Methanogens in the Digestive Tract of Termites

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Contents

Abstract Methanogenesis in termite guts is a product of symbiotic digestion, fueled by hydrogen and reduced one-carbon compounds that are formed during the fermentative breakdown of plant fiber and humus. Methanogens are restricted to the hindgut region and can be found in several distinct microhabitats. In lower termites, the methanogens belong almost exclusively to the genus *Methanobrevibacter*. They are either endosymbionts of flagellate protists or colonize the periphery of the hindgut, a habitat that is not fully anoxic. The oxygen-reducing capacities of the few isolates available so far indicate that they are well adapted to the continuous influx of oxygen across the gut wall. In higher termites, which lack gut flagellates, the hindgut is highly compartmented and characterized by strong differences in pH, redox potential, and other microenvironmental conditions. Here, the archaeal communities differ strongly between compartments and comprise not only *Methanobacteriales*, but also *Methanosarcinales*, *Methanomicrobiales*, and the recently discovered *Methanomassiliicoccales*. All methanogens in termite guts

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belong to distinct phylogenetic clusters that are restricted to the intestinal tracts of insects and millipedes. Only few representatives have been isolated in pure culture. The high methane emissions of termites, together with their enormous biomass in the tropics, make them a significant natural source of this important greenhouse gas.

1 Introduction

Most insects that thrive on a fiber-rich diet harbor microbial symbionts that participate in digestion, but only termites, cockroaches, and the larvae of scarab beetles have been found to emit methane (Hackstein and van Alen 2018). This methane is produced by methanogenic archaea, which represent the last link in an anaerobic feeding chain of microorganisms located in the enlarged hindgut of these insects—microbial bioreactors that transform lignocellulosic matter to short-chain fatty acids, the major energy source for the host (Fig. 1). A detailed account of symbiotic digestion in termites can be found in other reviews of this topic (Brune and Ohkuma 2011; Brune 2014).

This chapter will provide an overview of the diverse aspects of methanogenesis in termites, including the role of methanogens in symbiotic digestion, the diversity and structure of the methanogenic community in different termite lineages, and advances

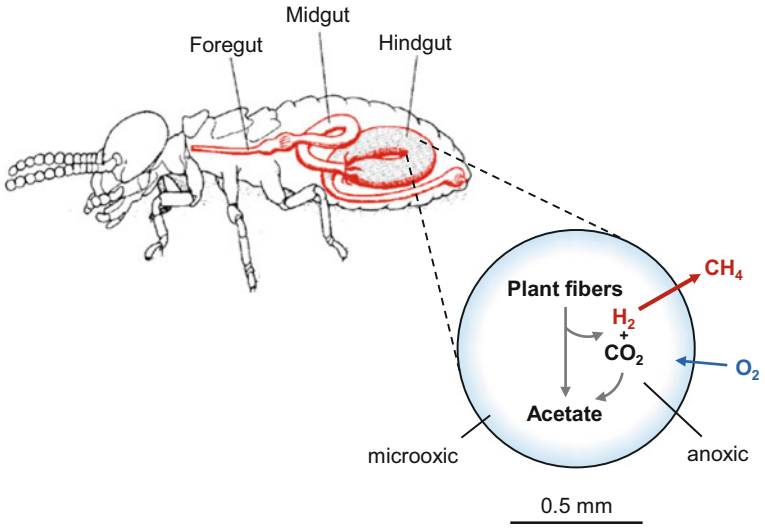

Fig. 1 The hindgut of termites is a microbial bioreactor that transforms lignocellulose to acetate and other short-chain fatty acids. Hydrogen formed during fermentation of plant fibers is the major substrate of both methanogenesis and reductive acetogenesis. The anoxic status of the hindgut lumen is maintained by the microorganisms colonizing the microoxic hindgut periphery, which consume the oxygen diffusing across the gut wall. Originally published in Brune (2010), reprinted with permission of ©Springer Nature

in understanding the interactions of methanogens with other gut microbiota and their physicochemical microenvironment. For detailed coverage of the older literature and broader surveys of methanogenesis in insects and millipedes and the associations between methanogens and termite gut flagellates, the reader is referred to other review articles (Breznak 2000; Ohkuma and Brune 2011; Brune 2018; Hongoh and Ohkuma 2018).

2 Methane as a Product of Symbiotic Digestion

Methane formation in the guts of termites had been suspected already more than 80 years ago. When Cook (1932) studied the respiratory gas exchange of *Zootermopsis nevadensis*, he found that the termite continued to form substantial amounts of an unidentified gas when the oxygen in the vessel was depleted. He was not able to analyze the gas, but—inspired by the situation in ruminants—proposed that the gas was most likely hydrogen or methane or a mixture of both. However, it took more than 40 years after Cook's initial observation until methane production in termite guts was finally recognized by Breznak and coworkers. While demonstrating nitrogenase activity in living termites and wood-feeding cockroaches with the acetylene reduction assay, the authors identified methane as an additional peak present in the gas chromatograms (Breznak et al. 1973, 1974)—a classic case of serendipity in science. Historical details have been reviewed elsewhere (Breznak 2000; Brune 2018).

Breznak (1975) had pointed out that the amount of methane produced by termites, if based on body weight, is in the same order of magnitude as that of ruminants. This observation immediately aroused the interest of atmospheric chemists studying the role of methane in radiative forcing of the atmosphere, who identified termites as a potential source of considerable strength of this greenhouse gas (see below). In the following years, methane production was documented for almost all termite species investigated (e.g., Brauman et al. 1992; Shinzato et al. 1992; Wheeler et al. 1996; Bignell et al. 1997; Sugimoto et al. 1998b), with marked differences in the methane emission rates between wood- and humus-feeding taxa (wood vs. humus; Fig. 2).

Methanogenic archaea form methane in two fundamentally different processes: (1) the reduction of CO_2 or methyl groups to CH_4 via the C_1 pathway (hydrogenotrophic methanogenesis); and (2) the cleavage of acetate to CH_4 and CO_2 via the acetyl-CoA pathway (aceticlastic methanogenesis) (Liu and Whitman 2008; Thauer et al. 2008). Interestingly, there is no evidence for aceticlastic methanogenesis in termite guts. As in the human gut and in the rumen, it is assumed that the relatively slow-growing aceticlastic species cannot cope with the short retention times of intestinal habitats (Liu and Whitman 2008). However, there is no explanation why they do not avoid washout by attaching to intestinal surfaces (see below).

The electron donors of hydrogenotrophic methanogenesis are hydrogen and reduced C_1 compounds, such as methanol and formate, which are formed during the fermentative breakdown of organic matter. In the hindguts of lower termites,

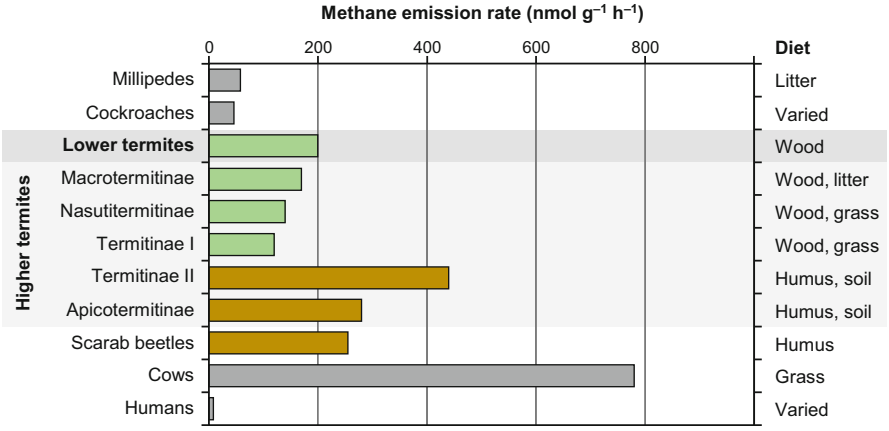

Fig. 2 Methane emission rates of lower and higher termites compared to those of other invertebrates, cows, and humans. The typical diet of the respective taxon is indicated; members of Termitinae were grouped into wood-feeding (green) and humus-feeding (brown) species. Values are averages, based on fresh weight, and were compiled from various sources (for details, see Brune 2018). Originally published in Brune (2010), adapted with permission of ©Springer Nature

hydrogen is the central free intermediate of lignocellulose degradation. It is a major fermentation product of cellulolytic flagellates and can accumulate to substantial concentrations (Ebert and Brune 1997; Pester and Brune 2007). Turnover rates are in the range of 7–16 μmol H_2 h^{-1} (g fresh weight)$^{-1}$ (Pester and Brune 2007)—on a volume basis, hydrogen fluxes in termite guts are in the same range as in the bovine rumen.

Methane production by lower termites strictly depends on the presence of (hydrogen-producing) gut flagellates (Odelson and Breznak 1983; Rasmussen and Khalil 1983; Messer and Lee 1989). However, the rates of methanogenesis are much lower than one would expect based on the large amount of hydrogen presumably produced by the microbial fermentations. If termites are fed with antibacterial drugs, their hydrogen and methane emission rates increase strongly, which indicates that methanogenic archaea compete with bacteria for hydrogen formed by the flagellates (Odelson and Breznak 1983).

In the phylogenetically higher termites (family Termitidae), which lack gut flagellates, the methanogenic substrates are most likely formed by fermenting bacteria. Here, methanogenesis in intact guts and gut homogenates is strongly stimulated by the supply of external hydrogen but also by formate (Brauman et al. 1992; Schmitt-Wagner and Brune 1999).

The most prominent process responsible for bacterial hydrogen oxidation in termite guts is the reduction of CO_2 to acetate (Breznak and Switzer 1986). It is a unique feature of termite guts that the bacteria responsible for reductive acetogenesis—at least in wood-feeding termite species—are members of the phylum *Spirochaetes* (Leadbetter et al. 1999; Ottesen and Leadbetter 2011). Although hydrogenotrophic methanogenesis occurs in most wood-feeding termites, it becomes

more important than reductive acetogenesis in the fungus-cultivating and humivorous taxa—both in gut homogenates and in situ (Breznak and Switzer 1986; Brauman et al. 1992; Tholen and Brune 1999, 2000; Pester and Brune 2007).

Despite the apparent substrate limitation of methanogenesis in termite guts, termites Hydrogen emission in considerable amounts (Zimmerman et al. 1982; Odelson and Breznak 1983; Ebert and Brune 1997; Sugimoto et al. 1998b; Schmitt-Wagner and Brune 1999; Pester and Brune 2007; Cao et al. 2010; Yanase et al. 2013), which indicates that production and consumption of hydrogen in the hindgut are not tightly coupled (see below).

3 Diversity of Methanogens in Termite Guts

The methanogens in termite guts belong to several genus-level lineages of the orders *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, and *Methanomassiliicoccales* (Fig. 3). However, not all lineages are represented by pure cultures. There are only three described species, all from the genus *Methanobrevibacter* (*Methanobacteriales*) and all from the same host species, namely, the lower termite *Reticulitermes flavipes* (Leadbetter and Breznak 1996; Leadbetter et al. 1998). Like other members of this genus found in the human gut or the rumen, they grow exclusively on $H_2 + CO_2$ (*Methanobrevibacter cuticularis* also grows, albeit poorly, on formate). Their genomes have been sequenced (Poehlein and Seedorf 2016).

Most of the *Methanobacteriales* clones from higher termites fall into the radiation of the genus *Methanobrevibacter*, but they are phylogenetically distinct from their relatives in lower termites and other insects. Several strains of *Methanobacteriales* in higher termites have been isolated in pure culture and characterized in some detail (Deevong et al. 2004). They comprise a *Methanobrevibacter* strain that is closely related to *Methanobrevibacter arboriphilus* and grows also on formate and several *Methanobacterium* strains that are close relatives of *Methanobacterium bryantii* and utilize also secondary alcohols. However, none of these strains were deposited in a culture collection.

The only other methanogen isolated from insect guts is *Methanomicrococcus blatticola* from the cockroach *Periplaneta americana*. It is the first cultivated representative of a lineage of *Methanosarcinales* and differs from the *Methanobrevibacter* species in its inability to grow on $H_2 + CO_2$. Instead, it is specialized on the hydrogen-dependent reduction of methanol or methylamines to methane (Sprenger et al. 2000). Its obligate requirement for hydrogen is explained by the inability to oxidize methyl groups to carbon dioxide (Sprenger et al. 2005). At low hydrogen concentrations, the use of methanol as the terminal electron acceptor in methanogenesis is thermodynamically more favorable than the use of carbon dioxide. As a consequence, the substrate affinity of *M. blatticola* for hydrogen is higher than those reported for hydrogenotrophic methanogens and that for methanol

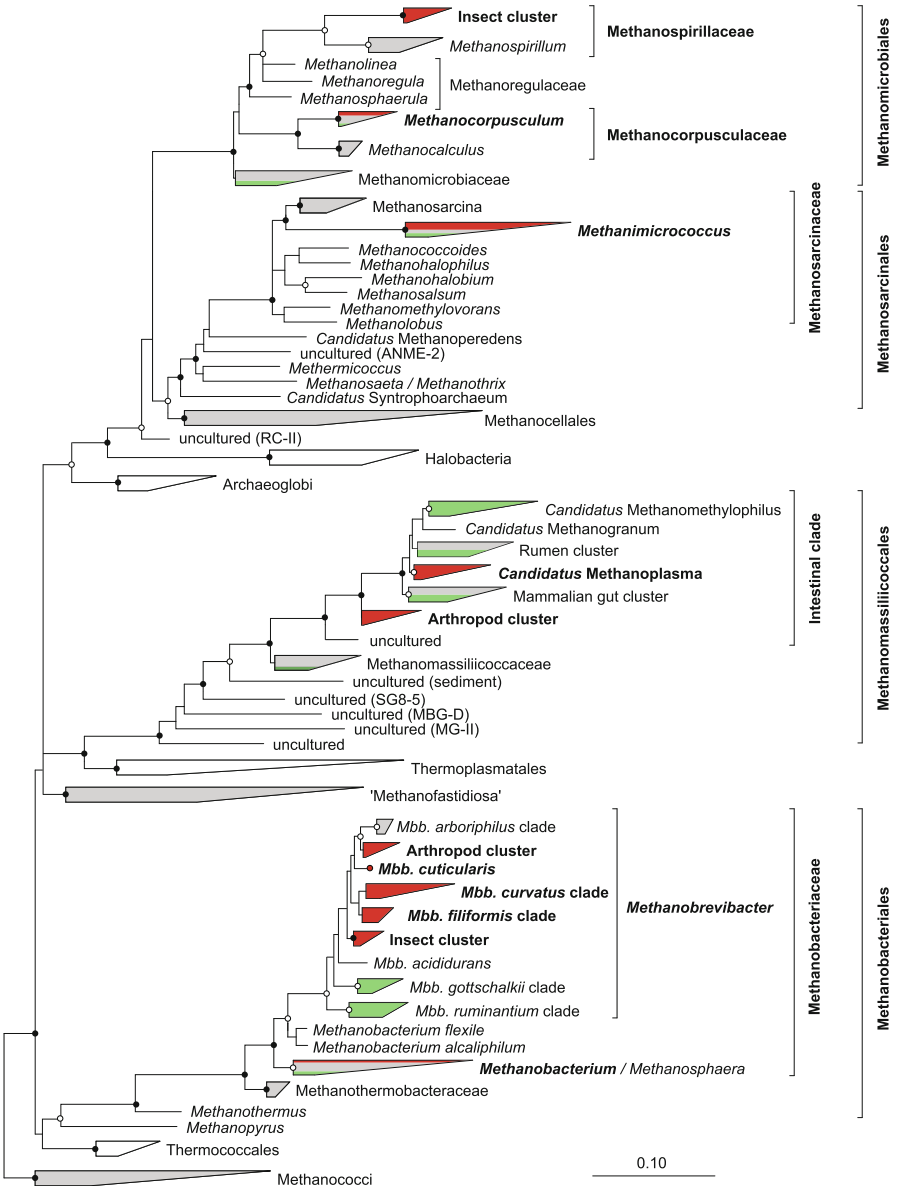

Fig. 3 Phylogenetic position of methanogens that occur in the guts of termites and other insects (red) or mammals (green) among other methanogenic (gray) and non-methanogenic (white) clades of *Euryarchaeota*. Taxa with representatives from insect guts are in boldface. Simplified version of a larger maximum-likelihood tree, based on a manually curated alignment of near full-length 16S rRNA gene sequences; symbols indicate node support (closed circle, >90%; open circle, >70%)

even surpasses those of other methylotrophic taxa (*Methanosphaera stadtmanae*, *Methanosarcina barkeri*) (Sprenger et al. 2007).

The uncultured members of *Methanosarcinales* encountered in higher termites (see below) fall into the radiation of the genus *Methanimicrococcus*, which comprises also clones recovered from other cockroaches and scarab beetle larvae (e.g., Hara et al. 2002; Egert et al. 2003). The same is true for the *Methanomicrobiales* clones obtained from higher termites, which form a sister group of the genus *Methanospirillum*; no representatives of this "insect cluster" have been brought into culture (Fig. 3).

Members of *Methanomassiliicocales* fall into the so-called intestinal clade and form several clusters that consist exclusively of clones from arthropod guts. A highly enriched culture has been obtained from the soil-feeding termite *Cubitermes ugandensis* (Paul et al. 2012). Physiological and ultrastructural characterization, combined with a comparative analysis of its genome, identified "*Candidatus* Methanoplasma termitum" as an obligately methyl-reducing hydrogenotroph without a cell wall (Lang et al. 2015). It shares a new mode of energy metabolism with the distantly related *Methanomassiliicoccales luminyensis* and other uncultured representatives of this order (Borrel et al. 2014; Lang et al. 2015). Since all *Methanomassiliicoccales* seem to lack coenzyme F_{420} (Lang et al. 2015), their cells cannot be visualized by epifluorescence microscopy.

4 Structure of the Methanogenic Communities

The methanogens colonizing the hindgut of lower termites belong almost exclusively to the genus *Methanobrevibacter* (*Methanobacteriales*). Cultivation-independent, 16S-rRNA-based surveys documented the presence of unique *Methanobrevibacter*-related phylotypes in each lower termite investigated (Ohkuma et al. 1995, 1999; Ohkuma and Kudo 1998; Shinzato et al. 1999, 2001). *Reticulitermes flavipes* harbors at least three species with distinct morphotypes, which colonize the hindgut cuticle and have been isolated in pure culture (Fig. 4). Also other lower termites harbor more than one lineage of *Methanobrevibacter*, and the phylotypes attached to the hindgut cuticle or to filamentous bacteria at the gut wall are phylogenetically distinct from those associated with the gut flagellates (Tokura et al. 2000; Hara et al. 2004; Inoue et al. 2008), which suggests an adaptation to the respective microhabitats (see below).

The methanogenic communities in the hindgut of higher termites are much more diverse and comprise members of *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, and *Methanomassiliicoccales*. Clone libraries of archaeal 16S rRNA genes are available for members of the genera *Alyscotermes* (Apicotermitinae), *Cubitermes* and *Ophiotermes* (Cubitermitinae), *Macrotermes* and *Odontotermes* (Macrotermitinae), *Nasutitermes* and *Trinervitermes* (Nasutitermitinae), *Pericapritermes*, and *Microcerotermes* (Termitinae) (Ohkuma et al. 1999; Friedrich et al. 2001; Donovan et al. 2004; Miyata et al. 2007; Paul et al. 2012;

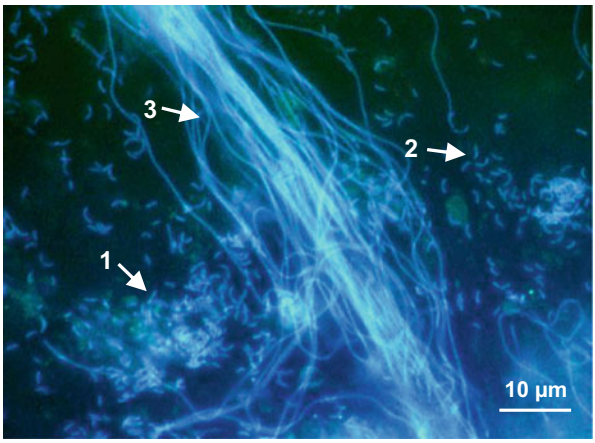

Fig. 4 Methanogens associated with the hindgut wall of *Reticulitermes flavipes*, visualized by the autofluorescence of their cofactor F_{420}. The arrows point to the characteristic morphotypes of *Methanobrevibacter cuticularis* (1), *Methanobrevibacter curvatus* (2), and *Methanobrevibacter filiformis* (3). Microphotograph courtesy of J. R. Leadbetter and J. A. Breznak. Originally published in Brune (2010), reprinted with permission of ©Springer Nature

Shi et al. 2015). Short-read amplicon libraries have been obtained for members of the genera *Nasutitermes* and *Tenuirostritermes* (Nasutitermitinae); *Drepanotermes*, *Gnathamitermes*, and *Macrognathotermes* (Termitinae); and *Syntermes* (Syntermitinae) (Rahman et al. 2015; Santana et al. 2015).

While representatives of all four orders have been recovered from Cubitermitinae, Syntermitinae, and Termitinae, clone libraries of Macrotermitinae yielded no *Methanomicrobiales*, and those of Apicotermitinae and Nasutitermitinae yielded no *Methanosarcinales*. The results obtained with short-read amplicon libraries differed between colonies of the same species and were not always consistent with those previously obtained for other members of the same subfamily, which indicates differences in community structure even between closely related taxa (Rahman et al. 2015). Coevolution between termites and methanogens is only diffuse and might be disturbed by rampant host switching, as observed for members of their bacterial microbiota (Bourguignon et al. 2018). However, the drivers of methanogenic community structure in termite guts remain unclear.

5 Differences in Methanogenic Activities and Populations

Information on the population sizes of methanogens in insect guts is scarce. Cultivation-based studies indicate that *Reticulitermes flavipes* harbors about 10^6 methanogens per gut, which is about 5% of the total cell count of prokaryotes (Leadbetter and Breznak 1996; Tholen et al. 1997). Such numbers are inherently inaccurate because of the uncertainties created by cultivation bias, the absence of

cofactor F_{420} from *Methanomassiliicoccales*, and the difficulties in enumerating prokaryotic cells attached to intestinal surfaces or intimately associated with the flagellate cells that occupy the bulk of the hindgut volume.

Hybridization of RNA extracted from the guts of a wide range of termite species with domain-specific oligonucleotide probes indicated that the average proportion of archaeal rRNA was only 1.5% of all prokaryotic rRNA (Brauman et al. 2001). The higher fraction of archaeal rRNA in soil-feeding species ($2.3 \pm 0.5\%$) than in wood-feeding and fungus-cultivating species ($0.9 \pm 0.5\%$) is in agreement with a general trend toward higher methane emission rates among termites with a humivorous lifestyle (Fig. 2), but it should be kept in mind that not all the archaea in termite guts are necessarily methanogenic (Friedrich et al. 2001).

Amplicon sequencing studies employing both universal and prokaryote primers indicated that archaeal reads obtained for *Reticulitermes* species range between 0.1 and 0.2% of the reads classified as prokaryotes (Boucias et al. 2013; Rahman et al. 2015). However, unrealistically high proportions of archaea obtained for termites from other genera (e.g., above 50% in *Porotermes*; Rahman et al. 2015) put into question the reliability of this approach.

Since soil-feeding termites—in contrast to their wood- and grass-feeding relatives—digest peptide-rich soil organic matter (Ji and Brune 2006; Brune and Ohkuma 2011), it is tempting to suggest that differences in methanogenic activity are diet related. However, information on the fermentative processes in the hindguts of humivorous insects is sparse, and also the substrate spectra of the mostly uncultivated methanogens in higher termites has to be better understood before a reasonable hypothesis can be proposed.

Such knowledge may also help to clarify whether the presence of methanogens provides benefits their respective hosts. So far, such evidence is lacking. In lower termites, methane emission rates can differ strongly among members of the same genus, and sometimes members of the same species are not consistently colonized by methanogens (e.g., Shinzato et al. 1992; Wheeler et al. 1996), indicating that the presence of methanogens provides no advantage. In *Zootermopsis angusticollis*, elimination of methanogens by feeding with bromoethanesulfonic acid (BES) does not affect the survival of the termites (Messer and Lee 1989).

6 Coexistence with Homoacetogens

The predominance of reductive acetogenesis over methanogenesis in most wood-feeding termites has puzzled microbiologists for the longest time. For thermodynamic reasons, methanogens should always outcompete homoacetogens for hydrogen, their common substrate—at least in a well-mixed system. However, the introduction of microsensor techniques into termite gut research led to the recognition that termite guts are spatially structured microenvironments characterized by steep diffusion gradients of metabolites (see Brune 1998; Brune and Friedrich 2000).

This conceptual advance allowed an explanation for the coexistence of methanogens and homoacetogens in this habitat.

Firstly, it turned out that hydrogen concentrations in termite guts are much higher than originally considered—far above the threshold concentrations at which methanogens can outcompete homoacetogens for hydrogen. At the hydrogen partial pressures observed in the hindgut proper of several lower termites (1–100 kPa; Ebert and Brune 1997; Pester and Brune 2007), both processes would operate at substrate saturation, and a direct competition for hydrogen cannot occur. Therefore, the previous hypothesis explaining the predominance of reductive acetogenesis in termite guts with an increased competitiveness of homoacetogens based on their ability to grow mixotrophically on H_2 and other substrates (Breznak 1994) was no longer tenable.

Secondly, high-resolution profiles of hydrogen concentration in the intestinal tracts of lower and higher termites (Ebert and Brune 1997; Schmitt-Wagner and Brune 1999; Pester and Brune 2007; Köhler et al. 2012) and rate measurements of reductive acetogenesis by microinjection of radiotracers (Tholen and Brune 1999, 2000; Pester and Brune 2007) documented that sources and sinks of hydrogen are not evenly distributed within the hindgut. The high hydrogen concentrations at the gut center of *Reticulitermes* spp., the steep hydrogen gradients toward the gut periphery, and the absence of any stimulatory effect of externally supplied hydrogen on the in situ rates of reductive acetogenesis indicate that the hydrogen-consuming, homoacetogenic spirochetes co-locate with the hydrogen-producing flagellates in the hindgut lumen. By contrast, the strong hydrogen sink at the hindgut wall, which is clearly caused by an anaerobic process (Ebert and Brune 1997), and the dense colonization of the cuticle with *Methanobrevibacter* species (Leadbetter and Breznak 1996; Leadbetter et al. 1998) explain the strong stimulation of methanogenesis by externally supplied hydrogen.

The spatial separation of the hydrogenotrophic processes—reductive acetogenesis in the gut lumen and methanogenesis in the periphery—avoids direct competition between homoacetogens and methanogens for their common substrate (Fig. 5). Nevertheless, it remains to be explained why the homoacetogens are able to colonize the hydrogen-rich gut lumen, whereas the methanogens (unless associated with gut flagellates) are not. In this context, it is important to recall that the termite gut is unusual not only with respect to the predominance of reductive acetogenesis over methanogenesis but also in the abundance of spirochetal life forms in wood-feeding termites (Lilburn et al. 1999; Breznak 2000). So far, the termite gut is the only habitat that harbors spirochetes capable of reductive acetogenesis (Leadbetter et al. 1999; Breznak and Leadbetter 2006).

Diversity studies and expression analysis of FTHFS genes, the functional markers of reductive acetogenesis, have revealed that termite gut treponemes predominate over homoacetogenic firmicutes in all wood-feeding termites studied to date (Salmassi and Leadbetter 2003; Ottesen et al. 2006; Pester and Brune 2006; Warnecke et al. 2007). Apparently, these highly motile spirochetes are well adapted to actively maintain their position in the hindgut lumen, whereas methanogens must attach to surfaces to prevent washout—they can colonize the gut lumen only by

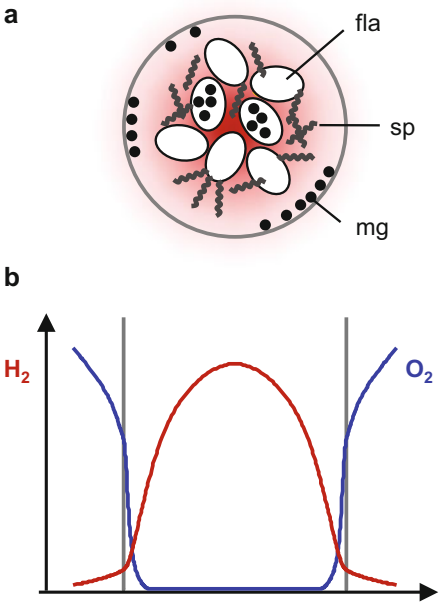

Fig. 5 Schematic cross section (**a**) of the hindgut of a wood-feeding lower termite, illustrating the location of methanogens (mg) at the hindgut wall and homoacetogenic spirochetes (sp) within the lumen of the paunch. In some termite species, methanogens are also associated with the gut flagellates (fla). Radial profiles (**b**) of oxygen and hydrogen partial pressure reveal that the respiratory activity of the gut microbiota maintains steep oxygen gradients within the gut periphery, rendering the center anoxic. Hydrogen formed by the flagellates accumulates at the gut center but is consumed toward the periphery. The strong hydrogen sink below the gut wall is probably caused by methanogens, which prevent larger amounts of H_2 from escaping into the atmosphere. Originally published in Brune (2010), reprinted with permission of ©Springer Nature

associating with the gut flagellates or (in some higher termites) by attaching to cuticular spines that protrude from the gut wall into the lumen (Bignell et al. 1980).

7 Association with Gut Flagellates

Methanogens colonizing intestinal environments are commonly associated with anaerobic protists (see Hackstein and van Alen 2018). The typical habitats of methanogens in termite guts are the hindgut cuticle and the surface of filamentous bacteria colonizing the hindgut wall (Hackstein and Stumm 1994; Leadbetter and Breznak 1996; Leadbetter et al. 1998), but also the gut flagellates of lower termites are frequently colonized by methanogenic symbionts (Odelson and Breznak 1985; Messer and Lee 1989; Shinzato et al. 1992; Hackstein and Stumm 1994; Radek 1994, 1997; Tokura et al. 2000; Hara et al. 2004).

Generally, only smaller species of termite gut flagellates are associated with methanogens. Lee et al. (1987) investigated the colonization of gut flagellates by methanogens in the hindgut of *Zootermopsis angusticollis* by epifluorescence microscopy and reported that only the small trichomonadid flagellates *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis* were associated with cells showing the characteristic F_{420} autofluorescence of methanogens. The larger hypermastigotes, which appeared to be the major hydrogen source (Messer and Lee 1989), usually lacked methanogenic symbionts. Similar observations were made by Tokura et al. (2000) with *Reticulitermes speratus*, where the methanogens were regularly associated with the oxymonadid *Dinenympha parva* and a small hypermastigote *Microjoenia* sp., and with *Hodotermopsis sjoestedti*, where the methanogens were associated with *Dinenympha* and *Microjoenia* spp. in large abundance. In all cases, the methanogens seemed to be located within the host cells, which is in agreement also with ultrastructural data reported by Lee et al. (1987).

Odelson and Breznak (1985) were the first to note that a putatively axenic culture of *Trichomitopsis termopsidis*, a gut flagellate isolated from a *Zootermopsis* species, contained a methanogenic symbiont. The symbiosis was not obligate because cultures continued to grow after they were cured of the methanogenic symbiont. Nevertheless, growth yields of *T. termopsidis* increased when the flagellate was cultivated in the presence of the methanogen *Methanospirillum hungatei*, which suggested that the flagellates may benefit in a similar manner from their methanogenic symbiont. There are reports that indicate that methanogens associated with eukaryotic partner organisms in other environments might benefit from interspecies hydrogen transfer, and the stimulation of fermentative processes by end product removal (hydrogen, formate) might even result in a mutual advantage (Schink 1997). However, considering the high hydrogen concentrations throughout the gut lumen of lower termites, it is not clear whether termite gut flagellates indeed benefit from the hydrogen-consuming activity of their methanogenic symbionts under in situ conditions. At the same time, this would mean that the methanogens associated with gut flagellates are never hydrogen limited as long as they can maintain their position in the hydrogen-rich gut lumen—no matter whether their particular host is producing hydrogen or not. From that perspective, the association of *Methanobrevibacter* species with gut flagellates might simply serve to maintain a stable position in the anoxic and hydrogen-rich hindgut lumen, an argument that may apply also to the hydrogenotrophic bacteria associated with such protists (see Hongoh and Ohkuma 2018).

8 Intercompartmental Transfer of Hydrogen

The guts of higher termites are characterized by the absence of cellulolytic flagellates and show (with the exception of the fungus-cultivating species) also a pronounced compartmentation, which goes hand in hand with remarkable dynamics of intestinal pH and redox potential (Brune and Kühl 1996; Kappler and Brune 2002; Köhler et al. 2012; Fig. 6). Since methanogenesis in termite guts is typically hydrogen

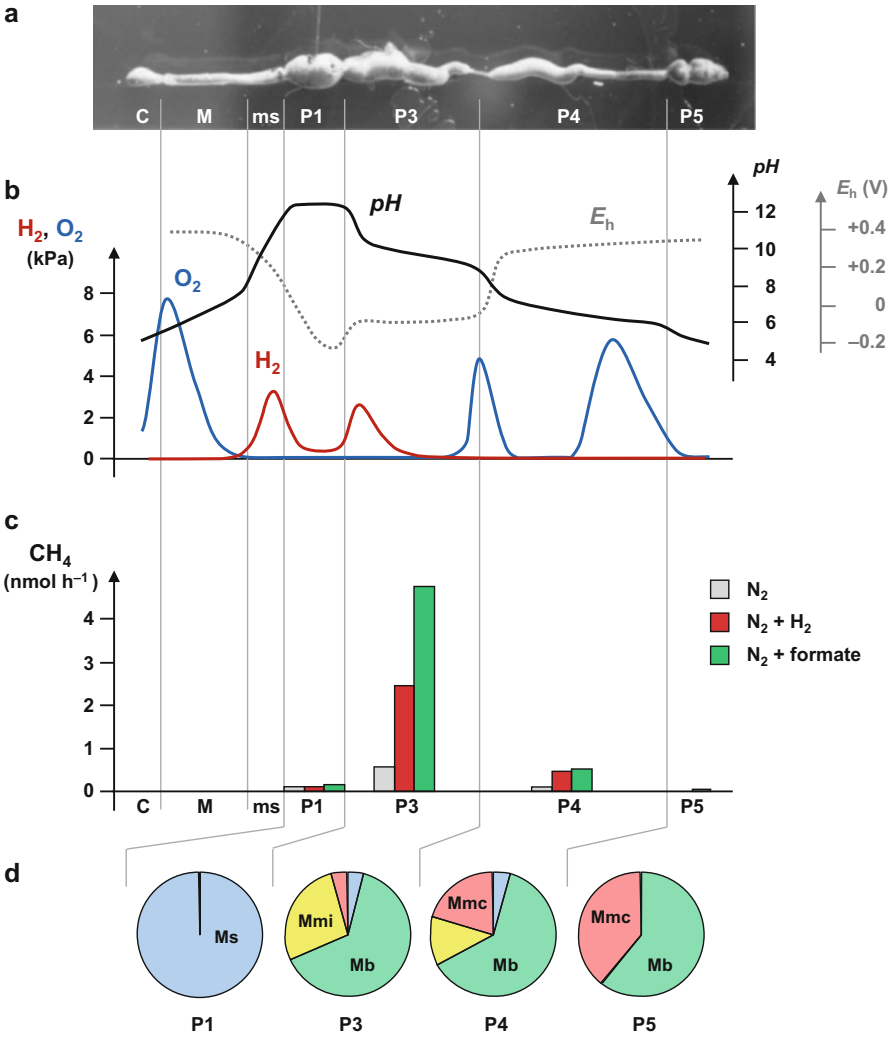

Fig. 6 Gut morphology (**a**) and microsensor profiles (**b**) of oxygen, hydrogen, pH, and redox potential (E_h) along the gut axis of a soil-feeding termite (*Cubitermes* spp.). Methanogenic capacities of individual compartments (**c**) were determined with isolated gut sections incubated under a N_2 headspace with or without addition of H_2 or formate. Relative abundance of methanogens (**d**) in 16S-rRNA-based clone libraries of the respective gut sections (Ms, *Methanosarcinales*; Mb, *Methanobacteriales*; Mmi, *Methanomicrobiales*; Mmc, *Methanomassiliicoccales*). Vertical lines indicate the borders between the different gut regions. Scheme based on various studies (Brune and Kühl 1996; Schmitt-Wagner and Brune 1999; Friedrich et al. 2001; Kappler and Brune 2002). Originally published in Brune (2010), reprinted with permission of ©Springer Nature

limited, Sugimoto et al. (1998b) suggested that differences in the rates of hydrogen and methane emission between termite species might reflect the particular location of the methanogens relative to the hydrogen source. In soil-feeding *Cubitermes* species, hydrogen production and consumption are spatially separated in different gut compartments (Schmitt-Wagner and Brune 1999; Tholen and Brune 1999). The strong stimulation of both methanogenesis and reductive acetogenesis in intact gut compartments by external hydrogen led to the hypothesis that hydrogen diffuses across the gut epithelia between hydrogen-producing and hydrogen-consuming gut regions, which are in close contact in situ. A cross-epithelial transfer of reducing equivalents has been experimentally documented in cockroaches and scarab beetle larvae (Lemke et al. 2001, 2003) and would explain the low hydrogen and high methane emissions of such soil-feeding termites. Since methanogenesis in the posterior hindgut is not only stimulated by hydrogen but also by formate, which accumulates to considerable concentrations in other gut compartments, there might also be intercompartmental transfer of reducing equivalents via the hemolymph (Schmitt-Wagner and Brune 1999).

A detailed analysis of the archaeal community structure in the gut compartmentation of *Cubitermes orthognathus* showed that the different phylogenetic groups are not evenly distributed among the different hindgut compartments (Friedrich et al. 2001). Each of the individual gut compartments harbors a distinct assemblage of Euryarchaeota (Fig. 6d). *Methanosarcinales* colonize the anterior, extremely alkaline compartment, whereas *Methanobacteriales*, *Methanomicrobiales*, and *Methanomassiliicoccales* are found in the posterior, less alkaline to neutral compartments. These gut regions harbor also the highest methanogenic capacities, and many of the microbial cells attached to the gut wall or to cuticular spines projecting from the hindgut wall into the lumen show the characteristic autofluorescence of methanogens (Schmitt-Wagner and Brune 1999).

9 Relationship to Oxygen

As obligate anaerobes, the methanogens in termites are restricted to the hindgut, the only gut region characterized by a negative redox potential (Ebert and Brune 1997; Kappler and Brune 2002; Köhler et al. 2012). It is not clear why they are regularly (in some cases exclusively) located at the hindgut wall, a microhabitat that experiences a constant influx of oxygen across the epithelium (Brune 1998; Brune and Friedrich 2000). Like all other methanogens, the three *Methanobrevibacter* species colonizing the gut epithelium of *Reticulitermes flavipes* (Leadbetter and Breznak 1996; Leadbetter et al. 1998) (and also *Methanomicrococcus blatticola* colonizing the hindgut epithelium of cockroaches; Sprenger et al. 2000) do not grow in media containing even traces of oxygen and are much more sensitive to oxygen accumulation than the homoacetogenic *Sporomusa* species isolated from termite guts (Boga and Brune 2003). However, *Methanobrevibacter* species remain metabolically active in dense cell suspensions that are exposed to controlled oxygen fluxes as

long as the influx of oxygen does not exceed their capacity for oxygen removal (Tholen et al. 2007), whereas reductive acetogenesis of *Sporomusa* species is inhibited even at the lowest oxygen fluxes (Boga and Brune 2003). It has been proposed that the redirection of electron flow from methanogenesis toward oxygen reduction enables *Methanobrevibacter* species to colonize the hindgut periphery of termites. The mechanisms of tolerance to reactive oxygen species and the biochemistry of oxygen reduction in *Methanobrevibacter* species have been discussed elsewhere (Brune 2018).

Nevertheless, the location of methanogens at the gut wall of lower termites, at the unfavorable end of the outwardly directed hydrogen gradient, remains enigmatic. It has been suggested that an attachment to the hindgut cuticle might protect against predation or prevent washout from the gut, which could compensate methanogens for the negative effects of hydrogen limitation and exposure to inflowing oxygen (Breznak 2000). In higher termites, the explanation for the colonization of the hindgut cuticle might lie also in the putative transfer of hydrogen between different compartments. The microorganisms located at the gut wall might be at the bottom end of the radial hydrogen flux from the gut proper but might benefit from external hydrogen entering the hindgut by cross-epithelial transfer from other compartments (see above).

10 Termites as a Source of Atmospheric Methane

Although the counter-gradients of methane and oxygen in the hindgut periphery provide seemingly ideal conditions for aerobic methane oxidation (Brune et al. 2000), there is no evidence for the presence of methanotrophic bacteria or their activities in termite guts (Pester et al. 2007). This means that the different methane emission rates of termites from different feeding guilds directly reflect differences in methane production within their intestinal tract. In the past, many attempts were made to extrapolate from the results of laboratory measurements of methane emissions to the contribution of termites to the global methane budget, but even the most recent estimates are still far from accurate and suffer from numerous biases (see Sanderson 1996; Bignell et al. 1997). Sugimoto and colleagues demonstrated that it is very important to consider methane oxidation in the mound material and the surrounding soil as an important factor mitigating methane production by termites at the environmental level (Sugimoto et al. 1998a, 2000). As a consequence, the net emissions of methane from intact colonies of soil-feeding termites are much lower than those of wood-feeding termites, even though the opposite would be predicted from the gross methane emission rates determined with individual termites in the laboratory.

In view of the grossly overestimated contribution of termites to global methane emissions into the atmosphere propagated in the older literature (reviewed by Collins and Wood 1984), it is important to note that the most recent estimates place these rates at probably less than 10 Tg/year (1.5–7.4 Tg; Sugimoto et al. 1998b) and

almost certainly below 20 Tg/year (a number that is still used in the last global budget published by the IPCC; Denman et al. 2007). Although termites remain a significant natural source of methane on the planet, their contribution to the total source strength (ca. 600 Tg/year) is certainly dwarfed by the sources under anthropogenic influence (such as the ruminants) (Kirschke et al. 2013). More detailed reviews of this subject can be found elsewhere (Bignell 2010; Brune 2018).

11 Conclusions

Termites are a significant source of methane in tropical ecosystems. Methane and short-chain fatty acids are formed from lignocellulosic matter by an anaerobic feeding chain of microorganisms located in the highly enlarged hindguts. However, termite hindguts are not purely anoxic fermentors. The gut habitat is characterized by the continuous influx of O_2 across the gut wall and steep hydrogen gradients between gut lumen and periphery. Despite the high hydrogen concentrations in the gut lumen, methanogens are not the predominant hydrogenotrophic microorganisms in lower termites. The ability to attach to biotic or abiotic surfaces or to colonize the cytoplasm of flagellate protists might be an important factor in the successful colonization of the intestinal tract. In higher termites, which lack gut flagellates, the increased methane production is correlated with a dietary shift from wood to humus. The assemblage of methanogenic archaea in higher termites is more diverse and changes between the major hindgut compartments, each of which differs with respect to the prevailing physicochemical conditions. The drivers determining archaeal community structure in the different microhabitats are not clear, but might involve the availability of and competition for methanogenic substrates and differences in adaptation to pH, oxygen, and other stresses imposed by the respective microenvironments. Since most of the methanogens in termite guts belong to lineages without any cultured representatives, more isolates are sorely needed to address these questions.

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