The symbioses of termite gut flagellates and their bacterial endo- and ectosymbionts: analysis of ultrastructure, phylogeny, and cospeciation

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Jürgen F. H. Strassert
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Erster Gutachter:	Prof. Dr. Klaus Hausmann Institut für Biologie Fachbereich Biologie, Chemie, Pharmazie Freie Universität Berlin
Zweiter Gutachter:	Prof. Dr. Andreas Brune Max-Planck-Institut für terrestrische Mikrobiologie Fachbereich Biogeochemie
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Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation

"The symbioses of termite gut flagellates and their bacterial endo- and ectosymbionts: analysis of ultrastructure, phylogeny, and cospeciation"

selbstständig und ohne unzulässige Hilfe sowie ohne Verwendung anderer als der genannten Hilfsmittel angefertigt habe. Des Weiteren habe ich mich keiner als der von mir ausdrücklich benannten Quellen bedient. Die Dissertation wurde noch bei keiner weiteren Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Berlin, den 22. Dezember 2009

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Jürgen Strassert

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1 General Introduction

Termites: biology and taxonomy

Termites are social insects occurring in tropical, subtropical, and temperate regions of the world. Currently, about 2,800 species are known. Several species of wood-feeding termites are renowned for causing serious economic damage. In the United States, the costs for damage repair and termite control may reach up to five billion dollars per year (NPMA, 2006). But termites are also beneficial in that they play an ecologically important role in the decomposition of organic matter, such as wood and leaf litter. They improve the nutrient content of the soil and increase the soil porosity, which leads to a higher water infiltration and a higher water-holding capacity. Their ability to digest lignocellulose makes termites the keystone animals in the global carbon cycle (Sugimoto *et al*., 2000). In the future, termites might play a role in producing more effective biofuels once the microbial metabolic pathways that take place in the termite guts and the cellulolytic enzymes synthesized there are fully elucidated (Schubert, 2006; Warnecke *et al*., 2007).

As social insects, termites divide labor among distinct castes. A colony consists of one or a few pairs of reproductives (kings and queens) and several hundred to several thousand workers and soldiers. Workers are the most abundant members. They forage for food, feed other cast members and the brood, and maintain the nest. Soldiers mainly defend the colony.

Termites were traditionally considered as a monophyletic order (Isoptera), but recent molecular phylogenetic studies suggest that they form a monophyletic group within the cockroaches (Blattodea; Inward *et al*., 2007). Termites can be divided into seven families [Fig. 1; downgrading of these families to subfamilies is proposed by Inward *et al*. (2007)]. The phylogenetically higher termites are represented by only one family (Termitidae), which comprises approximately 85% of all termite species (Ohkuma *et al*., 2001). The phylogenetically lower termites belong to the remaining six families. They do not form a monophyletic group. A main feature distinguishing lower termites from higher termites is the presence of symbiotic flagellates in the hindgut.

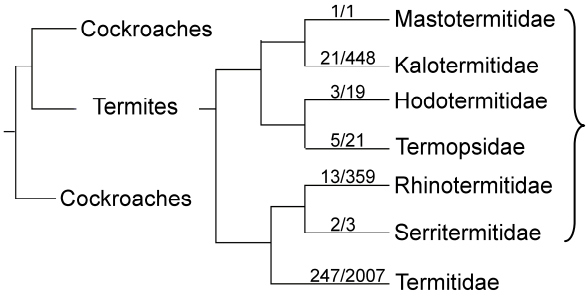

Figure 1. Phylogenetic scheme of the seven termite families (modified after Higashi and Abe, 1997). The numbers indicate the number of genera/species of the respective family.

The termite gut environment

The termite gut is divided into the foregut, midgut, and hindgut. The pH values in the midgut and hindgut of lower and higher termites range between 6 and 7.5 (O'Brien and Slaytor, 1982). More alkaline conditions can be found in the gut of soil-feeding species (Termitidae; e.g., Bignell and Anderson, 1980; O'Brien and Slaytor, 1982; Brune and Kühl, 1996). The midgut is aerobic. The hindgut was long assumed to be anaerobic, representing a purely anoxic fermentor (Bignell and Anderson, 1980; Veivers *et al*., 1980). In the 1990s, more precise measurements with microelectrodes revealed a steep oxygen gradient in the periphery of the hindgut (Brune *et al*., 1995). The periphery of the hindgut is aerobic because oxygen diffuses through the gut wall. Facultatively and obligately aerobic bacterial symbionts consume the oxygen, which leads to a strict anoxic hindgut center (Fig. 2). This in turn enables the simultaneous presence of a strictly anaerobic microbial community in the hindgut. Hydrogen, which is mainly produced by anaerobic flagellates, is another important environmental parameter. The highest hydrogen concentration is found in the center of the hindgut, whereas low concentrations are present in its periphery (Fig. 2). Here, methanogens form a major hydrogen sink (Brune, 1998). The gut environment provides several microhabitats, which allow colonization by microorganisms and flagellates adapted to different ecological niches. The structured environment is maintained by metabolites of these symbionts.

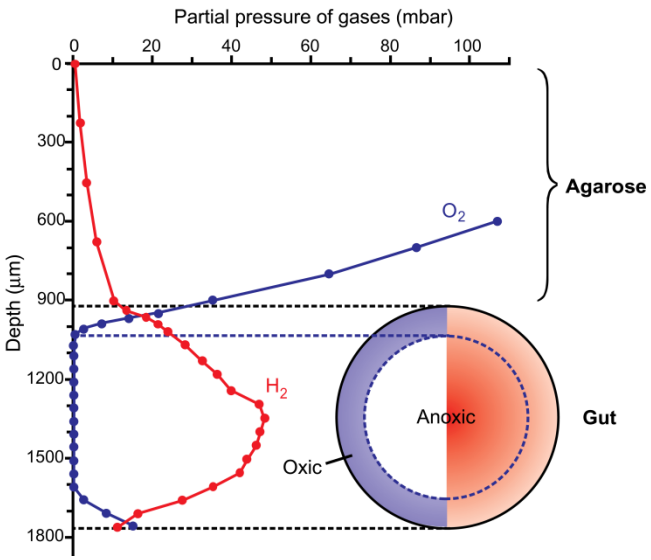

Figure 2. Radial profiles of oxygen and hydrogen in an agarose-embedded hindgut of *Reticulitermes flavipes*. Scheme from Brune (1998).

Symbiotic flagellates

Cellulose degradation

The hindgut of wood-feeding lower termites is inhabited by a multitude of anaerobic, symbiotic flagellates. The hindgut and its contents may constitute about 1/7 to 1/3 of the fresh weight of a termite (Hungate, 1955). The flagellates are involved in the digestion of the cellulose-rich diet of their hosts (e.g., Honigberg, 1970; Breznak and Brune, 1994). In contrast, higher termites, which also feed on cellulose-rich material (e.g., leaves, grass, roots, and humus), do not harbor symbiotic flagellates. Some of the higher termites (subfamily Macrotermitinae) cultivate fungi that predigest their food (Wood and Thomas, 1989). The non-fungus-cultivating higher termites produce their own cellulolytic enzymes in the midgut and salivary glands (Slaytor, 1992; Breznak and Brune, 1994). However, evidence for cellulose degradation by bacterial symbionts of a higher termite species belonging to the genus *Nasutitermes* has recently been presented (Warnecke *et al*., 2007).

Although cellulolytic enzymes (endoglucanases and cellobiases) are also produced in the salivary glands and midgut of lower termites, these termites cannot survive without their symbiotic flagellates. Experimental defaunation showed that the termites die within a few weeks, despite continued feeding (Honigberg, 1970). The first evidence for the cellulolytic activity of the gut flagellates was documented by Yamin and Trager (1979),

Yamin (1980), and Odelson and Breznak (1985a), who were able to cultivate the flagellate *Trichomitopsis termopsidis* in a bacteria-free, axenic medium. By using radiolabeled cellulose as a substrate, the production of CO_2, acetate, and hydrogen was demonstrated (Yamin, 1980).

A dual cellulose-digesting system has been suggested for the termite gut: one originating from the symbiotic gut flagellates and the other originating endogenously from the termite itself (Nakashima *et al*., 2002a; Tokuda *et al*., 2007). This assumption was confirmed by reverse transcription PCR using cellulase-specific primers. For the termite *Coptotermes formosanus*, expression of mRNA encoding cellulases from the glycoside hydrolase family (GHF) 9 was restricted to the salivary glands and the midgut, whereas cellulases from GHF7 were confined to the hindgut (Nakashima *et al*., 2002a) — an environment harboring thousands of cellulolytic flagellates. The lack of secreting epithelial cells in the hindgut (Breznak and Pankratz, 1977) excludes the possibility that the GHF7 cellulases are produced endogenously by the termite itself. Furthermore, additional cellulases (e.g., GHF5 and GHF45) as well as xylanases (GHF8, GHF10, and GHF11) and β-glucosidase (GHF3) have been obtained from various flagellate species, again confirming that cellulose degradation is mainly carried out by the symbiotic gut flagellates (Ohtoko *et al*., 2000; Nakashima *et al*., 2002b; Watanabe *et al*., 2002; Inoue *et al*., 2005; Todaka *et al*., 2007). Following these results, it can be assumed that initially amorphous cellulose is degraded by the endogenous endoglucanase of the termite, whereas the remaining crystalline cellulose is further depolymerized by cellulases (endo- and exoglucanases) originating from the flagellates. In the cytoplasm of the flagellates, the resulting glucose is then glycolytically converted to pyruvate, which is used for ATP synthesis in the hydrogenosomes.

Diversity, phylogeny, and cytology

Yamin (1979) listed more than 430 flagellate species in 205 investigated termite species, with each termite species carrying only a few to more than 20 flagellate species. Each termite species generally has a typical composition of flagellate species (Honigberg, 1970). The termite gut flagellates are affiliated to the lineages Parabasalia and Preaxostyla, which are affiliated to the Excavata (Adl *et al*., 2005).

A typical feature of the Parabasalia (parabasalids; Fig. 3A–I) is the parabasal apparatus. This structure is composed of specific cross-striated fibers arising at the basal bodies and is

attached to the dictyosomes of the Golgi apparatus. Other common features are the microtubular pelta-axostyle complex, the presence of hydrogenosomes (ATP- and molecular-hydrogen-generating organelles), and a pleuromitotic type of division (see Brugerolle and Lee, 2000a). The phylogeny of the parabasalids is still under discussion (e.g., Keeling, 2002; Gerbod *et al*., 2004; Ohkuma *et al*., 2005; Hampl *et al*., 2006; 2007; Noël *et al*., 2007). Based on morphological studies, the parabasalids were traditionally divided into the Trichomonada, which are characterized by 4–6 flagella per mastigont system (multiplication of the karyomastigont is possible), and the Hypermastigida, which have many flagella per mastigont system, a single nucleus, and multiple parabasal bodies (Brugerolle and Lee, 2000a). Recent studies, including sequence analyses of rRNA genes and other molecular markers, subdivide the parabasalids into the following major taxa: Trichomonadida, Cristamonadida, Trichonymphida, and Spirotrichonymphida (Adl *et al*., 2005).

- Trichomonadida (Fig. 3A, B): Have three to five anterior flagella in addition to one recurrent flagellum. Basal body of recurrent flagellum is orthogonal to basal bodies of anterior flagella. A sigmoid fiber and two parabasal fibers are connected with basal body 2. Recurrent flagellum is often associated with an undulating membrane underlain by a striated costal fiber (costa).
- Cristamonadida (Fig. 3C–F): Have three anterior flagella plus a recurrent flagellum (privileged flagella). Hundreds up to thousands of additional flagella originated by multiplication of the karyomastigont (Fig. 3E) or by multiplication of basal body 1 (Fig. 3F). No costa and no undulating membrane are present. Recurrent flagellum is associated with a microfibrillar structure named cresta. Recurrent flagellum and cresta are reduced in several species with multiplied flagella.
- Trichonymphida (Fig. 3G, H): Anterior rostrum is composed of two hemi-rostra associated in bilateral or tetraradiate symmetry. Each hemi-rostrum comprises a set of privileged basal bodies, a flagellar area, sigmoid fibers attached to basal body 2, axostylar fibers, and parabasal fibers. Post-rostral region is bare or flagellated.
- Spirotrichonymphida (Fig. 3I): Have two or more spiraled rows of flagella. Each row is associated with a parabasal fiber. First basal body of each line bears sigmoid fibers associated with the pelta-axostyle complex. Basal bodies are interconnected by a microfibrillar layer. Axostyles are multiple or grouped in a central bundle.

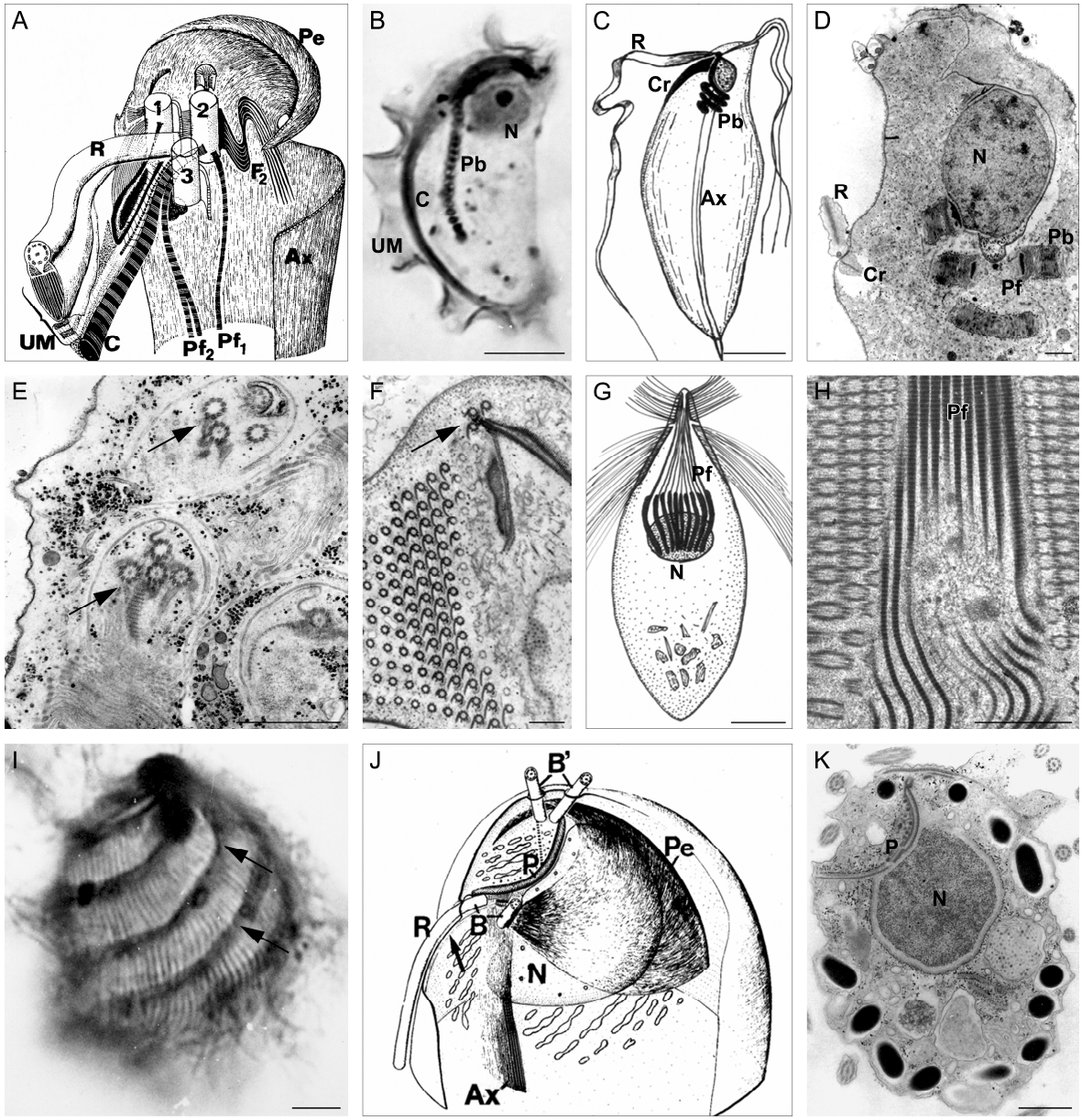

Figure 3. Morphological features of the termite gut flagellates. **A–I:** Parabasalids. **A, B:** Trichomonadida. **A:** Organization of basal bodies and associated fibers in *Tritrichomonas muris*. Basal body of recurrent flagellum (R) is orthogonal to basal bodies of anterior flagella (1, 2, 3). A sigmoid fiber (F_2) and two parabasal fibers (Pf_1, Pf_2) are attached to basal body 2. Two microtubular rows form a pelta (Pe) and axostyle (Ax). A costa (C) supports the undulating membrane (UM). **B:** *Trichomitopsis termopsidis* (protargol staining). Nucleus (N), parabasal body (Pb), costa (C), and undulating membrane (UM). **C–F:** Cristamonadida. **C:** Scheme of *Devescovina* showing the cresta (Cr) and recurrent flagellum (R). The parabasal body (Pb) is wound around the axostyle (Ax). **D:** Transmission electron micrograph of *Devescovina glabra*. Nucleus (N), parabasal body (Pb), parabasal fiber (Pf), cresta (Cr), and recurrent flagellum (R). **E:** Transmission electron micrograph of *Stephanonympha* sp. showing the basal bodies (arrows) of two karyomastigonts. **F:** Transmission electron micrograph of *Joenia annectens* showing three of the four privileged basal bodies (arrow) and the basal bodies of the flagellar area. Basal body of recurrent flagellum not in section plane. **G, H:** Trichonymphida. **G:** *Trichonympha* with rostral and subrostral flagella. Parabasal fibers (Pf) with attached dictyosomes, nucleus (N). **H:** Transmission electron micrograph of the rostrum of *Trichonympha*. Parabasal fibers (Pf) in a longitudinal section. **I:** Spirotrichonymphida. *Spirotrichonympha minor* (protargol staining). Arrows indicate spiraled flagellar rows

underlain by dictyosomes associated to parabasal fibers. **J, K:** Oxymonads. **J:** Scheme of the mastigont system of *Monocercomonoides*. Two pairs of basal bodies (B) are connected by the preaxostyle (P). The nucleus (N) is covered by a pelta (Pe). Axostyle (Ax), recurrent flagellum (R). **K:** Transmission electron micrograph of *Monocercomonoides hausmanni*. Nucleus (N), preaxostyle (P). Scale bars: 10 µm (**B, C**), 1 µm (**D–F**), 50 µm (**G**), 1 µm (**H**), 5 µm (**I**), 1 µm (**K**). Drawings and photographs are from Brugerolle and Lee (2000a; **A**; 2000b; **J**) and Brugerolle and Radek (2006; **C, G, H**), or were kindly provided by R. Radek (**B, D, E, F, I, K).**

The other group of flagellates occurring in the termite gut belongs to the Oxymonadida (oxymonads; Fig. 3J, K). Within the Preaxostyla, the oxymonads represent a sister group of *Trimastix* (Dacks *et al*., 2001; Moriya *et al*., 2003, Adl *et al*., 2005). The oxymonads lack hydrogenosomes and parabasal apparatuses. They have one or more karyomastigonts, each equipped with four flagella. The basal bodies of the flagella are arranged in two pairs that are connected by a preaxostylar lamina. The axostyle, which is contractile in some genera, is composed of parallel rows of microtubules. Anteriorly, the nucleus is usually covered by a single row of microtubules, the pelta (see Brugerolle and Lee, 2000b; Adl *et al*., 2005; Brugerolle and Radek, 2006).

Several taxa are attached to the cuticle of the gut by an anterior holdfast. An extensile rostellum may elongate the distance between holdfast and cell body. Yet the function of this structure is unclear. Since the rostellum can reach more than 250 µm in length, it is likely that it enables the cell body of flagellates to reach the anoxic area in the hindgut without energy-consuming swimming (compare Figs. 2, 4).

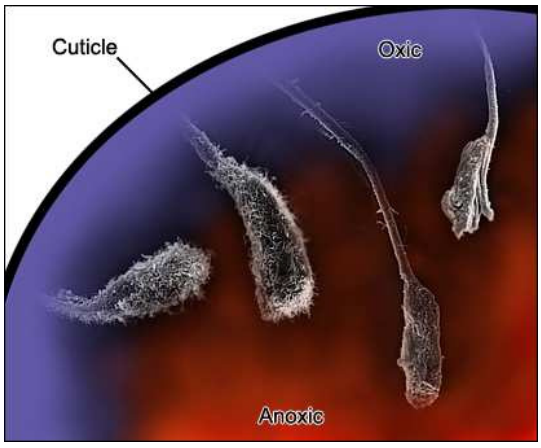

Figure 4. Photomontage of scanning electron micrographs showing *Oxymonas* sp. It is assumed that the long rostellum enables the cell bodies to reach the anoxic center of the hindgut.

Symbiotic bacteria

In lower termites, not only the flagellates but also the prokaryotes are essential for the survival of their hosts (Eutick *et al*., 1978). Although the bacteria are not believed to contribute considerably to the degradation of cellulose, they play a major role in maintaining the chemical environment of the termite gut. During ATP synthesis in the hydrogenosomes of the flagellates, acetate, H_2, and CO_2 are released. Whereas acetate is resorbed by the termites and is used as the major oxidizable energy source and as an important biosynthetic precursor (e.g., Hungate, 1943; Blomquist *et al*., 1979; 1982; Odelson and Breznak, 1983), H_2 and CO_2 are removed by the prokaryotic symbionts. Homoacetogens use H_2 and reduce CO_2 to acetate (e.g., Breznak and Switzer, 1986; Kane and Breznak, 1991; Brune, 2006). In this way, they contribute substantially to the nutrition of the termites. Methanogens use CO_2 or the methyl group of acetate as an electron acceptor (Odelson and Breznak, 1983; 1985b; Breznak and Brune, 1994). When CO_2 is used, H_2 acts as an electron donor. Thus, methanogenesis represents a further important hydrogen sink in the termite gut. Oxygen, which diffuses into the hindgut, is consumed by facultatively or even obligately aerobic bacteria in the gut periphery (Brune *et al*., 1995). The oxygen sink resulting from this activity is a prerequisite for the survival of the obligately anaerobic flagellates.

Since wood has a high C-to-N ratio, symbiotic dinitrogen-fixing bacteria are indispensable for the nutritional requirement of the termites. These bacteria are mainly affiliated to the enterobacteria (e.g., French *et al*., 1976; Potrikus and Breznak, 1977) and spirochetes (e.g., Lilburn *et al*., 2001; Graber *et al*., 2004). The bacteria fix atmospheric nitrogen and synthesize amino acids. Stable isotope analysis has revealed that up to 60% of nitrogen in a wood-feeding lower termite is derived from the atmosphere (Tayasu *et al*., 1998). Other bacteria recycle nitrogen from uric acid and urea, which leads to a high ammonia concentration in the hindgut. The ammonia is then assimilated into microbial biomass. Via proctodeal trophallaxis, this biomass can be digested in the foregut and midgut. The digestion of the bacteria thus closes the nitrogen cycle (for reviews, see Breznak, 2000; Brune, 2006).

Symbiotic bacteria in the hindgut of lower termites can be found free in the gut lumen, attached to the gut wall, or associated with the symbiotic flagellates. However, considering the dense colonization of the gut by symbiotic flagellates, it is not astonishing that the majority of the bacteria (about 90% in the hindgut of *Mastotermes darwiniensis*; Berchtold *et al*., 1999) are associated with these flagellates.

Symbiosis between flagellates and bacteria

Most of the termite gut flagellates are associated with bacterial symbionts (see reviews by Brune and Stingl, 2005; Ohkuma, 2008). The bacteria can be found attached to the plasma membrane of the flagellates (ectosymbionts) and/or in the cytoplasm or even the nucleus of their hosts (endosymbionts). The symbiosis between the flagellates and prokaryotes seems to be quite specific. Earlier light microscopy studies have already shown that distinct species of termite gut flagellates are always associated with identical morphotypes of bacterial symbionts; the presence or absence of these symbionts was even used as a distinguishing mark for species descriptions (e.g., Light, 1926; Kirby, 1945). Today, the specificity of the symbioses is documented by fluorescent *in situ* hybridization (FISH) using phylotype-specific oligonucleotide probes. With this method, several host–symbiont pairs and even cospeciation between the two partners have been detected (Ikeda-Ohtsubo *et al*., 2007; Noda *et al*., 2007; Ikeda-Ohtsubo and Brune, 2009). Since generally the hindgut microorganisms cannot be cultivated, little is known about the functional aspects of the symbiosis between termite gut flagellates and their bacterial symbionts (Brune and Stingl, 2005). However, the system is assumed to be stable because of an exchange of metabolites.

Since all methanogens of the genus *Methanobrevibacter* are known to utilize H_2 and CO_2 (methanogenesis), it is highly likely that also the ecto- and endosymbionts affiliated with *Methanobrevibacter* utilize H_2 and CO_2 (Tokura *et al*., 2000). The spirochetes from termite guts obtained so far belong exclusively to the genera *Treponema* (Lilburn *et al*., 1999; Ohkuma *et al*., 1999) and *Spirochaeta* (Dröge *et al*., 2006). Reports of acetogenesis in *Treponema* strains isolated from termite guts (Leadbetter *et al*., 1999; Graber *et al*., 2004) support the assumption that treponemes associated with the gut flagellates also carry out acetogenesis. The same argument applies to dinitrogen fixation, which was documented for treponemes isolated from termite guts by Lilburn *et al*. (2001) and Graber *et al*. (2004). An unequivocal assignment of this function to a bacterial symbiont was recently presented by Hongoh *et al*. (2008), who showed that the Bacteroidales endosymbionts of *Pseudotrichonympha grassi* are able to fix dinitrogen. Motility symbioses were documented for the spirochetal symbionts of *Mixotricha paradoxa* (Cleveland and Grimstone, 1964) and the "*Synergistes*" symbionts of *Caduceia versatilis* (Tamm, 1982; Hongoh *et al*., 2007). In both cases, the motile bacteria propel their eukaryotic host cells to which they are attached. Other hypothesized functions of the

ectobiotic symbionts comprise involvement in maintaining the cell form of the host flagellate (Radek *et al*., 1996; Leander and Keeling, 2004) and support in providing an anoxic environment to their host flagellate (Baughn and Malamy, 2004).

Generally, however, the roles of the symbionts are still obscure. To understand the nature of these symbiotic interactions, further characterizations of the two uncultivable partners on phylogenetic and ultrastructural levels are required.

Aims of the studies

The overall goal of my studies was to demonstrate the specificity of the flagellate/bacteria symbioses. To do this, the validity of the termite flagellate genera/species assignments first needed to be verified using morphological as well as molecular phylogenetic techniques since the phylogeny of termite gut flagellates was still not clear. This is not only true for the validity of the four major taxa of the parabasalids (see above), but also for classifications on the generic and species level.

The most recent example of an assignment error was given by Harper *et al*. (2009), who showed that *Coronympha octonaria* and *Metacoronympha senta* are neither different species nor distinct genera, but are structurally different life cycle stages of the same species. There are similar discrepancies in the assignment of the devescovinid flagellates, which occur in almost all dry-wood termites investigated (Yamin, 1979). In this case, the validity of the genus *Metadevescovina* was questionable. Light (1926) separated this genus from the genus *Devescovina* by the presence of twelve additional flagella. Later, these "flagella" were considered as spirochetal symbionts occurring in different numbers attached to the host flagellate (Grassé, 1938; Kirby, 1945). Consequently, *Metadevescovina* was considered as a synonym for *Devescovina* by Grassé (1952). In contrast, the validity of the genus *Metadevescovina* was still supported by Kirby (1945) and Pérez-Reyes and López-Ochoterena (1965). However, the morphological traits, which were used for this classification, were completely different. Additional discrepancies on the species level classification are conspicuous. For the termite *Incisitermes marginipennis*, Kirby (1945) described two species of *Metadevescovina*, whereas Pérez-Reyes and López-Ochoterena (1965) described ten devescovinid species belonging to the genera *Devescovina* and *Metadevescovina*.

One of my aims was to investigate the true diversity of the devescovinid flagellates in the termite *I. marginipennis*. Furthermore, the validity of the genus *Metadevescovina* needed to be clarified (Chapter 2). Molecular phylogenetic analysis of the small subunit (SSU) rRNA genes of the gut flagellates was combined with morphological techniques, including the first ultrastructural analysis of devescovinids classified as *Metadevescovina* symbionts. This work was linked to a study investigating the evolutionary history of the symbiosis between *Devescovina* spp. and their bacterial symbionts.

Another morphological trait distinguishing *Metadevescovina* from *Devescovina* proposed by Kirby (1941; 1945) was the presence of rod-shaped (filamentous) bacteria, which are always attached to *Devescovina* and are generally absent from *Metadevescovina*. However, little is known about the obligation of these associations.

Another aim was to determine the intimacy of the symbiosis between *Devescovina* spp. and the ectosymbionts. The phylogenies of the host flagellates and their bacterial symbionts were reconstructed and compared based on their SSU rRNA gene sequences (cospeciation analysis; Chapter 3). The flagellates and their ectosymbionts were finally identified by morphological analyses using scanning electron microscopy.

During the last decade, a number of bacterial gut flagellate symbionts have been identified based on molecular phylogenetic analyses. The ectosymbionts were affiliated to novel lineages of the Bacteroidales (Noda *et al*., 2006), methanogens (Tokura *et al*., 2000), and treponemes (Iida *et al*., 2000; Noda *et al*., 2003). The endosymbionts were also affiliated to the Bacteroidales (Noda *et al*., 2005) and methanogens (Tokura *et al*., 2000), and also to the candidate class 'Endomicrobia' (Stingl *et al*., 2005). Congruent diversification of the bacterial symbionts and the host flagellates leading to a cospeciation of the two partners was recently shown for endosymbionts of the Bacteroidales (Noda *et al*., 2007) and 'Endomicrobia' (Ikeda-Ohtsubo and Brune, 2009). In most cases, however, the phylogenetic affiliation of the symbionts and the specificity of the symbiosis are still obscure. One example is the multitude of bacterial symbionts associated with the termite

gut flagellate *Joenia annectens*, distinguished by their morphological characteristics by Radek *et al*. (1992).

Another aim of my studies was to characterize the multiple associations of *J. annectens* with its yet unidentified symbionts (Chapter 4). The following questions were answered: (i) To which lineages of bacteria are the symbionts affiliated? (ii) Do the subcellular locations of the diverse lineages differ? (iii) Do the bacterial symbionts have a host specificity for *J. annectens*? To answer these questions, the SSU rRNA gene sequences of the host flagellate and its bacterial symbionts were analyzed. To identify and localize the bacteria in and on the host cells, FISH using specifically designed oligonucleotide probes was combined with scanning and transmission electron microscopy.

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2 The true diversity of devescovinid flagellates in the termite *Incisitermes marginipennis*

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The true diversity of devescovinid flagellates in the termite *Incisitermes marginipennis*

Jürgen F. H. Strassert, Mahesh S. Desai, Andreas Brune, and Renate Radek

Summary

More than 40 years ago, ten species of devescovinid flagellates were described to occur in the gut content of the termite *Incisitermes marginipennis*. Based on light microscopic examinations, the flagellates were then classified into the two genera *Devescovina* and *Metadevescovina*. Here, we combined molecular phylogenetic analysis of the small subunit rRNA genes of the gut flagellates with the first ultrastructural investigation of the genus *Metadevescovina*. Our results suggest that *I. marginipennis* contains only one species of devescovinid flagellates, *Metadevescovina modica*, which comprises three variants of the same phylotype (≥99.5% sequence similarity). Monophyly of all *Metadevescovina* sequences obtained from *Pterotermes* and *Incisitermes* species in this and previous studies and the absence of filamentous bacterial epibionts typical of *Devescovina* species (*M. modica* is densely colonized with spirochetes) corroborate the validity of the genus *Metadevescovina* and allow its differentiation from other genera of devescovinid flagellates.

3 Strict cospeciation of devescovinid flagellates and *Bacteroidales* ectosymbionts in the gut of dry-wood termites (Kalotermitidae)

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Strict cospeciation of devescovinid flagellates and *Bacteroidales* ectosymbionts in the gut of dry-wood termites (Kalotermitidae)

Mahesh S. Desai, Jürgen F. H. Strassert, Katja Meuser, Horst Hertel, Wakako Ikeda-Ohtsubo, Renate Radek, and Andreas Brune

Summary

The surface of many termite gut flagellates is colonized with a dense layer of bacteria, yet little is known about the evolutionary relationships of such ectosymbionts and their hosts. Here we investigated the molecular phylogenies of devescovinid flagellates (*Devescovina* spp.) and their symbionts from a wide range of dry-wood termites (Kalotermitidae). From species-pure flagellate suspensions isolated with micropipettes, we obtained SSU rRNA gene sequences of symbionts and host. Phylogenetic analysis showed that the *Devescovina* spp. present in many species of Kalotermitidae form a monophyletic group, which includes also the unique devescovinid flagellate *Caduceia versatilis*. All members of this group were consistently associated with a distinct lineage of *Bacteroidales*, whose location on the cell surface was confirmed by fluorescence *in situ* hybridization. The well-supported congruence of the phylogenies of devescovinids and their ectosymbionts documents a strict cospeciation. In contrast, the endosymbionts of the same flagellates ("*Endomicrobia*") were clearly polyphyletic and must have been acquired independently by horizontal transfer from other flagellate lineages. Also the *Bacteroidales* ectosymbionts of *Oxymonas* flagellates present in several Kalotermitidae belonged to several distantly related lines of descent, underscoring the general perception that the evolutionary history of flagellate–bacteria symbioses in the termite gut is complex.

4 Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens*

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Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens*

Jürgen F. H. Strassert, Mahesh S. Desai, Renate Radek, and Andreas Brune

Summary

The hindgut of wood-feeding lower termites is densely colonized by a multitude of symbiotic microorganisms. While it is well established that the eukaryotic flagellates play a major role in the degradation of lignocellulose, much less is known about the identity and function of the prokaryotic symbionts associated with the flagellates. Our ultrastructural investigations of the gut flagellate *Joenia annectens* (from the termite *Kalotermes flavicollis*) revealed a dense colonization of this flagellate by diverse ecto- and endobiotic bacteria. Phylogenetic analysis of the small subunit rRNA gene sequences combined with fluorescence *in situ* hybridization allowed us to identify and localize the different morphotypes. Furthermore, we could show that *K. flavicollis* harbors two phylotypes of *J. annectens* that could be distinguished not only by their small subunit rRNA gene sequences, but also by differences in their assemblages of bacterial symbionts. Each of the flagellate populations hosted phylogenetically distinct ectosymbionts from the phylum Bacteroidetes, one of them closely related to the ectosymbionts of other termite gut flagellates. A single phylotype of 'Endomicrobia' was consistently associated with only one of the host phylotypes, although not all individuals were colonized, corroborating that the latter are not always cospeciating with their host lineages. Flagellates from both populations were loosely associated with a single phylotype of Spirochaetales attached to their cell surface in varying abundance. Current evidence for the involvement of Bacteroidales and 'Endomicrobia' symbionts in the nitrogen metabolism of the host flagellate is discussed.

5 General Discussion

My studies of termite gut flagellates focused on two main aspects: the identity and validity of flagellate genera/species and their multiple associations with prokaryotic ecto- and endosymbionts.

The validity of the genus *Metadevescovina*, which has long been discussed, was definitely confirmed by the combination of light microscopy with ultrastructural and molecular phylogenetic analyses. Our studies showed that based on morphological characteristics, the presence of different ectobiotic bacteria is the only reliable feature that allows the genera *Metadevescovina* and *Devescovina* to be differentiated. Spirochetes are attached to *Metadevescovina*, whereas rod-shaped Bacteroidales symbionts, which cospeciate with their hosts, are attached to *Devescovina*. Also in another model system of flagellate/bacteria interactions, i.e., with the parabasalid *Joenia annectens* and diverse bacteria, typical associations were found. By combining molecular and morphological techniques, my colleagues and I demonstrated that there are two phylotypes of *J. annectens* that can be distinguished not only by their SSU rRNA gene sequences, but also by differences in their assemblages of bacterial symbionts.

Individual results obtained in our studies have already been discussed in the respective chapters. In the following, general aspects of the determination of the true diversity of termite gut flagellates are presented. Moreover, this chapter addresses general as well as thesis-related not-yet discussed aspects about the specificity of the bacterial symbionts for their host flagellates and their possible functions in the flagellate–bacteria symbiosis. Finally, I propose possible candidates for dinitrogen fixation in the bacterial symbionts investigated in the studies.

Determination of the true diversity of termite gut flagellates

In 205 investigated termite species, more than 430 flagellate species, which were affiliated to approximately 80 different genera, were listed by Yamin (1979). However, for termite gut flagellates, the species definition as a group of interbreeding organisms (biological species concept; Mayr, 1963) cannot be generally applied because reports of sexual reproduction are extremely rare (e.g., Messer and Lee, 1989). Furthermore, termite gut

flagellates have only rarely been successfully cultivated (e.g., Trager, 1934; Yamin, 1978a; 1978b; 1981). Owing to methodological reasons, most of these flagellates have to date been identified solely based on light microscopy using the species concept of distinct morphospecies (Ruse, 1969). The morphological changes in populations from one generation to the next can be gradual or erratic; therefore, this concept can lead to misidentification of diverse species. Several such misidentifications have recently been revealed by molecular phylogenetic studies. For instance, *Pyrsonympha* and *Dinenympha*, which were considered as morphotypes of the same organism, were shown by SSU rRNA gene sequence analysis to form two separate genera (Moriya *et al*., 2003; Stingl and Brune, 2003). Using the same phylogenetic marker, the opposite (the fusion of two genera that appear to be morphologically distinct) was documented for *Coronympha* and *Metacoronympha* (Harper *et al*., 2009). Furthermore, cryptic species diversities were shown e.g., for *Coronympha octonaria* obtained from different termite species (Harper *et al*., 2009) and oxymonads from *Reticulitermes flavipes* (Stingl and Brune, 2003).

In view of these results, one could conclude that solely molecular phylogenetic analyses are sufficient to determine the diversity of termite gut flagellates. However, also the application of molecular phylogenetic techniques can lead to incorrect interpretations of protozoan diversity. For instance, with respect to the biological species concept, these techniques only barely allow the determination of the moment when a population splits. Moreover, multiple copies of the SSU rRNA gene (the most common marker gene used for diversity studies of termite gut flagellates) showing slight variations may cause microheterogeneity within a distinct species, making identification of a single population more difficult (e.g., Caraguel *et al*., 2007; O'Mahony *et al*., 2007). Finally, different interpretations of the phylogeny of parabasalids can be inferred from phylogenetic analyses using different molecular markers, such as glyceraldehyde-3-phosphate dehydrogenase, enolase, α-tubulin, and β-tubulin gene sequences (Gerbod *et al*., 2004).

The above examples clearly document that either morphological or molecular phylogenetic evidence alone can lead to misinterpretations of the diversity of termite gut flagellates. Nevertheless, in combination, these techniques should complement each other by verifying the results obtained. Especially the excellent light microscopy studies of L. R. Cleveland, F. De Mello, I. F. De Mello, and H. Kirby — conducted during the first half of the 20th century — often agree with molecular phylogenetic evidence, confirming the results of both tools. Since the 1960s, ultrastructural analyses using electron microscopy

improved the quality of morphological descriptions of termite gut flagellates. Based on such studies, the order Cristamonadida, including devescovinid, calonymphid, and lophomonad flagellates, was established (Brugerolle and Patterson, 2001). The monophyly of this order was later supported by analyses of the parabasalid SSU rRNA gene sequences (e.g., Keeling, 2002; Hampl *et al*., 2004; Noël *et al*., 2007), which verified the earlier result. Another example is given in my study (Chapter 2), where the results obtained by light and electron microscopy agree with the results obtained by molecular phylogenetic analyses. The application of both techniques clearly showed that there is only one species of *Metadevescovina* in the termite *Incisitermes marginipennis*.

Therefore, in conclusion, I strongly suggest that for studying the diversity of termite gut flagellates, a combination of molecular phylogenetic and morphological analyses be used. Molecular phylogeny is a powerful tool for detecting cryptic species diversity or morphological variations within a single species, and morphological analyses can provide information about the reliability of the marker gene used. In some cases, the termite gut flagellate phylogeny based on molecular analyses differs from that based on morphological characteristics (Gerbod *et al*., 2004; Hampl *et al*., 2004; Noël *et al*., 2007). To resolve these discrepancies, I recommend using different methods of light microscopy (e.g., protargol and DAPI staining, immunostaining of different proteins) as well as ultra-structural and molecular phylogenetic analyses using various marker genes.

Specificity of the bacterial symbionts for their host flagellates

Most of the termite gut flagellates are closely associated with bacterial symbionts. Even in early light microscopic descriptions, the presence and arrangement of distinct morphotypes of bacteria were used as criteria to distinguish different flagellate species or genera (e.g., Light, 1926; Kirby, 1941; 1945). In recent studies, molecular phylogenetic analyses including FISH allowed the bacterial symbionts to be identified. Host specificity of several symbionts has been shown (e.g., Ikeda-Ohtsubo *et al*., 2007). As a result of a strict association between flagellates and their bacterial symbionts, cospeciation of the two partners was documented. The first report was that of *Pseudotrichonympha* species with "*Azobacteroides pseudotrichonymphae*" (Noda *et al*., 2007). However, since *Pseudo-trichonympha* spp. also cospeciate with their host termites, it cannot be excluded that cospeciation with their bacterial symbionts is merely caused by a spatial separation of the

flagellate hosts within their respective termite species. To date, cospeciation between flagellates not cospeciating with the host termites and their bacterial symbionts was documented only for *Trichonympha* spp. with "*Endomicrobium trichonymphae*" (Ikeda-Ohtsubo and Brune, 2009). In this case, a strict vertical transmission of the symbionts within their host lineage is a prerequisite for explaining cospeciation of the symbiotic pairs.

We showed that the bacterial symbionts of *Devescovina* and *Metadevescovina* represent the only reliable distinguishing mark that allow these two devescovinid genera to be separated based on morphological characteristics (Chapters 2 and 3). According to previous reports of ectobiotic spirochetes associated with *Metadevescovina* spp. (Kirby, 1945), the ectosymbionts of *Metadevescovina modica* were clearly identified as spirochetes using transmission electron microscopy, which showed special attachment structures (Chapter 2). The typical associations with spirochetal ectosymbionts raise the question whether these symbionts have been acquired independently by multiple horizontal transfers from other gut flagellates or free-living spirochetes in the termite guts, or whether they have been acquired by a common ancestor of *Metadevescovina* spp. covered by spirochetes, i.e., by vertical transmission. The latter requires strict host specificity of the spirochetal symbionts.

Reports of single spirochetal phylotypes attached to host flagellates of different genera (Iida *et al*., 2000; Noda *et al*., 2003) do not necessarily exclude a host specificity of spirochetes attached to *Metadevescovina* species. Whereas host specificity and even cospeciation was clearly shown for *Devescovina* spp. with "*Armantifilum devescovinae*" (Chapter 3) and *Trichonympha* spp. with "*Endomicrobium trichonymphae*" (Ikeda-Ohtsubo and Brune, 2009), polyphyly indicating a horizontal transfer between different flagellate lineages was documented, for example, for Bacteroidales symbionts of oxymonads (Noda *et al*., 2009; Chapter 3) and 'Endomicrobia' symbionts of *Devescovina* spp. (Chapter 3). However, an argument questioning the acquisition of spirochetes by a common ancestor of all *Metadevescovina* species is given by the report of "spirochete-like" Bacteroidales being attached to *Metadevescovina cuspidata* (Hongoh *et al*., 2007). Although also spirochetes are attached to *M. cuspidata*, this finding raises the question whether there are *Metadevescovina* species lacking spirochetal symbionts. If so, it is also possible that host specificity of spirochetes is valid only for a distinct cluster of *Metadevescovina* species, comparable to "*Endomicrobium trichonymphae*", where only the *Trichonympha* flagellates of "*Trichonympha* cluster I" harbored 'Endomicrobia' symbionts

(Ikeda-Ohtsubo and Brune, 2009). To clarify such questions about vertical or horizontal transfer of spirochetal symbionts of *Metadevescovina* spp., analyses comparing the molecular phylogenies of both partners are required.

We showed a strict cospeciation of termite gut flagellates with their bacterial symbionts, i.e., of *Devescovina* spp. with their Bacteroidales ectosymbionts ("*Armantifilum devescovinae*"; Chapter 3). This was the first report of cospeciation of an ectobiotic symbiont with its host protist. In contrast to endosymbionts, vertical transmission of ectosymbionts is hampered by the possibility of dissociation of loosely attached symbionts reattaching to another host flagellate. Despite the ectobiotic association of "*A. devescovinae*" and the horizontal transfer of the host flagellates between different termite species belonging to the Kalotermitidae, a strict cospeciation of the symbiotic pairs was detected. This indicates that symbiosis between these two partners provides a selective advantage against the non-associated *Devescovina* and Bacteroidales cells occurring in the hindguts of the Kalotermitidae. Based on the evolutionary rates of the SSU rRNA genes, the beginning of the symbiosis between the common ancestor of *Devescovina* spp. and the common ancestor of Bacteroidales ectosymbionts was calculated to be 50–100 million years ago (Desai, 2008).

The Bacteroidales ectosymbionts of the two *Joenia annectens* phylotypes did not show strict host specificity (Chapter 4). Although generally each phylotype of *J. annectens* was covered by only the canonical ectosymbionts (one phylotype), in rare cases; symbionts of both Bacteroidales phylotypes were attached to a single host flagellate, which indicated horizontal transfer of the symbionts. Since it was shown for parabasalid flagellates not only that the Bacteroidales symbionts are specific for their host but also that each flagellate species is associated with only one Bacteroidales phylotype (Noda *et al*., 2009), these findings represent a special case. The two closely related phylotypes of *J. annectens* (99.2% sequence similarity) probably provide only slightly different habitats for their symbionts, thereby allowing also non-canonical Bacteroidales symbionts to colonize. However, more effective symbiotic interactions between the two phylotypes of *J. annectens* and their respective canonical ectosymbionts could explain the rare occurrence of this exceptional interaction.

One of the two detected phylotypes of 'Endomicrobia' was found exclusively in the cytoplasm of one of the two *J. annectens* phylotypes. However, about half of the potential host flagellates did not harbor these symbionts. A probable reason explaining the absence

of the endosymbionts is discussed below (see "Possible candidates for dinitrogen fixation"). The spirochetal symbionts of *J. annectens* were not localized by FISH. Based on ultrastructural analysis, a host specificity of the spirochetal symbionts is not likely. Different morphotypes could not be found attached to the two phylotypes of *J. annectens*. Thus, a strict functional interdependence between the spirochetal symbionts and their host flagellate species is not assumed.

Until now, strict host specificity leading to a cospeciation of termite gut flagellates and their bacterial symbionts was exclusively documented for the bacteria affiliated to the Bacteroidales and 'Endomicrobia' (see above). However, there are also members of Bacteroidales and 'Endomicrobia' symbionts that were independently acquired by the host flagellates from a pool of diverse bacteria in the gut community or were horizontally transferred between the host flagellates (Noda *et al*., 2007; Ikeda-Ohtsubo and Brune, 2009; Chapter 3), which indicates that the evolutionary history of symbioses between termite gut flagellates and these bacterial symbionts is complex. The mechanisms leading to a strict host specificity of the bacterial symbionts are still mostly unclear. Physiological interactions causing a selective advantage and a mutual dependence of the two partners are highly likely. To clarify such questions, we have recently started studies addressing the function of the bacterial symbionts.

Functional aspects of the bacterial symbionts

In these studies, the assignment of various bacteria to a single flagellate host species gives an idea of the complex interactions presumably taking place between prokaryotes and their eukaryotic host cell. The functional aspects of the symbiosis between termite gut flagellates and their bacterial symbionts have long remained obscure because the hindgut microorganisms generally could not be cultivated (Brune and Stingl, 2005). These bacteria were finally identified and localized using molecular techniques, specifically FISH. The resulting reports of host specificity or cospeciation (Ikeda-Ohtsubo *et al*., 2007; Noda *et al*., 2007; Ikeda-Ohtsubo and Brune, 2009) of Bacteroidales or 'Endomicrobia' with the host flagellates lead to the assumption that an exchange of metabolites is responsible for the symbiosis. The hypothesis that these bacterial symbionts are involved in nitrogen metabolism was recently confirmed by complete genome sequence analyses in other studies. For instance, Hongoh *et al*. (2008a) showed that the Bacteroidales endosymbionts

of *Pseudotrichonympha grassi* are able to fix dinitrogen, and the authors suggested that the fixed nitrogen can be assimilated and subsequently used to synthesize diverse amino acids. A transamination (upgrading) of the amino acid glutamine, which is provided by the host flagellate, was proposed for the 'Endomicrobia' symbionts of *Trichonympha agilis* (Hongoh *et al*., 2008b). The new amino acids may then be used by the flagellates. In return, it is likely that the bacteria ferment sugars, which are imported from the cytoplasm of the host flagellates (Hongoh *et al*., 2008a, b). The ability to fix dinitrogen was also documented for termite gut spirochetes affiliated to the genus *Treponema* (Lilburn *et al*., 2001; Graber *et al*., 2004). An involvement in the nitrogen nutrition of the termites, whose food is low in nitrogen, is assumed. Besides dinitrogen fixation, acetate production from H_2 and CO_2 was reported for these treponemes (Leadbetter *et al*., 1999); acetate provides a major carbon and energy source for the termites (Breznak, 1994).

Possible candidates for dinitrogen fixation

The termite gut flagellates investigated in these studies showed typical associations with distinct bacterial symbionts. Assuming that the flagellates rely on an additional nitrogen source, it is highly likely that these symbionts, which are indispensable for their hosts, fix dinitrogen and supply nitrogenous compounds to their host flagellates. This would mean that for *Metadevescovina*, which often completely lack rod-shaped ecto- or endosymbionts, possibly the ectobiotic spirochetes that densely cover the cells (Kirby, 1945; Chapter 2) are involved in dinitrogen fixation. However, caution must be taken because some bacterial symbionts of *Metadevescovina* have been described only using light microscopy, and some of the spirochetal symbionts of *Metadevescovina cuspidata* were later shown by molecular methods not to be spirochetes but rather "spirochete-like" (bristle-shaped) Bacteroidales (Hongoh *et al*., 2007); identification of such symbionts by molecular means is required to postulate whether ectobiotic spirochetes or other bacterial species are involved in dinitrogen fixation. To assign the function to a symbiont, a useful approach would be the complete genome sequence analysis of the symbiont, which would allow putative metabolic pathways to be identified. Another approach is to amplify genes involved in dinitrogen fixation (*nifH*, *anfH*). An unambiguous approach is to detect the activity of these genes using simultaneous FISH of mRNA and rRNA (Pernthaler and Amann, 2004; Pernthaler and Pernthaler, 2005; Pilhofer *et al*., 2009).

In contrast to *Metadevescovina*, flagellates of the genus *Devescovina* are densely covered by filamentous rods, whereas spirochetes are absent or present only in low numbers (Kirby, 1941; Radek *et al*., 1996; Chapter 3). The ectobiotic rods affiliated to the Bacteroidales cospeciate with their host flagellates. This cospeciation is maintained despite a horizontal transfer of flagellates among several termite species belonging to the Kalotermitidae (Chapter 3). This indicates that cospeciation is not merely caused by spatial separation of the flagellates in their host termites, but is a result of an obligate symbiosis leading to strict vertical transmission of the symbionts by their host flagellates. With respect to these findings, the Bacteroidales ectosymbionts represent likely candidates for symbionts that exchange nutrients with their host flagellates. The 'Endomicrobia' endosymbionts of *Devescovina*, on the other hand, probably are involved in non-obligatory interactions because they were probably acquired by horizontal transfers from other termite gut flagellates. Thus, it is hypothesized that the Bacteroidales ectosymbionts either fix dinitrogen or supply nitrogenous compounds to their host flagellates or both. This hypothesis is currently being tested by M. Desai from the MPI for Terrestrial Microbiology, Marburg.

The termite gut flagellate *Joenia annectens* is associated with bacterial symbionts belonging to the Bacteroidales, 'Endomicrobia', and *Treponema* (Chapter 4). Here, the irregular occurrence of the treponemes (none, few, or many cells) associated with *J. annectens* indicates the lack of functional interdependence between the two partners. One of the two detected 'Endomicrobia' phylotypes was present only in one of the two host flagellate phylotypes (see Chapter 4), which, however, also occurred just as frequently without these symbionts. Considering the specificity of this symbiosis, a mutualistic relationship between the 'Endomicrobia' and their host flagellates is likely. Nevertheless, the benefit gained by each partner is probably low since the relationship can be ended by loss of the symbionts. Whether the other *J. annectens* phylotype lost its 'Endomicrobia' symbionts or whether it was never associated with them remains unclear. Since the second detected phylotype of 'Endomicrobia' is closely related to free-living 'Endomicrobia' and only distantly related to intracellular members of 'Endomicrobia' (Ikeda-Ohtsubo *et al*., 2010), it is highly likely that this phylotype has never been associated with *J. annectens*. 'Endomicrobia' could be lost or not even ever acquired if there are other symbionts that supply nitrogen compounds to the host. Likely candidates for this function are the two Bacteroidales phylotypes that generally cover the surface of the two phylotypes of *J.*

annectens. I am currently examining the evidence for dinitrogen fixation in these Bacteroidales symbionts. So far, reverse transcription PCR has revealed the expression of *nifH* genes of *Treponema* and Bacteroidales in *Kalotermes flavicollis*, the host termite of *J. annectens*. To link *nifH* gene expression to the symbionts, I have recently begun simultaneous FISH of mRNA and rRNA.

The functions of the symbiosis between termite gut flagellates and bacteria are not yet fully understood. In the studies described in this thesis, the identification of the partners as well as localization of the bacterial symbionts fulfill prerequisites for understanding the complex interactions taking place between flagellates and their bacterial symbionts.

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Summary

The complex mechanisms leading to a tripartite symbiosis involving bacteria, flagellates, and host termites are not yet fully understood. While the flagellates are known to play a major role in the degradation of the cellulosic food of the termites, in most cases, the functions of the diverse flagellate-associated bacteria are completely obscure. Unambiguous identification of the mostly uncultivable prokaryotes and eukaryotes is an important step in understanding the mutual interactions between the two partners. For this purpose, in the studies described in my thesis, morphological investigations (light microscopy and electron microscopy) were combined with molecular phylogenetic analyses (full-cycle-rRNA approach).

In two earlier light microscopy studies, other authors reported contradicting numbers of devescovinid flagellates occurring in the hindgut of the dry-wood termite *Incisitermes marginipennis*. We clearly and unambiguously documented the presence of only one devescovinid species (*Metadevescovina modica*) inhabiting the gut of *I. marginipennis* using a combination of various light and electron microscopy techniques and molecular phylogenetic analysis of the small subunit (SSU) rRNA gene sequences. Moreover, we confirmed the validity of the genus *Metadevescovina*, which had long been discussed as being the same as the genus *Devescovina*; monophyly of each of the genera was revealed by molecular phylogenetic analyses. *Metadevescovina* could not be distinguished from *Devescovina* solely by morphological characteristics of the flagellates themselves, but the two flagellate genera could be differentiated by examining their bacterial symbionts. The cell surface of *Metadevescovina* flagellates is densely colonized with spirochetes, and that of *Devescovina* flagellates is densely covered with filamentous bacteria affiliated to the Bacteroidales.

Molecular phylogenetic analyses of *Devescovina* spp. and their bacterial symbionts from a wide range of Kalotermitidae revealed that the termites acquired two bacterial symbionts by two different routes: vertical transmission and horizontal transmission. The ectosymbionts of *Devescovina* spp. form a monophyletic group within the Bacteroidales ("*Candidatus* Armantifilum devescovinae"). Congruence analyses of the phylogenetic trees of *Devescovina* spp. and "*Candidatus* Armantifilum devescovinae" documented a strict cospeciation of the partners, which indicated an obligate symbiosis, leading to a

vertical transmission of the bacteria within their host lineages. The 'Endomicrobia' endosymbionts of *Devescovina* spp. are most closely related to endosymbionts of phylogenetically unrelated termite gut flagellates, which indicated that these symbionts were acquired by horizontal transmission between different flagellate species present in the same termite gut.

In a further study documented in this thesis, the multiple symbionts of the flagellate *Joenia annectens* from the dry-wood termite *Kalotermes flavicollis* were identified, localized using a full-cycle-rRNA approach, and morphologically described at the ultrastructural level. Two populations of *J. annectens* could be distinguished not only by their SSU rRNA gene sequences (0.8% sequence divergence), but also by differences in their assemblages of bacterial symbionts. Each of the flagellate populations hosted phylogenetically distinct ectosymbionts from the phylum Bacteroidetes, while a single phylotype of 'Endomicrobia' was consistently associated with only one of the host phylotypes. However, not all individuals were colonized, once again corroborating that 'Endomicrobia' are not always cospeciating with their host lineages.

The results reported in my thesis provide important information about the specificity of the symbioses between termite gut flagellates and their bacterial symbionts. This information is necessary for further studies of the function of these symbioses. A possible involvement of bacterial symbionts in the nitrogen metabolism of the host flagellates is discussed.

Zusammenfassung

Die komplexen Mechanismen, welche der Dreiersymbiose zwischen Bakterien, Flagellaten und ihren Wirtstermiten zugrundeliegen, sind bis heute noch weitgehend unverstanden. Während den Flagellaten eine wesentliche Rolle beim Abbau der cellulosehaltigen Nahrung der Termiten zugeschrieben wird, sind die Funktionen der diversen, mit den Flagellaten assoziierten Bakterien in den meisten Fällen komplett unbekannt. Ein wichtiger Schritt für die Erforschung der wechselseitigen Beziehungen zwischen den in der Regel nicht kultivierbaren pro- und eukaryotischen Symbionten stellt deren eindeutige Identifizierung dar. Dazu wurden in den vorliegenden Studien morphologische Untersuchungen (Licht- und Elektronenmikroskopie) beider Partner mit molekular-phylogenetischen Analysen kombiniert (full-cycle-rRNA approach).

In zwei vorangegangenen lichtmikroskopischen Studien anderer Autoren wurde für die Trockenholztermite *Incisitermes marginipennis* eine widersprüchliche Artenanzahl von devescoviniden Flagellaten dokumentiert. Durch den Einsatz verschiedener licht- und elektronenmikroskopischer Techniken sowie durch die Analyse der Sequenzvariabilität der Gene der kleinen ribosomalen Untereinheit (SSU rRNA) konnten wir in der vorliegenden Arbeit eindeutig zeigen, dass *I. marginipennis* lediglich eine Art devescovinider Flagellaten (*Metadevescovina modica*) im Darm beherbergt. Gleichzeitig konnten wir die Gültigkeit der Gattung *Metadevescovina*, welche bis zum heutigen Tag stark umstritten war und häufig als Synonym zu *Devescovina* angesehen wurde, bestätigen. Molekularphylogenetische Analysen zeigten, dass beide Gattungen jeweils eine separate monophyletische Gruppe bilden. Eine Unterscheidung der beiden Gattungen an Hand morphologischer Merkmale der Flagellaten selbst war nicht möglich, konnte jedoch unter Berücksichtigung ihrer bakteriellen Symbionten erfolgen. Während Flagellaten der Gattung *Metadevescovina* einen dichten Besatz von Spirochaeten auf ihrer Oberfläche zeigen, sind *Devescovina* spp. vollständig von filamentösen Bakterien bedeckt, welche den Bacteroidales zugeordnet werden.

Molekularphylogenetische Analysen von *Devescovina* spp. von verschiedenen Vertretern der Kalotermitidae und ihren bakteriellen Symbionten ergaben zwei verschiedene Szenarien bezüglich des Erwerbs dieser Symbionten: Eine vertikale Weitergabe und eine horizontale Weitergabe. Es konnte gezeigt werden, dass die

Ektosymbionten eine monophyletische Gruppe innerhalb der Bacteroidales bilden („*Candidatus* Armantifilum devescovinae“). Kongruenzanalysen der Stammbäume von *Devescovina* spp. und „*Candidatus* Armantifilum devescovinae“ dokumentierten eine strikte Kospeziation der Partner. Eine obligate Symbiose der beiden Partner, und somit eine vertikale Weitergabe der Bakterien innerhalb ihrer Wirtsflagellaten, konnte demnach belegt werden. Der Erwerb von Symbionten durch horizontale Weitergabe von anderen Wirtsflagellaten wurde dagegen für die im Zytoplasma vorkommenden ‚Endomicrobia‘ dokumentiert. Hier waren die nächsten Verwandten der mit den *Devescovina* spp. assoziierten ‚Endomicrobia‘ Endosymbionten von phylogenetisch nicht verwandten Termitenflagellaten.

In einer weiteren Studie dieser Arbeit wurden die multiplen Symbionten des Flagellaten *Joenia annectens* aus der Trockenholztermite *Kalotermes flavicollis* identifiziert und lokalisiert (full-cycle-rRNA approach). Ultrastrukturelle Untersuchungen ermöglichten eine morphologische Beschreibung der gefundenen Phylotypen. Basierend auf den Assoziationen mit phylogenetisch verschiedenen Symbionten konnten zwei Populationen von *J. annectens* unterschieden werden. Gestützt wurde das Ergebnis durch die Analyse der SSU rRNA Gensequenzen von *J. annectens* (0,8% Sequenzunterschied zwischen beiden Populationen). Beide Flagellatenpopulationen waren jeweils mit eigenen Ektosymbionten des Phylums Bacteroidetes assoziiert. Dahingegen beherbergte nur eine der beiden Populationen von *J. annectens* einen Vertreter der ‚Endomicrobia‘ im Zytoplasma. Das Fehlen von ‚Endomicrobia‘-Symbionten bei vielen Flagellaten der gleichen Population zeigt ein weiteres Beispiel dafür, dass diese Symbionten nicht immer mit ihren Wirtsflagellaten kospeziieren.

Die Ergebnisse meiner Arbeit haben wichtige Erkenntnisse zur Spezifität der Symbiosen zwischen Termitenflagellaten und ihrer bakteriellen Symbionten gebracht. Sie stellen somit eine Grundvoraussetzung für die anstehende Erforschung der funktionellen Aspekte dieser Symbiosen dar. Eine Beteiligung der bakteriellen Symbionten am Stickstoffstoffwechsel der Flagellaten wird diskutiert.

Abgrenzung der Eigenleistung

Chapter 2:

Strassert, J. F. H., M. S. Desai, A. Brune & R. Radek (2009): The true diversity of devescovinid flagellates in the termite *Incisitermes marginipennis*. *Protist* 160, 522–535.

Licht- und elektronenmikroskopische Untersuchungen wurden geplant und durchgeführt von J. S. Die Betreuung dieser Arbeiten erfolgte durch R. R. Molekularphylogenetische Untersuchungen wurden von M. D. durchgeführt und von A. B. betreut. Das Manuskript wurde von J. S. angefertigt und von R. R. sowie A. B. überarbeitet.

Chapter 3:

Desai, M. S., J. F. H. Strassert, K. Meuser, H. Hertel, W. Ikeda-Ohtsubo, R. Radek & A. Brune (2009): Strict cospeciation of devescovinid flagellates and *Bacteroidales* ectosymbionts in the gut of dry-wood termites (Kalotermitidae). *Environ. Microbiol.* (published online; doi:10.1111/j.1462-2920.2009.02080.x).

Molekularphylogenetische Untersuchungen wurden geplant und durchgeführt von M. D. und betreut von A. B. Ergänzt wurden diese Arbeiten von W. I.-O. und K. M. Morphologische Untersuchungen erfolgten durch J. S. unter der Betreuung von R. R. Termiten wurden bereitgestellt von H. H. Die Anfertigung des Manuskriptes erfolgte durch M. D. in Zusammenarbeit mit A. B.

Chapter 4:

Strassert, J. F. H., M. S. Desai, R. Radek & A. Brune (eingereicht): Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens*. Eingereicht bei *Microbiology*.

Molekularphylogenetische Untersuchungen wurden geplant und durchgeführt von J. S. und betreut von A. B. Die morphologischen Untersuchungen erfolgten durch R. R. und J. S. Das Isolieren der Flagellaten ("capillary picking of *J. annectens*") erfolgte durch M. D. Das Manuskript wurde von J. S. angefertigt und von R. R. sowie A. B. überarbeitet.

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