termites. This section reviews the recent achievements in the study of those ecto- and endosymbionts of the flagellates on their phylogeny, localization, coevolutionary history, and functions. The functional analysis using genomics is described in Sect. 4.

3.1 Phylogeny of Ectosymbiotic Eubacteria

Most of the flagellates in the termite gut harbor ectosymbiotic eubacteria, which attach to the surface of the host cells laterally or with a tip. Numerous morphological studies have described the presence of ectosymbionts on the gut flagellates (Radek 1999), and a specific apparatus to hold the ectosymbionts has occasionally been found (Tamm 1980; Radek et al. 1996; Radek and Tischendorf 1999). The application of culture-independent molecular techniques has enabled researchers to identify these ectosymbionts phylogenetically, which is otherwise almost impossible due to the unculturability.

Among the ectosymbionts, the most frequently found groups are the genus *Treponema* in the phylum Spirochaetes and the order Bacteroidales in the phylum Bacteroidetes. In various flagellate species in the termite gut, both groups of ectosymbionts are observed on a single host cell simultaneously (Fig. 5a, b) (Hongoh et al. 2007b). Further, multiple phylotypes of treponemes have occasionally been detected on a single host flagellate cell (Noda et al. 2003), whereas only a single phylotype of Bacteroidales ectosymbionts is found on a single host cell in most cases (Stingl et al. 2004; Noda et al. 2006b, 2009; Desai et al. 2010). In the parabasalid flagellate *Caduceia versatilis* from the termite *Cryptotermes cavifrons*, an eubacterial phylotype belonging to the phylum Synergistetes has been discovered as a motility ectosymbiont (Hongoh et al. 2007a). All the cells of the host *C. versatilis* are covered with this ectosymbiont, named “*Candidatus Tammella caduceiae*”, and also simultaneously covered with a Bacteroidales phylotype (Fig. 5c–f).

Two studies attempted to elucidate the evolutionary history between the Bacteroidales ectosymbionts and their flagellate hosts. Noda et al. (2009) surveyed the gut flagellate community in several termite species for the presence of Bacteroidales ectosymbionts by FISH analysis using a Bacteroidales-specific probe. The Bacteroidales-associated flagellate species were collected using a micromanipulator and subjected to 16S rRNA gene clone analysis. In total, combined with previously published data, 31 taxa of Bacteroidales ectosymbionts from 17 flagellate genera in 10 families were used for phylogenetic analysis. The results clearly indicated multiple, independent acquisitions of the Bacteroidales ectosymbionts by different

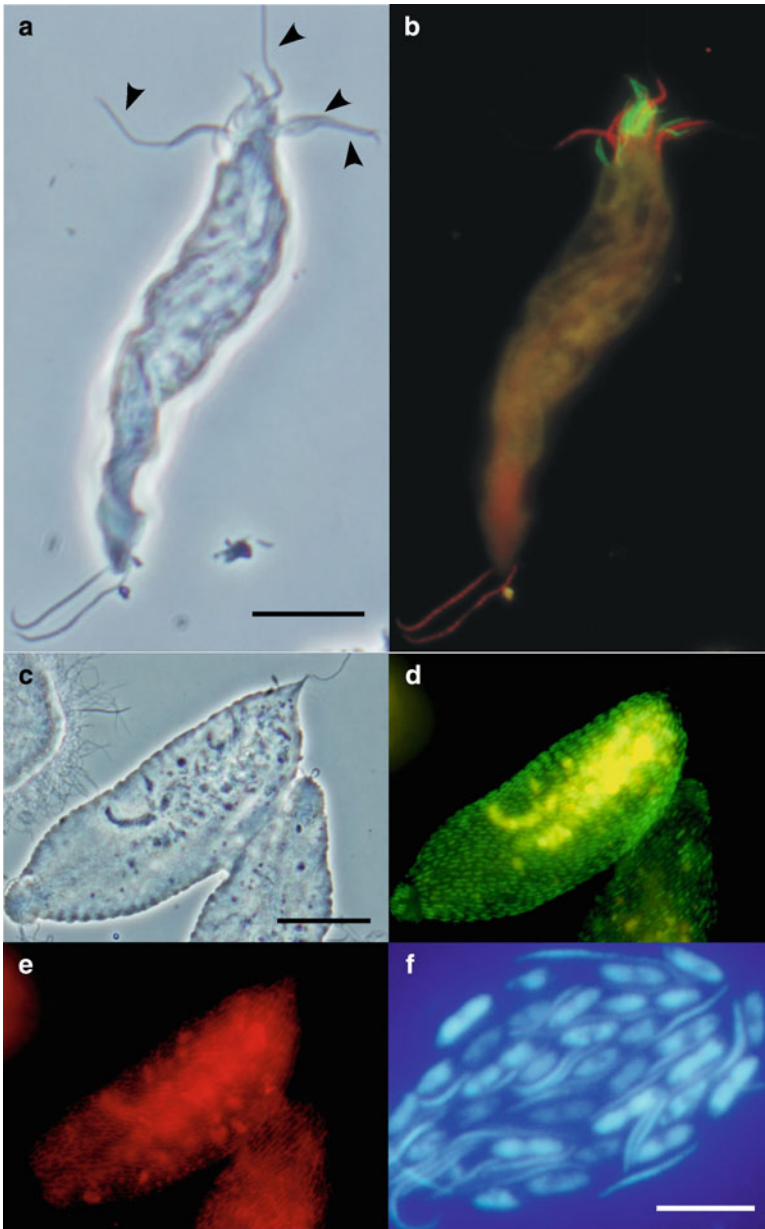


Fig. 5 Ectosymbiotic eubacteria discovered on the surface of flagellate cells from the gut of lower termites. (a) Phase contrast image of the oxymonad flagellate *Dinenumpha porteri* from the gut of the termite *Reticulitermes speratus*. (b) FISH analysis of the ectosymbiotic spirochetes (red) and Bacteroidales bacteria (green) using taxon-specific probes. Arrowheads in panel (a) indicate the true flagella of the flagellate host. Bar = 10 μ m in panel (a). (c) Phase contrast image of the parabasalid flagellate *Caduceia versatilis* from the gut of the termite *Cryptotermes cavifrons*.

flagellate genera. Within a host genus, however, the ectosymbionts appear to have cospeciated with their host flagellates.

The cospeciation within a host genus was examined in detail by Desai et al. (2010). They focused on the relationship between the parabasalid flagellates belonging to the genus *Devescovina* and their Bacteroidales ectosymbionts and demonstrated that the devescovinids and the ectosymbionts have strictly cospeciated. On the other hand, they also found that the Bacteroidales ectosymbionts of the oxymonad flagellates *Oxymonas* spp. do not constitute a monophyletic cluster but are derived from distantly related lineages. The latter result was consistent with a previous report that *Oxymonas* sp. from the termite *Neotermes koshunensis* harbors two distinct lineages of Bacteroidales ectosymbionts (Noda et al. 2006a). Hongoh et al. (2007b) found that a single phylotype, designated as “*Candidatus Symbiothrix dinenymphae*”, resides on the cells of several distinct flagellate species belonging to the genus *Dinenympha*. These data indicate a complex evolutionary history of the symbiosis between the gut flagellates and their ectosymbiotic eubacteria.

3.2 Phylogeny of Endosymbiotic Eubacteria

In addition to the ectosymbionts, the majority of the termite gut flagellates harbor endosymbiotic eubacteria. A novel-uncultured phylum-level cluster, named Termite Group 1 (TG1), was reported for the first time in 1996 by Ohkuma and Kudo, based on 16S rRNA sequences that were obtained by PCR amplification from the gut homogenate of *R. speratus*. The specific localization of a TG1 phylotype, later named as Rs-D17 (Hongoh et al. 2003), was identified and reported in the 97th Annual Meeting of the American Society for Microbiology by Eldridge et al. (1997) and also in the 9th International Symposium on Microbial Ecology by Ohkuma et al. (2001): the TG1 phylotype is an endosymbiont of the parabasalid flagellate *Trichonympha agilis* (Fig. 6a–c).

Stingl et al. (2005) confirmed the result and further identified another TG1 phylotype as an endosymbiont of the oxymonad flagellate *Pyrsonympha veterans* in the gut of *R. flavipes*. Moreover, they found that diverse lower termites and a *Cryptocercus* cockroach harbor TG1 phylotypes specific to each host species. On the basis of the phylogenetic distance from other bacterial groups, they described this group as the candidate phylum “Endomicrobia” (later corrected from phylum to

←

Fig. 5 (continued) (d) FISH analysis of ectosymbiotic eubacteria belonging to a phylotype in the phylum Synergistetes (*green*). (e) FISH analysis of ectosymbiotic eubacteria belonging to a phylotype in the phylum Bacteroidetes. Bar = 50 μ m in panel (c). (f) DAPI-stained ectosymbiotic bacteria on the surface of a host flagellate cell. Bar = 5 μ m in panel (f). Panels (a) and (b) were originally published in Hongoh et al. (2007a). Panels (c–f) were originally published in Hongoh et al. (2007b)

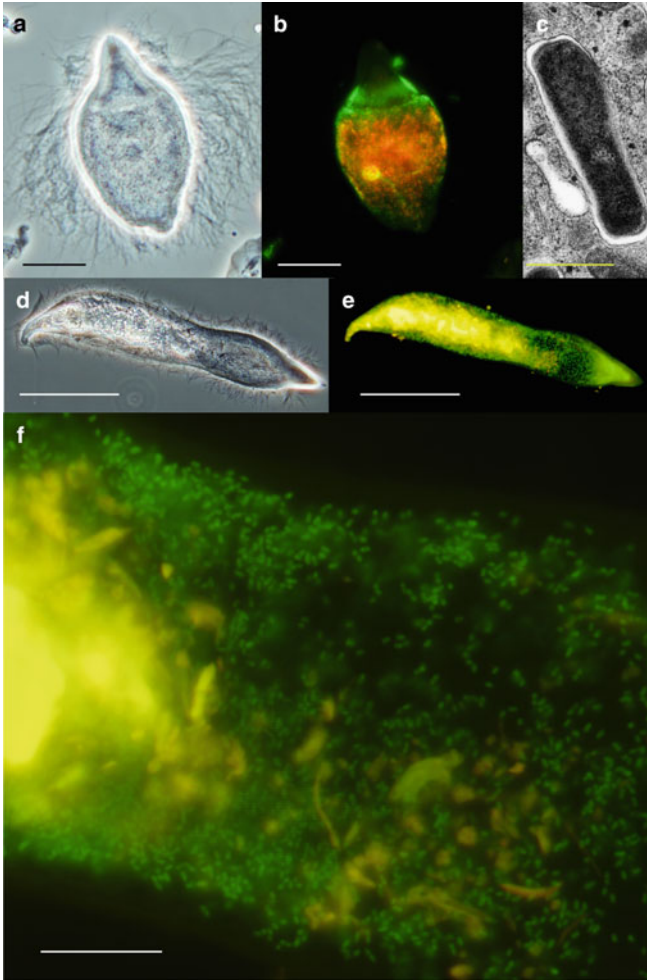


Fig. 6 Endosymbiotic eubacteria discovered inside the cells of flagellate in the gut of lower termites. **(a)** Phase contrast image of the parabasalid flagellate *Trichonympha agilis* from the gut of the termite *Reticulitermes speratus*. **(b)** FISH analysis of the endosymbiotic Termite Group 1 (“Endomicrobia”) bacteria (orange) and other eubacteria (green) using taxon-specific probes. The majority of the eubacteria detected with the green signals comprise endosymbiotic *Desulfovibrio* bacteria. Bars = 20 μm . **(c)** Transmission electron microscopy of the TG1 bacteria. Bar = 0.5 μm . **(d)** Phase contrast image of the parabasalid flagellate *Pseudotriconympha grassii* from the gut of the termite *Coptotermes formosanus*. **(e)** FISH analysis of the endosymbiotic Bacteroidales bacteria (green). The yellow color indicates the autofluorescence from phagocytosed wood particles. Bars = 50 μm . **(f)** Magnified image of (e). Bar = 10 μm . Panels **(a–c)** were originally published in Hongoh et al. (2008a) and panels **(d–f)** were originally published in Hongoh et al. (2008b) as supporting online materials

class; see below), and on the basis of 16S rRNA sequences and transmission electron micrographs, the phylotypes found inside *T. agilis* and *P. veterans* in *R. flavipes* guts as “*Candidatus* Endomicrobium trichonymphae” and “*Candidatus* Endomicrobium pyrsonymphae”, respectively.

The localizations of TG1 phylotypes were further examined by FISH and clone analysis, and it is now recognized that TG1 bacteria are species-specific endosymbionts of various flagellates in diverse lower termites (Ikeda-Ohtsubo et al. 2007; Ohkuma et al. 2007). In general, a single host flagellate cell contains 10–1,000 of the cells of a single TG1 phylotype (Stingl et al. 2005; Ohkuma et al. 2007). Similar to the case of the Bacteroidales ectosymbionts, the evolutionary history of the symbiosis between TG1 bacteria and their flagellate hosts appears to be complicated. While it has been demonstrated that TG1 bacteria have strictly cospeciated with their host *Trichonympha* flagellates (Ikeda-Ohtsubo and Brune 2009), multiple acquisitions or horizontal transfers of TG1 bacteria are suggested in other host lineages and at the level of host families (Ikeda-Ohtsubo et al. 2007; Ohkuma et al. 2007; Desai et al. 2010).

In contrast to the TG1 bacteria, which are endosymbionts of various species of gut flagellates, the endosymbionts belonging to the Bacteroidales inhabit only the cells of the parabasalid flagellates belonging to the genus *Pseudotriconympha* (Fig. 6d–f) (Noda et al. 2007). Further, *Pseudotriconympha* flagellates are unique to termites in the family Rhinotermitidae (Yamin 1979; Kitade and Matsumoto 1998). Hence, the distribution of the endosymbiotic Bacteroidales bacteria is restricted. Their population, however, is huge and they are predominant in the prokaryotic gut microbiota of rhinotermitid termites. In the Formosan subterranean termite *C. formosanus*, a single cell of *Pseudotriconympha grassii* contains up to 10⁵ cells of the Bacteroidales endosymbiont, designated as phylotype CfPt1-2, and in total, it accounts for two-thirds of the prokaryotic cells in *C. formosanus* guts (Noda et al. 2005).

Noda et al. (2007) examined the evolutionary history of the symbiosis among rhinotermitid termites, the *Pseudotriconympha* flagellates, and their Bacteroidales endosymbionts. Phylogenetic analyses showed that these three have almost completely cospeciated, implying the importance of the Bacteroidales endosymbionts to the host *Pseudotriconympha*, as well as the importance of the *Pseudotriconympha* to the host termites. Indeed, it has been demonstrated by selective elimination of *P. grassii* from the gut microbiota that *P. grassii* is essential for the host *C. formosanus* to feed on wood materials (Yoshimura 1995).

As in the ectosymbiosis, multiple species of endosymbionts occasionally inhabit a single flagellate cell. For example, a *Desulfovibrio* phylotype, Rs-N31, always coexists with the TG1 phylotype, Rs-D17, inside the cells of the flagellate *T. agilis* in *R. speratus* guts (Fig. 6b) (Sato et al. 2009). A similar association among *Trichonympha* flagellates, TG1 endosymbionts, and *Desulfovibrio* endosymbionts has also been discovered in *H. sjoestedti* and *Z. nevadensis* guts. The Rs-N31 bacterium has been described as “*Candidatus* *Desulfovibrio trichonymphae*” on the basis of the 16S rRNA sequence and transmission electron micrographs (Sato et al. 2009).

3.3 Predicted Functions of Eubacterial Symbionts

Although the eubacterial symbionts associated with gut flagellates have never been cultured thus far, certain functions and symbiotic roles have been proposed on the basis of their taxonomic positions, localizations, functional gene marker analyses, and fragmental physiological information.

The most intriguing and clearly demonstrated function of ectosymbionts is to provide the host flagellates with the motility. The parabasalid flagellate *Mixotricha paradoxa* in the gut of the termite *Mastotermes darwiniensis* harbors three species of treponemes on the surface of its cell (Wenzel et al. 2003). Surprisingly, *M. paradoxa* cannot swim by its own flagella, but is propelled solely by a synchronized movement of the ectosymbiotic treponemes (Cleveland and Grimstone 1964). Similarly, the parabasalid flagellate *C. versatilis* in the gut of *C. cavifrons* swims only by the movement of the bundled flagella of the Synergistetes ectosymbionts “*Candidatus Tammella caduceiae*” (Fig. 5c–f) (Tamm 1982; Hongoh et al. 2007a). The detailed symbiotic mechanism, however, is totally unknown due to the unculturability of these flagellates and bacteria.

The motility symbiosis has never been observed in other treponemal ectosymbionts, and instead, other functions have been hypothesized on the basis of the physiological data obtained from cultured strains of the genus *Treponema* isolated from termite guts. Leadbetter et al. (1999) have succeeded in axenically culturing *Treponema* strains from termite gut for the first time in the world. The strains, ZAS-1 and ZAS-2 isolated from the gut of *Z. angusticollis*, later described as *Treponema primitia* (Graber and Breznak 2004; Graber et al. 2004), are homo-acetogens that grow by mixotrophy using sugars or H₂ plus CO₂, and exhibit a low level of nitrogen-fixing activity. Another strain, ZAS-9 isolated also from a *Z. angusticollis* gut, later described as *Treponema azotonutricum* (Graber et al. 2004), grows by heterotrophy fermenting sugars to acetate, ethanol, CO₂, and H₂, and it exhibits a strong activity of nitrogen fixation (Lilburn et al. 2001). Recently, the third species, *Treponema isoptericolens*, has been isolated from the gut of the termite *Incisitermes tabogae*, which grows by heterotrophy, fermenting sugars to ethanol and CO₂ as main products (Dröge et al. 2008).

Functional gene marker analyses suggested that the H₂-dependent reductive acetogenesis, as exhibited by *T. primitia*, is mainly conducted by treponemes in termite guts. Genes encoding formyl tetrahydrofolate synthetase (FTHFS), a key enzyme in the reductive acetogenesis, have been detected by PCR from the gut of diverse lower termites, and the majority of the FTHFS genes were identified to be originating from treponemes (Salmassi and Leadbetter 2003; Ottesen et al. 2006; Pester and Brune 2006). From these data, one can speculate that the ectosymbiotic treponemes might be involved in mutualism mediated by interspecies H₂ transfer as suggested in the endosymbiosis with methanogens and/or they might contribute to the nitrogen metabolism of the host flagellates and termites by nitrogen fixation, though no direct evidence exists.

The functions of the Bacteroidales ectosymbionts are more difficult to predict because they are only distantly related to cultured Bacteroidales strains (Noda et al. 2009). Since phagocytosis of Bacteroidales ectosymbionts by the host *Devescovina* flagellates have been observed (Noda et al. 2006b), it is conceivable that they serve as a nutrient source for the flagellate hosts. Other hypothesized functions are maintenance of the cell structure of the flagellate host by acting as exoskeletons (Radek et al. 1996; Leander and Keeling 2004) and protection of the anaerobic hosts from penetrating oxygen (Noda et al. 2006b). Inoue et al. (2007) tested hydrogenase activity of the hydrogenosome fraction and endosymbiont fraction, respectively, for the flagellate *P. grassii* from *C. formosanus* guts. As a result, the fraction of the Bacteroidales endosymbiont CfPt1-2 (Fig. 6d–f) exhibited a strong uptake-type hydrogenase activity. Thus, phylotype CfPt1-2 may contribute to the elimination of H₂ together with other H₂-consuming prokaryotes.

Sato et al. (2009) described the uncultured endosymbiotic species “*Candidatus Desulfovibrio trichonymphae*” and characterized its basic properties by functional gene marker analysis. They demonstrated that *D. trichonymphae* has potential to conduct sulfate respiration that uses H₂ as an electron donor. In addition, they speculated that *D. trichonymphae* might contribute to the detoxification of penetrating oxygen as suggested in desulfovibrios isolated from termite guts (Kuhnigk et al. 1996), which share 95% sequence similarity with *D. trichonymphae*. The physiological functions of the coexisting TG1 endosymbionts within the cells of *T. agilis* (Fig. 6b), however, had been impossible to predict until the genome analysis was performed (see below), because there has been no cultured strain closely related to the phylotypes, whereas a distantly related strain has recently been cultured and described as *Elusimicrobium minutum*, isolated from the gut of a larva of the cetoniid beetle *Pachnoda ephippiata* (Geissinger et al. 2009). Upon the description of *E. minutum*, the TG1 phylum has been described as the phylum Elusimicrobia and the endosymbiotic cluster as the candidate class “Endomicrobia” (“TG1” is used throughout this text to avoid confusion) (Geissinger et al. 2009; Herlemann et al. 2009).

4 Genomics of Endosymbionts

To analyze the functions of uncultivable microbiota, metagenomics has been applied to diverse environmental samples, including the hindgut luminal fluid of a higher termite, *Nasutitermes ephratae*. In the metagenomic study, Warnecke et al. (2007) discovered numerous genes of eubacterial origins involved in degradation of cellulose and hemicellulose, H₂ production, reductive acetogenesis, and nitrogen fixation. However, although this type of conventional metagenomics can provide a comprehensive view of the whole microbiota, the functions of individual species in the community remain almost unknown due to the enormous diversity of bacterial species and strains.

Hongoh et al. (2008a,b) proposed an alternative method, aiming to acquire the complete genome sequence of an uncultivable bacterial species from a complex community. They applied isothermal whole genome amplification (WGA) technique using Phi29 DNA polymerase (Dean et al. 2002) to obtain enough DNA from only 10^2 to 10^3 bacterial cells, while $\geq 10^{10}$ cells are required for a standard genome analysis. Two species of endosymbiotic eubacteria, Rs-D17 in the TG1 phylum (Fig. 6a–c) and CfPt1-2 in the order Bacteroidales (Fig. 6d–f), were targeted in the genome analysis.

Single cells of the host flagellates, *T. agilis* from a *R. speratus* gut and *P. grassii* from a *C. formosanus* gut, respectively, were physically isolated by micromanipulation, and the membrane was ruptured using detergent. The bacterial cells that leaked out from the single host cell were collected and subjected to WGA. From the amplified samples, a single circular chromosome (1.1 Mb) and three circular plasmids for Rs-D17 and a single circular chromosome (1.1 Mb) and four circular plasmids for CfPt1-2 were successfully reconstructed, respectively, without ambiguity (Hongoh et al. 2008a,b).

The functional annotation of both genomes revealed their basic metabolic pathways and suggested the roles in each symbiotic system. In Rs-D17, the chromosome encodes 761 protein-coding genes and additionally 121 pseudogenes. The pseudogenes comprise genes involved in functions such as DNA replication/repair, lipopolysaccharide (LPS) biosynthesis, transports, and defense mechanisms. In contrast, genes required for biosynthesis of amino acids and cofactors are abundantly retained (Hongoh et al. 2008a). These characteristics are consistent in the genome of CfPt1-2, but in addition, CfPt1-2 possesses genes for nitrogen fixation from the atmosphere and those for recycling putative nitrogen waste products of the flagellate host. Thus, it is strongly suggested that these endosymbionts of the termite gut flagellates play essential roles in the nitrogen metabolism of the flagellate hosts. Based on the genome analysis and other information, phylotype CfPt1-2 has been described as “*Candidatus Azobacteroides pseudotrichonymphae*” (Hongoh et al. 2008b).

The process of nitrogen fixation and biosynthesis of amino acids and cofactors conducted by the endosymbionts are considered to be much more stable and efficient than those conducted by free-swimming gut bacteria. The endosymbionts can utilize the ample carbon and energy sources without competition and their genomes have been reduced, streamlined, and specialized for nitrogen metabolism. Particularly in CfPt1-2, its ability to directly couple nitrogen fixation to cellulolysis enables a highly efficient growth of the host cellulolytic protist, the termite, and the termite colony, without the limitation of a nitrogen deficiency. The schematic view of the predicted roles of the endosymbiotic eubacteria is shown in Fig. 7.

5 Concluding Remarks and Future Perspectives

Most of the flagellates in termite guts harbor ecto- and endosymbiotic eubacteria and/or methanogenic archaea. During this decade, their phylogenetic and spatial structures have been revealed to some extent by using molecular analyses based on

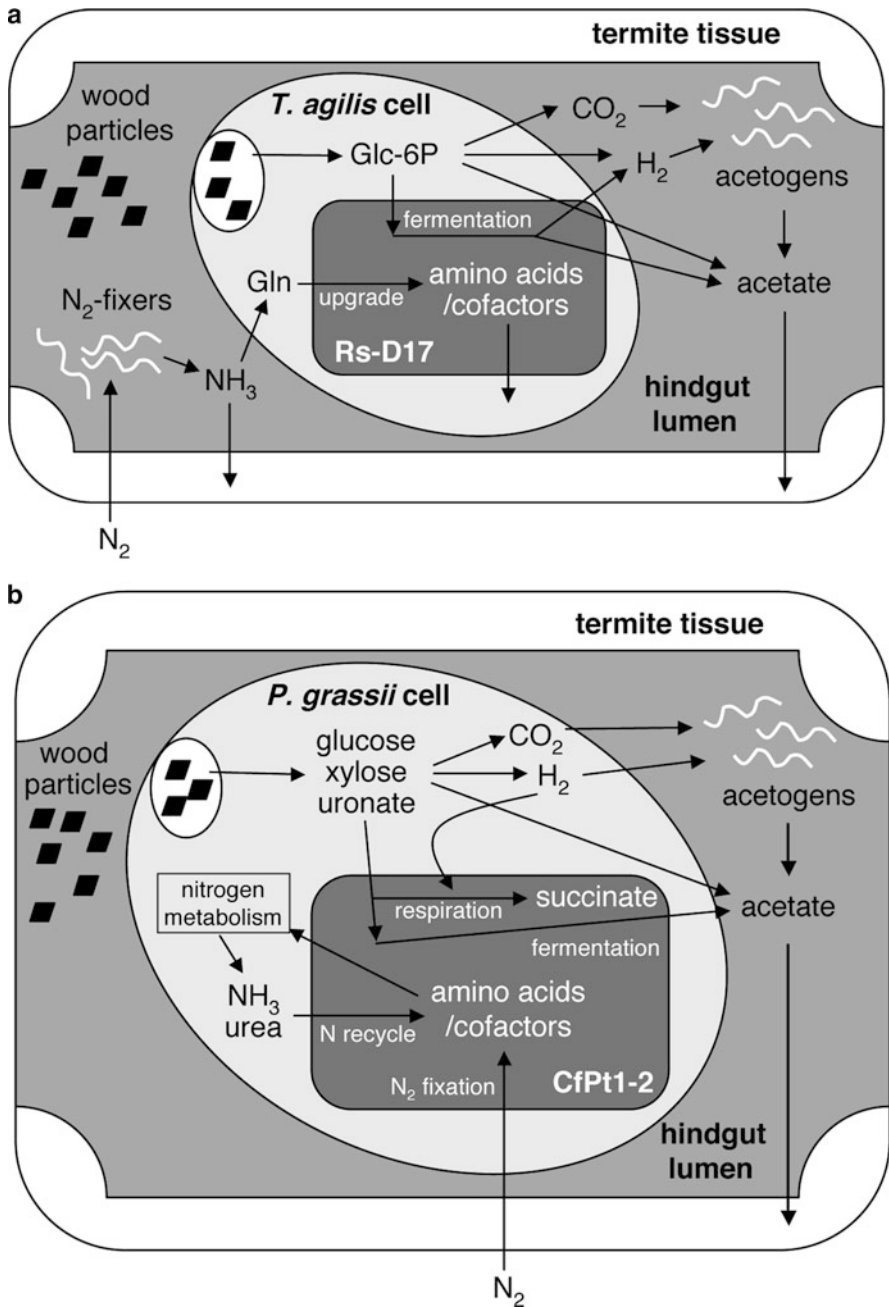


Fig. 7 Predicted functions of endosymbiotic eubacteria based on the complete genome sequences. (a) Predicted functions of phylotype Rs-D17 belonging to the phylum Termite Group 1 (Hongoh et al. 2008a). (b) Predicted functions of phylotype CfPt1-2 belonging to the order Bacteroidales (Hongoh et al. 2008b)

16S rRNA sequences. However, for these as-yet-uncultivable microbes, effective methodologies to analyze their functions had not been well developed. To overcome the difficulty, metagenomics has recently emerged and been a powerful tool to unveil the functions of bacterial communities comprising both uncultivable and cultivable strains, and it is greatly useful to capture a comprehensive view of the microbiota for their functions and diversity.

More recently, another innovative methodology for the functional analysis of uncultivable microbiota has been developed: whole genome amplification to acquire the complete genome sequence of an individual strain or a cluster of very similar strains from a small number of cells. Applying this strategy, hitherto unknown functions of eubacterial endosymbionts of termite gut flagellates have successfully been revealed: the endosymbionts contribute to the nitrogen metabolism of the flagellate host by provision of amino acids and cofactors and by nitrogen fixation from the atmosphere.

Although “single-cell genomics”, which aims to obtain the complete genome sequence from a single bacterial cell, is the best way to analyze the functions of uncultured bacterial species, it takes more time (hopefully a short time) to be established for practical use. However, there are diverse endo- and ectosymbionts of flagellates in termite guts from which 10–100 cells comprising a single strain or very similar strains can be collected. Using a similar method to that described by Hongoh et al. (2008a,b), the complete genome sequences of these symbiotic prokaryotes including the endosymbiotic methanogens might be acquired. It is expected that this novel genomic approach will provide a great progress in understanding this complex, multilayered symbiotic system in termite gut.

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