# Archaeal diversity and community structure in the compartmented gut of higher termites

**Doctoral thesis**

submitted in partial fulfillment for the award of a doctoral degree “Doktorgrad der Naturwissenschaften (Dr. rer. nat.)” to the Faculty of Biology, Philipps-Universität Marburg

by

**James Oluoch Nonoh**

from Siaya County, Kenya

Marburg/Lahn

2013

This thesis was part of the major project on “Environmental drivers of methanogenic community structure in termite guts” and was funded by the Deutsche Forschungsgemeinschaft (DFG) under the topic Sonderforschungsbereich (SFB 987). The theme of my study was developed by Prof. Dr. Andreas Brune, the Group leader and main supervisor. The work described in this thesis was carried out in the Laboratory of Prof. Dr. Andreas Brune, at the Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany, between October 2009 and March 2013, under the direct supervision of Prof. Dr. Andreas Brune.

The thesis was submitted to the Dean, Faculty of Biology, Philipps-Universität, Marburg, on: 08.04.2013

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| First reviewer | Prof. Dr. Andreas Brune |
| Second reviewer | Prof. Dr. Ralf Conrad |
| Date of oral examination | 21.05.2013 |

# Declaration

I do hereby certify that my doctoral thesis titled:

"Archaeal diversity and community structure in the compartmented gut of higher termites"

was carried out with legally and professionally authorized scientific methods and devices. All samples were legally and procedurally obtained from Kenya by myself, with permission from the Kenya National Council of Science and Technology (NCST) and the Kenya Wildlife Service (KWS), and with due approval from all the other relevant government authorizing agencies in Kenya. Unless specifically indicated, the experimental work described in this thesis was executed by me. Information obtained from published work is appropriately acknowledged, both in the text and in references therein appended. This thesis is the culmination of my original work and the contents herein, either in full or in part, have not been previously submitted for examination to any university for any award.

Marburg/Lahn, April 2013

James O. Nonoh

# Acknowledgements

With sincerity, I would like to thank my supervisor Prof. Dr. Andreas Brune for his support during my doctoral work.

I sincerely thank the German Academic Exchange Service (DAAD) for the PhD fellowship. Your support, including the many trainings and capacity building workshops organized for the DAAD scholarship holders ensured that I had a focused but smooth stay in Germany and strengthened my resolve to participate effectively in nation building upon completion of my studies.

I am grateful to IMPRS for organizing numerous trainings and support in workshops that all complemented very well with my research work.

I am grateful to the Kenya National Council for Science and Technology (NCST) for facilitating my PhD study by granting me research permit to get samples from Kenya. On the same strength, I acknowledge the support from the Kenya Wildlife Service (KWS) for giving me permission to collect samples from Kenya for my research study. Your policy in promoting capacity building in science through the numerous fees waivers on my student research permit is highly commendable.

I acknowledge the support of Harrison Kibogo and David Wainaina (both of ICIPE) during sampling, without which this work would not have been possible. On numerous occasions, Harrison Kibogo accepted to use his personal car to take me to the field for sampling upon request, despite the difficulty in accessing some sampling sites due to bad weather. David Wainaina, of Molecular Biology Department (ICIPE), always devoted his time, way outside his official commitments, to prepare and package the samples for shipment to our laboratories.

I would like to thank all my colleagues in the “Termite Group” for their immense support during my stay at MPI. Many thanks to all of you for lending me a helping hand whenever I was in need, either during actual experimental work, or through the numerous

discussions. Special thanks to Roey Angel, Kristof Brenzinger and Franziska Brandt (Department of Biogeochemistry) for their support in qPCR analysis.

I am deeply indebted to my late loving parents for all the support they gave me and for laying in me a good foundation that has no doubt contributed immensely to my successful achievements not only in academics, but in all aspects of my life. Thank you Dad and Mum for inculcating in me strong values that continues to shape and motivate my opinions in life. You always reminded me to work hard, but objectively, to believe strongly in myself despite contrary views and to trust none but myself and above all, to always take good care of all that I have worked so hard to achieve, for in the end, it will benefit not only myself, but all in need. These are the ideals that I strongly hold to date.

With pleasure, I would like to express my sincere gratitude to my loving wife Lilian, and my sons Joshua and Emmanuel for their prayers, endless love and overwhelming support throughout my PhD work. It was not an easy decision to stay away from the people I love so dearly in my life, for such a long period of time. Your strength and understanding, and your commitment to support me at whatever cost, always made me to be even stronger, and in the end, it ensured that the only reason I sacrificed everything to be away from you, is well accomplished.

**The following manuscripts were published, submitted or in preparation by the date of submission of this thesis**

**Published**

Paul, K., **Nonoh, J.O.,** Mikulski, L., Brune, A. (2012). "*Methanoplasmatales*," Thermoplasmatales-related archaea in termite guts and other environments, are the seventh order of methanogens. Appl. Environ. Microbiol. **78:** 8245–8253.

**In preparation**

**Nonoh, J.O.,** Paul, K., Boga, H.I., Köhler, T., Meuser, K., Kamanda, D.K. and Brune, A. Diversity and community structure of archaeal populations in the gut of higher termites (Blattodea: Termitidae).

**Nonoh, J.O.,** Dietrich, C., and Brune, A. Distribution of archaeal populations and methanogenic potentials in the highly compartmented gut of soil-feeding termites (*Cubitermes ugandensis* and *Ophiotermes* sp.).

**Nonoh, J.O.,** Dietrich, C., and Brune, A. Microenvironment and community structure of Archaea and Bacteria in the highly compartmented gut of *Amitermes* sp. (Isoptera: Termitidae).

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## Chapter One

# General introduction

**James O. Nonoh**

## Ecological importance of termites

Termites are social insects that belong to the order Isoptera (Nutting, 1990). The order Isoptera contains six families of lower termites and one family of higher termites (Noirot, 1992). The order Isoptera comprise about 2600 described species (Kambhampati and Eggleton, 2000). Termites are the most abundant insects in many tropical regions and may constitute up to 95% of all insect biomass in soil in certain habitats with density estimates of up to 50–100 g $m^{-2}$ in some habitats (Eggleton *et al.*, 1996). They have a strong impact on soil fertility through their influence on structure and properties (Bignell and Eggleton, 2000) and other important soil processes such as plant decomposition, carbon mineralization, as well as stimulation of microbial activity (Wood and Sands, 1978; Bignell *et al*., 1997; Lavelle *et al.*, 1997). Termites are considered to play a major role in the biogeochemical cycles in their ecosystems (Lopez-Hernandez, 2001) particularly in the tropical and sub-tropical regions in which they are more abundant. They are also potential sources of the greenhouse gas methane (Zimmerman *et al*., 1982; Wandiga and Mugedo, 1987; Shinzato *et al*., 1992; Brauman *et al.*, 1992; Darlington *et al*., 1997) and they are currently estimated to contribute between 2–4% to the global budget of atmospheric greenhouse gas methane (Sugimoto *et al*., 2000).

## Higher termite phylogeny and feeding behavior

Termites are phylogenetically classified into lower and higher termites. Lower termites, which are mostly wood feeding, are associated with cellulolytic protists (Inoue *et al*.,

1997; Inoue *et al*., 2000 Noirot, 2001) that colonize their hindgut paunch, accounting for over 60% of the hindgut paunch volume. On the other hand, the higher termites, which feed on a wide diversity of diet including wood, grass, litter, or soil (Noirot, 1992; Bignell *et al*., 1997; Eggleton and Tayasu, 2001), have lost symbiotic gut flagellates. Instead, they rely on a dense community of prokaryotic symbionts which colonize their highly structured and compartmented guts (Inoue *et al*., 1997, 2000; Eggleton and Tayasu, 2001; Brune 2010). Higher termites are abundant, diverse and ecologically more important (Wood and Johnson, 1986), and are represented by only one family, the Termitidae (Noirot, 1992; Donovan *et al*., 2000). Members of this family constitute the majority of all known termite species (Noirot, 1992, 2001; Inward *et al*., 2007). They exhibit wide species diversity and have a much more complex gut structure than their lower termite counterparts, although the morphology of the gut also varies between species. They have great variations in feeding behavior; ranging from foraging for grass or plant material in sound wood or dead wood, to feeding on mineral soil or soil containing organic matter at different stages of humification (Wood and Sands, 1978; Wood and Johnson, 1986; Noirot, 1992, 2001; Donovan *et al*., 2001). The family Termitidae comprises four sub-families of diverse characteristics (Noirot, 1992). Members of sub-family Macrotermitinae cultivate fungal gardens which they consume together with wood litter (Sands, 1969; Rouland *et al*., 1993; Darlington *et al*., 1997; Bignell, 2011) deriving most of the cellulases for degradation of plant material from the symbiotic fungi (Noirot, 1992; Rouland *et al*., 1993). The sub-family Nasutitermitinae consists of wood and grass feeding species, but 44% of the genera are soil-feeders (Noirot, 1992). In the sub-family Termitinae, the majority of the members (over 70% of the genera) are soil feeders (Noirot, 1992; Bignell *et al*., 1997) while the rest are wood feeders. Members of the sub-family Apicotermitinae are mostly soil-feeding (Noirot, 1992). Soil-feeding is not known among the fungus-cultivating members of the sub-family Macrotermitinae. More than half of termite genera in the phylogenetically higher termites are humivorous, feeding on soil organic matter which consists of wood and litter at different stages of decay and humification (Noirot, 1992; Bignell *et al*., 1997). In higher termites, cellulose degradation is mainly mediated by the endogenous cellulases

produced by the termite (Ji and Brune, 2001) and includes endo-β-1,4-glucanases and β-1,4-glucosidases produced by the salivary glands and midgut epithelium in wood-feeding termites (Tokuda *et al*., 2012). In wood-feeding *Nasititermes* spp., Tokuda and Watanabe (2007) provided evidence for presence of significant hindgut cellulase activity which was assumed to originate from the symbiotic bacteria (Tokuda and Watanabe, 2007). In soil-feeding termites, it is not clear whether they are produced exclusively by the midgut epithelium, but degradation of labeled cellulose incubated in soil was shown to increase more than 20-fold in presence of soil-feeding termites (Ji and Brune, 2001) suggesting that termite gut processes mediate cellulase activity. Termites in the sub-family Macrotermitinae, in addition to hosting prokaryotes in their gut, form special association with eukaryotic fungi, mainly *Termitomyces* spp. (Sands, 1969; Rouland *et al*., 1993; Eggleton and Tayasu, 2001), which they cultivate in fungal gardens within the comb and consume together with their wood diet.

Soil-feeding termites strongly influence many soil properties (Brauman *et al*., 2000; Eggleton and Tayasu, 2001), including the structural stability of soil organic matter (Mora *et al*., 2003), affect the distribution of organic matter and bacterial community structure in different particle size fractions (Fall *et al*., 2004), and are considered potentially important sources of micro-habitat heterogeneity in tropical forest soils (Donovan *et al*., 2001b). In contrast to wood-feeding termites, the diet of soil-feeding species consists of a heterogeneous mixture of different organic and inorganic components, where all potential substrates are present in a strongly stabilized form (Kappler and Brune, 2002). There is strong evidence that structural polysaccharides of plants and bacteria and microbial biomass are carbon and energy sources for soil-feeding termites (Ji and Brune, 2001). It has been demonstrated that soil-feeding termites do not mineralize the aromatic components of synthetic humic model compounds to significant extent, but instead they degrade peptidic components which likely forms part of their carbon and energy source (Ji *et al*., 2000). Among the soil-feeding termites, the true soil-feeders ingest degraded mineral soil and are able to thrive on the most recalcitrant soil components (Donovan *et al*., 2001). The true soil-feeders comprise the *Cubitermes* group

of the *Termitinae* and they form important component of the soil macro-fauna particularly in tropical forests and savannahs (Eggleton and Tayasu, 2001). The ability of these termites to feed on soil, an organic nutrient poor resource, is correlated with a highly lengthened and compartmented gut structure which, together with the extreme gut alkalinity, aids in efficient digestion of organic matter present in the diet (Noirot, 1992; Brune and Kühl, 1996; Bignell *et al*., 1997). The highly extended and compartmented hindgut of soil-feeding termites (Fig. 1) has pronounced axial dynamics of $O_2$ and $H_2$ partial pressure (Schmitt-Wagner and Brune, 1999), and intestinal pH (Brune and Kühl, 1996; Brune, 2010) and these dynamics potentially influence the distribution of microbial communities along the gut axis. Gut-microbiota-dependent processes such as $H_2$ production, reductive acetogenesis and methanogenesis have been demonstrated to be heterogeneously distributed in the gut (Schmitt-Wagner and Brune, 1999; Tholen and Brune, 1999).

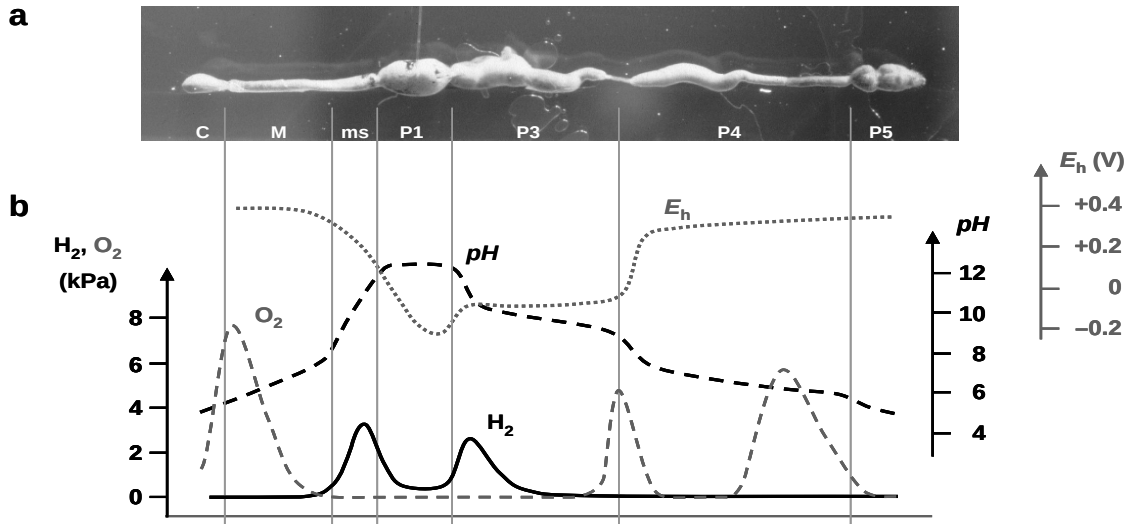


Figure 1: Gut morphology of a soil-feeding termite (*Cubitermes orthognathus*) (a) and the physicochemical gradients of oxygen, pH and redox potential (b) in the gut of *Cubitermes* spp. (figure from Brune *et al*., 2010).

## Methanogenesis in higher termites

Currently, there are six phylogenetic orders of methanogens in the phylum Euryarchaeota. These include Methanobacteriales, Methanococcales, Methanopyrales (Class I), Methanomicrobiales Class II) and Methanosarcinales and Methanocellales (Class III) (Bapteste *et al*., 2005; Sakai *et al*., 2008). Lineages of these methanogenic orders are widely distributed in various environments including those of extreme temperatures, salinity and pH (Liu and Whitman, 2008). They colonize paddy soils, fresh water sediments, marine sediments, anaerobic digestors, geothermal vents, landfills, xylems of trees, animal gastro-intestinal tracts and termite guts (Liu and Whitman, 2008, Brune 2010). The termite hindgut provides a good habitat for methanogenic archaea and is colonized by various lineages of methanogens. The termite gut methanogens fall into three major phylogenetic groups; Methanobacteriales, Methanosarcinales and Methanomicrobiales (Brune, 2010). However, a deep-branching cluster of uncultured archaea distantly related to non-methanogenic Thermoplasmatales have been recovered in the guts of investigated higher termites. This cluster of uncultured Thermoplasmatales comprise archaeal lineages from higher termite guts, the guts of other arthropods, animal rumen and various other environments, and is presently represented by a cultivated isolate *Methanomassiliicoccus luminyensis* isolated from human stool (Dridi *et al*., 2012). They form distinct phylogenetic clusters of mostly uncultivated archaea that are often unique to the intestinal tract of termites and other animals (Brune, 2009; Mihajlovski *et al*., 2010). It is presently unclear whether this group is methanogenic.

Both phylogenetically lower and higher termites harbor methanogenic archaea in their hindguts (Brauman *et al*., 1992; Shinzato *et al*, 1992; Shinzato *et al*., 1999; Brauman *et al*., 2001). These methanogenic archaea are responsible for methane emission by termites. Together with cockroaches, millipedes and beetles, termites are among the few terrestrial arthropods known to emit this greenhouse gas (Hackstein and Stumm, 1994). It presently estimated that termites contribute about 2–4% to the global budget of this greenhouse gas (Sugimoto *et al*., 2000). Termite gut methanogenic archaea play a role as

terminal "$H_2$ sink" organisms for the fermentation reactions in the hindgut (Leadbetter and Breznak, 1996; Schmitt-Wagner and Brune, 1999). Most methanogens are capable of performing hydrogenotrophic methanogenesis, using hydrogen as terminal electron donor. There is a strong difference in methane emission rates between termite feeding groups as well as between termite species (Bignell *et al*., 1997), but the reasons for these differences remain fully uninvestigated. Soil-feeding termites generally emit more methane and have lower potential rates of $H_2$-dependent acetogenesis than wood-feeding species. Because termites exist in large biomass densities and play a keystone role in decomposition processes in tropical and sub-tropical ecosystems, their $CH_4$ emission activities are considered to contribute significantly to the global fluxes of this atmospherically relevant trace gas (Leadbetter and Breznak, 1996). In lower termites, methanogens have been observed to colonize intestinal surfaces at the micro-oxic hindgut periphery of the paunch, where they may be free living, or attached to the gut wall, or to filamentous prokaryotes or occur as endosymbionts of protists (Leadbetter and Breznak, 1996; Brune, 2009). The situation is still not clear in the phylogenetically higher termites mainly because there is still no clear information on the specific localization and density of methanogens in the compartmented gut. This is further complicated by the fact that no isolate representative of the higher termite gut methanogen is currently available to help understand their specific morphological and physiological traits.

## Substrate range of methanogenic archaea

Methanogenic archaea are a large and diverse group of prokaryotes forming methane as the major product of their energy metabolism (Balch *et al*., 1979; Daniels *et al*., 1984; Oremland 1988). They are obligate methane producers that obtain all or most of their energy from methanogenesis (Balch *et al*., 1979; Liu and Whitman, 2008).They are strict anaerobes and belong to the domain archaea. Methanogens can only metabolize a restricted number of substrates (Balch *et al*., 1979; Schink, 1980; Liu and Whitman, 2008). The major substrates for methanogenesis are $H_2$ + $CO_2$, formate, and acetate (Liu and Whitman, 2008). Some methanogens are capable of utilizing some other C-1

compounds, such as methanol, trimethylamine, and dimethylsulfides, and some alcohols, such as isopropanol, isobutanol, cyclopentanol, and ethanol, are substrates for some methanogens (Schink, 1997; Liu and Whitman, 2008). It has been shown that methanogenesis from C-1 compounds is common where methyl containing C-1 compounds are abundant (Schink, 1997). Most organic substances such as carbohydrates, long-chain fatty acids and alcohols, are not metabolized directly by methanogenesis (Schink *et al*., 1980; Schink, 1997; Liu and Whitman, 2008). In this case, methanogens form syntrophic associations with anaerobic bacteria or eukaryotes which ferment these compounds first into intermediates that can then be directly utilized by the methanogens (Schink, 1997; Liu and Whitman, 2008). In most anaerobic environments, methanogens often associate with heterotrophic $H_2$-producing bacteria, which catalyze the oxidation of a variety of substrates (fatty acids, alcohols, and aromatic compounds) (Schink, 1997; Stams *et al.*, 1994). Through interspecies substrate transfer, methanogens use the $H_2$ produced by these syntrophic heterotrophic gut microbiota hence keeping hydrogen partial pressures below 10 Pa (Schink, 1997), and in return, the bacterial symbionts benefit from the removal of the $H_2$ that would otherwise inhibit their growth (Mclnerney *et al*., 2008).

## Methanogenic substrates utilized by termite gut methanogens

Many oxidation reactions occur in the termite hindguts during degradation of diet-derived organic matter and they result in a number of intermediates, but not all can be directly utilized by gut methanogens. Hydrogen is one of the major intermediates linking fermentative breakdown of sugars with methanogenesis as well as reductive acetogenesis during cellulose and lignocellulose degradation in termite hindguts (Ebert and Brune, 1997; Schmitt-Wagner and Brune, 1999). It has been speculated that methanogenesis activity in the hindgut of termites is not only fueled by hydrogen, but also reduced one carbon compounds that are formed during the fermentation breakdown of organic matter and humus (Brune, 2009). It has been postulated that the $H_2$ consumption of methanogens is expected to promote anaerobic cellulose decomposition in the termite hindgut

(Shinzato *et al*., 1999) mainly by keeping hydrogen partial pressures below 10 Pa and allowing fermenting bacterial syntrophs to carry out reactions that favor more ATP synthesis for growth (Schink, 1997). Competition for hydrogen by both methanogens and bacteria may lead to its in limited supply in some hindgut compartments. Conrad and Babel (1989) observed that in natural microbial methanogenic environments such as wetland soil or animal rumen, there is little hydrogen emission because of its efficient transfer from producing organism to the methanogens.

In lower termites however, hydrogen is a major fermentation product of the cellulolytic gut flagellates and it can accumulate to substantial amounts (Ebert and Brune, 1997; Pester and Brune, 2007). Phylogenetically higher termites (family Termitidae) in which methanogenesis outcompetes reductive acetogenesis as the major hydrogen sink reactions, also still accumulate and emit hydrogen (Schmitt-Wagner and Brune, 1999) potentially as a product of bacterial fermentation in the gut (Brune, 2009). But in these termites, particularly in soil-feeding termites, hydrogen only accumulates to detectable levels in specific gut compartments whereas it is below detection levels in other compartments (Schmitt-Wagner and Brune, 1999). That hydrogen is one of the key substrates supporting methanogenesis in termite guts (Brune, 2009) is supported by the hydrogenotrophic nature of all available termite isolates; *Methanobrevibacter cuticularis*, *Methanobrevibacter curvatus* and *Methanobrevibacter filiformis*, all of which are isolated from the wood-feeding lower termite *Reticulitermes flavipes*. All the isolates reduces $CO_2$ with hydrogen during methanogenesis (Table 1), but are also capable of growing on formate (Leadbetter and Breznak, 1996) demonstrating that gut methanogens may utilize potential alternative substrates in methanogenesis. Most Methanobacteriales clones from the investigated higher termites (Ohkuma *et al*., 1999; Friedrich *et al*., 2001; Donovan *et al*., 2004; Miyata *et al*., 2007), and those from lower termites cluster within *Methanobrevibacter* lineages related to known hydrogenotrophic methanogens. Likewise, Methanobacteriales clones from the humivorous cockroaches *Periplaneta americana* (van Hoek *et al*., 2000), xylophagous cockroaches *Panesthia angustipennis* and *Salganea esakii* (Hara *et al*., 2002) and the humus-feeding beetle *Pachnoda ephippiata* (Egert *et*

*al*., 2003) also cluster with termite gut-derived clones and they form close relationships to known hydrogenotrophic methanogens in the genus *Methanobrevibacter*. The close relationship between *Methanobrevibacter* related lineages from termites and cockroaches probably suggest that hydrogenotrophic methanogenesis has some genetic origin from the termite ancestors.

In the highly compartmented gut of higher termites, hydrogen is only accumulated in the anterior gut compartments while the posterior methanogenic gut regions do not accumulate any significant amounts of hydrogen (Schmitt-Wagner and Brune, 1999). This was a paradox, mainly because it was not clear how methanogens in the more substrate limited, but highly methanogenic posterior gut sections get their substrates. While inter-compartmental hydrogen transfer has been postulated to nourish archaea in the non hydrogen accumulating posterior gut sections in which hydrogen partial pressures was below detection limit (Tholen and Brune, 1999; Schmitt-Wagner and Brune, 1999), the varied nature of the higher termite diet potentially provides avenues for gut methanogenic substrates other than merely hydrogen. Further, considering the diffusion limitations of hydrogen, it seems reasonable to speculate that other mechanism may be available for transport of methanogenic substrates to the methanogen communities in different gut compartments. Schmitt-Wagner and Brune, (1999) observed that methanogenesis in the posterior hindgut of soil-feeding (*Cubitermes* spp.) was not only stimulated by hydrogen but also by formate. In the hindgut of the scarab beetle larva *Pachnoda ephippiata*, formate and methanol also stimulated methane emission (Lemke *et al*., 2003). This was later corroborated by the presence of considerable concentrations of formate (2.6 mM) in other gut compartments and in the haemolymph, and led to the speculation that methanogenesis in the hindgut may be driven also by an inter-compartmental transfer of reducing equivalents via the haemolymph (Schmitt-Wagner and Brune, 1999). The speculation over involvement of methanol in gut methanogenesis continued to build up simply because Methanosarcinales sequences recovered from termite guts are closely related to the cockroach isolate *Methanimicrococcus blatticola* from *Periplaneta americana*. So far, *Methanimicrococcus blatticola* is the only cultivated

representative in the cluster of the family *Methanosarcinaceae*- related clones recovered from insect guts, and it is an obligately methylotrophic methanogen using $H_2$ as an electron donor only in the presence of methanol or methylamines (Sprenger *et al*, 2000). Recently, *Methanomassiliicoccus luminyensis*, a methylotrophic methanogen, growing on methanol in presence of hydrogen was isolated from human stool (Dridi *et al*., 2012), and is the only cultivated representative of the deep-branching cluster of uncultured Thermoplasmatales, which is dominant in the gut of the few investigated higher termites. Although this isolate forms a different cluster (Rice cluster III) from that of termites, its metabolism provides an insight into the physiology of this apparently novel group of uncultured euryarchaeota to which little is presently understood. The requirement of formate in the growth media of *Methanomassiliicoccus luminyensis* (Dridi *et al*., 2012) leads to further speculations over the role of alternative substrates in the metabolism of archaea including termite-derived methanogenic archaea. It is becoming increasingly clear that the environmental energy sources for methanogenic archaea, particularly those of the termite gut methanogens, are still poorly understood. Because of the considerable variations in methane emission rates and feeding behavior observed in different species of higher termites, it remains an open question if methanogenesis in termite guts is not supported by substrates other than merely hydrogen. Even still less understood is how the substrates are partitioned in the highly compartmented gut of higher termites and how this affects the distribution of archaea along the gut axis. However, based on the different physicochemical conditions in the gut, it is highly likely that substrate gradients exist in the compartments. Such gradients may influence the distribution and localization of methanogens in the highly compartmented gut of higher termites and this may potentially result in evolution of diverse metabolic groups of gut methanogens.

Table 1: Pathways for utilization of methanogenic substrates by representatives of methanogen isolates, including those from termite and cockroach guts.

| Pathway | Methanogen |
|---|---|
| **Hydrogenotrophic pathway** | |
| $4H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$ | Most methanogens; |
| | *Methanobrevibacter cuticularis*[a] |
| | *Methanobrevibacter curvatus*[a] |
| | *Methanobrevibacter filiformis*[a] |
| **Methylated C1 pathway** | |
| Methylotrophic pathway | |
| $4CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$ | *Methanosarcina* |
| | *Methanosphaera stadtmanae* |
| Methyl reduction pathway | |
| $CH_3OH + H_2 \rightarrow CH_4 + H_2O$ | *Methanimicrococcus blaticola*[b] |
| | *Methanomassiliicoccus luminyensis* from human feces |
| **Aceticlastic pathway** | |
| $H_3C\text{-}COO^- + H^+ \rightarrow CH_4 + CO_2$ | *Methanosaeta* |
| | *Methanosarcina* |
| | No aceticlastic methanogen isolate from termites |

[a] Isolated from the wood-feeding lower termite *Reticulitermes flavipes*

[b] Isolated from the litter-feeding cockroach *Periplaneta americana*

## Methane emission rates in termites

Methane is a global greenhouse gas. Major sources of atmospheric methane include rice paddy soils, natural wetlands, energy generation, fermentations in ruminant animals, landfills, biomass burning, gas reservoir leakages and, intestinal tracts of arthropods (Zimmerman *et al*., 1982). About 74% of methane is derived from biological sources occurring in anaerobic environments (Rasmussen and Khalil 1983). Termites have attracted attention as sources of methane because of their enormous abundance especially in tropical and subtropical regions and because of the difficulty in quantification of their exact numbers on earth. They are currently estimated to contribute about 2–4% to the global atmospheric emissions of this greenhouse gas (Sugimoto *et al*., 2000). Methane production by termites, first observed by Cook (1932) in *Zootermopsis nevadensis* is now documented through numerous data resulting from both laboratory and field estimations in various species of lower and higher termites. Breznak (1975) provided the first quantitative data on methane production by termites when he investigated methane formation by the wood-feeding lower termites *Reticulitermes flavipes*, *Coptotermes formosanus* and *Cryptotermes brevis* as well as in the wood-feeding cockroach *Cryptocercus panctulatus*. He observed a body weight based differences in methane production between the three species of lower termites. The importance of termites as global sources of methane was observed by Zimmerman *et al*. (1982) when they estimated methane production from laboratory colonies of the lower termite *Reticulitermes tibialis* and the higher termite *Gnathamitermes perplexus*, as well as from the nest of a Nasutitermitinae species. Although they did not observe any significant difference in methane production rates between the two groups of termites, they could show global significance of termites as potential source of the trace gas methane. Later, Rasmussen and Khalil (1983 estimated methane production in five different colonies of *Zootermopsis angusticollis* each with twenty five termites and they observed variability in methane production per termite per day both within species and among different species. Since most of these studies were based on laboratory colonies of only a few termite species, it was difficult to get true estimates of methane production rates because

the results did not take into consideration the varied nature of the termite diet, particularly in higher soil-feeding termites. Also methane oxidation potential of the mound was not factored in. Seiler *et al*., (1984) provided data for the first field estimates of methane emission rates by different species of higher termites as well as a species of lower termite. They measured efflux of methane and $CO_2$ from the mounds of representative species of higher termites *Macrotermes* and *Odontotermes* sp. (Macrotermitinae), *Trinervitermes* sp. (Nasutitermitinae) and, *Amitermes* and *Cubitermes* sp. (Termitinae) as well as that of the lower termite *Hodotermes* sp. (Hodotermitidae). They observed variations in flux rates of methane from the mounds of the termites. However, these values were only estimates of net effluxes from the mounds and therefore the results could have been underestimated since it could not be established the exact number of termites in the mound at the time of the experiment. The study by Brauman *et al*. (1992) provided a comprehensive analysis of methane emission in diverse termites by investigating six and eighteen species of freshly sampled and laboratory maintained colonies of lower and higher termites respectively. From the results, it became evident that there were several variations in methane emission rates between different species of termites and among feeding groups. Highest methane emission rates were observed in members of soil-feeding and some species of fungus-cultivating higher termites as compared to wood-feeding higher and lower termite species. Additional data on methane emission rates in termites was provided by Shinzato *et al*. (1992) who investigated both lower and higher termites collected from Japan. He observed variations in methane emission rates between the species of the lower termites *Neotermes Koshunensis*, *Reticulitermes speratus,* and *Coptotermes formosanus* as well as the higher termites *Odontotermes formosanus* and *Nasutitermes takasagoensis*. However, he did not observe methane emission in some colonies of *Neotermes Koshunensis* demonstrating that methane emission may be dependent on factors such as feeding, nature of the diet and that it may be underestimated in some cases. Hackstein and Stumm (1994) performed laboratory screening of several representatives of different taxa of arthropods for methane production. They observed that other than the termites (Isoptera) which generally produce more methane than the other arthropods, cockroaches (Blattidae), millipedes (Diplopoda)

and scarab beetles (Scarabaeidae) are the only other insects that emit methane and harbor methanogenic archaea in their hindguts. Their estimation of methane emission rates in these arthropods revealed variations between individuals, sexes, the different developmental stages and even variations among populations. Bignell *et al.* (1997) estimated methane emission rates in various field collected termites and in undisturbed mounds, and they observed variations in methane emission rates not only between termite species but also between colonies. They observed high rates in soil-feeding and wood/soil interface feeding species than in wood-feeding termites. Sugimoto *et al.* (1998) investigated methane emission rates in 2–35 laboratory individual workers and soldiers of representatives of all the four sub-families of higher termites and five of the seven families of lower termites collected from Australia, Thailand and Japan. They observed extremely high methane emission rates in workers of the soil-feeding *Termes* group from both regions, while workers of *Macrotermes*, *Odontotermes*, *Microcerotermes*, and *Globitermes* species produced relatively low amount of methane.

In general, these studies show that methane emission rates vary between termite species, with lower termites generally emitting less methane than their higher termite counterparts (Collins and Wood, 1984; Brauman *et al*., 1992; Shinzato *et al*., 1992; Bignell *et al*., 1997; Sugimoto *et al*., 1998). Nevertheless, some species, for example *Zootermopsis anguisticollis*, a lower termite showed unusually high rates in the range of 1300 nmol $g^{-1}h^{-1}$. Estimates of methane emission rates by the worker caste termites based on the taxa level and on feeding group of the family Termitidae (Sugimoto *et al.* 2000) showed that members of the soil-feeding termites in the sub-family Termitinae and Apicotermitinae emit high amounts of methane than members of the other sub-families (Figure 2), with exceptionally high rates, exceeding 1000 nmol $g^{-1}h^{-1}$ reported in the soil-feeding *Thoracotermes macrothorax*. This corroborated the findings of the previous studies in which high methane emission rates among higher termites was observed in soil-feeding and some fungus-cultivating termite species, with highest rates reported in the *Cubitermes* group of Termitinae (Brauman *et al*., 1992; Bignell *et al*., 1997), suggesting influence of termite diet on methane emission rates . Although these studies

employed various approaches to estimate methane emission rates by termites, there is a general concurrence that weight-specific rates of methane emission differ across termite phylogenies and feeding groups as well as between species. The reasons for these differences remain unclear. The high rates observed in some termite feeding taxa, particularly in soil-feeding species suggest that these variations are probably influenced by the diet and the physicochemical gut environment. However, information on community structure and population density of archaea in the guts of diverse feeding groups of termites is still very deficient.

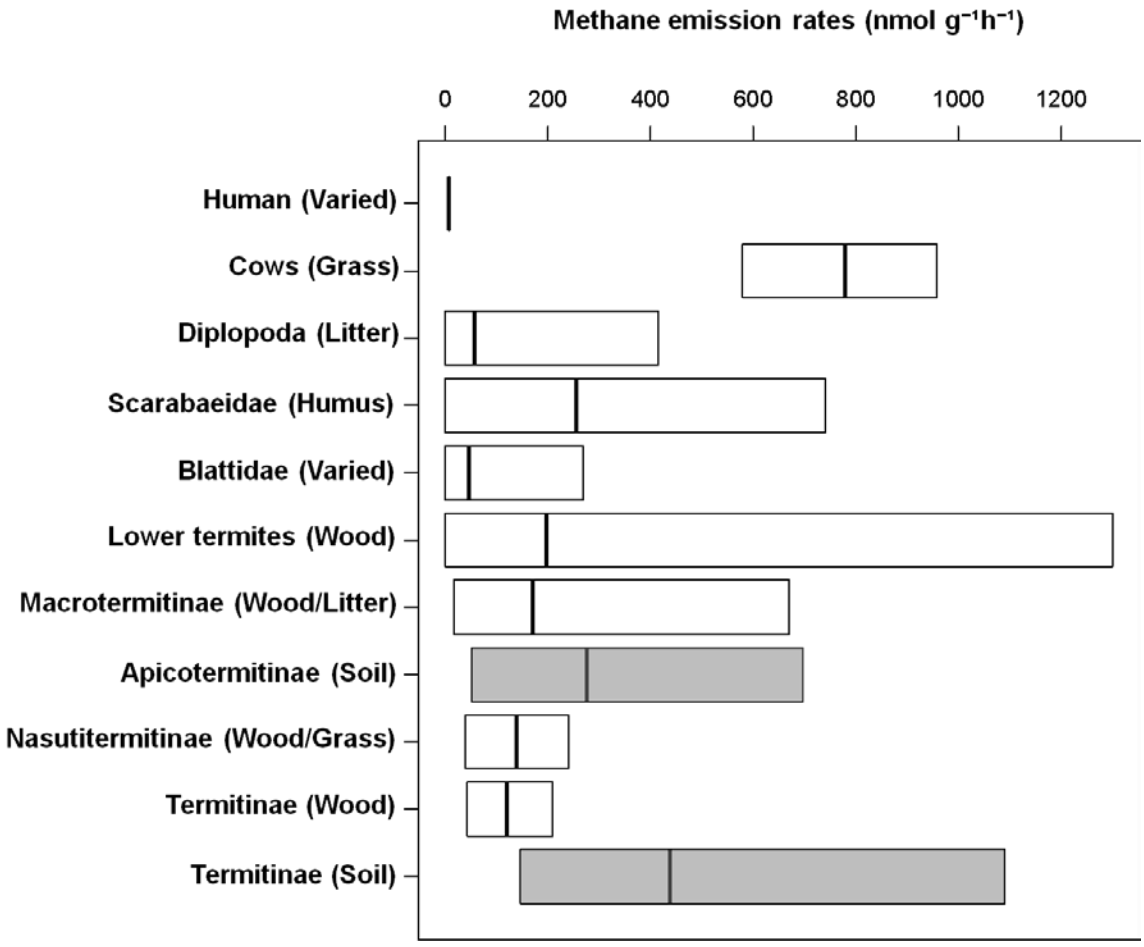


Figure 2: Methane emission rates in different higher termite taxa as compared to those of lower termites, other arthropods, cows and humans. The diet of the respective taxon is given in brackets. The values shown are fresh weight based averages from different studies.

Minimum and maximum rates are also shown for each taxon. Members of the soil-feeding termite taxa (gray bars) generally show high methane emission rates than those of other feeding groups. Data was obtained from Brune *et al.*, 2010.

## Community composition of archaea in higher termites

Although archaea and specifically methanogens are present in the guts of nearly every termite investigated to date, an indication that they form an important termite gut microbiota, much is still unknown about the community structure, their distribution in the gut and the specific roles they play in the gut microhabitats especially in the compartmented gut of higher termites. A few studies have investigated archaeal community structure in both lower and higher termites, but most of the studies have focused on selected species, mainly because of the challenges involved in getting the samples. Despite the great diversity and abundance of members of the higher termite family Termitidae which accounts for 75% of the termite species, only a limited number of species have been investigated, with most of them being biased towards the members of particular taxa. Clone analysis of 16S rRNA gene sequences of archaeal gut symbionts from the whole gut DNA of wood-feeding lower termites *Hodotermopsis sjöestedt* (Ohkuma *et al*., 1999; Tokura *et al*., 2000), *Cryptotermes domesticus* (Ohkuma and Kudo, 1998) and *Reticulitermes speratus* (Ohkuma *et al*., 1995; Shinzato *et al*, 1999; Tokura *et al*., 2000) revealed low archaeal diversity in these termites, with only sequences belonging to Methanobacteriales detected. However, archaeal community structure profiling in the lower wood-feeding termite *Reticulitermes speratus* (Kalotermitidae) revealed a higher diversity especially in one colony (colony RS1) in which majority of the clones were Methanobacteriales, one clone fell within uncultured Thermoplasmatales while another clone belonged to the genus *Methanocorpusculum* in the order Methanomicrobiales (Shinzato *et al*., 1999). In a different colony (colony RS4) of *Reticulitermes speratus* in the same study, majority of the clones were Methanobacteriales, while two clones belonged to uncultured Thermoplasmatales, with no Methanomicrobiales or Methanosarcinales being recovered, an indication of inter-colony variations. Using domain-specific nucleic acid probes targeting 16S rRNAs,

Brauman *et al.* (2001) profiled prokaryotic communities in representative species of two families of lower termites as well as representatives of all the four sub-families of higher termites. Quantitative hybridization with family-level probes targeting the 16S rRNA of methanogens detected members of only two families, Methanobacteriaceae and Methanosarcinaceae, with the most abundant archaeal community Methanobacteriaceae being detected in the guts of almost every termite investigated irrespective of diet or taxonomy (Brauman *et al*, 2001). Methanosarcinaceae was detected in only half of the species investigated, being the dominant archaea in only four species, all of which were higher termites. But in some termites, the combined signals for group-specific probes were less than that of archaeal domain probes, with Methanobacteriaceae and Methanosarcinaceae signal representing less that 20% of the archaeal probe signal in some termite species, suggesting a bias in probe coverage.

Culture-independent studies based on clone sequencing of archaeal 16S rRNA gene in the investigated higher termites revealed that higher termite guts are colonized by much more diverse groups of symbiotic archaea than their lower termite counterparts. In the wood-feeding *Microcerotermes crassus* and the soil-wood inter-phase feeding *Termes comis* (all Termitinae), only Methanobacteriales lineages was recovered (Deevong *et al*., 2004). Although only clones belonging to Methanomicrobiales were recovered in a field collected sample of the wood-feeding *Nasutitermes takasagoensis* (Nasutitermitinae) (Ohkuma *et al*., 1999), a much higher diversity of archaea was recovered in a laboratory colony of *Nasutitermes takasagoensis* (Nasutitermitinae) maintained on various carbon sources, with members of Methanosarcinales, uncultured Thermoplasmatales, Methanobacteriales and Crenarchaeota being represented (Miyata *et al*., 2007). In the fungus cultivating *Odontotermes formosanus* (Macrotermitinae), only members of *Methanimicrococcus* (Methanosarcinales) was recovered (Ohkuma *et al*., 1999) whereas *Methanobacterium* spp. (Methanobacteriales) were the only archaea recovered from the fungus-cultivating *Macrotermes gilvus* (Macrotermitinae) (Deevong *et al*., 2004). In the soil-feeding *Pericapritermes nitobei* (Termitinae), Ohkuma *et al*. (1999) recovered diverse genera of methanogenic euryarchaeota, comprising *Methanimicrococcus* (Methanosarcinales), *Methanospirillum* (Methanomicrobiales) and *Methanobrevibacter*

(Methanobacteriales), whereas only *Methanobacterium* spp. (Methanobacteriales) was recovered in the soil-feeding *Pericapritermes* sp. (Deevong *et al*., 2004). Highest diversity was reported in the soil-feeding members of the *Cubitermes* group of Termitinae including *Cubitermes orthognathus* (Friedrich *et al*, 2001) and *Cubitermes fungifaber* (Donovan *et al*., 2004) (all Termitinae) with most sequences being affiliated with known taxa of methanogenic euryarchaeota comprising the orders; Methanobacteriales, Methanosarcinales and Methanomicrobiales. A large number of sequences from the soil-feeding *Cubitermes orthognathus* (Friedrich *et al*., 2001) fell within the deep-branching cluster of uncultured Thermoplasmatales. These studies contributed greatly to our understanding of archaeal community composition in both lower and higher termites. However, in most of these previous studies, only a very minimum number of clones were analyzed and therefore the low diversity reported in some cases may be due to under sampling hence underrepresentation of diversity. The differences in the diversity coverage could also be as a result of primer bias. Since most of these sequences were recovered from the whole gut DNA, it could not be established in which specific gut compartments the recovered methanogenic communities were localized.

TRFLP studies combined with clone analysis of 16S rRNA gene of euryarchaeota in the whole gut of the soil-feeding *Cubitermes fungifaber* (Donovan *et al*. 2004) revealed higher archaeal diversity with major methanogenic lineages comprising Methanosarcinales, Methanomicrobiales, Halobacteriales and Methanobacteriales being recovered. However, like many other studies, it did not provide an insight into the axial distribution of these major archaeal communities in the highly compartmented gut. For example their discovery of the new group of Halobacteriales in termite guts was a novel observation, but since the group was recovered from whole gut DNA, it was not possible to establish the specific gut compartment localization of this archaeal group whose cultured relatives are known to tolerate high salt concentrations. Their failure to recover any sequences belonging to the deep-branching group of uncultured Thermoplasmatales, and members of Crenarchaeota which are abundant in soil-feeding *Cubitermes orthognathus* (Friedrich *et al*., 2001), was perhaps as a result of limitations in primer

coverage. A more comprehensive insight into the axial distribution of archaea in the highly compartmented gut of soil-feeding *Cubitermes orthognathus* was provided by Friedrich *et al*., (2001), in which they analyzed the archaeal community structure patterns within each individually sectioned gut compartments. They observed a large phylogenetic diversity and pronounced axial differences in the composition of the archaeal gut microbiota, which they correlated to the morphological and physicochemical heterogeneity of the gut. However, considering the high methane emission rates in higher termites and the great variations in feeding behavior, the diet potentially plays a role in influencing the distribution of methanogenic archaea in the gut, potentially by influencing the availability of methanogenic substrate gradients in specific gut compartments. The observation that some termites are collected from abandoned mounds of others, or colonize the same mound or nest of another species may also have implications on the nature of the diet consumed and hence influence the overall community structure of archaeal gut microbiota. Considering the number of described termite species, and that even more still remains un-described, it is reasonable to believe that the community composition of archaea has been investigated in only a few termite species, particularly in higher termites. The previous studies contributed greatly to the understanding of the archaeal community composition in higher termites. However, these studies may have been limited in sample coverage, and in some cases would appear to be biased towards specific taxa. Members of some taxa such as Apicotermitinae have not been well investigated yet they form a significant component of the family Termitidae, whereas other taxa have been underrepresented despite the observed variations in gut morphology, feeding behavior and even methane emission rates. Information on numerical abundance of gut archaeal communities is critical in understanding specific colonization of the gut compartments by methanogens, as well as in estimating methanogenic potentials in the compartments in which they are localized, yet such knowledge is still largely unavailable. Also, knowledge of the micro-environmental factors influencing the distribution of archaea in the highly varied gut structure of higher termites is still limited, yet that information is critical to understanding the preferential colonization of specific micro-habitats in the gut. It is therefore hypothesized that the varied methane emission rates

between higher termites may be reflected in the diversity and density of methanogenic archaea colonizing specific gut micro-habitats, a phenomenon that may be influenced not only by micro-environmental conditions, but also by the availability of utilizable substrates potentially derived from degradation of the host diet. This study provides a comprehensive coverage of the community composition and population size of archaea in the guts of representative species of higher termite sub-families with reference to the varied methane emission rates.

## Objectives of the study

Higher termites (family Termitidae) have diverse feeding behavior and show variations in gut morphology. These differences are also observed in methane emission rates which vary between termite species. Phylogenetically higher termites generally emit much more methane than lower termites (Shinzato *et al*., 1992, Brauman *et al*., 1992, Bignell *et al*., 1997). Only a few higher termites have been investigated in archaeal community structure studies (Ohkuma *et al*., 1999; Friedrich *et al*., 2001; Deevong *et al*., 2004; Donovan *et al*., 2004) in which only a limited number of clones were analyzed. To get an understanding of the dynamics in methane emission rates of archaeal communities, a comprehensive analysis of archaeal community composition in representative species of the four sub-families of higher termites is necessary.

The highest rates observed in the soil-feeding species (Brauman *et al*., 1992; Bignell *et al*., 1997), which have a highly compartmented gut, with varied physicochemical conditions, suggest that the distribution of archaea is also influenced by the host diet. In *Cubitermes orthognathus*, it was previously shown (Schmitt-Wagner and Brune, 1999) that formate stimulated methanogenesis in the posterior gut sections more than hydrogen. However, owing to the diverse diets of higher termites, it is possible that substrates other than merely hydrogen, derived from degradation of diet, may be responsible for methanogenesis in the gut. The range of substrates supporting methanogenesis in the gut is still not clearly understood. Our knowledge of the community structure, diversity, localization of archaea and the potential rates of methanogenesis in the specific gut

compartments of the highly compartmented gut of soil-feeding termites is still very deficient.

Finally, information on the population density of archaea in the highly compartmented and physicochemically diverse guts of higher termites is still lacking. Previous studies on the soil-feeding termite *Cubitermes orthognathus* demonstrated high relative abundance of methanogens in the posterior gut sections (Tholen and Brune, 1999; Friedrich *et al*. 2001). However, no study has quantitatively investigated the absolute abundance and the population size variations of archaea in the highly varied and compartmented guts of higher termites. Such information will help in estimating methanogenic potential of individual gut compartments and of the host termite.

In order to understand the community structure, diversity, and population densities of archaea and the methanogenic activities in the highly compartmented guts of higher termites, my PhD study was structured with the following objectives:

- To do comparative analysis of archaeal community structure in representative species of higher termites in order to understand whether the variations observed in methane emission rates between higher termite species is reflected in the diversity and community composition of archaea in the guts of the host termites,
- To investigate the distribution and localization of archaea in the highly compartmented gut of soil-feeding higher termites in relation to the existing *in situ* physicochemical characteristics in the gut, and to evaluate the potential substrates supporting methanogenesis in the gut and,
- To quantify the abundance of archaea in the gut of higher termites and the population size variations of methanogens along the gut axis of the highly compartmented gut of soil-feeding termites in order to understand methanogenic potential of the gut.

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## Chapter Two

# Diversity and community structure of archaeal populations in the gut of higher termites (Blattodea: Termitidae)

**James O. Nonoh, Kristina Paul, Hamadi I. Boga, Tim Köhler, Katja Meuser, David K. Ngugi and Andreas Brune**

Max Planck Institute for Terrestrial Microbiology, Department of Biogeochemistry, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany.

Author contributions:

J.O.N. designed experiments, obtained the samples, prepared the DNA, made clone libraries, performed qPCR, performed phylogenetic analysis, analyzed and visualized the data, interpreted the results, and wrote the manuscript.

K.P. made some 16S rRNA clones and some qPCR for *Cubitermes* and *Ophiotermes* sp.

H.I.B. obtained the sample, prepared DNA, and made clone libraries for *Macrotermes* sp.

T.K. contributed to phylogenetic analysis.

K.M. made clone libraries for *Ophiotermes* sp.

D.K.N. obtained the sample and prepared DNA for *Ophiotermes* sp.

A.B. conceived the study, designed experiments, interpreted the results and secured funding.

# Abstract

Methanogenesis in termite guts contributes 2–4% to the global budget of this greenhouse gas. There are large variations in methane emission rates, with higher termites emitting more methane than their lower termite counterparts. Highest rates are observed in soil-feeding taxa but little is known about the community structure and diversity of archaea in the highly compartmented guts of higher termites. We used a comparative 16S rRNA-based sequencing and qPCR analysis of archaeal communities in various higher termites, including wood-feeding, grass-feeding, soil-feeding, and fungus-cultivating species. Our results showed that higher termites host a diverse assemblage of methanogens comprising representatives of four orders: Methanobacteriales, Methanosarcinales, Methanomicrobiales and the recently discovered Methanoplasmatales. Highest diversity and density of archaea was observed soil-feeding termites, in which lineages from all the four orders of methanogens were recovered. Other than methanogenic euryarchaeota, soil-feeding termites also host uncultured members of other archaeal phyla, comprising lineages closely related to ammonia-oxidizing Thaumarchaeota and a deep-branching termite specific group of uncultured archaea loosely affiliated to Crenarchaeota, whose role in termite guts is still not clear. The presence of higher termite specific lineages, suggest that archaeal gut microbiota seems to co-evolve with the host termite. Diversity was much lower in the fungus-cultivating, grass-feeding and in the wood-feeding termites. Highest archaeal diversity observed soil-feeding termites suggests that availability of substrates and the differences in micro-environmental conditions in the gut influence methane emission rates.

# Introduction

Termites are phylogenetically divided into lower and higher termites (Inward *et al*., 2007). The higher termites, which constitute three quarter of all known termite species, are represented by only one family Termitidae, which exist in great biomass densities especially in tropical ecosystems (Bignell and Eggleton, 2000). The higher termites show a great diversity in feeding behavior, feeding on plant material at different stages of decomposition, from wood to true soil with the majority being soil feeders (Donovan *et al*., 2001). Together with cockroaches, millipedes and beetles, termites are among the few terrestrial arthropods known to produce methane (Hackstein and Stumm, 1994). Termites produce significant amounts of methane (Sugimoto *et al*., 2000), and this is attributed to the presence of members of methanogenic archaea in their hindguts. However, methane emission rates vary between termite taxonomy as well as between species (Shinzato *et al*., 1992; Brauman *et al*., 1992; Bignell *et al*., 1997). Soil-feeding higher termites emit more methane than wood-feeding higher ones, with some wood feeding termites emitting little or no methane (Brauman *et al*., 1992). Also, among the soil-feeders, members of soil-feeding Termitinae emit more methane than the other soil feeding taxa. Generally higher termites emit more methane than their lower termite counterparts and the reason for these differences has not been very clear. Methanogenic archaea, which are responsible for methane production in termites, have been detected in all termites investigated to date (Purdy, 2007). While the methanogenic community in lower termites exists exclusively of members of Methanobacteriales, a more complex archaeal community seems to colonize the guts of higher termites (Ohkuma *et al*., 1999, Friedrich *et al*., 2001, Donovan *et al*., 2004). Using domain specific probes, Brauman *et al*., (2001) observed high relative abundance of archaea in the guts of soil feeding termites compared to those of wood feeding species in the same family. However, his family level probes detected only members of two families; Methanobacteriaceae and Methanosarcinaceae in higher termites. Molecular studies have shown that the highly compartmented guts of higher termites host more diverse archaeal communities than in lower termites (Friedrich *et al*., 2001, Donovan *et al*., 2004). However, only a few higher termites have so far been

investigated, most of which have been biased towards members of specific taxa. Little is still known about the community composition, the diversity and population size of methanogenic archaea in the gut of individual species of higher termite taxa. Moreover, the population density of archaeal gut microbiota and specifically the methanogens, which contributes to methanogenesis in termite guts, has not investigated. The functional roles of termite archaeal gut microbiota is not clearly understood partly because they have not been studied in detail and also because attempts to cultivate higher termite methanogens have not been very successful. The implications of the varied nature of the higher termite diet on the structure of its archaeal gut microbiota remains to be fully understood. Also, the implications of the differences in gut morphology observed in members of higher termites on community structural patterns and physicochemically highly structured hindgut of the soil-feeding species on the diversity and distribution of methanogenic archaeal populations need to be clearly understood. Such information is necessary as it will not only help in understanding the important metabolic relationship between termites and their gut symbionts, but will also help in understanding the important role that this insects play in influencing ecological processes in global ecosystems.

# Methods

## Termites

We investigated representative species of higher termite sub-families (Table 1). We used soil-feeding termites *Cubitermes ugandensis*, *Ophiotermes* sp. and *Amitermes* sp. (Termitinae), the wood-feeding *Microcerotermes* sp. (Termitinae), grass-feeding *Trinervitermes* sp. (Nasutitermitinae), soil-feeding *Alyscotermes trestus* (Apicotermitinae) and fungus-cultivating *Macrotermes michaelseni*, *Macrotermes* sp. and *Microtermes* sp. (all Macrotermitinae). All the termites used in this study were collected from Kenya. The soil-feeding *Cubitermes* and *Ophiotermes* sp. were collected from Liranda hills in Kakamega forest. *Trinervitermes* sp., *Alyscotermes* sp., *Amitermes* sp.

and *Microcerotermes* sp. were collected from JKUAT farm in Juja while *Macrotermes michaelseni*, *Macrotermes* sp. and *Microtermes* sp. were collected from Kajiado district in Kenya. Live termites together with their nest and soil food material collected from the vicinity of the respective mounds were brought to our laboratories for further analysis. The termites with their nest material were kept in plastic containers in our rearing room at 25°C. Only the worker castes were used for this study. The termites were investigated within 1-2 months after collection.

Table 1: Representative species of the sub-families of the higher termite family (Termitidae) investigated in this study. The feeding guild of each species and the collection area is indicated.

| Code | Termite | Sub-family | Feeding guild | Sampling location |
|---|---|---|---|---|
| TD110 | *Cubitermes ugandensis* | Termitinae | Soil-feeding | KK[a] |
| TD79 | *Ophiotermes* sp. | Termitinae | Soil-feeding | KK |
| TD121 | *Amitermes* sp. | Termitinae | Soil-feeding | JKU[b] |
| TD118 | *Microcerotermes* sp. | Termitinae | Wood-feeding | JKU |
| TD114a | *Trinervitermes* sp. | Nasutitermitinae | Grass-feeding | JKU |
| TD117 | *Alyscotermes trestus* | Apicotermitinae | Soil-feeding | JKU |
| TD115 | *Macrotermes michaelseni* | Macrotermitinae | Fungus-cultivating | KJ[c] |
| TD116 | *Microtermes* sp. | Macrotermitinae | Fungus-cultivating | KJ |
| Mm | *Macrotermes* sp. | Macrotermitinae | Fungus-cultivating | KJ |

[a] Collected from Kakamega forest reserve, Kenya.

[b] Collected from JKUAT farm, Kenya.

[c] Collected from Kajiado, Kenya.

## DNA extraction and purification

DNA from the gut of worker caste termites (10-20) was extracted as follows; the gut of each termite was pulled out with sterile, fine tipped forceps on a clean sterile Petri-dish. For *Cubitermes Ophiotermes* sp. and *Amitermes* sp., the whole guts were separated into different compartments; crop (C), midgut including mixed segment (M), and proctodeal sections P1, P3, P4 and P5 with the aid of a stereomicroscope (Zeiss, Jena, Germany). Gut sections 10-20 each for *Cubitermes* and *Ophiotermes* sp., and the same number of

whole guts, including the midgut and crop, for *Alyscotermes*, *Trinervitermes*, *Microcerotermes* and *Microtermes* species were pooled together into a sterile 2 ml eppendorf tubes filled with 750 µl of sterile phosphate buffer (120 mM; pH 8.0). The pooled whole guts or gut sections were then homogenized using a sterile pestle (Eppendorf, Hamburg, Germany). DNA was extracted following the Zirconium bead-beating method combined with phenol-chloroform (Lueders *et al*., 2004). The whole gut and compartment homogenates were transferred to 2 ml bead-beating vials, followed by addition of 250 µl sodium dodecyl sulfate (SDS) solution (10% SDS; 0.5 M Tris-HCl, pH 8.0; 0.1 M NaCl), and 0.7 g heat-sterilized zirconium-silica beads (0.1 mm diameter, Carl Roth, Karlsruhe, Germany) and the cells were lysed using cell disruptor (FastPrep-24 MP Biomedicals, Ilkirch, Germany) for 45 s at 6.5 m/s after which the cell debris were removed by centrifugation at 20000 × g for 4 minutes. The supernatant, containing the DNA was extracted with one volume of phenol-chloroform-isoamylalcohol (25:24:1) and eventually with 1 volume of chloroform/isoamyl alcohol (24:1, vol/vol) after centrifugation for 1 minute, prior to precipitation of the DNA by mixing the aqueous phase with 2 volumes of polyethylene glycol (PEG) solution (30% PEG 6000 in 1.6 M NaCl) and then centrifugation at 20000 × g and 4 °C. After washing with ice-cold ethanol (70%), the recovered pellet was dried at 30 °C for 5 minutes. The DNA was re-suspended in 50 µl elution buffer (MinElute PCR Purification Kit, Qiagen, Hilden, Germany). The extracted DNA was run on a standard 1% agarose gel in order to verify extraction efficiency and quality of the extracted DNA. The purity of the extracted DNA was checked photometrically (Nanodrop, PeqLab, Erlangen, Germany), then quantified fluorimetrically (Qubit, Invitrogen, Eugene, OR, USA), and then stored at –20 °C.

## Amplification of archaeal SSU 16S rRNA genes

PCR amplification was carried out using primer pairs that target archaeal small sub unit (SSU) 16S rRNA genes from position 109 to 934 as described by Großkopf *et al*., (1998) and Friedrich *et al*., (2001). Oligonucleotide primers specific for archaeal 16S rDNA used were Ar109F and Ar912R (CTCCCCCGCCAATTCCTTTA) (Escherichia coli 16S

rRNA numbering) [Brosius *et al*., 1978]. Each PCR reaction (50 µl) contained reaction buffer, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase (all Invitrogen, Carlsbad, CA, USA), 50 µM deoxynucleoside triphosphate mix, 0.3 µM of each primer, 0.8 mg/ml bovine serum albumin, and 1 µL DNA template. PCR (30 cycles for P1, P3, P4 and P5 compartments) were carried out at initial denaturation step (94 °C for 3 min), followed by denaturation (94 °C for 20 s), annealing step (52 °C for 30 s), extension (72 °C for 45 s), and a final extension step (72 °C for 7 min). Our attempt to amplify the crop and midgut DNA under the same conditions did not result in any amplification. We had to increase the number of cycles to 36 cycles for C and M sections under the same conditions to get an amplicon. Aliquots of the 16S rDNA amplicons (5 µl) were analyzed by electrophoresis on a 1% agarose gel and visualized after staining with ethidiun bromide.

## Generation of clone libraries

Clone analysis of 16S rRNA genes was done for the investigated representative species of higher termite sub-families. Cloning analysis was performed by generating clone libraries from archaeal small sub-unit (SSU) 16S rDNA amplicons obtained from termite gut community DNA. Purified PCR products (~ 800 bp [Ar109f-Ar912r] long) were ligated into the pGEMT easy plasmid vector (Promega), and *E. coli* JM109 (Promega) was transformed with the recombinant plasmids according to the manufacturer's instructions. Clones were given codes which were derived from the termite ID numbers for whole gut generated clones and based on termite ID number plus the gut compartments where the clone was originated e.g.; C (crop section), M (mid-gut gut section including mixed segment), P1 (P1 gut section), P3 (P3 gut section), P4 (P4 gut section) and P5 (P5 gut section) for clones from gut compartment. Randomly picked clones were further analyzed as described previously (Großkopf *et al.*, 1998, Friedrich *et al*., 2001 and Donovan *et al*., 2004). Clones were checked for the correct insert size by vector-targeted PCR and standard agarose gel electrophoresis. Forty (40) randomly selected clones per library containing the correct inserts were sent for sequencing commercially in both directions using vector based primers.

## Phylogenetic analysis of sequence data

Sequence data were analyzed and edited by Seqman (DNAStar) software. Phylogenetic analysis was done with the current ARB-SILVA database (version 106; Pruesse *et al*., 2007; http://www.arb-silva.de) using the ARB software package (Ludwig *et al*., 2004). The SSU 16S rRNA gene sequences obtained in this study were added into the database using the ARB software package tool (Ludwig *et al*., 2004) after editing and alignment with SINA software tool (v1.2.9) using SILVA seed (Pruesse *et al*., 2007). For comparative analysis, sequences from other studies that were relevant to our study and that were not present in the Silva database were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/) and added to the database accordingly. The alignments were corrected manually where necessary in all cases. A 20% order level consensus filter was used to exclude highly variable positions. We constructed the Phylogenetic trees with ARB maximum likelihood method using RAxML algorithm (Stamatakis, 2006). Aligned sequences were also analyzed by ARB maximum-parsimony (DNAPARS) methods to check for tree topology and best support for the nodes (1000 bootstraps). Phylogeny was inferred by comparing sequences to the main lines of descent within the archaeal phyla; Euryarchaeota, Thaumarchaeota and Crenarchaeota. A threshold (>97% similarity) was set to assign sequence to the same operational taxonomic units (OTUs).

## Quantification of abundance of archaea in the gut

Quantitative PCR was performed to estimate the relative density of archaeal populations in both whole guts and gut compartments of higher termites. The abundance of archaeal as compared to bacterial 16S rRNA genes in the whole gut and gut compartments of higher termites used in this study were measured by quantitative 'real-time' PCR (qPCR) following the method described by Kemnitz *et al*. (2005). Copy numbers of archaeal 16S rRNA genes were estimated using the primers A364aF (5'-CGGGGYGCASCAGGCGCGAA-3'; Burggraf *et al*., 1997) and A934b (5'-GTGCTCCCCCGCCAATTCCT-3'; Grosskopf *et al*., 1998). The Bacterial 16S rRNA gene copy numbers were quantified as previously described by Stubner (2002) using the

primer pairs 519fc (5'-CAGCMGCCGCGGTAANWC-3') and 907r (5'-CCGTCAATTCMTTTRAGTT-3') (Lane, 1991).

### Accession numbers

The sequences described in this work will be submitted to Genbank.

## Results

### Phylogenetic analysis of the 16S rRNA genes

Phylogenetic analysis revealed four major taxonomic groups of methanogenic euryarchaeota; Methanobacteriales, Methanomicrobiales, Methanosarcinales and the recently discovered Methanoplasmatales in the guts of higher termites. In addition, an apparently termite specific group of Thaumarchaeota and a deep-branching uncultured archaeal group loosely affiliated to Crenarchaeota were mainly found in the soil-feeding termite species. Methanosarcinales group (Fig. 1) formed two distinct phylotypes. These includes *Methanimicrococcus* cluster, an insect cluster consisting of clones from both the anterior and posterior gut sections of soil-feeding *Cubitermes ugandensis* and *Ophiotermes* sp., as well as clones from the whole gut homogenates of fungus-cultivating termites *Macrotermes michaelseni*, *Macrotermes* sp. and *Microtermes* sp. They clustered together with clones from xylophagous cockroaches *Panesthia angustipennis* and *Salganea esakii*. Only two clones from the food soil of *Cubitermes ugandensis* clustered in this phylotype. Members of this group clustered closely with clones previously recovered from the soil-feeding termites *Cubitermes orthognathus* (Friedrich *et al*., 2001) and *Cubitermes fungifaber* (Donovan *et al*., 2004), fungus-cultivating *Odontotermes formosanus* (Ohkuma *et al*., 1999) as well as clones from the humivorous beetle *Pachnoda ephipiata* (Egert *et al*., 2003) and the xylophagous cockroach *Panesthia angustipennis* (Hara *et al*., 2002). Members of this phylotype were closely related to the methanogen symbiont *Methanimicrococcus blaticola* (96-99% similarity) isolated from a litter-feeding cockroach *Periplaneta americana* (Sprenger *et al*., 2000). The second

phylotype, *Methanimicrococcus* related cluster, formed a higher termite specific cluster consisting of clones from the posterior gut compartments of the soil-feeding termites *Cubitermes ugandensis*, *Ophiotermes* sp., and *Amitermes* sp. These clones clustered together with clones previously obtained from *Cubitermes orthognathus* (Friedrich *et al*., 2001), *Nasutitermes takasagoensis* (Miyata *et al*., 2007) and *Pricapritermes nitobei* (Ohkuma *et al*., 1999). Their closest cultivated relative was *Methanimicrococcus blaticola* (94-96% sequence similarity). The termite related Methanosarcinales group was distantly related (90-93%) to clones from bovine rumen (Tajima *et al*., 2001; Whitford *et al*., 2001) and clones from compost manure.

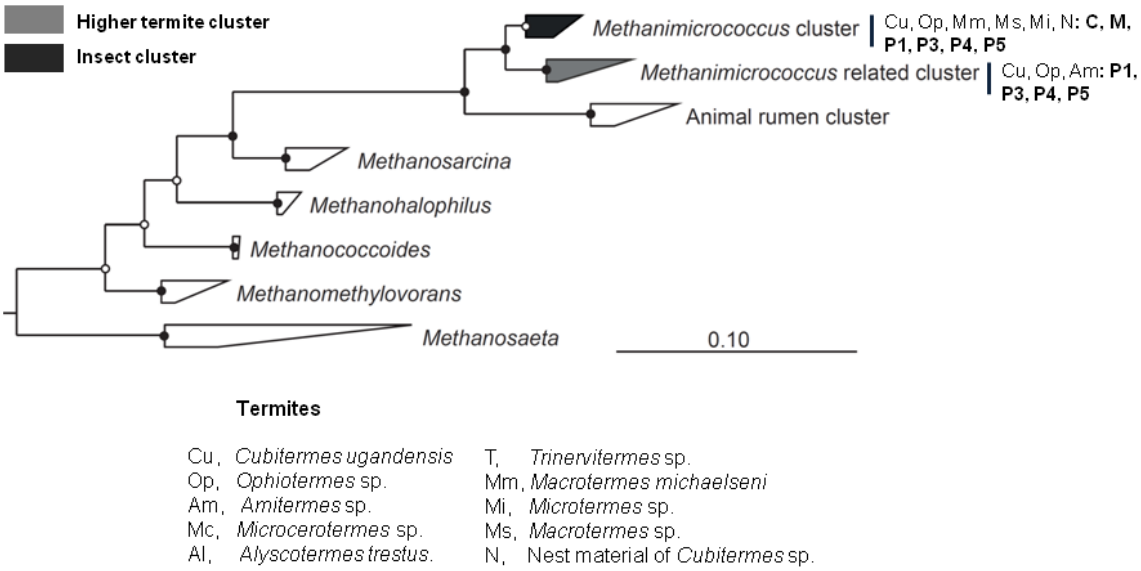


Fig. 1: A maximum-likelihood tree showing the phylogenetic positions of archaeal lineages related to *Methanosarcinales* recovered from higher termites as compared to those from other different environments as well as representative isolates in this group. The phylotypes containing clones from different higher termites in this study are indicated. Clones from representative species of higher termites used in this study are indicated. Clones from individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] are marked in bold. Bullets indicate bootstrap support ●, >90%; ○, >70%). The scale bar indicates rate of substitutions per nucleotide position. The tree was rooted with euryarchaeote *Methanogenium marinum.*

Methanomicrobiales group formed a monophyletic higher termite specific cluster (Fig. 2). The only phylotype in this group, the higher termite euryarchaeal cluster consisted mainly of clones from the posterior gut of the soil-feeding *Cubitermes ugandensis*, as well as clones from whole guts of the soil-feeding *Alyscotermes trestus*, the grass-feeding *Trinervitermes* sp., and the fungus-cultivating *Microtermes* sp. Members of this phylotype clustered together with clones previously obtained from *Cubitermes orthognathus* (Friedrich *et al*., 2001) and *Cubitermes fungifaber* (Donovan *et al*., 2004), *Pericapritermes nitobei* (Ohkuma *et al*., 1999) and *Nasutitermes takasagoensis* (Ohkuma *et al*., 1999; Miyata *et al*., 2007). The higher termite euryarchaeal gut cluster of Methanomicrobiales formed a distinct clade of sequences only specific to higher termites and they were phylogenetically radiating away from their only closest cultivated relative *Methanospirillum hungatei* (87-91% sequence similarity).

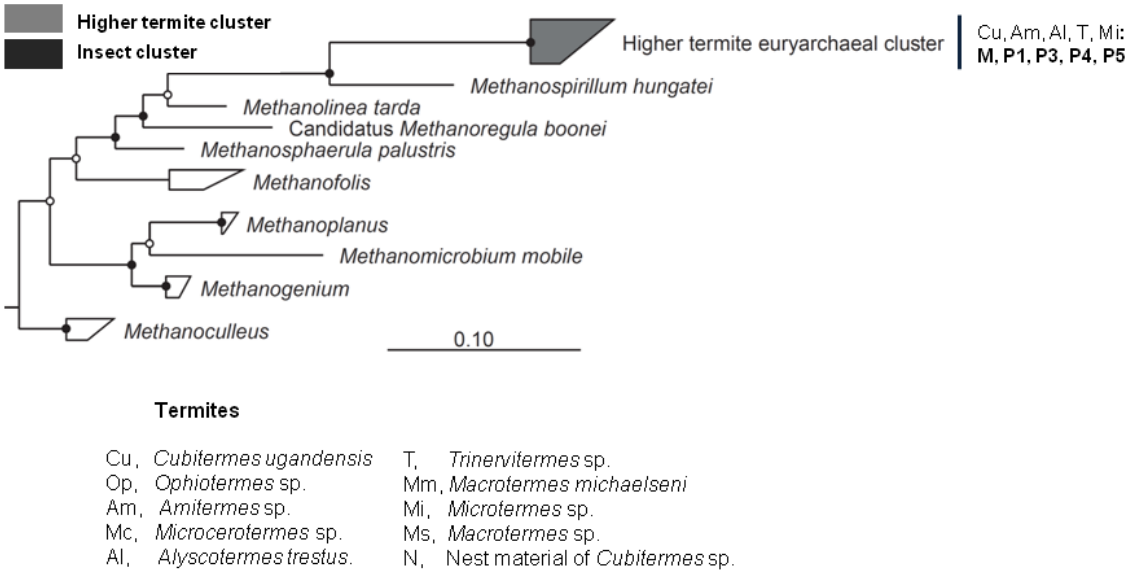


Fig. 2: A maximum-likelihood tree showing the phylogenetic positions of archaeal lineages related to *Methanomicrobiales* recovered from higher termites as compared to representative isolates in this group. The phylotypes containing clones from different higher termites in this study are indicated. Clones from representative species of higher termites used in this study are indicated. Clones from individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] are marked in bold. Bullets indicate

bootstrap support ●, > 90%; ○, >70%). The scale bar indicates rate of substitutions per nucleotide position. The tree was rooted with euryarchaeote *Methanosaeta harundinacea*.

The recently discovered Methanoplasmatales (Fig. 3) formed a diverse group of archaea recovered from all the termites investigated in this study. Members of this clade appear to be highly established in higher termite guts (7-30% proportional abundance in termites investigated) and they formed two main groups each consisting of sequences from the guts of arthropods. Both groups consisted of clones from both the anterior and posterior guts of higher termites as well as clones from other insects and they were distantly related (90-95% sequence similarity) from each other. In the first group (Gut cluster II) was only one phylotype, Insect gut cluster consisting mainly of clones from posterior gut sections of soil-feeding termites *Cubitermes ugandensis*, *Ophiotermes* sp. and *Amitermes* sp., as well as clones from whole gut of soil-feeding *Alyscotermes trestus*, grass-feeding *Trinervitermes* sp. and fungus-cultivating *Macrotermes michaelseni*. Members of this group clustered with clones previously obtained from the soil-feeding termite *Cubitermes orthognathus* (Friedrich *et al*., 2001), and the wood-feeding termite *Nasutitermes takasagoensis* (Miyata *et al*., 2007) as well as clones from xylophagous cockroaches *Salganea esakii* and *Panesthia angustipennis* (Hara *et al*., 2002). Their close cultivated relative was methylotrophic Methanoplasmatales enrichment culture strain MpT1 isolated from *Cubitermes ugandensis* (Paul *et al*., 2012). They were distantly related (91-96% sequence similarity) to clones from animal rumen (Wright *et al*., 2007; Sundset *et al*., 2009), anaerobic digesters as well as clones from human stool (Mihajlovski *et al*., 2008; 2010). In the second group (Gut cluster I) were three phylotypes: Macrotermitinae and xylophagous cockroach gut cluster consisted of clones from fungus-cultivating termites *Macrotermes* sp. and *Microtermes* sp. They clustered together with clones previously obtained from xylophagous cockroaches *Panesthia angustipennis* and *Salganea esakii* (Hara *et al*., 2002). The second phylotype, *Cubitermes* gut cluster consisted of clones from the posterior gut section of *Cubitermes ugandensis*. One clone from the nest material of *Cubitermes* sp. also fell within this phylotype. These clones clustered together with clones previously obtained from the soil-feeding *Cubitermes orthognathus* (Friedrich *et al*., 2001). The last phylotype, Higher termite gut cluster consisting of

clones from both anterior and posterior gut section of soil-feeding *Cubitermes ugandensis*, *Ophiotermes* sp., and *Amitermes* sp., as well as clones from whole guts of soil-feeding *Alyscotermes trestus*, grass-feeding *Trinervitermes* sp., and the wood-feeding *Microcerotermes* sp. Members of this phylotype clustered with clones previously obtained from *Cubitermes orthognathus* (Friedrich *et al*., 2001) and *Nasutitermes takasagoensis* (Miyata *et al*., 2007). They did not have any close cultivated relative. The termite gut derived Methanoplasmatales formed distinct clusters from those derived from other animals including the foregut of Wallabies (Evans *et al*., 2009), human stool (Mihajlovski *et al*., 2010), cattle rumen (Wright *et al*., 2007), and from anaerobic sludge digestors and landfills (Huang *et al*., 2002). The termite derived lineages were also very distantly related (87-89% sequence similarity) to the only cultured representative of this order of Methanoplasmatales, *Methanomassiliicoccus luminyensis* isolated from human feces (Dridi *et al*., 2012) as well as clones from human gut (Mihajlovski *et al*., 2008) which are part of the Rice Cluster III group. Only two clones of Methanoplasmatales has been previously reported from the wood-feeding lower termite *Reticulitermes speratus* (Shinzato *et al*., 1999), but because of the differences in the sections of the sequence analyzed, we could not add them to the tree.

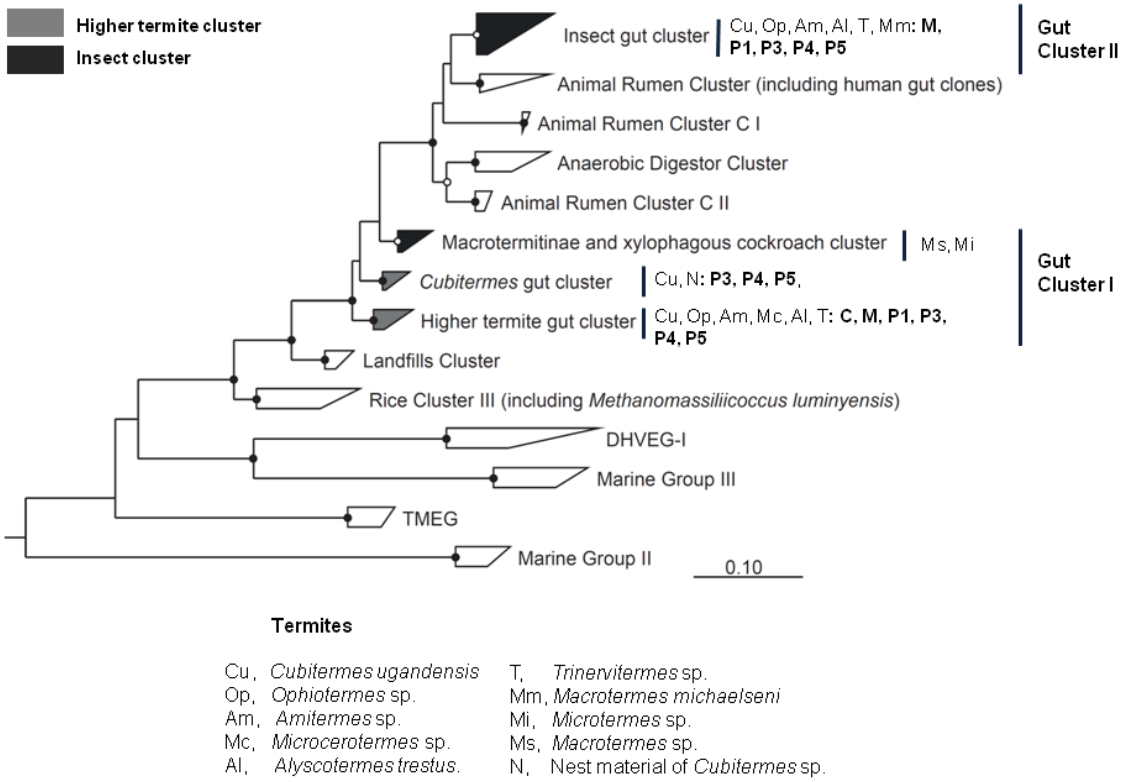


Fig. 3: A maximum-likelihood tree showing the phylogenetic positions of archaeal lineages related to *Methanoplasmatales* recovered from higher termites as compared to those from other animals and different environments as well as representative isolates in this group. The phylotypes containing clones from different higher termites in this study are indicated. Clones from representative species of higher termites used in this study are indicated. Clones from individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] are marked in bold. Bullets indicate bootstrap support (●, >90%; ○, >70%). The scale bar indicates rate of substitutions per nucleotide position. The tree was rooted with euryarchaeote *Archaeoglobus fulgidus*.

The highest diversity was observed within the Methanobacteriales group, recovered from all termite species investigated (Fig. 4). Majority of phylotypes within this clade formed gut clusters consisting of clones from the guts of higher termites, cockroaches, beetles, animal rumen and endosymbionts of ciliates. Methanobacteriales lineages from lower termites formed a distinct cluster (93-95% sequence similarity) from those of higher termites. The most diverse phylotype within Methanobacteriales was Insect cluster III

consisting of clones from both anterior and posterior gut sections of the soil-feeding termites *Cubitermes ugandensis*, *Ophiotermes* sp., and *Amitermes* sp., as well as clones from whole guts of soil-feeding *Alyscotermes trestus*, wood-feeding *Microcerotermes* sp., grass-feeding *Trinervitermes* sp., and fungus-cultivating *Macrotermes* sp. Members of this phylotype clustered together with clones previously obtained from *Cubitermes orthognathus* (Friedrich *et al*., 2001), *Cubitermes fungifaber* (Donovan *et al*., 2004), *Nasutitermes takasagoensis* (Miyata *et al*., 2007), *Pericapritermes nitobei* (Ohkuma *et al*., 1999), *Microcerotermes* sp. (Deevong *et al*., 2004), as well as clones from beetles *Melolontha melolontha* (Egert *et al*., 2005), *Pachnoda ephippiata* (Egert *et al*., 2003), cockroaches *Periplaneta americana* (Van Hoek *et al*., 2000), and clones from endosymbiont of free-living ciliate *Trimyema compressum* (Shinzato *et al*., 2007) and *Nyctotherus ovalis* from *Blaberus* sp. and *Periplaneta americana* (Van Hoek *et al*., 2000). Members of this cluster were closely related to *Methanobrevibacter arboriphilus* (97-99% sequence similarity). The next phylotype, Insect cluster II consisted only of clones from the soil-feeding *Amitermes* sp. and they clustered together with clones from xylophagous cockroaches *Panesthia angustipennis* and *Salganea esakii*. These clones clustered together with clones previously recovered from the endosymbiont *Nyctotherus ovalis* from cockroaches *Blaberus* sp. and *Periplaneta Americana* (Van Hoek *et al*., 2000). Their closest cultivated relative (96-98% sequence similarity) was *Methanobrevibacter cuticularis* from the wood-feeding lower termite *Reticulitermes flavipes* (Leadbetter and Breznak, 1996). The next phylotype, Lower termite related cluster consisted of clones from the posterior gut section of *Cubitermes ugandensis*. These clones clustered together with clones previously recovered from *Cubitermes orthognathus* (Friedrich *et al*., 2001) and *Nasutitermes takasagoensis* (Miyata *et al*., 2007). These sequences were distantly related (94-96% sequence similarity) to clones from lower termites *Hodotermopsis sjoestedti* (Tokura *et al*., 2000), *Hodotermopsis japonica* (Ohkuma *et al*., 1999), *Cryptotermes domesticus* (Ohkuma *et al*., 1995) and *Reticulitermes speratus* (Ohkuma *et al*., 1998). Their closest cultivated relatives (94-96% sequence similarity) were *Methanobrevibacter curvatus* and *Methanobrevibacter filiformis* (Leadbetter and Breznak, 1996; Leadbetter *et al*., 1998).The next phylotype,

Insect cluster I, consisting of clones from the posterior gut sections of *Cubitermes ugandensis*, *Ophiotermes* sp., and *Amitermes* sp, as well as clones from whole gut homogenates of *Alyscotermes trestus* and *Trinervitermes* sp. Members of this phylotype clustered with clones from xylophagous cockroach *Panesthia angustipennis*. This group were related to clones previously obtained from posterior gut section of *Cubitermes orthognathus* (Friedrich *et al*., 2001) and whole gut of *Cubitermes fungifaber* (Donovan *et al*., 2004) as well as the wood-feeding termite *Nasutitermes takasagoensis* (Miyata *et al*., 2007) and humivorous beetle *Pachnoda ephippiata* (Egert *et al*., 2003). One clone from nest material of *Cubitermes* sp. fell within this cluster. Members of this phylotype were not closely affiliated to any cultured relatives. The next phylotype formed a novel distinct Animal rumen phylotype comprising *Methanobrevibacte*r-related clones from the fungus-cultivating *Microtermes* sp. These clones clustered together with clones from rumen of Tammar wallaby *Macropus eugenii* (Evans *et al*., 2009), clones from sheep (Wright *et al*., 2004) as well as clones from other animals (Sundset *et al*., 2007). Their closest cultivated relative (99% sequence similarity) was *Methanobrevibacter thaueri* (Lin and Miller, 1998) and the human gut isolate *Methanobrevibacter smithii* (98% sequence similarity) (Miller *et al*., 1982). Except for one clone (97% sequence similarity) previously isolated from a lower termite *Reticulitermes speratus* (Tokura *et al*., 2000), there were no other clones from any termite within this phylotype. The last phylotype, Macrotermitinae and xylophagous insects cluster formed a new novel phylotype consisting of clones from the wood-feeding *Microcerotermes* sp. and the fungus cultivating *Macrotermes michaelseni*. Members of this phylotype clustered together with clones from a xylophagous cockroach *Salganea esakii*. They were not closely related to any cultivated relative.

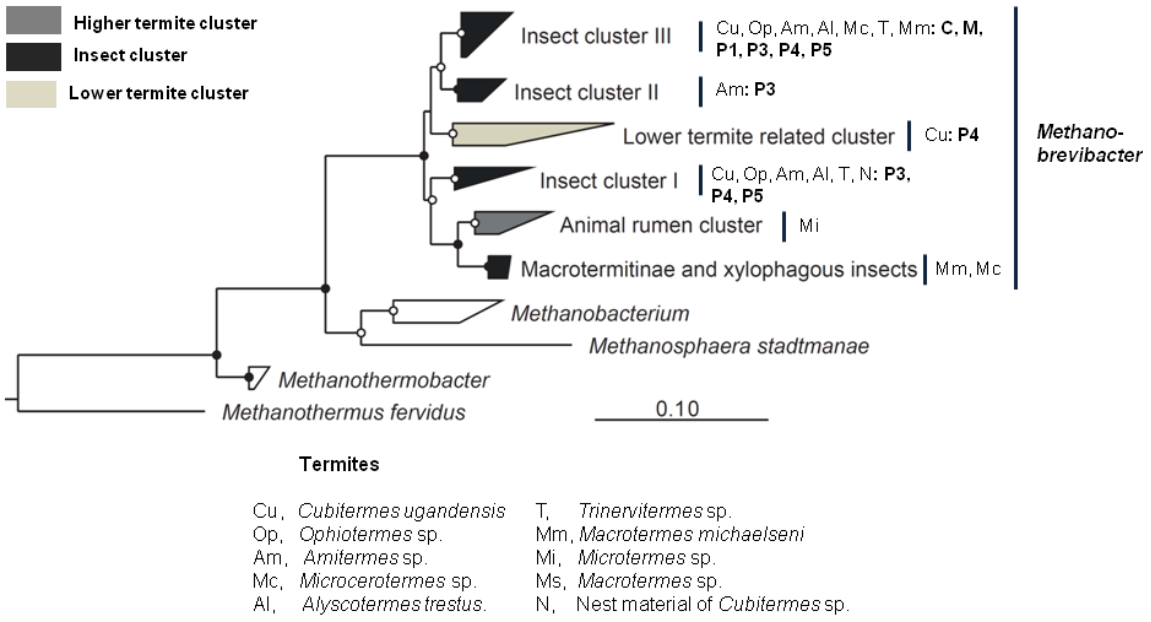


Fig. 4: A maximum-likelihood tree showing the phylogenetic positions of archaeal lineages related to *Methanobacteriales* recovered from higher termites as compared to those from other insects as well as representative isolates in this group. The phylotypes containing clones from different higher termites in this study are indicated. Clones from representative species of higher termites used in this study are indicated. Clones from individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] are marked in bold. Bullets indicate bootstrap support ◖, > 90%; ○, >70%). The scale bar indicates rate of substitutions per nucleotide position. The tree was rooted with euryarchaeote *Methanothermococcus thermolithotrophicus*.

Other than the Euryarchaeota, termite-derived members of Thaumarchaeota (Fig. 5) formed a cluster consisting of clones from the crop, midgut and rectum of soil-feeding termites *Cubitermes ugandensis* and *Amitermes* sp. as well as clones from whole gut of soil-feeding *Alyscotermes trestus* and the fungus-cultivating *Microtermes* sp. Members of this group clustered with clones previously recovered from the anterior gut compartment of soil-feeding termite *Cubitermes orthognathus* (Friedrich *et al.*, 2001) and whole gut of humivorous cockroach *Pachnoda ephippiata* (Egert *et al.*, 2003). All the clones from the food soil of *Cubitermes ugandensis* and *Ophiotermes* sp. (in this study), and clones from food soil of *Cubitermes orthognathus* (Friedrich *et al.*, 2001), as well as most clones from the nest material clustered in this group. Members of this group were affiliated to the

aerobic ammonia-oxidizing Thaumarchaeota belonging to the *Nitrososphaera* cluster. Their closest cultivated relatives were *Nitrososphaera gargensis* isolated from biomass of hot spring (Hatzenpichler *et al*., 2008) and *Nitrososphaera viennensis* (Tourna *et al*., 2011) isolated from the soil.

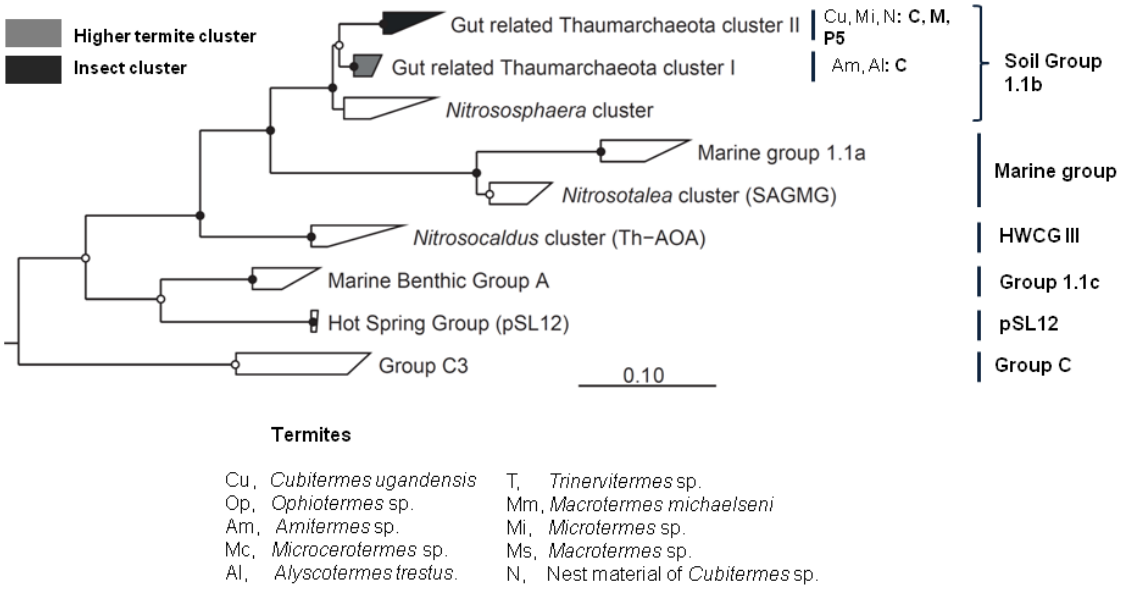


Fig. 5: A maximum-likelihood tree showing the phylogenetic positions of archaeal lineages related to the phylum *Thaumarchaeota* recovered from higher termites as compared to those from different environments as well as representative isolates in this group. The phylotypes containing clones from different higher termites in this study are indicated. Clones from representative species of higher termites used in this study are indicated. Clones from individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] are marked in bold. Bullets indicate bootstrap support (●, >90%; ○, >70%). The scale bar indicates rate of substitutions per nucleotide position. The tree was rooted with korarchaeote *Korarchaeum cryptofilum.*

The last group recovered mainly from soil-feeding higher termites was a deep-branching group of uncultured archaea loosely related to Crenarchaeota (Fig. 6). This group formed a termite-specific monophyletic cluster consisting of clones from the posterior hind gut compartments of soil-feeding termites *Cubitermes ugandensis*, *Ophiotermes* sp. and *Amitermes* sp., as well as clones from whole gut of *Alyscotermes trestus*. Members of this

group clustered with clones previously obtained from soil feeding termite *Cubitermes orthognathus* (Friedrich *et al*., 2001) and *Cornitermes cumulans* (Grieco *et al*., 2012). They were distantly affiliated to clones from various other environments comprising anaerobic sludge digester, fresh water sediments, Obsidian pool hot springs, sulfidic springs and ponds and their role in the gut is presently not clear. This group was very distantly related (74-80%) to extremophilic sulfur metabolizing crenarchaeota belonging to Thermoprotei.

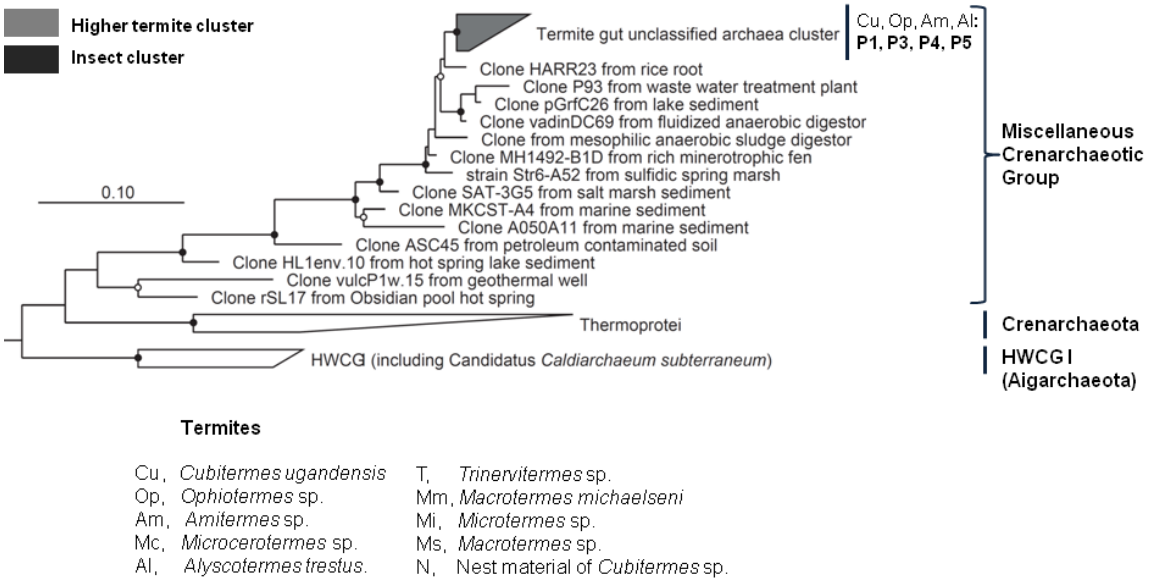


Fig. 6: A maximum-likelihood tree showing the phylogenetic positions of archaeal lineages loosely related to *Crenarchaeota* recovered from higher termites as compared to those from other environments. The phylotypes containing clones from different higher termites in this study are indicated. Clones from representative species of higher termites used in this study are indicated. Clones from individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] are marked in bold. Bullets indicate bootstrap support ◖, > 90%; ○, >70%). The scale bar indicates rate of substitutions per nucleotide position. The tree was rooted with euryarchaeote *Archaeoglobus fulgidus*.

## Community structure and population density of archaea in higher termites

Except for the crop and midgut of *Cubitermes ugandensis* (C=21; M=15) *Ophiotermes* sp. (C=30; M=29) and *Amitermes* sp. (C=2; M=30), approximately 40 clones each were screened from each library of whole gut or gut compartments of the termites used in this study (Table 1). There were wide variations in the abundances of the archaeal as well as bacterial 16S rRNA gene copy numbers in the termites investigated (Table 2). Archaeal diversity and community structure was more complex in soil-feeding termites than the wood-feeding, grass-feeding and fungus-cultivating species (Fig.7). Within the Termitinae, the soil-feeding *Cubitermes ugandensis Ophiotermes* sp. and *Amitermes* sp. showed a higher diversity with nearly all the major archaeal groups represented. These termites harbor methanogenic euryarchaeota comprising representative of the orders: Methanosarcinales, Methanobacteriales, Methanomicrobiales, and Methanoplasmatales as well as members of other archaeal phyla comprising Thaumarchaeota and a deep-branching lineage of uncultured Archaea loosely affiliated to Crenarchaeota. Except for the fungus-cultivating *Macrotermes michaelseni*, highest abundances of archaeal 16S rRNA gene copies were observed in soil-feeding termites. The results of the density of both Archaeal and Bacterial16S rRNA gene copies are summarized in Table 2. Only members of Thaumarchaeota were recovered from the food soil of both *Cubitermes ugandensis* and *Ophiotermes* sp., and the same was true for the nest of *Cubitermes ugandensis* except for a few clones of Methanosarcinales, Methanobacteriales and Methanoplasmatales recovered from the nest material.

Table 2: Absolute abundances of 16S rRNA gene of Archaea and Bacteria in whole guts of representative species of each of the sub-family of the higher termite family Termitidae.

| **Taxa** | **Absolute copy numbers of 16S rRNA gene ($10^3$ copies/gut)[a]** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ***C. ugandensis*** | ***Ophiotermes* sp.** | ***Amitermes* sp.** | ***Microcerotermes* sp.** | ***Trinervitermes* sp.** | ***A. trestus*** | ***M. michaelseni*** | ***Microtermes* sp.** |
| Total Archaea[b] | 73 ± 0.83[e] | 403 ± 86.9[f] | 72.7 ± 18.4[e] | 54.2 ± 1.98 | 55.4 ± 1.56 | 721 ± 48.6 | 342 ± 104 | 57.4 ± 2.77 |
| Total Bacteria[c] | 4840 ± 517 | 7610[g] | 7780 ± 542 | 108000 ± 750 | 14200 ± 3580 | 12200 ± 350 | 228000 ± 27200 | 16800 ± 750 |
| Arch/Prokaryotes (%)[d] | 1.49 | 5.04 | 0.926 | 0.0501 | 0.388 | 5.54 | 1.48 | 0.341 |

[a] Values shown are mean ± mean deviation of two independent measurements (shown only to 3 significant figures)

[b] Values are obtained by multiplying the copy numbers of archaeal 16S rRNA gene by the relative abundance of archaeal taxa recovered in the whole gut of each higher termite investigated.

[c] Values are bacterial 16S rRNA gene copy numbers per whole gut of each higher termite investigated

[d] Abundance of archaea expressed as a percentage over that of total prokaryotes.

[e] Sum of abundance of archaea in all individual gut compartments crop, midgut including mixed segment, and proctodeal compartments.

[f] Sum of abundance of archaea in all the gut compartments except the P4 compartment in which qPCR data was not available.

[g] Values shown are only for one measurement.

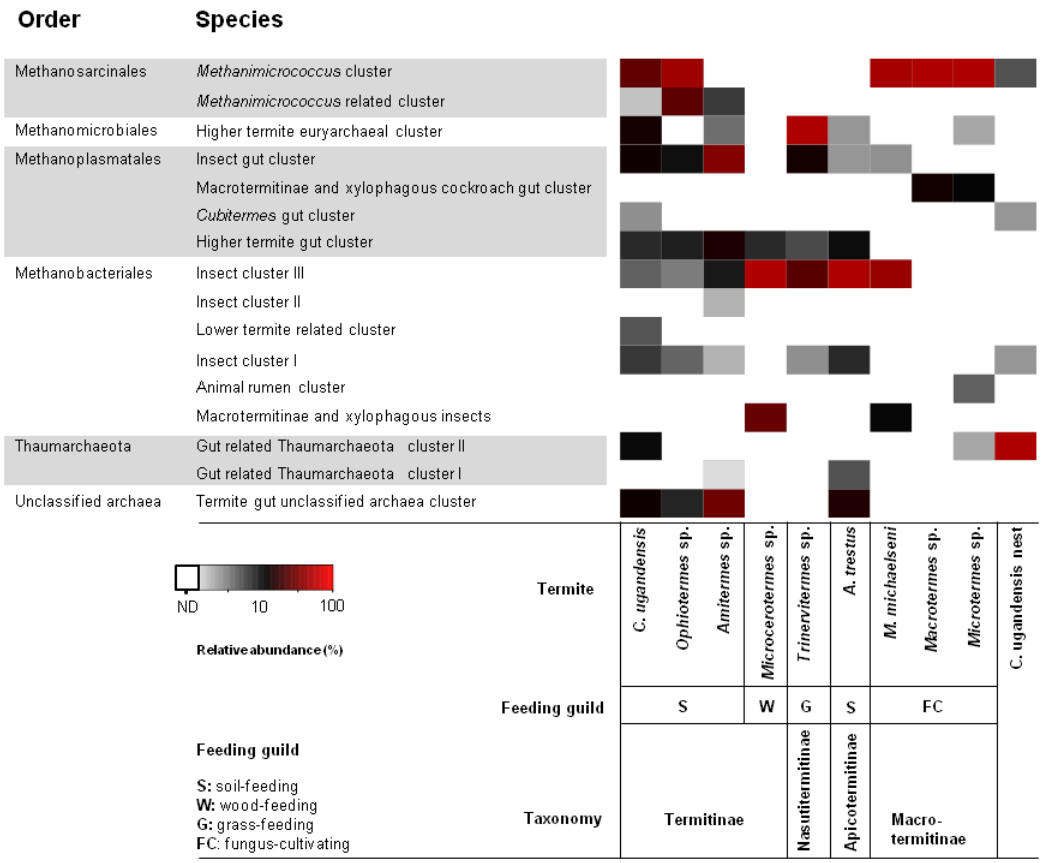


Fig. 7: Relative abundance of archaeal taxa recovered in whole guts of representative species of different sub-families of the higher termite family (Termitidae). The classification was shown down to species level (based on assignment of OTUs at a threshold of 97% sequence similarity following analysis of 750 bp fragment of archaeal SSU 16S rRNA gene after filtering with order level filters). The values shown in the heat map are log transformations of the abundances to show low abundance groups.

## Community similarities

Community similarity between different pairs of the major archaeal lineages recovered from the different higher termites in this study was calculated with Bray-Curtis indices. Pair wise comparison showed that communities in the soil-feeding termites were more similar to each other (Fig. 8). Within the Termitinae, the communities in the soil-feeding termites; *C. ugandensis* and *Amitermes* sp. were highly similar. They were also closely similar to communities in the grass-feeding *Trinervitermes* sp. (Nasutitermitinae), but

were less similar to those of the soil-feeding *Alyscotermes trestus* (Apicotermitinae). The archaeal communities in the soil-feeding *A. trestus* (Apicotermitinae) were more similar to those of the wood-feeding *Microcerotermes* sp. (Termitinae). The communities in these two species were also closely similar to those of the fungus-cultivating *Macrotermes michaelseni* (Macrotermitinae). However, communities in soil-feeding Termitinae were totally different from those of the wood-feeding *Microcerotermes* sp. (also Termitinae) with very low Bray-Curtis indices. There was low Bray-Curtis index between the *C. ugandensis* and its nest material showing that the communities in the gut were different from those of the nest material. The communities in the grass-feeding *Trinervitermes* sp. were almost similar to *C. ugandensis*, but were different from those of *Ophiotermes* sp. The communities in the fungus-cultivating termites *Macrotermes* sp. and *Microtermes sp*. (Macrotermitinae) were more similar to each other, and they both had a high Bray-Curtis indices with the soil-feeding *Ophiotermes* sp., demonstrating high similarity.

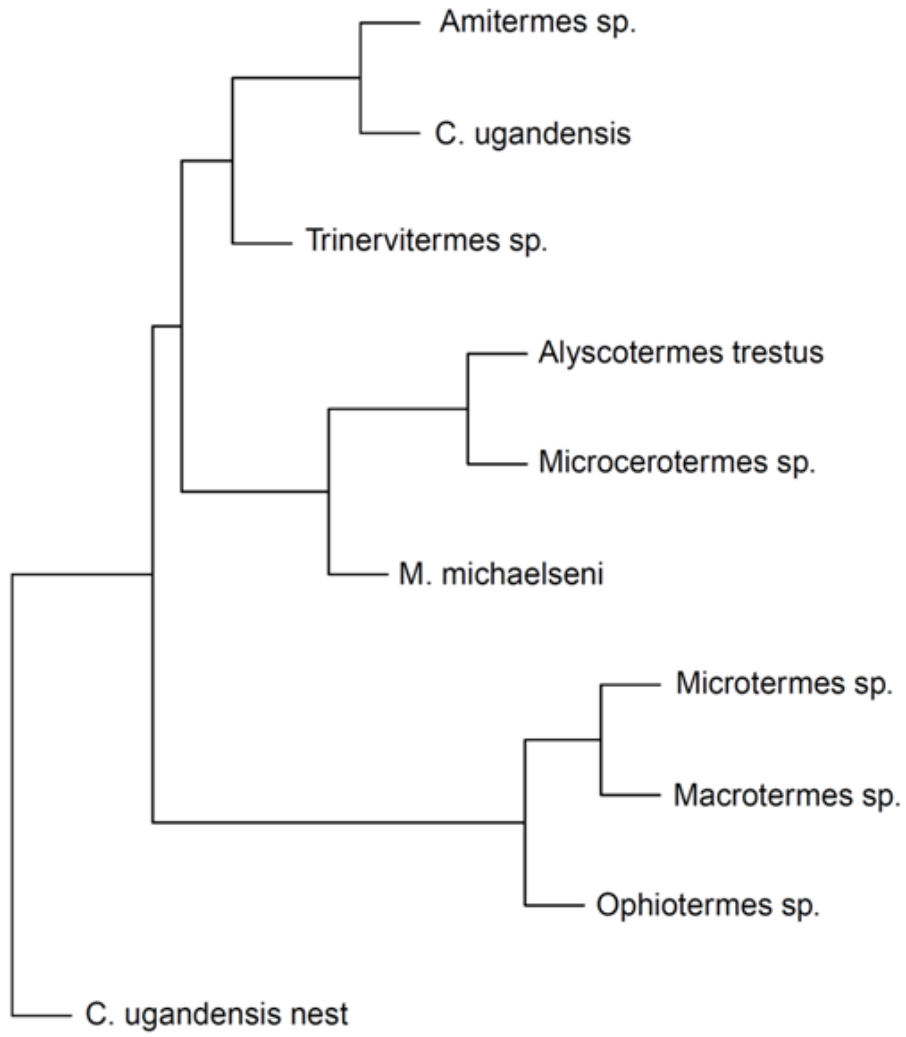


Fig. 8: A dendrogram showing similarity of archaeal communities in representative species of the different sub-families of the higher termite family Termitidae. Bray–Curtis coefficient was used to calculate the similarity indices based on relative abundance of the major archaeal taxa recovered from the higher termites. For feeding guild of the respective termites, refer to Table 1.

## Discussion

Phylogenetically higher termites emit more methane than their lower termite counterparts (Brauman *et al*., 1992; Shinzato *et al*., 1992; Bignell *et al*., 1997). However, the community composition of archaea in the highly compartmented guts of higher termites has been investigated by only a few studies (Ohkuma *et al*., 1999; Friedrich *et al*., 2001; Donovan *et al*., 2004). We report results of a comparative study of archaeal community structure in representative species of the four sub-families of higher termites. Our results show high archaeal diversity in the guts of higher termites, particularly in soil-feeding taxa. As compared to the lower termites, which host primarily members of Methanobacteriales (Leadbetter and Breznak, 1996; Ohkuma *et al*., 1998, 1995; Tokura *et al*., 2000; Shinzato *et al*., 1999), higher termites host a diverse assemblage of methanogenic archaea, comprising mainly higher termite-specific lineages. Highest diversity and density of methanogenic euryarchaeota was observed in soil-feeding *Cubitermes ugandensis*, *Ophiotermes* sp., *Amitermes* sp., and *Alyscotermes trestus* (Table 2 and Fig. 7), which harbor representatives of four orders; Methanobacteriales, Methanomicrobiales, Methanosarcinales and the recently discovered Methanoplasmatales. In addition, the soil-feeding termites also host uncultured archaeal phyla comprising lineages closely related to ammonia-oxidizing Thaumarchaeota and a deep-branching termite-specific group of uncultured archaea loosely affiliated to Crenarchaeota, whose role in the gut still remains unclear. Previous studies on the soil-feeding termites *Cubitermes orthognathus* (Friedrich *et al*., 2001), *Cubitermes fungifaber*, (Donovan *et al*., 2004), and *Pericapritermes nitobei* (Ohkuma *et al*., 1999) also reported high archaeal diversity in soil-feeding species. This pattern was also observed in humus-feeding beetles *Pachnoda ephippiata* (Egert *et al*., 2003) in which new phylotypes were recovered. Like the differences in methane emission rates, variations in community structure and density of archaea was observed even in members of the same feeding group, suggesting that these differences may be linked to not only to the nature of the diet, but also to the conditions in the gut, which potentially results in different gradients of methanogenic substrates. No clear pattern was observed to link

community structure to taxonomy or feeding behavior. For example, *Cubitermes* and *Ophiotermes* sp. showed differences in archaeal diversity. While Methanomicrobiales forms about 13 % of the overall archaeal community in the whole gut of *Cubitermes ugandensis*, it was not recovered in *Ophiotermes* sp. even with our most exhaustive clone analysis, probably suggesting that either the gut micro-environmental conditions in *Ophiotermes* sp. are not favorable for this group, or the potential influence of diet on archaeal diversity. Clones from soil-feeding termites clustered together, but there was host-specificity observed in higher termite archaeal gut microbiota, probably indicating separate co-evolution with the host. The high density of archaeal 16S rRNA genes (about one order of magnitude higher) in *Ophiotermes* sp. than in *Cubitermes* sp. (Table 2) is probably due to Methanosarcinales, the most dominant group (67% of overall gut archaea) in the former containing more than one copy of 16S rRNA genes. Clones from *Alyscotermes trestus* formed distinct sub-clusters, probably demonstrating separate co-evolution with the host in the evolution process of soil-feeding behavior. Similarity indices showed a weak similarity between communities in members of soil-feeding Termitinae and *A. trestus* (Fig. 8). The more basal gut structure in *Alyscotermes trestus* probably explains the lower archaeal diversity than in Termitinae, whose members have a much more complex gut structure. The failure to detect members of Methanosarcinales lineages in *Alyscotermes trestus* by our clone analysis suggest either their presence in very low abundance or complete absence in the gut, a phenomenon that may influence methane emission. However, archaeal density in the gut of in *A. trestus* (Table 2) was one order of magnitude higher than in *Cubitermes* sp., potentially suggest that Methanobacteriales, the most dominant methanogen in the former (66% of overall gut archaea) may have lineages with more than one copy numbers leading to overestimation of abundance. However, it is not clear whether this pattern is true in all members of Apicotermitinae. *Alyscotermes trestus*, collected in subterranean tunnels near abandoned nests of *Cubitermes* sp., may be feeding on a diet that is already modified by the activities of *Cubitermes* species. This may influence the gut community structure. Since not much is known about members of this sub-family, it may be interesting to investigate the physicochemical characteristics and localize methanogenic activities in the gut. Soil-

feeding termites have a highly compartmented gut structure, and it has been hypothesized (Bignell *et al*., 2011) that this extension of the gut creates several micro-habitats providing a selection for different microbial communities. This, together with their highly humified diet consisting of recalcitrant aromatic compounds whose breakdown possibly results in availability of alternative methanogenic substrates, may create specialist archaeal groups and may explain the high archaeal diversity and hence high methane emission rates.

Contrary to the soil-feeding species, the wood-feeding higher termites which emit comparatively low amounts of methane (Brauman *et al*., 1992), harbor low diversity of archaea (Fig. 7) despite their gut compartmentation. The predominant colonization of the gut of wood-feeding *Microcerotermes* sp. (Termitinae) by Methanobacteriales (93% of overall gut archaea), a group comprising of hydrogenotrophic lineages, probably suggests hydrogen as major driver of methanogenesis in this termite whose diet consists purely of wood. However, the presence of Methanoplasmatales (7% of the gut archaea) which comprise methylotrophic lineages may suggest potential role of substrates other than merely hydrogen. The absence of other members of methanogenic euryarchaeota in this termite may negatively influence methane emission rates in wood-feeding Termitinae. Our archaeal 16S rRNA copy numbers were higher in the soil-feeding than wood-feeding termites, suggesting that methanogenesis rates in the gut may also be influenced by density of methanogens density. Using domain level probes, Brauman *et al*., (2001) also reported higher relative abundances of 16S-like archaeal rRNA in the guts of soil-feeding termites as compared to wood-feeding species in the same family. Interestingly, bacterial 16S rRNA gene copies was about two orders of magnitude higher in the wood-feeding *Microcerotermes* sp. than in the soil-feeding species potentially suggesting competition for methanogenic substrates by bacterial counterparts. The low archaeal diversity in most fungus-cultivating species may be explained by their more basal gut structure probably because their diet is mostly modified by the fungal symbiotic partner rather than by the archaeal gut microbiota. Surprisingly, archaeal abundance in *M. michaelseni* was two orders of magnitude higher than in the high methane emitting *Cubitermes* sp. This may be because methanogens probably face competition for substrates, leading to low

methanogenic potential or Methanobacteriales and Methanosarcinales lineages dominant in this termite (51% and 46% of overall gut archaea respectively) probably have more than one copies of 16S rRNA gene leading to overestimation of abundance. The surprisingly high archaeal diversity in *Microtermes* sp., in which almost all the major archaeal groups were recovered, may be explained by its more elaborate and compartmented gut structure (Bignell and Eggleton, 1995) as compared to its fungus-cultivating counterparts, probably demonstrating the influence of gut compartmentation on creating microbial micro-habitats.

Based on our results, higher termites harbor four major groups of methanogenic euryarchaeota. Methanobacteriales, present in all termites investigated in this study (Fig. 4), and in previous other studies, is the only group documented in all lower termites. The presence of this largely hydrogenotrophic methanogens in high abundance (up to 93% of overall gut archaea in some termites), suggest that it forms a core archaeal microbiota in termites. Like their lower termite counterparts, the higher termite related Methanobacteriales lineages clusters within the genus *Methanobrevibacter* whose cultured relatives are mainly hydrogenotrophic, suggesting that hydrogenotrophic methanogenesis is conserved phenomenon in termites and may have been acquired from termite ancestors. It has been observed (Bapteste *et al*., 2005) that all methanogens seems to have an inherent ability to perform hydrogenotrophic methanogenesis having vertically acquired the genes involved in the all the steps in methanogenic reduction of carbon dioxide from a common ancestor and that these genes are generally conserved. Shinzato *et al*., (1999) had even hypothesized that *Methanobrevibacter*-related methanogens may be the major group of symbiotic methanogens in termites. The insect gut specific phylotypes showed some homogeneity in which clones from higher termites and other insects clustered together may be a case of vertical transfer of the lineages from common ancestor to subsequent generations during the evolutionary process. However, within these clusters, was a feeding guild-based homology which further radiated away in a host-specific clustering, suggesting a co-evolution probably influenced by the host diet as is evidenced by distinct sub-clusters between communities from soil-feeding termites and grass-feeding Nasutitermitinae, even though they show some overlap. The close

association between sequences from fungus cultivating termites and xylophagous insects suggest vertical transfer from cockroach ancestors and co-evolution with the host based on diet. There was a very strong Bray-Curtis index between community from the fungus cultivating *M. michaelseni* and the wood-feeding *Microcerotermes* sp. demonstrating high similarity. The distinct phylotypes (93-95%) between Methanobacteriales related clones from wood-feeding lower termites and those of higher termites potentially indicates community differences, a phenomenon probably shaped by the diet and the *in situ* physicochemical conditions in the gut, suggesting separate co-evolution with their *Methanobrevibacter*-related symbionts. The animal rumen related *Methanobrevibacter* phylotype comprising only clones from *Microtermes* sp., is novel observations in higher termites. Since members this phylotype cluster with methanogens from animal rumen, it is possible that they were horizontally acquired from the environment. *Microtermes* sp. was collected from a *Macrotermes michaelseni* mound but members of this species also forage on animal dung probably creating specialist communities in their guts. Together with the closely related Macrotermitinae and the xylophagous insect phylotype consisting of clones from *Microcerotermes* sp. and *Macrotermes* sp., demonstrate the complex nature of the higher termite archaeal community. The high archaeal diversity in our study may be due to our adequate sequencing of relatively large number of clones than in previous studies and the different primer sets used in these studies, but may also be caused by species specific variations as well as differences in ecosystems from which the termites were collected. Leadbetter *et al*., (1998) observed that *Methanobrevibacter filiformis* cells, a dominant methanogen in *Reticulitermes flavipes* collected from Woods Hole USA was not consistently observed in *Reticulitermes flavipes* collected from different locations.

Methanosarcinales seems to be a core gut archaeal community in the fungus cultivating Macrotermitinae and in the soil-feeding Termitinae (Table 2). Soil-feeding and fungus-cultivating termites are associated with high methane emission rates (Brauman *et al*., 1992) and the high abundance of Methanosarcinales, which comprise lineages with wide substrate range may be potential candidates involved in high methanogenesis rates in the guts of humus or litter-feeding termites, a habitat which may also experience alternative

substrate gradients based on the complex nature of their diet. Methanosarcinales lineages from fungus-cultivating termites clustered together with those of the anterior gut of soil-feeding Termitinae (Fig. 1) in which methanogenesis rates are low, and may explain low methanogenesis rates in fungus-cultivating than in soil-feeding termites. Pair wise calculation of similarity showed strong indices between communities in soil-feeding *Ophiotermes* sp. (Termitinae) and fungus-cultivating termites suggesting the communities are similar. In most cases, there was a clear difference between communities in *Macrotermes* spp. and *Microtermes* sp., which was collected from the former's mound, suggesting diet related differences. Methanosarcinales lineages from posterior gut and from whole gut of many soil-feeding termites formed a distinct phylotype (Fig. 1 and 7) potentially suggesting they are most active methanogenic communities. It seems that Methanosarcinales, recovered from all fungus-cultivating and soil-feeding Termitinae, but so far not reported in lower termites, may have been acquired by Macrotermitinae, the most basal group of the higher termite taxa, probably through evolution in their feeding process and then passed on to subsequent generations as they shifted from a purely wood to litter or humified diet. However, somehow they were lost in some higher termite species in the process of their evolution, probably based on a shift in diet. *Alyscotermes trestus* is one such example in which Methanosarcinales may have been lost, but the group was then regained in soil-feeding members of the subsequent taxa. Instead, *A. trestus* is dominated by members of Methanobacteriales, a pattern also observed in wood-feeding lower and higher termites. Bray-Curtis similarity indices showed strong community similarity between *A. trestus* and *Microcerotermes* sp. (Fig. 8). The absence of Methanosarcinales in *A. trestus* may probably influence methane emission rates in this termite. Likewise, the wood-feeding *Microcerotermes* sp. (Termitinae), and the grass-feeding *Trinervitermes* sp. (Nasutitermitinae), which generally emit low amounts of methane than soil-feeders (Brauman *et al*., 1992), seems to have lost Methanosarcinales lineages probably due to the nature of their diet rich in organic matter but less humic.

Methanomicrobiales, which predominantly colonized the grass-feeding *Trinervitermes* sp. (55% of overall gut archaea) (Fig. 2 and 7) may be a core methanogenic microbiota in

the gut of termites feeding on lignocellulosic diet, but it is not clear why they are absent in the gut of wood-feeding *Microcerotermes* sp. and fungus-cultivating termites. Their equally high abundance in *Cubitermes* species, but absence in lower termites, suggest it as a group establishing in higher termites. Methanomicrobiales are known hydrogenotrophic methanogens whose cultured relatives require formate supplements for growth and have been predominantly recovered in anaerobic digesters and other anaerobic environments in which syntrophic fermenting bacteria have also been isolated (Sakai *et al*., 2009). The presence of a distinct deep-branching higher termite specific lineage of Methanomicrobiales (Fig. 2), phylogenetically radiating away (87% sequence similarity) from their closest cultivated neighbor *Methanospirillum hungatei* (Methanospirillaceae) potentially suggests a novel higher termite specific family of Methanomicrobiales, unique only to the termite gut environment and may have probably co-evolved with the host termite based on diet. Methanomicrobiales sequences from *Trinervitermes* sp. overlapped with those of *Cubitermes* spp., but formed their own distinct host-specific sub-cluster suggesting co-speciation with the host, probably based on diet. There was a weak Bray-Curtis index between communities in *Trinervitermes* sp. and *Cubitermes ugandensis* suggesting low similarity. Previous studies (Ohkuma *et al*., 1999; Miyata *et al*., 2007) on *Nasutitermes takasagoensis*, a wood-feeding Nasutitermitinae observed that the gut is almost exclusively colonized by Methanomicrobiales lineages and together with our results demonstrates Methanomicrobiales as a core archaeal microbiota in the sub-family Nasutitermitinae.

The recently discovered Methanoplasmatales (Paul *et al*., 2012) is represented in all the higher termites investigated irrespective of food diet (Fig. 3) suggest that probably yet to be discovered methanogens are present in termite guts and may explain the high methane emission rates in higher termites. Previous, this archaeal group was discovered in the gut of other termites (Friedrich *et al*., 2001, Hara *et al*., 2002, Ohkuma *et al*., 1999), in other insects (Egert *et al*., 2003) and in various other environments. In termites, Methanoplasmatales lineages form two groups both of which consist of higher termite sequences and clusters with archaea from cockroaches, suggesting adaptation to the gut and vertical transfer from termite ancestors. But the presence of *Cubitermes*-specific and

Macrotermitinae-specific phylotypes suggest host specialization. The methylotrophic nature of the isolate *Methanomassiliicoccus luminyensis* (Dridi *et al*., 2012) and highly enriched culture MpT1 from termite gut (Paul *et al*., 2012) suggest that termite gut methanogenesis is driven by alternative substrates besides hydrogen.

Our results provide the first evidence for the presence of members of Thaumarchaeota (Fig. 5) in termites, particularly in soil-feeding termites *Cubitermes ugandensis*, *Amitermes* sp. and *Alyscotermes trestus*. Although currently, no culture is available to elucidate the physiology of the termite gut related Thaumarchaeota, their close relationship with *Nitrososphaera* cluster, which are known aerobic ammonia-oxidizing Thaumarchaeota comprising *Nitrososphaera gargensis* from a moderately thermophilic environment (Hatzenpichler *et al*., 2008) and *Nitrososphaera viennensis* from soil (Tourna *et al*., 2011) may carefully lead to speculation of their potential involvement in nitrification processes in termite guts. In the anterior gut sections in which termite gut-derived Thaumarchaeota were recovered, ammonia concentrations have been detected in the lower milimolar range (Pester *et al*., 2007), and together with the high oxygen partial pressures in these gut sections (Brune *et al*., 1995), the tubular anterior gut compartments and rectum provides potential environments for aerobic ammonia oxidation. Cultures of ammonia-oxidizing archaea have been isolated from diverse environments including soils (Tourna *et al*., 2011), marine sediments (Könneke *et al*., 2005), acidic agricultural soils (Lehtovirta-Morley *et al*., 2011;), hot springs (de la Torre *et al*., 2008; Hatzenpichler *et al*., 2008). Recently, Kim *et al*., (2012) isolated strain JG1, a mesophilic Thaumarchaeota whose affinity for ammonia and oxygen was much higher than those of ammonia-oxidizing bacteria from an agricultural soil. The close relationship between termite gut Thaumarchaeota and lineages from the food soil of termites and other environments indicates that this group is not specific to the termite gut, but may have been acquired by the termite during its feeding activities. This may be corroborated by their low abundance in the gut potentially suggesting they are still establishing in the gut. Since termites excrete large concentrations of ammonia in their nest, it can be speculated that termite gut processes influence the activities of ammonia oxidizing Thaumarchaeota and probably bacteria in the surrounding soils and hence N-metabolism in soil. This may be a relevant

subject worth investigating. Nevertheless, the presence of Thaumarchaota mainly in soil-feeding termites which are also among the highest methane emitters is interesting and it is presently not clear whether their metabolic activities influences gut methanogenesis in any way or they are merely a transient group from food soil of the termites.

The deep-branching higher-termite-specific cluster of uncultured archaea (Fig. 6 and 7) recovered mainly from the posterior guts of soil-feeding *Cubitermes*, *Ophiotermes*, and *Amitermes* sp. as well as in whole gut of *Alyscotermes* species are distantly related (74-80% sequence similarity) to known sulfur metabolizing extremophilic Crenarchaeota in the class Thermoprotei and probably represents a novel group of archaea. This termite gut related uncultured group of archaea clusters distantly with archaeal lineages recovered from diverse environments including rhizospheres of rice (Lu *et al*., 2005), mesophilic anaerobic sludge digestors (Godon *et al*., 1997), lake sediment (Hershberger *et al*., 1996), marine sediments (Harrison *et al*., 2009), cold sulfidic spring marsh (Koch *et al*., 2006) and rich minerotrophic fen (Cadillo-Quiroz *et al*., 2008). However, the termite lineages of this group form a monophyletic deep-branching termite-specific cluster which is distinct from those of other environments. Previously, this group of archaea, then classified as Crenarchaeota, was also recovered in the gut of the soil-feeding termite *Cubitermes orthognathus* (Friedrich *et al*., 2001) as well as in the litter-feeding *Cornitermes cumulans* (Grieco *et al*., 2012). It is presently not clear what role they play in the termite gut, but their presence in high relative abundance (12-50% of the total archaea in the gut) seems to suggest that they are highly established members of the gut micro-biota with presently unknown functional role.

## Conclusion

Unlike lower termites which mainly harbor members of Methanobacteriales, higher termite hosts an assemblage of methanogens comprising four methanogenic lineages. Highest diversity was found in soil-feeding termites. Archaeal diversity in the wood feeding *Microcerotermes* sp. and in the grass-feeding *Trinervitermes* sp., which emit much less methane, was much lower. Highest density of methanogenic archaea in the gut

of soil-feeding termites may influence the high methane emission rates in these termite species. It seems that depending on the diet, termite gut methanogens evolve from hydrogenotrophic methanogenesis observed in Methanobacteriales, the core gut methanogenic microbiota, by developing adaptive mechanisms for withstanding *in situ* gut physicochemical conditions and acquiring methanogenesis genes necessary to utilize alternative substrates derived from fermentation of organic matter in the diet, resulting in more diversity in some termites than others. Termites maintain a unique lineage of host-specific archaeal gut microbiota which seem to co-evolve with the host probably based on diet, and are different from those in the food soil, nest material and from other environments. This was corroborated by low Bray-Curtis indices (Table 2) between *C. ugandensis* gut and those of its nest soil, indicating community differences.

## Acknowledgments

This work was supported by grant from DFG. James Nonoh received a doctoral fellowship from DAAD. We are grateful to Kenya Wildlife Service (KWS) for giving us permission to collect termites from Kenya for this study.

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## Chapter Three

# Distribution of archaeal populations and methanogenic potentials in the highly compartmented gut of soil-feeding termites (*Cubitermes ugandensis* and *Ophiotermes* sp.)

**James O. Nonoh, Carsten Dietrich and Andreas Brune**

Max Planck Institute for Terrestrial Microbiology, Department of Biogeochemistry, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany.

Author contributions:

J.O.N. designed experiments, obtained the samples, prepared the DNA, made clone libraries, performed qPCR, performed phylogenetic analysis, measured methanogenesis rate, analyzed and visualized the data, interpreted the data, and wrote the manuscript.

C.D. determined metabolite pool sizes, conducted statistical analysis and visualized the data.

A.B. conceptualized the study, designed experiments, interpreted the data and secured funding.

## Abstract

Soil-feeding termites, which have a highly compartmented gut with varied physicochemical conditions, emit more methane than other feeding guilds. Highest rates in these termites suggest that the diet influence the distribution of methanogens in the specific compartments. However, little is still known about the diversity, localization and population density of archaea in their highly compartmented guts. We used 16S rRNA gene-based cloning and qPCR analysis to investigate the distribution and population size variations of methanogens along the gut axis. Results show that community structure differed strongly among the different compartments, a phenomenon that is also reflected in the different micro-environmental conditions in the specific compartments. Density and diversity of archaea was highest in posterior gut compartments, which also harbored most of the methanogenic activities. The highly alkaline anterior gut compartments were preferentially colonized by Methanosarcinales. Besides the euryarchaeotal lineages, the gut contained also lineages closely related to ammonia-oxidizing Thaumarchaeota and a deep-branching termite specific group of uncultured archaea loosely affiliated to Crenarchaeota. Experimental stimulation of methanogenesis in isolated gut sections of *Cubitermes ugandensis* revealed significant activities of hydrogenotrophic methanogens that are obligately dependent on methanol and formate. Our results suggest that community structure in the different microhabitats is shaped by exogenous factors, such as pH, oxygen status and the availability of methanogenic substrates.

# Introduction

Methanogens are strictly anaerobic archaea producing methane as the end-product of anaerobic metabolism, obtaining all or most of their energy from methanogenesis. Methanogens have a narrow range of substrates in anaerobic metabolisms. They catalyze the terminal step in anaerobic food chain by converting various methanogenic substrates to methane (Hackstein and van Alen, 2010) and play a key role as the terminal electron sink organisms in most anaerobic environments. They are found in a wide variety of anaerobic habitats including those with extreme temperatures, salinity and pH (Liu and Whitman, 2008). Termites harbor methanogenic archaea in their guts and all termites investigated to date, both phylogenetically lower and higher ones, emit methane albeit in varied quantities (Brauman *et al*., 1992). Because of the morphological heterogeneity of the termite gut, the distribution of methanogenic archaea in the gut potentially influences methane emission potentials. Unlike in lower termites in which the gut is a simple structure consisting mainly of the hindgut paunch, the higher termite gut is much more complex (Noirot, 2001) showing considerable variations in compartmentation. In soil feeding termites, which emits more methane than members of the other feeding guilds, the gut is highly compartmented with steep gradients of physicochemical parameters such as oxygen, hydrogen, pH and redox potential (Brune *et al*., 1995; Brune, 2010). Culture independent studies in soil-feeding termites have shown more a much more complex community structure (Nonoh *et al*., chapter 2; Friedrich *et al*., 2001; Donovan *et al*., 2004), but the exact localization of methanogens in the gut and population size variations of methanogens along the gut axis is not clear. It is known that $H_2$, $CO_2$ and acetate are major products of fermentation reactions in the hindgut of termites (Brune and Friedrich, 2000) and these products provide an important link between fermentative breakdown of carbohydrates with methanogenesis and reductive acetogenesis (Schmitt-Wagner and Brune, 1999; Brune, 2009). Cultured methanogen isolates from lower termites, which generally emits low amounts of methane, are hydrogenotrophic, reducing $CO_2$ with hydrogen as electron donor (Leadbetter and Breznak, 1996), but are also capable of growing on formate. In lower termites, hydrogen is a major fermentation product of the

cellulolytic gut flagellates and it can accumulate to substantial amounts (Pester and Brune, 2007). Phylogenetically higher termites (family Termitidae), which do not harbor flagellates also accumulate and emit hydrogen (Collins *et al*., 1984; Schmitt-Wagner and Brune, 1999), probably as a product of fermenting bacteria (Brune, 2009) suggesting that hydrogen may be a major methanogenic substrate for most termite gut methanogens. Hydrogenotrophic methanogens have been recovered from both higher and lower termites and it has been speculated that $H_2$ consumption by gut methanogens promote anaerobic decomposition of organic matter in the termite hindgut (Shinzato *et al*, 1999). However, considering the complex nature of the soil diet, and the high methane emission rates in soil-feeders, a phenomenon that is correlated with high density and diversity of archaea in their guts, (Nonoh *et al*., chapter 2), it seems possible that availability of alternative substrates influence localization of archaea in the specific gut compartments. The presence of considerable concentrations of formate (2.6 mM) in other gut compartments and in the haemolymph led to a speculation that methanogenesis in the hindgut may be driven also by an inter-compartmental transfer of reducing equivalents via the haemolymph (Lemke *et al*., 2003). Methanogenesis in the posterior hindgut of soil-feeding (*Cubitermes* spp.) was stimulated by hydrogen as well as by formate (Schmitt-Wagner and Brune, 1999) and in the larval hindgut of the scarab beetle *Pachnoda ephippiata*, by formate and methanol (Lemke *et al*., 2003). Speculation over the potential role of methanol in driving gut methanogenesis has continued to grow because sequences recovered from various higher termites by our investigation (Nonoh *et al*., companion paper), and from *C. orthognathus* (Friedrich *et al*., 2001) clusters closely with methylotrophic isolates from insects, *Methanimicrococcus blatticola* (Methanosarcinaceae) from the litter-feeding cockroach *Periplaneta americana* (Sprenger *et al*., 2000) and strain MpT1 from Cubitermes ugandensis (Paul *et al*., 2012). These sequences also cluster closely with *Methanomassiliicoccus luminyensis*, a methylotrophic Methanoplasmatales isolate from human feces, which also require formate in its growth media (Dridi *et al*., 2012). However, to date, no study has directly localized methylotrophic activity in the specific gut compartments of the highly compartmented guts of soil-feeding termites and the methanogenic potential of the highly

compartmented gut of soil-feeding termites is still less understood. The influence of highly compartmented gut together with its varied physicochemical conditions on community structure needs to be understood. In this study, different exogenously supplied substrates and substrate mixtures were used to investigate methanogenic potentials in whole guts and gut compartments of soil feeding *Cubitermes ugandensis*. Population size variations of archaea along the gut axis were also investigated using clone analysis and quantitative PCR.

# Methods

## Termites

Two soil feeding termites *Cubitermes ugandensis* and *Ophiotermes* sp. were used in this study. The termites were collected in Kakamega forest reserve in Kenya and carried together with their nest material to Germany. Only worker caste termites were used. The termites were maintained in the laboratory by regularly feeding on a soil diet which was collected in the vicinity of their mounds.

## DNA extraction and purification

Worker caste termites (10-20) were used for the study. Using sterile fine tipped forceps, whole guts of *Cubitermes ugandensis* and *Ophiotermes* sp. were pulled out for compartmentation. The whole guts were separated into different individual compartments; crop (C), midgut including mixed segment (M), and proctodeal compartments P1, P3, P4 and P5 with the aid of a stereomicroscope (Zeiss, Jena, Germany). About 10-20 gut sections each were then pooled together into a sterile 2 ml eppendorf tubes (Qiagen, Germany) filled with 750 µl of sterile phosphate buffer (120 mM; pH 8.0). The pooled gut sections were then homogenized using a clean sterile pestle (Eppendorf, Hamburg, Germany). DNA was extracted following the Zirconium bead-beating method combined with phenol-chloroform (Lueders *et al*., 2004). The gut compartment homogenates were transferred to 2 ml bead-beating vials, followed by

addition of 250 µl sodium dodecyl sulfate (SDS) solution (10% SDS; 0.5 M Tris-HCl, pH 8.0; 0.1 M NaCl), and 0.7 g heat-sterilized zirconium-silica beads (0.1 mm diameter, Carl Roth, Karlsruhe, Germany) and the cells were lysed using cell disruptor (FastPrep-24 MP Biomedicals, Ilkirch, Germany) for 45 s at 6.5 m/s after which the cell debris were removed by centrifugation at 20000 × g for 4 minutes. The supernatant, containing the DNA was extracted with one volume of phenol-chloroform-isoamylalcohol (25:24:1) and eventually with 1 volume of chloroform/isoamyl alcohol (24:1, vol/vol) after centrifugation for 1 minute, prior to precipitation of the DNA by mixing the aqueous phase with 2 volumes of polyethylene glycol (PEG) solution (30% PEG 6000 in 1.6 M NaCl) and then centrifugation at 20000 × g and 4 °C. After washing with ice-cold ethanol (70%), the recovered pellet was dried at 30 °C for 5 minutes. The DNA was re-suspended in 50 µl elution buffer (MinElute PCR Purification Kit, Qiagen, Hilden, Germany). The extracted DNA was run on a standard 1% agarose gel in order to verify extraction efficiency and quality of the extracted DNA. The purity of the extracted DNA was checked photometrically (Nanodrop, PeqLab, Erlangen, Germany), and then quantified fluorimetrically (Qubit, Invitrogen, Eugene, OR, USA), and stored at –20 °C.

## Amplification of archaeal SSU 16S rRNA genes

PCR amplification was carried out using primer pairs that target archaeal small sub unit (SSU) 16S rRNA genes from position 109 to 934 as described by Großkopf *et al*., (1998) and Friedrich *et al*., (2001). Oligonucleotide primers specific for archaeal 16S rDNA used were Ar109F and Ar912R (CTCCCCCGCCAATTCCTTTA) (Escherichia coli 16S rRNA numbering) [Brosius *et al*., 1978]. Each PCR reaction (50 µl) contained reaction buffer, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase (all Invitrogen, Carlsbad, CA, USA), 50 µM deoxynucleoside triphosphate mix, 0.3 µM of each primer, 0.8 mg $ml^{-1}$ bovine serum albumin, and 1 µL of DNA template. PCR (30 cycles for P1, P3, P4 and P5 compartments) were carried out at initial denaturation step (94 °C for 3 min), followed by denaturation (94 °C for 20 s), annealing step (52 °C for 30 s), extension (72 °C for 45 s), and a final extension step (72 °C for 7 min). Our attempt to amplify the crop and midgut

DNA under the same conditions did not result in any amplification. We had to increase the number of cycles to 36 cycles for C and M sections under the same conditions to get an amplicon. Aliquots of the SSU 16S rDNA amplicons (5 µl) were analyzed by electrophoresis on a 1% agarose gel and visualized after staining with ethidiun bromide.

## Generation of clone libraries

Clone analysis of 16S rRNA genes was done for each of the six gut compartments including the crop, mid-gut, P1, P3, P4 and P5. Cloning analysis was performed by generating clone libraries from archaeal small sub-unit SSU 16S rDNA amplicons obtained from termite gut compartment community DNA. Purified PCR products (~ 800 bp [Ar109f-Ar912r] long) were ligated into the pGEMT easy plasmid vector (Promega), and *E. coli* JM109 (Promega) was transformed with the recombinant plasmids according to the manufacturer's instructions. Clones were given codes according to termite ID number plus the gut compartment as; C (crop gut section), M (midgut gut section including mixed segment), P1 (P1gut section), P3 (P3 gut section), P4 (P4 gut section) and P5 (P5 gut section). Randomly picked clones were further analyzed as described previously (Großkopf *et al*., 1998, Friedrich *et al*., 2001 and Donovan *et al*., 2004). Clones were checked for the correct insert size by vector-targeted PCR and standard agarose gel electrophoresis. About 40 randomly selected clones per library with the correct inserts were sent for sequencing commercially in both directions using vector based primers.

## Phylogenetic analysis of sequence information

Sequence data were analyzed and edited by Seqman (DNAStar) software. Phylogenetic analysis was done with the current ARB-SILVA database (version 106; Pruesse *et al*., 2007; http://www.arb-silva.de) using the ARB software package (Ludwig *et al*., 2004). The SSU 16S rRNA gene sequences obtained in this study were added into the database using the ARB software package tool (Ludwig *et al*., 2004) after alignment with SINA software tool (v1.2.9) using SILVA seed (Pruesse *et al*., 2007). The Sequences from

other studies that were not present in the Silva database were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/) and added to the database. The alignments were corrected manually where necessary. A 20% order level consensus filter was used to exclude highly variable positions. Phylogenetic trees were constructed with ARB maximum likelihood method using RAxML algorithm (Stamatakis, 2006). Aligned sequences were also analyzed by ARB maximum-parsimony (DNAPARS) methods to check for tree topology and node support (1000 bootstraps). Phylogeny was inferred by comparing sequences to the main lines of descent within the archaeal phyla; Euryarchaeota, Thaumarchaeota and Crenarchaeota. A threshold (>97% similarity) was set to assign sequence to the same OTUs.

## Quantification of abundance of archaea in the gut

Quantitative PCR was performed to estimate the relative density of archaeal populations along the gut compartments of the soil-feeding termites. The abundance of archaeal as compared to bacterial 16S rRNA genes in the specific gut compartments were measured by quantitative 'real-time' PCR (qPCR) following the method described by Kemnitz *et al.* (2005). Copy numbers of archaeal 16S rRNA genes were estimated using the primers sets A364aF (5'-CGGGGYGCASCAGGCGCGAA-3'; Burggraf *et al.*, 1997) and A934b (5'-GTGCTCCCCCGCCAATTCCT-3'; Grosskopf *et al.*, 1998). The bacterial 16S rRNA gene copy numbers were quantified as described by Stubner (2002) using the primer pairs 519fc (5'-CAGCMGCCGCGGTAANWC-3') and 907r (5'-CCGTCAATTCMTTTRAGTT-3') (Lane, 1991).

## Preparation of guts for potential rates of methanogenesis

The guts were prepared as previously described by Schmitt-Wagner and Brune, 1999. Fifteen (15) live termites (*Cubitermes ugandensis*) were weighed and then placed in 15 ml glass vials. The vials were then immediately covered with butyl rubber stoppers (Fisher, Germany). Methane emission rates from live termites were measured both under aerobic atmosphere and under nitrogen headspace over a 1–2 hour period. In both cases,

the methanogens were stimulated with 20% and 50% hydrogen substrate in the headspace. For the whole gut and gut sections, the termites were degutted using sterile fine tipped forceps in an anaerobic glove box (MECAPLEX, Switzerland) under $N_2$ headspace. To obtain intact whole guts, 15 non-compartmentalized whole guts were pooled together using a sterile fine tipped forceps without homogenization into a vial containing a shallow layer of buffered salt solution (BSS) in a 15 ml serum bottle. For gut compartments, 15 gut sections each were pooled together into a vial containing a shallow layer (0.3 ml) of BSS buffer. For the treatments, 20 mM formate, 10 mM methanol or 20% $H_2$ was added as substrates for methanogenesis, to the shallow layer of BSS buffer or to the headspace in the glass vial prior to placement of intact whole guts, and gut compartments. A control experiment was appropriately set up as above, but without any substrate added to the BSS/ buffer solution and with only $N_2/CO_2$ gas in the headspace. The vials were covered with butyl rubber stoppers (Fisher, Germany) and immediately flushed with $N_2/CO_2$ gas. Where necessary, gas headspace was exchanged with desired gas mixtures. For whole gut and whole gut homogenates, 1 ml BSS solution was used with $N_2$ as headspace gas. For gut sections (C, M, P1, P3, P4 and P5 compartments), a shallow layer (0.3 ml) BSS buffer was used with $N_2$ gas in the headspace. All the vials were incubated on the bench for a period of 1 to 2 hours without stirring. Gas headspace samples (200 µl) were taken at intervals of 30 minutes each and immediately replaced with equal volume of the original headspace mixtures. Methane concentrations were measured on GC.

## Methane measurements

Methane emission rates from live termites, intact whole guts, and individual gut compartments were monitored by measuring the concentration of methane gas in the headspace of the incubation vials with gas chromatography. Using a glass syringe fitted with a gas tight valve (Hamilton, Nevada, USA), 200 µl of the headspace gas samples from each vial were sampled every 30 minutes for methane analysis. The sample was injected through the septum of gas chromatograph (GC-8A Shimadzu, Japan) equipped

with Flame Ionization Detector (FID) (McWilliam *et al.*, 1958) and column (6 m, Ø 1/8″) packed with Porapak Q (50/80). The carrier gas was $H_2$ at a flow rate of 10 ml $min^{-1}$ and the column temperature was at 40 °C with the injector temperature at 110 °C. Before sampling, the sample vial was gently tapped to ensure uniform distribution of methane gas in the headspace.

### Metabolite pool size measurements in gut compartments

The pool sizes of different metabolites in gut were analyzed with HPLC. 10 pooled gut compartments of *Cubitermes ugandensis* were homogenized in 50 µl of BSS buffered and processed following the methods described in Tholen and Brune (2000). Pool size measurements were also performed as described in Tholen and Brune (2000).

## Results

### Metabolite pool size measurements in gut compartments

Highest concentration of most metabolites was detected in the posterior P3 and P4 compartments of the worker castes of *Cubitermes ugandensis*. In the anterior gut sections, the metabolite pool sizes were very low. Table 1 summarizes the metabolite pool sizes in each gut section.

Table 1: Metabolite pool sizes in selected gut sections [midgut, including mixed segment (M/ms), and proctodeal compartments P1–5] of *Cubitermes ugandensis*.

| Gut section | Pool sizes of different metabolites (nmol section$^{-1}$)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Acetate | Propionate | Butyrate | Lactate | Succinate | Ethanol | Methanol |
| M/ms | 0.4 ± 0.2 | 0.05 ± 0.02 | _[b] | _ | 0.05 ± 0.03 | 0.05 ± 0.02 | 0.11 ± 0.05 |
| P1 | 0.8 ± 0.3 | 0.07 ± 0.04 | _ | _ | 0.12 ± 0.08 | 0.08 ± 0.05 | 0.23 ± 0.09 |
| P3 | 7.1 ± 1 | 3.2 ± 0.5 | 0.1 ± 0.0 | 0.4 ± 0.2 | 0.23 ± 0.12 | 0.11 ± 0.06 | 0.85 ± 0.12 |
| P4 | 6.5 ± 1.2 | 2.4 ± 0.7 | 0.2 ± 0.0 | 0.6 ± 0.1 | 0.12 ± 0.05 | 0.23 ± 0.08 | 1.2 ± 0.4 |
| P5 | 1.2 ± 0.5 | 0.5 ± 0.09 | _ | 0.23 ± 0.08 | 0.03 ± 0.01 | 0.45 ± 0.02 | 0.6 ± 0.2 |

[a] Values are mean ± mean deviation of two independent assays.

[b] Below detection limit (0.02 nmol section$^{-1}$).

## Potential rates in the gut

Live termites used in this study showed methane production activity at a steady rate of 0.30 ± 0.04µmol $g^{-1}h^{-1}$ (Table 2), a slightly higher value than that obtained in previous studies with *Cubitermes orthognathus* (0.180 ± 0.048µmol $g^{-1}h^{-1}$) (Schmitt-Wagner and Brune 1999). The differences may be as a result of our constant feeding of the termites at least two days prior to the experiments, but may also be caused by inter-specific variations between the termites. When exogenous hydrogen (20%) was supplied to the headspace, there was about four-fold increase in methane emission rate from live termites. However, when exogenous hydrogen concentration in the headspace was increased to 50%, there was about ten-fold increase in methane emission rates, an indication that gut methanogens are highly substrate limited in situ.

Table 2: Methane emission rates of living worker castes of *Cubitermes ugandensis* as compared to the values obtained for *Cubitermes orthognathus*.[a]

| Termite | $CH_4$ emission rates (nmol $g^{-1}h^{-1}$)[b] | | | | | |
|---|---|---|---|---|---|---|
| | Air | $N_2$ | Air + $H_2$[c] | Air + $H_2$[d] | $N_2$ + $H_2$[c] | $N_2$ + $H_2$[d] |
| *C. ugandensis* | 300 ± 0.04 | 320 ± 0.03 | 1150 ± 0.16 | 2870 ± 0.52 | ND | 3000 ± 0.49 |
| *C. orthognathus*[e] | 158 ± 28 | 160 ± 29 | 1123 ± 88 | ND[f] | 1176 ± 254 | ND |

[a] Living termites were incubated under air or nitrogen for 2 hours.

[b] Values are mean ± mean deviation of two independent assays.

[c] 20% $H_2$ in the headspace.

[d] 50% $H_2$ in the headspace.

[e] Data obtained from Schmitt-Wagner and Brune, 1999.

[f] Not determined.

Our results showed a positive trend in methane emission when exogenous substrates was supplied to the gut compartments, probably an indication of substrate limitation. Methane

emission was greatly enhanced by addition of substrate mixtures. All the substrates tested in this study stimulated methane production in whole guts and in intact individual gut compartments, with highest stimulation observed with formate and methanol substrates (Table 3). There was constant methane production from the compartments during the experiments but reached a plateau towards the end of the two-hour incubation. Hydrogen also showed stimulation mainly in the posterior gut compartments. When hydrogen was supplied with each of the substrates, there was a sudden increase in methane emission in all the gut compartments. In the anterior gut sections, methanogenesis was mainly stimulated by methanol and formate. Hydrogen did not show stimulation in the anterior gut sections. In the highly alkaline P1 compartment, hydrogen showed a stronger stimulation of methane emission (27% over control) than methanol substrate (18% over control). However, when methanol was supplemented with hydrogen, there was one-fold (36%) increase in methane emission rates over control. Addition of exogenous formate to the P1 compartment resulted in over one-fold (47%) increase in methane emission rates. However, the highest methane emission rate in this compartment was observed when exogenous formate was supplemented with hydrogen in which there was a two-fold (72%) increase in methane emission. The highest stimulation in the dilated P3 compartment was obtained with exogenous formate substrate in which a two-fold increase in methane emission rates (124%) was observed over control. When exogenous formate was supplied together with hydrogen in the headspace, methane emission rates increased over two-fold (147%). There was only minimal (8%) increase in methane emission rates with exogenous methanol. However, when methanol was supplied together with exogenous hydrogen in the headspace, there was almost a two-fold (58%) increase in methane emission rates. In the tubular P4 gut compartment, like in the P3 gut section, the highest stimulation was observed with exogenous formate which resulted in over two-fold (117%) increase in methane emission rate. When exogenous formate was supplied together with exogenous hydrogen in the headspace of the P4 compartment, methane emission rates increased three-fold (172%) providing the highest stimulation by a substrate in the whole gut. All the substrates stimulated methane emission in the P5 compartment with more than one-fold (34%) and two-fold (88%) with exogenous

methanol and formate substrates respectively. Potential rates of methanogenesis in specific compartments are shown in table 3.

Table 3: Methane emission rates of whole guts and individual gut compartments [crop (C), midgut, including mixed segment (M), and proctodeal compartments P1–5] of *Cubitermes ugandensis* using exogenous substrates.[a]

| Gut section | $CH_4$ emission rate (nmol termite$^{-1}$h$^{-1}$)[b] | | | | | |
|---|---|---|---|---|---|---|
| | $N_2$ | $H_2$ | Methanol | $H_2$ + Methanol | Formate | $H_2$ + Formate |
| C | 0.66 ± 0.08 | 0.68 ± 0.05 | 0.83 ± 0.18 | 0.72 ± 0.21 | 0.81 ± 0.05 | 0.91 ± 0.05 |
| M[c] | 0.57 ± 0.12 | 0.60 ± 0.06 | 0.64 ± 0.21 | 0.65 ± 0.08 | 0.81 ± 0.07 | 0.80 ± 0.06 |
| P1 | 0.97 ± 0.13 | 1.24 ± 0.15 | 1.15 ± 0.03 | 1.32 ± 0.09 | 1.43 ± 0.63 | 1.67 ± 0.61 |
| P3[d] | 2.90 ± 0.16 | 3.5 ± 0.47 | 3.15 ± 0.17 | 4.58 ± 0.59 | 6.49 ± 0.31 | 7.17 ± 0.09 |
| P4 | 1.48 ± 0.09 | 2.03 ± 0.16 | 1.72 ± 0.31 | 2.38 ± 0.04 | 3.21 ± 0.74 | 4.03 ± 0.99 |
| P5 | 0.59 ± 0.05 | 0.72 ± 0.06 | 0.79 ± 0.14 | 0.97 ± 0.04 | 1.11 ± 0.14 | 1.25 ± 0.08 |

[a] Whole guts and individual gut sections were incubated in a shallow layer of BSS solution for 2 hours. Headspace ($N_2$) or incubation buffer was amended with the following substrates: $H_2$ (20 kPa), methanol (10 mM), formate (20 mM).

[b] Values are mean ± mean deviation of two independent assays.

[c] The midgut included the alkaline mixed segment.

[d] Values for P3 may be an underestimation as a third of the pooled guts collapsed during sectioning, potentially leading to low measured methanogenesis rates.

## Population density and axial distribution of archaea along the gut

Axial distribution and diversity of archaea, and the population size variations was investigated in individual gut compartments of two soil-feeding species (*Cubitermes* sp. and *Ophiotermes* sp.). The community structure was quite diverse between the termites. There were differences in axial distribution of archaeal populations between the two soil-feeding termites investigated (Table 4 and 5 and Fig. 1). The crop of *Cubitermes ugandensis* was dominated by members of Thaumarchaeota (1% of overall gut archaea). However, in *Ophiotermes* sp., there was very low archaeal density in the crop with the dominant group being Methanosarcinales. Methanosarcinales were the dominant group

recovered in the anterior alkaline midgut and P1 gut compartments of *C. ugandensis* (1% and 5% of overall gut archaea respectively), while in *Ophiotermes* sp., the alkaline midgut and P1 gut compartment were exclusively colonized by members of Methanosarcinales (1% and 20% of overall gut archaea respectively). The P3 gut compartment of *C. ugandensis* was dominantly colonized by members of Methanomicrobiales and uncultured archaea loosely affiliated to Crenarchaeota (18% each of overall gut archaea). In contrast, the P3 compartment in *Ophiotermes* sp., was dominated Methanosarcinales (46% of the overall gut archaea). Methanoplasmatales was the dominant group in the P4 gut compartment of *C. ugandensis* (14% of overall gut archaeal population). In *Ophiotermes* sp., we did not manage to quantify the copy numbers of archaea in the P4 compartment due to lack of sample, but Methanosarcinales were the most dominant group in terms of relative abundance. In the rectum of *C. ugandensis*, Methanoplasmatales was the dominant group while in *Ophiotermes* sp., Methanosarcinales was the dominant archaea. A summary of the population density of archaea as compared to bacteria in the respective gut compartments is provided in table 4 and 5.

Table 4: Absolute abundances of Archaea and Bacteria in individual gut sections [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] of *Cubitermes ugandensis*.

| **Taxa** | **Absolute copy numbers of 16S rRNA gene ($10^3$ copies/gut)[a]** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **C** | **M** | **P1** | **P3** | **P4** | **P5** | **Total/whole gut** |
| Total Archaea[b] | 1.03 ± 0.05 | 1.39 ± 0.12 | 6.34 ± 1 | 43.8 ± 0.06 | 20.2 ± 0.1 | 0.25 ± 0.02 | 73 ± 1.18 |
| Total Bacteria[c] | 36.6 ± 19.2 | 37.7 ± 27.4 | 164 ± 57.1 | 3530 ± 475 | 1030 ± 37.5 | 42.3 ± 15.1 | 4840 ± 517 |
| Arch/Prokaryotes (%)[d] | 2.72 | 3.55 | 3.73 | 1.23 | 1.92 | 0.59 | 1.49 |

[a] Values shown are mean ± mean deviation of two independent measurements (shown only to 3 significant figures).

[b] Values are obtained by multiplying the copy numbers of archaeal 16S rRNA gene by the clone abundance of archaeal taxa recovered in each compartment.

[c] Values are bacterial 16S rRNA gene copy numbers in each compartment.

[d] Abundance of archaea expressed as a percentage of that of total prokaryotes in each compartment.

Table 5: Absolute abundances of archaea and bacteria in individual gut sections [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] of *Ophiotermes* sp.

| Taxa | Absolute copy numbers of 16S rRNA gene ($10^3$ copies/gut)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | C[b] | M[b] | P1 | P3 | P4 | P5 | Total/whole gut |
| Total Archaea[c] | 0.1 | 5.15 | 84 ± 4.5 | 311 ± 77.3 | ND[f] | 2.76 ± 1.05 | 403 ± 84.8 |
| Total Bacteria[d] | ND | 510 | 5800 | ND | ND | 1300 | 7610 |
| Arch/Prokaryotes (%)[e] | NA[g] | 1 | 1.43 | NA | NA | 0.21 | 5.04 |

[a] Values shown are mean ± mean deviation of two independent measurements (shown only to 3 significant figures).

[b] Values are only for one measurement.

[c] Values are obtained by multiplying the copy numbers of archaeal 16S rRNA gene by the clone abundance of archaeal taxa recovered in each compartment.

[d] Values are bacterial 16S rRNA gene copy numbers in each compartment (values are only for one measurement).

[e] Abundance of archaea expressed as a percentage of that of total prokaryotes in each compartment.

[f] Not determined.

[g] Not applicable.

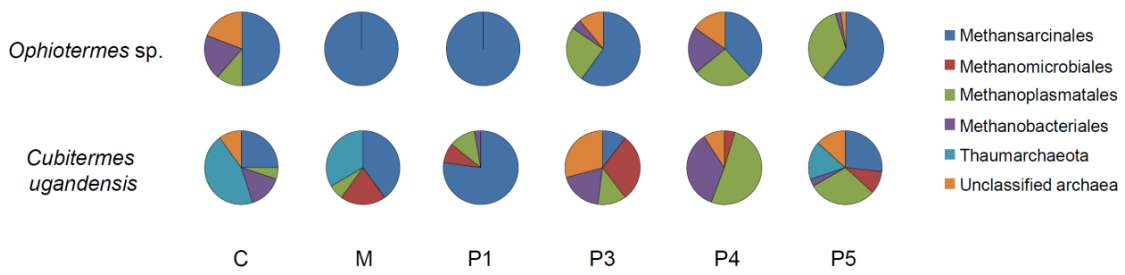


Fig. 1: Relative abundance and localization of major taxonomic groups of methanogenic euryarchaeota and other archaeal phyla along the gut axis of *Cubitermes ugandensis* and *Ophiotermes* sp. Values are relative abundance of major archaeal taxa in individual gut compartments [(C), midgut including mixed segment (M), and proctodeal compartments P1–5] of each termite, based on clone sequence analysis of archaeal SSU 16S rRNA gene.

## Phylogeny of communities in both termites

There was an overlap between archaeal communities in both termites. In most of the archaeal groups recovered, the communities from the two termites clustered together. However, the communities from *Ophiotermes* sp. formed a distinct sub-cluster from that of *Cubitermes ugandensis*, suggesting host-specific co-evolution (Fig. 2). There were also community differences among the compartments, with those in the anterior gut being distinct from those in the posterior gut (Fig. 2 and 3). The soil-feeding termites harbored high diversity of archaea, but Methanomicrobiales was not recovered in *Ophiotermes* sp. (Fig. 3).

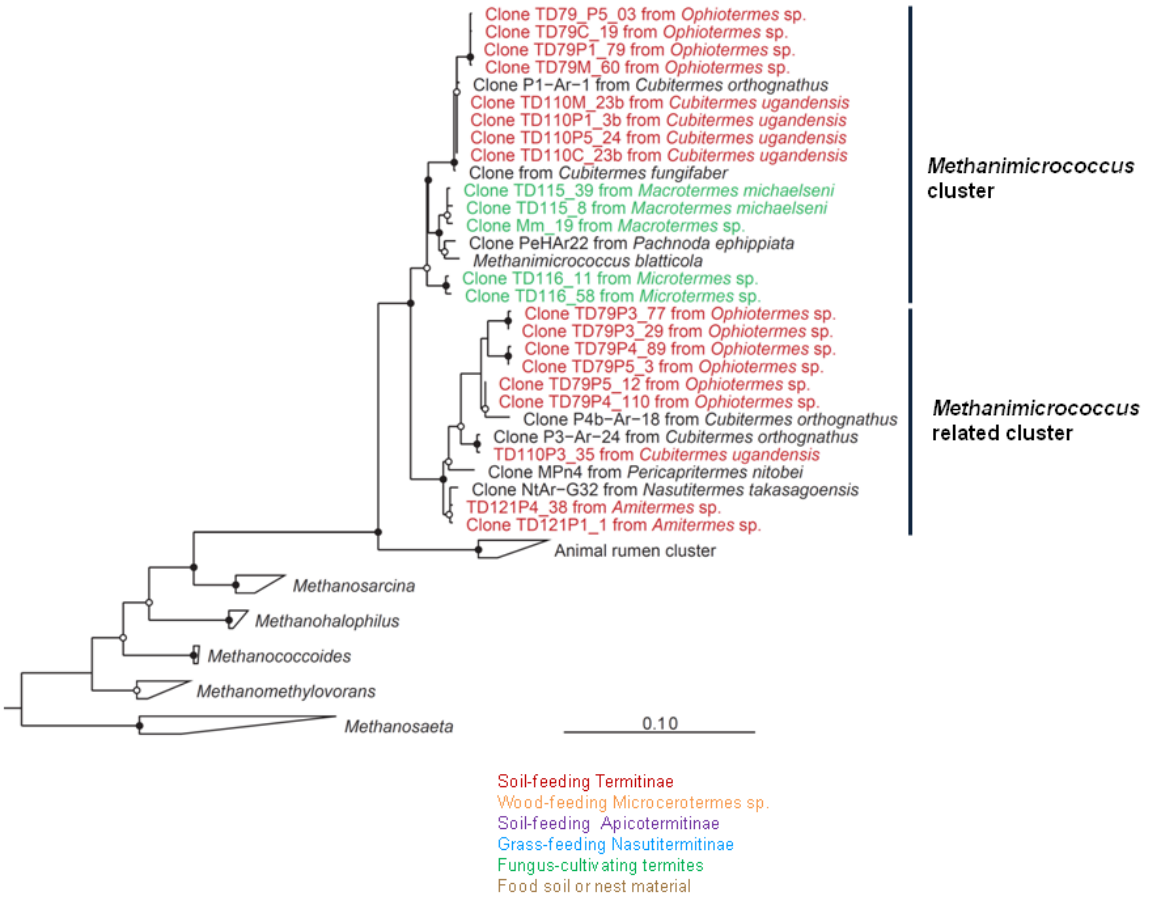


Fig. 2: A maximum-likelihood tree showing the phylogenetic positions of archaeal lineages related to Methanosarcinales recovered from higher termites as compared to those from other insects and different environments as well as representative isolates in this group. The phylotypes containing clones from different higher termites in this study are indicated. Sequences from representative species of higher termites used in this study are marked in colors. Sequences from individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] are also indicated. Bullets indicate bootstrap support (●, >90%; ○, >70%). The scale bar indicates rate of substitutions per nucleotide position. The tree was rooted with euryarchaeote *Methanogenium marinum*.

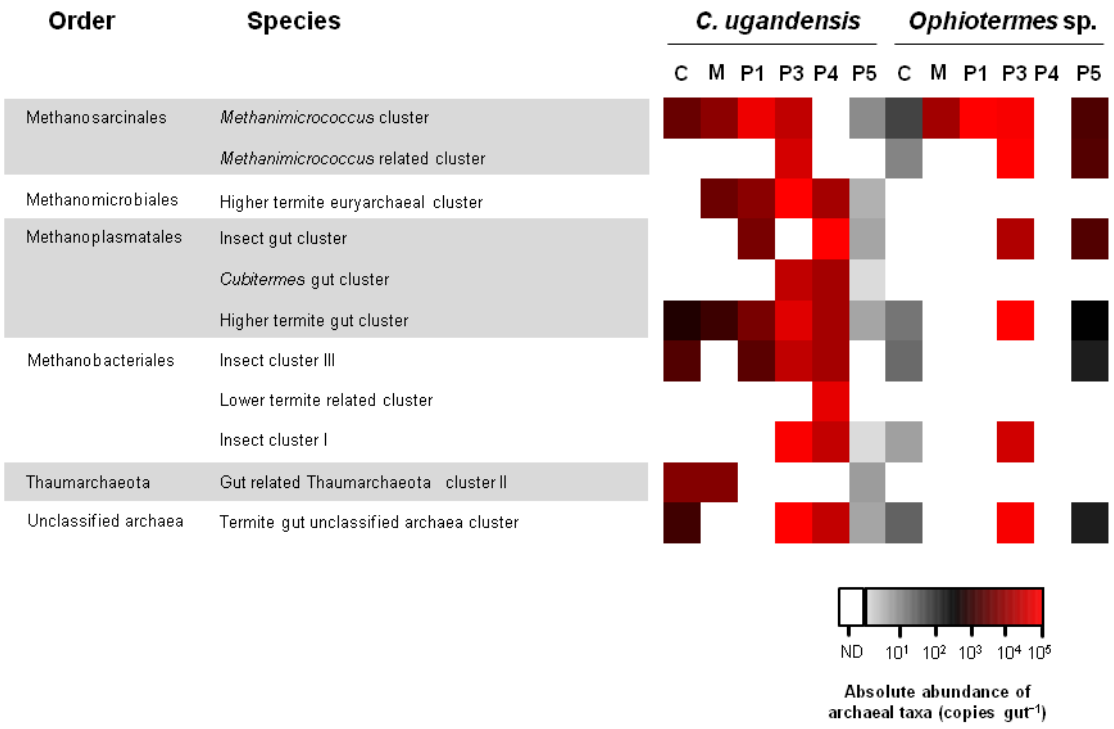


Fig. 3: Absolute abundance of major archaeal taxa recovered in individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] of *Cubitermes ugandensis* as compared to that of *Ophiotermes* sp. The classification is shown down to species level (based on assignment of OTUs at a threshold of 97% sequence similarity following analysis of 750 bp fragment of archaeal SSU 16S rRNA gene after filtering with order level filters). The values were log transformed to visualize the low abundance taxa. No values are shown in the P4 compartment of *Ophiotermes* sp. as we did not have enough DNA to run a quantitative PCR. (ND, below detection limit).

## Discussion

Highest rates of methane emission have been found in soil-feeding taxa, but only little is known about the diversity of the archaeal communities in their highly compartmented guts. Our results show that in the highly substrate limited gut (Table 2), archaeal populations are heterogeneously distributed along the highly compartmented gut axis of both *Cubitermes ugandensis* (Table 4) and *Ophiotermes* sp. (Table 5). The uneven inter-

compartmental distribution (Fig. 1) and community differences between anterior and posterior gut (Fig. 2 and 3) may be influenced by the gut morphology and the different micro-environmental conditions in the specific compartments, including availability of substrates. There seems to be a niche differentiation in archaeal colonization of specific gut compartments, with communities in the anterior gut being different from those in the posterior gut.

**Anterior crop and midgut**

The mildly acidic crop and the tubular alkaline midgut of both termites were predominantly colonized by Methanosarcinales, suggesting a possible adaptation to the extreme conditions in these sections. The limited availability of substrates in the anterior gut (Table 1) may influence the low abundance of other methanogens in these compartments, but it may also be due to inhibitive pH and the high oxygen concentrations in these compartments. The exclusive colonization of the anterior gut of *Ophiotermes* sp. by Methanosarcinales may be influenced by the gut pH and availability of substrates. The communities in the anterior gut of both termites were largely similar. The stimulation of methane emission in the anterior compartments of *C. ugandensis* by exogenous methanol and formate substrates may be explained by the presence of endogenous substrates. The minimal rates of endogenous methanogenesis in the anterior gut of *C. ugandensis* may be explained by the low archaeal abundances (1% and 2% of overall gut archaea) in the crop and midgut respectively suggesting that only specialist methanogens colonize this gut sections. The lack of stimulation by hydrogen in the anterior gut may be due to most of it being used in oxygen removal activities. The close proximity of the mid-gut to the mixed segment where hydrogen accumulates to high concentration probably facilitates hydrogen diffusion into the mid-gut. However, since low pH is known to inhibit hydrogenotrophic methanogens *in situ* (Schink, 1997), the acidic nature of the anterior gut may result in inhibition of hydrogenotrophic methanogenesis.

**Alkaline P1 compartment**

The predominant colonization of the highly alkaline P1 compartment of both termites by Methanosarcinales, suggest that they are adapted to alkaline conditions in the anterior gut. Previous studies on the soil-feeding *Cubitermes orthognathus* (Friedrich *et al*., 2001) recovered mainly Methanosarcinales and Crenarchaeota in the P1 compartment. However, in a companion paper (Nonoh *et al*, chapter 2), we showed that Methanosarcinales were the dominant archaea in whole gut of fungus cultivating termites which do not exhibit high hindgut pH like in soil-feeders, suggesting that some lineages of this group probably colonize less alkaline micro-habitats. In *C. ugandensis*, all major methanogenic euryarchaeota colonized the P1 compartment, suggesting a possible presence of less alkaline micro-habitats as well as availability of specific methanogenic substrates. However, the low density of other methanogenic groups in this compartment, together with the fact that only 9% of the overall gut archaea colonize this dilated compartment (Table 4), suggest inhibitory effect of the high alkalinity. This may also be explained by the low availability of substrates. Microscopic examination revealed only a few filamentous, rod-shaped and coccoid cells in this compartment. Like in the crop and midgut, highest stimulation in the highly alkaline P1 compartment of *C. ugandensis* was observed with formate and methanol, suggesting that lineages of Methanosarcinales, the dominant group may be dependent on these substrates. Presently, no representative of Methanosarcinales is known to grow on formate, but the strong stimulation by formate is suggestive of some lineages in this group being formate utilizers. It is also possible that Methanoplasmatales comprises some unidentified formate utilizing lineages. But the strong effect by exogenous formate could be caused by Methanomicrobiales, whose cultured lineages require formate for growth. The near two-fold increase in methane emission rates when each of these substrates was supplemented with hydrogen demonstrates hydrogenotrophic nature of formate and methanol utilizing gut methanogens. Hydrogenotrophic methanogens seems to be present in all gut compartments and agree with the observation that most methanogens may have conserved hydrogenotrophic capacities (Bapteste *et al*., 2005). The stimulation by hydrogen in P1 gut compartment is surprising because it is sandwiched between mixed segment and P3 compartments in which hydrogen accumulates to high partial pressures

(Schmitt-Wagner and Brune, 1999). This indicates hydrogen limitation either because of high competition for it or diffusion related setbacks. There was only minimal methane emission in the non-stimulated P1 compartment. The lower potential rates of methanogenesis with exogenous substrates in P1 gut section than in the posterior gut compartments is probably due to its colonization by only a few specialist methanogens as is evidenced by the low archaeal density in this compartment.

**Dilated posterior P3 compartment**

Highest abundance of archaea in the P3 compartment of both *C. ugandensis* and *Ophiotermes* sp. (60% and 77% of overall gut archaea) suggest availability of substrates and less alkaline micro-habitats. The preferential colonization of posterior P3 gut section of *Cubitermes ugandensis* by Methanomicrobiales (Table 4) may be influenced by specific substrates whose availability is probably dependent on the diet. Since this archaeal group, not recovered in *Ophiotermes* sp. (Nonoh *et al*., chapter 2), was previously recovered in posterior gut of *Cubitermes orthognathus* (Friedrich *et al*., 2001) and in whole gut homogenate of *Cubitermes fungifaber* (Donovan *et al*., 2004) and *Alyscotermes trestus* (Nonoh *et al*., chapter 2), their presence in the gut may be linked to the diet. It is presently not clear what specific role the Methanomicrobiales play in the guts of *Cubitermes* species, but their high abundance in the P3 gut section, in which all substrates stimulated methanogenesis may suggest involvement in metabolism of fermentation intermediates. Due to the high abundance of Methanomicrobiales in Nasutitermitinae (Nonoh *et al*., chapter 2), one may speculate a role in metabolism of substrates in the gut of termites feeding on lignocellulosic and humified diet. The high abundance of methanogens in the P3 compartment conforms to its highest concentration of metabolite pools in the gut and may explain the high methane emission rates in this gut compartment. The P3 gut compartment of *C. ugandensis* was already emitting methane at a steady rate in the non-stimulated guts, demonstrating availability of endogenous substrates and colonization by high density of diverse groups of methanogenic archaea. The high archaeal density, also corroborated by high density of diverse morphotypes of $F_{420}$ auto-fluorescing cells explains the highly substrate limitation in this compartment as

was evidenced by increased stimulation by all exogenous substrates. The highest stimulation with formate in the P3 compartment potentially suggests that it may be an important intermediate in the gut and that its utilization may be widespread among gut methanogens. Cultivated lineages of Methanomicrobiales, the dominant group in this compartment, require formate in their growth media although the exact role of formate still remains unclear. Methanobacteriales, the second most dominant methanogenic group in this compartment may also contribute to strong stimulation by formate. However, it is a paradox that the cultured lineages of this order grow only poorly on formate. Considering the equally high abundance of Methanoplasmatales, the high stimulation by formate substrate in this compartment, may also be due to some lineages in this order being formate utilizers. Interestingly, the only cultured Methanoplasmatales isolate *Methanomassiliicoccus luminyensis* requires formate in its growth media (Dridi *et al*., 2012), suggesting that lineages of this group are involved in formate metabolism. Our qPCR measurements showed high densities of bacterial 16S rRNA gene (Table 4) in the P3 compartment, suggesting most fermentation reactions may be localized here. The very strong stimulation with methanol, particularly when supplied with hydrogen demonstrates the hydrogenotrophic nature of gut methylotrophs and explains the high concentration of methanol pools in this compartment. Hydrogen is a limiting substrate despite the P3 being hydrogen accumulating compartment, probably due to rapid consumption by different competing groups. The low stimulation with methanol alone suggests that most gut methylotrophic methanogens are incapable of methanol disproportionation and the consumption of endogenously produced methanol is dependent on hydrogen probably emanating from neighboring compartments. Despite the Methanosarcinales being the dominant archaea in the more alkaline anterior gut sections, equally high proportion of this group (6%) was observed in the P3 compartments of *C. ugandensis*. In *Ophiotermes* sp., the highest density of Methanosarcinales (46% of overall gut archaea respectively) observed in the P3 compartment suggest that some lineages of this group potentially colonize less alkaline micro-habitats. Highest densities of Methanobacteriales were also localized in the P3 compartment of both termites (Table 4 and 5). Quantitative PCR demonstrated almost two-fold increase in density of archaeal 16S rRNA copies in the P3b

than the P3a sections respectively (chapter 6). The same was also true for bacterial copy numbers which was about one order of magnitude higher in the P3b than the P3a section, suggesting that most cells potentially inhabit the less alkaline micro-habits in the gut. The less alkaline P3b section is also less dilated and prone to inward oxygen diffusion and it seems that gut alkalinity influences localization more than oxygen stress, probably because the archaeal inhabitants have developed oxygen withstanding mechanisms or they rely on bacterial counterparts for that service. In the lower termite *Reticulitermes flavipes*, Leadbetter and Breznak (1996) observed that *Methanobrevibacter* species attached at the micro-oxic periphery of the hindgut paunch may be involved in oxygen reduction in the gut. *Methanobrevibacter cuticularis* from a lower wood-feeding termite *Reticulitermes flavipes* has been shown to have catalase enzymes for oxygen detoxification (Brune 2010). In higher termites, their role is not very clear but highest abundances of Methanobacteriales in the posterior P3 and P4 compartments, which also experiences high oxygen gradients, suggest their ability to control inflowing oxygen. Fluorescence microscopy revealed presence of $F_{420}$ auto-fluorescing rod-shaped cells attached to the gut wall of P3b section of *Cubitermes ugandensis* (see chapter 6). Most archaea seems to be localized in the posterior P3 and P4 gut sections where virtually most of the methanogenic activities are localized. This agrees with the results of Tholen and Brune, (1999) who also reported high abundances of methanogens and bacteria in the posterior guts of *Cubitermes* spp. The highest abundance observed in the dilated P3 compartment of both termites, may not only be influenced by availability of substrates, but also the anoxic status of P3 compartment, especially at the gut center. There was some overlap between the communities in the P3 and P4 compartments (Fig. 2). However, the P3 compartment formed distinct communities from those of the other neighboring compartments suggesting the differences in the micro-environmental conditions, including availability of substrates influence compartment-based specialization. Another reason for the high density of archaea in the P3 compartment may be the un-uniform pH in this compartment suggesting presence of less alkaline micro-habitats. It is possible that due to the high oxygen gradients in the tubular P4 compartment, oxygen sensitive cells tend to specialize in the dilated P3 section in which

there would be oxygen diffusion barriers and in which substrates may be available. The P3 compartment likely represents the most functional methanogenic gut compartment in this soil-feeding termite. Köhler *et al*., (2012) also observed high bacterial activity in highly dilated P3 compartment of *Nasutitermes takasagoensis* suggesting fermentation reactions to be influenced by the size.

**Tubular P4 compartment and rectum**

The minimal methane emission observed in the non-stimulated P4 gut compartment (Table 3) and the strongest stimulation with all the exogenously supplied substrates potentially makes it the most substrate limited. The lower archaeal density in the P4 as compared to the P3 compartment, may be attributed to available substrates in the P3 compartment resulting from fermentation reactions, but may also be due to oxygen stress in the tubular P4 compartment. The highest stimulation in P4 compartment was observed with formate, and especially when it was supplied with hydrogen, demonstrating hydrogenotrophic nature of formate utilizing methanogens. Methanobacteriales and Methanomicrobiales, abundant in this compartment, comprise hydrogenotrophic formate utilizing lineages and may be responsible for hydrogen limitation in this compartment. Schmitt-Wagner and Brune (1999) showed that hydrogen concentration was always below the detection limit (<100 Pa) in the posterior hindgut of *Cubitermes* spp. The predominant colonization of the tubular P4 gut compartment of *Cubitermes ugandensis* by Methanoplasmatales and the abundance of other methanogenic groups explains the high methanogenic nature of the posterior gut sections. This is corroborated by high metabolite pool sizes in the posterior gut (Table 1). The strong effect by exogenous methanol in this compartment may be due to stimulation of Methanoplasmatales, which comprise methylotrophic lineages (Paul *et al*., 2012; Dridi *et al*., 2012). Since methanol concentration was highest in this compartment, the strong effect may be due to stimulation of other methylotrophic methanogens present in this compartment. Since our culture-independent studies and those by previous investigators did not recover methanol utilizing *Methanosarcina* or *Methanosphaera* in the P4 gut compartment of *Cubitermes* species, it is also possible that some lineages of *Methanobrevibacter*

(Methanobacteriales) which were the second most abundant methanogens in this compartment may have acquired methylotrophic characteristics. The colonization of the P4 gut compartment by different metabolic groups of archaea and bacteria may be attributed to the moderate pH as well as availability of substrates. Like most compartments, the rectum was also substrate limited as evidenced by very low methane emission in the non-stimulated gut. Methane emission was however restored with exogenous substrates especially when supplied with hydrogen. The low methane emission rates in P5 section as compared to other hind-gut compartments may be attributed to low archaeal density colonizing this gut section, a phenomenon that may be attributed to the low substrate availability, its acidic nature and effect of high oxygen gradients.

The colonization of the gut and the rectum by uncultured archaea closely related to aerobic ammonia-oxidizing Thaumarchaeota (Fig. 5) in the cluster *Nitrososphaera* suggests presence of nitrifying activities in the gut. The low ammonia concentrations in the anterior gut as compared to the posterior gut sections (Pester *et al.*, 2007) and the high oxygen partial pressures in these sections (Schmitt-Wagner and Brune, 1999) provides a good environment for ammonia-oxidation activities to occur. Since the termite gut Thaumarchaeota were only recovered in soil-feeding termites, which also emits high amounts of methane, it is reasonable to speculate if their activities does not influence the methanogenesis processes in the gut. The deep branching group of unclassified archaea affiliated with Miscellaneous Crenarchaeotic group, were only distantly related to hyperthermophilic Crenarchaeota in the class Thermoprotei. This group, predominantly colonizing the posterior gut with highest density in the P3 gut compartment in which most methanogenic activities is also localized, formed a monophyletic higher termite-specific cluster (Nonoh *et al.*, chapter 2) distinct from related archaea from other environments. Previously, a few clones of this group were recovered in the gut of a soil-feeding termite *Cubitermes orthognathus* (Friedrich *et al.*, 2001) and in the litter-feeding *Cornitermes cumulans* (Grieco *et al.*, 2012). It is still not clear what role these uncultivated archaea play in the gut but because of their high abundance in the posterior methanogenic gut sections, it is speculative if their activities are not influenced by the

activities of methanogens. It would be important to understand their physiological and functional roles in the gut.

The clear methanogenic community differences between the anterior and posterior gut sections potentially suggest that the high methanogenic potential of the posterior gut is strongly influenced by micro-environmental conditions, including availability of utilizable substrates. This may explain the high diversity of archaea observed in the posterior gut. The methanogen lineages in the anterior gut sections may be inactive, probably due to the inhibitive gut conditions and low substrate concentrations. The anterior gut methanogens may have lost most of their methanogenesis genes hence low methanogenic potential. Our results show that substrates other than hydrogen potentially drive methanogenesis in the gut of soil-feeding termites.

Our results provide direct link of exogenous methanol to methanogenic activity in all the gut compartments of *Cubitermes ugandensis* (Table 3), demonstrating methanol as methanogenic substrate in the gut, a phenomenon that is explained by high methanol concentrations especially in the posterior gut. The strong stimulation observed when methanol was supplied with hydrogen indicates hydrogenotrophic nature of gut methanogens. Microscopic examination of hydrogen-supplemented methanol enrichment culture showed presence of mainly spherical cells, but there were also rod-shaped, and filamentous cells in the culture (chapter six), suggesting that methylotrophic methanogens may be widespread in the gut. It is presently not clear the source of methanol in the gut of termites. However, given the humified nature of the soil diet, methanol is most likely an intermediate from degradation of methoxylated aromatic compounds like pectin and xylans. Pectin, a highly esterified methyl polysaccharide in form of α (1-4) linked galacturonic acid, constitute substantial proportion (1% of dry weight) of middle lamella of plant cell wall (Killham, 1994; Harholt *et al*., 2010). De-esterification of pectin by pectin esterases (pectin methyl esterase) yields pectate and methanol as products. In soil, chain terminating pectinases are produced by known pectinolytic bacteria such as *Pseudomonas* sp., *Arthrobacter* sp., *Clostridium* sp., *Bacillus* sp and *Streptomyces* sp. and many soil fungi (Killham, 1994). Lineages of these

bacteria present in the gut of termites may be responsible for methanol genesis. Another possible source of methanol may be xylan, a major hemicellulose in the cell wall of most plants. The exact site of methanolgenesis in the termite gut is not clear, but previous studies in honey bees suggested that pectin of ruptured pollen grains is digested in the honey bee midgut in which γ-proteobacterial species are present (Klungness and Peng, 1984). In the gut of honey bee *Apis mellifera*, Engel *et al*., (2012) also detected genes encoding for pectin degrading enzymes, including pectate lyases targeting polygalacturonic acid backbone of pectin and debranching enzymes. Owing to the volatile nature of methanol and its stimulation of methanogenesis in nearly all the gut compartments, its presence in almost all gut compartments is suggestive of some form of transport, may be inter-compartmental transfer via the haemolymph. The utilization of methanol in most gut compartments and in whole gut without exogenous hydrogen may be an indication that some methanogenic lineages are capable of methanol disproportionation. However, it may also be due to the use of endogenously available hydrogen for the reduction of methanol.

The strong stimulation of methanogenesis by formate in most compartments indicates potential formate oxidation by some gut methanogens, probably those with formate dehydrogenases but lacking hydrogenases. It was previously observed (Pester and Brune, 2007; Tholen and Brune, 2000) that when labeled formate was injected in to the hindguts of the wood-feeding lower termites *Cryptotermes secundus* and *Reticulitermes flavipes*, it was quickly converted to $CO_2$ and acetate, with some methane emission observed, indicating that some methanogens may be capable of formate oxidation. However, the increased stimulation by formate when it is supplied with hydrogen may indicate that some formate utilizing methanogens requires hydrogen probably as electron donor for the reduction of $CO_2$ derived from formate oxidation. Formate may be preferentially metabolized by many methanogens over methanol as it does not require additional pathways to oxidize, whereas methanol utilization requires additional substrate-specific methytransferases (Liu and Whitman, 2008). Our results concur with those of Schmitt-Wagner and Brune, (1999), who also observed higher stimulation by formate over hydrogen in posterior gut compartments of the soil-feeding *Cubitermes orthognathus*.

Hydrogen and formate are important products resulting from fermentative degradation of organic matter (Schink, 1997) and are potential drivers of methanogenic activities in the gut. Formate has been detected in haemolymph of *C. orthognathus* at concentrations of 2.6 mM (Tholen and Brune, 1999 and it may also be inter-compartmentally transferred to most gut compartments. Since acetate is also an intermediate in the fermentation reactions in the gut, it is a potential energy source for gut methanogens. However, the host termite requires acetate as carbon and energy source, and it is not clear if it will host competing acetate utilizing methanogens. Nevertheless, all methanogen isolates from termite guts requires acetate in their growth media as a carbon source. Methanogenesis in both stimulated and non-stimulated intact whole guts was always higher than that of individual homogenized gut compartments (data not provided), but was almost fully recovered with intact gut compartments. Since we did not expect any strong variations in pH of the buffer or oxygen inhibition during preparation, the disparity may be caused by biasness due to homogenization resulting in disorganization of community structure, or some cells attach to the wall of homogenization tubes leading to underestimation of methanogenic potential in homogenized compartments. The results for the P3 gut compartments may be underestimated because a third of the compartments collapsed during sectioning and loss of in situ populations cannot be overlooked. Methane emission rates with exogenous substrates were higher in the posterior gut compartments than the anterior sections. This was corroborated by high densities and archaeal diversity in the posterior segments, probably due to availability of utilizable substrates and favorable in situ physicochemical conditions. Highest densities of bacterial detected in the posterior gut suggest that substrate limitation in these compartments may also be as a result of bacterial activities. Previous studies (Tholen and Brune, 1999) also reported highest MPNs of homoacetogenic bacteria and methanogenic archaea in the posterior P3/P4a and P4b/P5 hindgut sections.

## Conclusions

Methanogenic archaea are heterogeneously distributed in the gut. Community structure differed strongly among gut compartments. Most methanogens are localized the posterior gut compartments, which harbor most methanogenic activities. The highly alkaline anterior gut compartments were preferentially colonized by Methanosarcinales. Thaumarchaeota colonized the crop, midgut and rectum while the unclassified archaeal group predominantly colonized the posterior gut. Experimental stimulation of methanogenesis in isolated gut sections of soil-feeding termites revealed significant activities of hydrogenotrophic methanogens that are obligately dependent on methanol and formate. Our results suggest that community structure in the different microhabitats is shaped by exogenous factors, such as pH, oxygen status and the availability of methanogenic substrates.

# Acknowledgments

This work was supported by grant from DFG. James Nonoh received a doctoral fellowship from DAAD. We are grateful to Kenya Wildlife Service (KWS) for giving us permission to collect termites from Kenya for this study.

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## Chapter Four

# Microenvironment and community structure of Archaea and Bacteria in the highly compartmented gut of *Amitermes* sp. (Isoptera: Termitinae)

**James O. Nonoh, Carsten Dietrich and Andreas Brune**

Author contributions:

J.O.N. designed experiments, obtained the samples, prepared the DNA, made clone libraries, performed qPCR, performed phylogenetic analysis, performed microsensor measurements, analyzed and visualized the data, interpreted the data, and wrote the manuscript.

C.D. contributed to microsensor measurements, conducted pyrotag analysis and visualized data.

A.B. conceived the study, interpreted data and secured funding.

# Abstract

We report an insight on the physicochemical characteristics of the gut and the prokaryotic community composition in *Amitermes* sp., a previously uninvestigated termite in the sub-family Termitinae. Microsensor measurements were taken for $O_2$, $H_2$ and pH gradients in the specific gut compartments of the worker castes of soil feeding *Amitermes* sp. Prokaryotic community structure was investigated using clone-based and 454 sequencing of 16S rRNA gene combined with qPCR analysis. Oxygen partial pressures were high in the tubular anterior and posterior gut compartments, while the more dilated P1 and P3 compartments were anoxic, especially at the gut centre. Highest hydrogen concentration was detected P1 compartment. The gut of *Amitermes* sp. was unusually alkaline, with majority of the gut compartments having pH values above six. Elevated pH was already observed at the anterior midgut-mixed segment inter-phase and increased to reach pH maxima in the P1 gut compartment (pH 11.4) before taking a negative trend towards the rectum. Most of the archaea were localized in the tubular P4 compartment. Members of Methanoplasmatales and the uncultured archaeal group loosely affiliated to Crenarchaeota were the dominant archaea in most compartments. High diversity of bacteria was recovered, with major phyla comprising Firmicutes, Spirochetes, Bacteroidetes, Actinobacteria, and Proteobacteria being represented. Firmicutes were the dominant phylum representing over 70% of the bacteria while a significant proportion (4%) represented unclassified bacteria. The population size variations along the gut axis suggest the distribution and localization of prokaryotic communities in the gut is influenced by the environmental conditions *in situ*.

# Introduction

*Amitermes* sp. falls within the *Amitermes group* of the sub-family Termitinae. Members of this group are very heterogeneous with varied feeding types. They feed on diverse diet including sound or dead wood, soil, humic soil and nest of other species (Noirot, 1992; Eggleton, 2000). Other than Apicotermitinae, *Amitermes* is the only other group that comprise soldier-less termites (Eggleton, 2000; Donovan *et al*., 2000). Some described genera in this group include *Microcerotermes*, *Protohamitermes*, *Prohamitermes*, *Gnathamitermes*, *Globitermes*, *Pseudhamitermes*, *Drepanotermes*, *Ahamitermes*, *Amitermes*, *Synhamitermes* species among others. Some members of *Amitermes* sp. feed on decaying wood, while some members of *Drepanotermes* and *Gnathamitermes* species feed on grass (Eggleton, 2000). However, unlike in *Cubitermes* group of Termitinae, members of *Amitermes* group remain largely uninvestigated. Except for the wood-feeding *Microcerotermes* sp. (Nonoh *et al*., chapter 2; Deevong *et al*., 2004), and the litter-feeding *Cornitermes cumulans* (Grieco *et al*., 2012), there is no data on the community composition of prokaryotes in the soil-feeding members of *Amitermes* group. Moreover, the physicochemical characteristics in the highly compartmented gut of members of this group, particularly those of the soil-feeding species, and the influence of such gradients on the functional colonization of the gut by prokaryotic communities are presently not well investigated. Bignell, (2000) observed that like in other higher termites, the gut of described members of this group is compartmentalized and varies between species, but is relatively simple than in members of soil feeding *Cubitermes* group. The P1 compartment in most members of the *Amitermes* group is long and tubular, but appears dilated and globular in some species, although it is much shorter and less dilated than in *Cubitermes* species (Noirot, 2001). The mixed segment is present and well developed but appears varied in most species (Noirot, 2001). There are limited studies on prokaryotic community composition in the gut of a representative species of this group. Grieco *et al*., (2012) performed culture-independent studies on the community structure of prokaryotes in the whole gut of the South American litter-feeding *Cornitermes cumulans* in which they reported low diversity of archaea with mainly Crenarchaeota lineages recovered.

However, previous studies on other soil-feeding termites (Nonoh *et al*., chapter 2; Friedrich *et al*., 2001; Donovan *et al*., 2004) as well as litter-feeding cockroaches (Egert *et al*., 2003) have revealed very high archaeal diversity in insects feeding on humified diet. It is possible that there exist interspecies variations in community composition. Information on the axial distribution and the exact localization of the prokaryotic communities in *Amitermes* spp. is still largely lacking. Whereas most studies on axial dynamics of prokaryotic community composition in the gut have been done on the soil-feeding higher termites, little is still known about the soil-feeding members of *Amitermes* group (Termitinae). In order to link structure of microbial populations to host gut micro-habitats, the knowledge of gut physicochemical environment becomes very critical. Not only will it help in understanding the spatial distribution patterns of prokaryotic communities in the highly compartmented guts, but also the diversity of the gut microbiota. Accordingly, workers castes from a colony of previously uninvestigated *Amitermes* sp., with reduced number of individual worker termites in the colony and highly reduced number of soldiers, were collected from subterranean galleries and investigated for prokaryotic community structure and population size variations relative to gut morphology and physicochemical gradients in the gut.

# Methods

## Termites

We collected worker castes of the Savannah dwelling soil-feeding *Amitermes* sp. (Termitinae) from a university farm in Kenya. The termites were collected in subterranean galleries in the neighborhood of *Trinervitermes* sp. mounds in JKUAT farm, Kenya. The termites were collected together with the nest material and carried in plastic containers to our laboratories in Germany. The collected termites were maintained in our laboratories on a soil diet which was collected within one meter from their mounds. All measurements were made within one month of termite collection. For all the experiments described in this study, only the worker caste termites were used.

## Microsensor studies

For microsensor measurements, the termites were dissected using sterile fine tipped forceps and then the entire intact whole gut including the crop, midgut together with mixed segment, P1, P3, P4 and P5 compartments were analyzed for physicochemical gradients.

Oxygen micro-electrodes with guard cathodes and a tip diameter of 10 µM were purchased commercially from Unisense (Aarhus, Denmark). The electrodes were pre-polarized overnight in air bubbled de-ionized water before starting the measurements. Calibration was done by taking electrode current measurements in air, in Ringer's solution (Brune *et al.*, 1995) bubbled with 21% $O_2$ as well as in Ringer's solution reduced completely with sodium dithionate or Ringer's solution bubbled with 100% $N_2$ gas. Calibration was checked periodically before and during each experimental measurement. Current was measured with ammeter connected to chart reader.

Hydrogen micro sensors with a tip diameter of 10 µM were also obtained commercially from Unisense (Aarhus, Denmark). Prior to use, the microelectrode was pre-polarized as above. Testing and calibration was done routinely as described previously (Schmitt-Wagner and Brune, 1999) with measurements taken when the microelectrode was in air, nitrogen, Ringers solution, as well as Ringers solution sparged with 5% $H_2$ in $N_2$. Current measurements were recorded with an ammeter connected to chart reader.

Glass pH micro electrodes with tip diameters of 10-30 µM and tip length of 100 µM were obtained commercially from Unisense (Aarhus, Denmark). Electrode potentials were measured against a Ag-AgCl reference electrode as described previously (Brune and Kühl, 1996). We performed calibration of the electrode using commercial pH standard 4.0, 7.0, 10.0 and 11.0 buffers (Carl Roth, Karlsruhe, Germany) as previously described (Brune and Kühl, 1996) before, between and after experimental measurements. Potential measurements were recorded by high-impedance electrometers.

## Gut physicochemical profile measurements

The experiment was set up as described previously (Brune *et al*., 1995) with slight modifications. The bottom layer of the chamber was filled up with molten 2% agarose (w/v, 60 ℃) in insect Ringers solution (Brune *et al*., 1995) and allowed to solidify. The dissected termite gut including the major hindgut compartments was carefully spread on the solidified agarose using fine tip forceps as described previously (Schmitt-Wagner and Brune, 1999) then covered immediately with a shallow layer of molten 0.5% agarose (w/v, 40 °C) in insect Ringers solution. The agarose over layer was allowed to solidify before measurements were made. The chamber and the agarose were electrically grounded to get rid of static charges. During the measurements, the microelectrodes were adjusted into place using micromanipulator (Märzhäuser, Wetzler, Germany) while the tip being carefully monitored with a stereomicroscope (Zeiss, Jena, Germany). All measurements were done on the bench at room temperature and in each case, triplicate assays were performed.

## DNA extraction and purification

For clone analysis and qPCR, DNA was extracted from gut compartment homogenates of twenty (20) worker caste termites. With the aid of a stereomicroscope (Zeiss, Jena, Germany), the entire gut was pulled out with sterile, fine tipped forceps, and then separated into different compartments; crop, midgut, P1, P3, P4 and P5 sections. Gut sections (20 each) were then pooled together into a sterile 2 ml eppendorf tubes filled with 750 µl of sterile phosphate buffer (120 mM; pH 8.0). The pooled gut sections were then homogenized using a sterile pestle (Eppendorf, Hamburg, Germany). The gut compartment homogenates were transferred to 2 ml bead-beating vials, followed by addition of 250 µl sodium dodecyl sulfate (SDS) solution (10% SDS; 0.5 M Tris-HCl, pH 8.0; 0.1 M NaCl), and 0.7 g heat-sterilized zirconium-silica beads (0.1 mm diameter, Carl Roth, Karlsruhe, Germany) and the cells were lysed using cell disruptor (FastPrep-24 MP Biomedicals, Ilkirch, Germany) for 45 s at 6.5 m/s after which the cell debris were removed by centrifugation at 20000 × g for 4 minutes. The supernatant, containing the

DNA was extracted with one volume of phenol-chloroform-isoamylalcohol (25:24:1) and eventually with 1 volume of chloroform/isoamyl alcohol (24:1, vol/vol) after centrifugation for 1 minute, prior to precipitation of the DNA by mixing the aqueous phase with 2 volumes of polyethylene glycol (PEG) solution (30% PEG 6000 in 1.6 M NaCl) and then centrifugation at 20000 × g and 4 °C. After washing with ice-cold ethanol (70%), the recovered pellet was dried at 30 °C for 5 minutes. The DNA was re-suspended in 50 µl elution buffer (MinElute PCR Purification Kit, Qiagen, Hilden, Germany). The extracted DNA was run on a standard 1% agarose gel in order to verify extraction efficiency and quality of the extracted DNA. The purity of the extracted DNA was checked photometrically (Nanodrop, PeqLab, Erlangen, Germany), and then quantified fluorimetrically (Qubit, Invitrogen, Eugene, OR, USA), and stored at –20 °C.

## Amplification of archaeal SSU 16S rRNA genes

PCR amplification was carried out using primer pairs that target archaeal small sub unit (SSU) 16S rRNA genes from position 109 to 934 as described by Großkopf *et al*., (1997) and Friedrich *et al*., (2001). Oligonucleotide primers specific for archaeal 16S rDNA used were Ar109F and Ar912R (CTCCCCCGCCAATTCCTTTA) (Escherichia coli 16S rRNA numbering) [Brosius *et al*., 1978]. Each PCR reaction (50 µl) contained reaction buffer, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase (all Invitrogen, Carlsbad, CA, USA), 50 µM deoxynucleoside triphosphate mix, 0.3 µM of each primer, 0.8 mg/ml bovine serum albumin, and 1 µL DNA template. PCR (30 cycles for P1, P3, P4 and P5 compartments) were carried out at initial denaturation step (94 °C for 3 min), followed by denaturation (94 °C for 20 s), annealing step (52 °C for 30 s), extension (72 °C for 45 s), and a final extension step (72 °C for 7 min). Our attempt to amplify the crop and midgut DNA under the same conditions did not result in any amplification. We had to increase the number of cycles to 36 cycles for C and M sections under the same conditions to get an amplicon. Aliquots of the SSU 16S rDNA amplicons (5 µl) were analyzed by electrophoresis on a 1% agarose gel and visualized after staining with ethidiun bromide.

## Generation of clone libraries

Clone libraries were generated from archaeal small sub-unit SSU rDNA amplicons obtained from termite gut community DNA of each compartment. Purified PCR products (~ 800 bp [Ar109f-Ar912r] long) were ligated into the pGEMT easy plasmid vector (Promega), and *E. coli* JM109 (Promega) was transformed with the recombinant plasmids according to the manufacturer's instructions. Clones were given codes according to the gut compartment as; C (crop gut section), M (midgut gut section including mixed segment), P1 (P1gut section), P3 (P3 gut section), P4 (P4 gut section) and P5 (P5 gut section) of *Amitermes* sp. Randomly picked clones were further analyzed as described previously (Großkopf *et al.*, 1998, Friedrich *et al.*, 2001 and Donovan *et al.*, 2004). Clones were checked for the correct insert size by vector-targeted PCR and standard agarose gel electrophoresis. Forty (40) randomly selected clones per library with correct inserts were sent for sequencing commercially in both directions using vector based primers.

## Phylogenetic analysis of the sequence data

Sequence data were analyzed and edited by Seqman (DNAStar) software. Phylogenetic analysis was done with the current ARB-SILVA database (version 106; Pruesse *et al.*, 2007; http://www.arb-silva.de) using the ARB software package (Ludwig *et al.*, 2004). The SSU 16S rRNA gene sequences obtained in this study were added into the database using the ARB software package tool (Ludwig *et al.*, 2004) after alignment with SINA software tool (v1.2.9) using SILVA seed (Pruesse *et al.*, 2007). The Sequences from other studies that were not present in the in Silva database were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/) and added to the database. The alignments were corrected manually where necessary. A 20% order level consensus filter was used to exclude highly variable positions. Phylogenetic trees were constructed with ARB maximum likelihood method using RAxML algorithm (Stamatakis, 2006). Aligned sequences were also analyzed by ARB maximum-parsimony (DNAPARS) methods to check for tree topology and node support (1000 bootstraps). Phylogeny was inferred by comparing sequences to

the main lines of descent within the archaeal phyla Euryarchaeota, Thaumarchaeota and Crenarchaeota. A threshold (>97% similarity) was set to assign sequence to the same OTUs.

## 454 sequencing

20 gut compartments each of worker caste termites of Amitermes sp. were pooled together and homogenized. The DNA was then extracted with phenol-chloroform method as described above. The 16SrRNA genes was then amplified using primer pairs (343F and 784R) targeting the V3–V4 region as described by Köhler *et al*., 2012). Pyrosequencing (454 GS FLX Titanium; Roche, Mannheim, Germany) was done commercially. Sequence processing and classification was done as described by Köhler *et al*., 2012).

## Quantification of abundance of archaea and bacteria in the gut

Quantitative PCR was performed to estimate the relative density of archaeal cells in the respective gut compartments of *Amitermes* sp. The abundance of archaeal as compared to bacterial 16S rRNA genes in the gut compartments of *Amitermes* sp. was measured by quantitative 'real-time' PCR (qPCR) following the method described by Kemnitz *et al*. (2005). Copy numbers of archaeal 16S rRNA genes were estimated using the primers A364aF (5'-CGGGGYGCASCAGGCGCGAA-3'; Burggraf *et al*., 1997) and A934b (5'-GTGCTCCCCCGCCAATTCCT-3'; Grosskopf *et al*., 1998). Bacterial 16S rRNA gene copy numbers were quantified as described by Stubner (2002) using the primer pairs 519fc (5'-CAGCMGCCGCGGTAANWC-3') and 907r (5'-CCGTCAATTCMTTTRAGTT-3') (Lane, 1991).

# Results

## Oxygen and hydrogen gradients

The experiment was performed with well maintained termites and the agarose embedded guts of *Amitermes* sp. were active during the experimental measurements as observed by periodic contractions. Stable gradients were observed at the beginning of the measurements and remained so for about 15 minutes before beginning to fluctuate. Micro sensor measurements of oxygen and hydrogen profiles along the gut showed that other than the more dilated P1 and P3 compartments (Fig. 1) which were anoxic at the gut center, the rest of the gut, particularly the less dilated anterior and posterior gut compartments were highly oxic, experiencing high oxygen partial pressures in the lumen (Fig. 2A). There was always a negative trend in oxygen profile (data not shown) from the agarose towards the embedded gut particularly in the posterior gut suctions. At the P1 and P3 periphery, a strong reduction in oxygen profile from the agarose layer was observed. Hydrogen on the other hand accumulated to detectable levels mainly from the midgut to the anterior portion of the P3 gut compartment (P3a), with values rising sharply at the mixed segment to reach a peak at the gut center of the dilated P1 compartment. However, hydrogen could not be detected in the posterior gut sections P4 and P5.

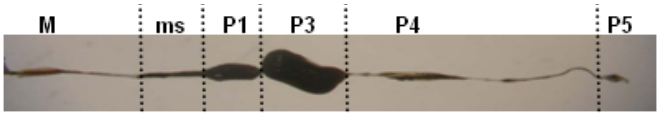


Fig. 1: Gut morphology of *Amitermes* sp. showing major gut compartments midgut (M), mixed segment (ms), and proctodeal compartments P1–5 (P).

## pH gradients

The luminal gut pH of this termite is generally alkaline, with no gut compartment having a pH values less than six (Fig. 2B). The crop was mildly acidic (pH 6) while the midgut was mildly alkaline. However, the pH begun to rise at the midgut-mixed segment junction with a steep increase in the mixed segment and continued so to reach maximum

levels in the P1 gut compartment, which also accumulated highest amount of hydrogen. At the P1-P3 junction, the pH then begun to drop briefly but then regained a stable status (pH 9.8) in the P3 compartment except at the junction with the P4 compartment in which it dropped further before stabilizing briefly in the P4 section (7.9) after which it dropped again to attain a neutral status in the rectum (pH 6.9). All of the posterior gut sections had pH values above neutrality.

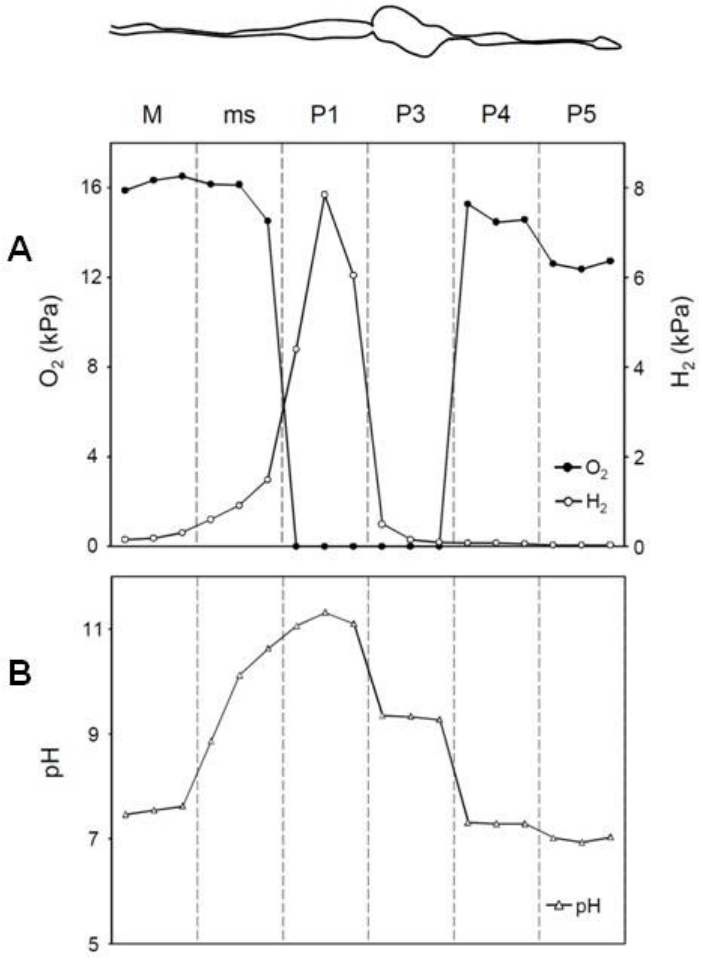


Fig. 2: Physicochemical profiles of [A] oxygen (●) and hydrogen (○) partial pressures, and [B] pH (△) along the gut axis of *Amitermes* sp., measured at the gut center. Profiles for each of the parameters (oxygen, hydrogen and pH) were measured in triplicates, but only representative profiles are shown in each case.

## Axial distribution and densities of archaea along the gut

The archaeal community distribution patterns along the gut of *Amitermes* sp. (Fig. 3) is quite unique from that observed in other soil feeding termites. The gut is highly dominated by members of uncultured archaea loosely affiliated to Crenarchaeota and Methanoplasmatales (51% and 30% of gut archaea respectively). In the mildly acidic crop, only Thaumarchaeota were recovered (0.2% of archaea in the gut). Unlike in *Cubitermes* sp., in the tubular midgut, only Methanoplasmatales (2% of total gut archaea) and Methanobacteriales (0.1% of gut archaea) were recovered. In the highly alkaline P1 compartment, the deep-branching archaeal group loosely affiliated to Crenarchaeota (4% of gut archaea) and Methanoplasmatales (2% of gut archaea) were the most dominant archaeal groups. In the P3 compartment, Methanoplasmatales (2% gut archaea) and Methanobacteriales (1% of archaea) were the dominant archaeal groups. In the P4 gut compartment in which highest density of archaea (84% of overall gut archaea) were localized (Table 1), nearly all major archaeal groups were recovered (Fig. 3), with the most dominant being the uncultured archaea loosely affiliated to Crenarchaeota (46% of gut archaea) followed by Methanoplasmatales (23% of gut archaea) and Methanosarcinales (11% of archaea). In the P5 compartment, there was very low density of archaea (1% of gut archaea) as well as low diversity with only three archaeal groups recovered, with the dominant group being Methanoplasmatales. The summary of density of archaea in the specific gut compartments of *Amitermes* sp. is provided in table 1.

Table 1: Absolute abundances of Archaea and Bacteria in individual gut sections [crop (C), midgut (M), and proctodeal compartments P1–5] of *Amitermes* sp.

| **Taxa** | **Absolute copy numbers of 16S rRNA gene ($10^3$ copies $gut^{-1}$)[a]** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **C** | **M** | **P1** | **P3** | **P4** | **P5** | **Total/whole gut** |
| Total Archaea[b] | 0.17 ± 0.03 | 1.81 ± 0.4 | 5.01 ± 1.2 | 2.14 ± 0.31 | 62.9 ± 20.5 | 0.73 ± 0.14 | 72.7 ± 19.5 |
| Total Bacteria[c] | 17.2 ± 1.86 | 575 ± 0.0 | 1020 ± 0.0 | 3800 ± 375 | 1990 ± 839 | 1180 ± 206 | 8580 ± 256 |
| Arch/Prokaryotes (%)[d] | 1 ± | 0.31 | 0.49 | 0.06 | 3.07 | 0.06 | 0.84 |

[a] Values shown are mean ± mean deviation of two independent measurements (shown only to 3 significant figures).

[b] Values are obtained by multiplying the copy numbers of archaeal 16S rRNA gene by the clone abundance of archaeal taxa recovered in each compartment.

[c] Values are bacterial 16S rRNA gene copy numbers in each compartment.

[d] Abundance of archaea expressed as a percentage of that of total prokaryotes in each compartment.

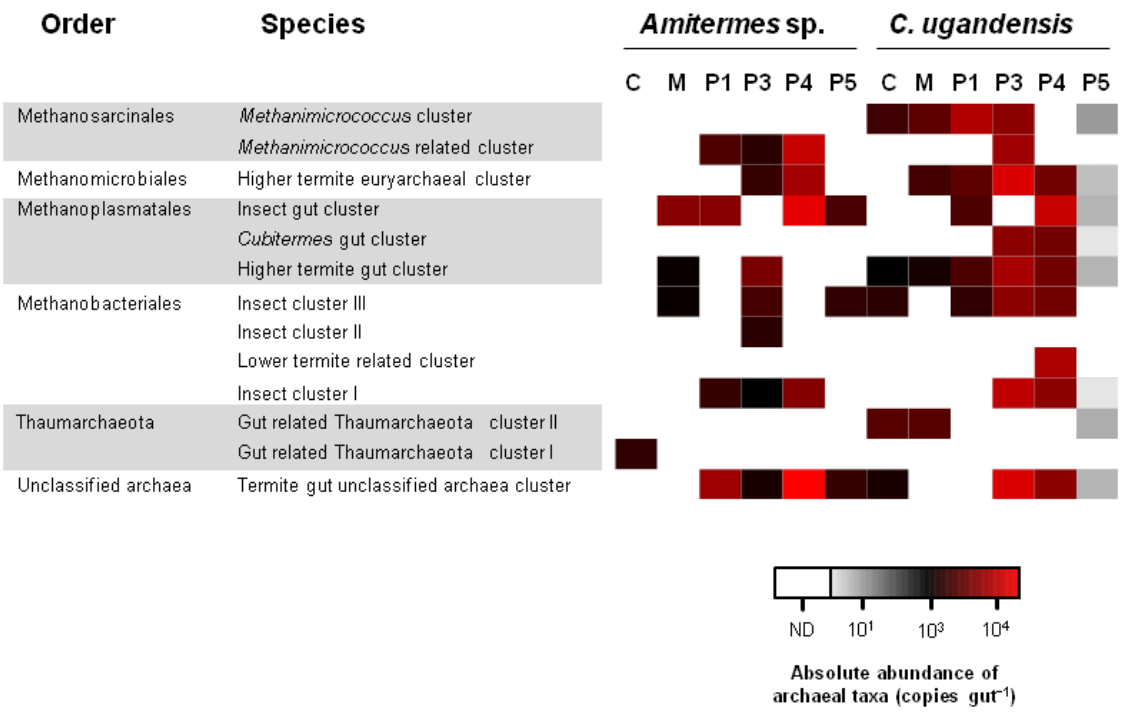


Fig. 3: Absolute abundance of major archaeal taxa recovered in individual gut compartments crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5 (P) of *Amitermes* sp. as compared to that of *Cubitermes ugandensis*. The data for *C. ugandensis* are the same as in Chapter 3. The classification is shown down to species level (based on assignment of OTUs at a threshold of 97% sequence similarity following analysis of 750 bp fragment of archaeal SSU 16S rRNA gene after filtering with order level filters). The values used in the heat map were log transformed to visualize the low abundance taxonomic groups. (ND, below detection limit).

## Bacterial community composition in the gut of *Amitermes* sp.

The major bacterial phyla recovered in the gut of *Amitermes* sp. (Fig. 4), were Firmicutes comprising 70% of the overall gut bacteria, Spirochetes (10%), Bacteroidetes, Proteobacteria and Actinobacteria (3% each), TG3 (2%) while a substantial proportion of bacteria (4%) belong to yet unclassified group. The rest were members of lineages present in the gut in very low abundances.

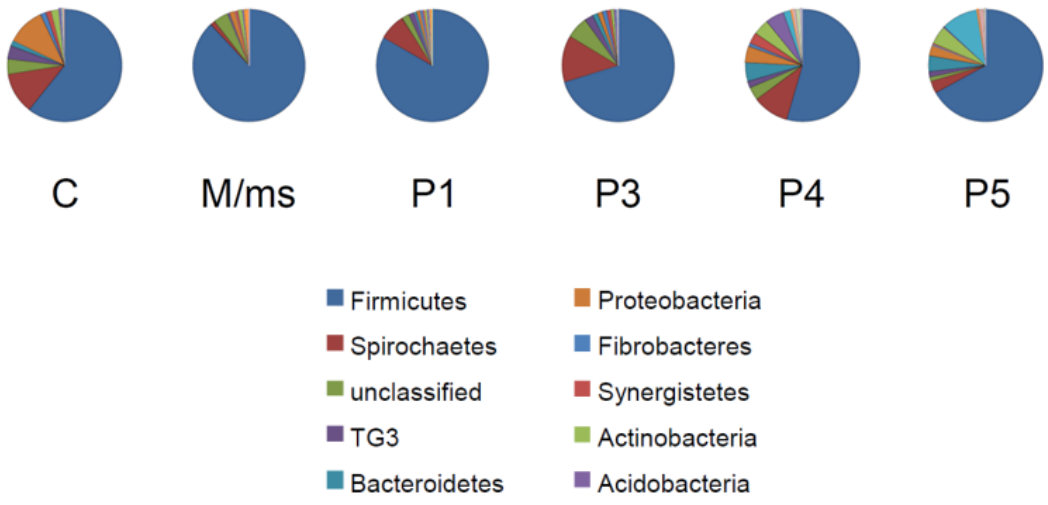


Fig. 4: Relative abundance of major bacterial phyla along the gut axis of *Amitermes* sp., based on pyrotag analysis of the V3–V4 region of the 16S rRNA genes. Values are relative abundance of major bacterial phyla in individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5 of the termite gut.

In terms of axial distribution in the gut, there was heterogeneous distribution of the major bacterial phyla throughout the gut (Fig. 5). Most of the bacteria (44% of the overall gut bacteria were localized in the highly dilated P3 compartment. The neighboring tubular P4 compartment equally hosted high bacterial abundance (23% of the overall gut bacteria), while the midgut host 7% of the overall gut bacteria. In the rectum, 14% of the overall bacteria were localized, while the highly alkaline P1 gut compartment host 12% of the bacteria. There was a low abundance of bacteria in the crop (0.2% of the gut bacteria). Firmicutes were the dominant group in all the gut compartments (Fig. 4 and 5), with high densities (31% and 13% of the overall gut bacteria) in the dilated P3 compartment and in the tubular P4 compartment. The most dominant families of Firmicutes, Ruminococcaceae and Lachnospiraceae and unclassified Clostridiales were also unevenly distributed in the gut. While Lachnospiraceae were dominant in the anterior alkaline sections, Ruminococcaceae were dominant in the posterior gut, particularly in the P3 compartment (Fig. 5). Members of the Lactobacillales were also relatively abundant mainly in the posterior P3 compartment. The Spirochetes were more abundant in the posterior gut compartments P3 and P4 (6% and 2% of the overall gut bacteria

respectively). The major families of Spirochetes were Spirochaetaceae and Leptospiraceae which were all dominant mainly in the posterior gut. Highest abundance of Proteobacteria was localized in the posterior P4 and the P3 compartments (1% each of the overall gut bacteria). Major families of Proteobacteria were Rhodocyclaceae and Cystobacteraceae which were dominant in the posterior P4 compartment and Enterobacteriaceae which was dominant in the midgut. Desulfovibrionaceae were also relatively dominant mainly in the posterior P3 and P4 compartments. The Bacteroidetes were dominant mainly in the posterior gut P3, P4 and P5 compartments (1% each). The major lineages of Bacteroidetes were termite-specific phylotypes comprising COBP4-1, M2PB4-65, Rs-E47, Porphyromonadaceae and Rikenellaceae. The actinobacteria were mainly dominant in the P4 and P5 compartments (1% each overall gut bacteria respectively). Highest abundance of TG3 was also localized in the posterior P3 and P4 compartments respectively. The rest of the other bacterial groups were also unevenly distributed along the gut axis. Table 1 summarizes the abundance of bacterial 16S rRNA gene copy numbers in the gut of *Amitermes* sp.

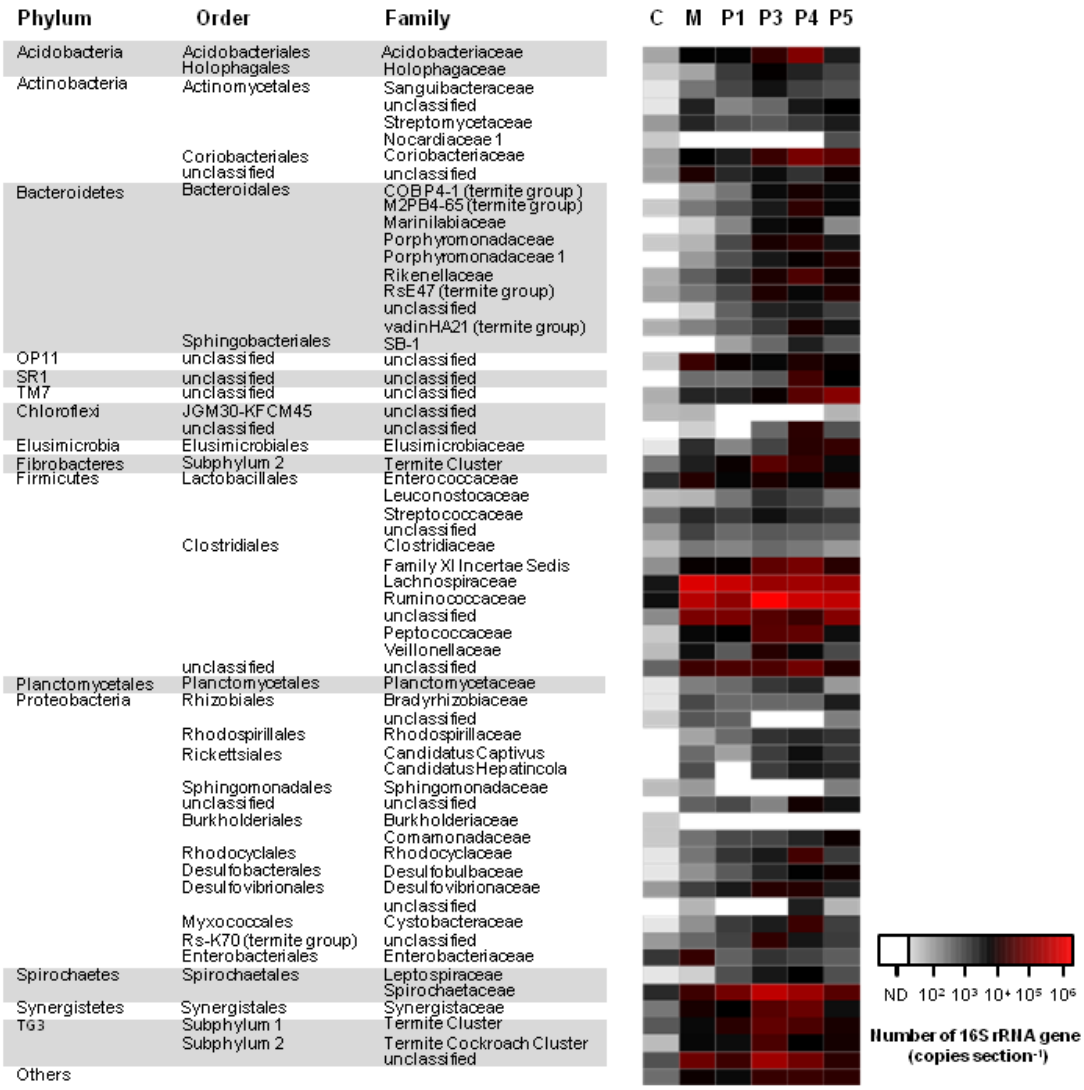


Fig. 5: Absolute abundance of major bacterial taxa recovered in individual gut compartments [crop (C), midgut including mixed segment (M), and proctodeal compartments P1–5] of *Amitermes* sp. The classification is shown only to family level. The values were log transformed to visualize the low abundance taxonomic groups. The other remaining sequences comprised diverse groups of bacteria, but were less than 0.1% of the bacterial populations in each compartment. (ND, below detection limit).

# Discussion

The results provide an insight into the structure of prokaryotic communities and their localization in the gut relative to the gut micro environmental conditions in *Amitermes* sp. The highly compartmented gut of *Amitermes* sp. (Fig. 1), with varied physicochemical conditions (Fig. 2A and B), hosts a diverse assemblage of methanogenic euryarchaeota comprising the orders: Methanosarcinales, Methanomicrobiales, Methanoplasmatales and Methanobacteriales (Fig. 3). Besides members of euryarchaeota, *Amitermes* sp. also host lineages closely related to ammonia-oxidizing Thaumarchaeota. A deep branching termite-specific cluster of unclassified archaea loosely affiliated to Crenarchaeota, whose role in the gut is not clear, were the most dominant archaea in this termite. Our results show that the gut of *Amitermes* sp. host much higher diversity than was previously reported for the related litter-feeding *Cornitermes cumulans* in which most 16S rRNA-based sequence were all affiliated to Crenarchaeota (Grieco *et al*., 2012). The differences could be due to interspecies specific variations, but may also be caused by the differences in the primer pairs used for the analysis. Previous studies on soil-feeding termites (Nonoh *et al*., companion paper; Friedrich *et al*., 2001; Donovan *et al*., 2004) as well as litter-feeding cockroaches (Egert *et al*., 2003) also revealed very high archaeal diversity. Like other soil-feeding termites, the gut of *Amitermes* sp. is highly compartmented (Fig. 1), with a long tubular midgut radiating into a slightly distended mixed segment. The P1 compartment is much reduced in length, but is highly globular. The P3 compartment appears uniformly dilated structure with no clear distinction between P3a and P3b, but is very elongated and slightly curved at the ends. The P4 is a highly extended narrowly tubular structure with moderate dilations which ends into a globular rectum. There exists a compartmentation of prokaryotic communities in the gut, a phenomenon that is also reflected in the differences in gut physicochemical gradients.

### Crop and midgut

The only less alkaline gut compartment, the crop, was mildly acidic and was colonized exclusively by Thaumarchaeota. However, because we were only able to sequence two

clones from this section, it is possible that other archaeal lineages colonize this compartment. Lineages of Thaumarchaeota have been recovered in acidic environments (Lehtovirta-Morley *et al*., 2011). The termite gut Thaumarchaeota, closely related to aerobic ammonia-oxidizing lineages in the cluster *Nitrososphaera*, is potentially involved in oxygen consumption in this compartment. The low bacterial abundance in this section (Fig. 5) may be influenced by its acidic nature. Major groups, Ruminococcacea and Lachnospiraceae (0.04% of the overall gut bacteria each) are Firmicutes which have been associated with cellulose degradation in lower termites. Other major lineages like Enterococcaceae may be involved in activities responsible for the lower pH in this compartment while the Spirochaetaceae have previously been linked to reduction of oxygen using hydrogen as electron donor. The low archaeal densities in the mildly alkaline midgut is probably caused by the high oxygen partial pressures due to its tubular nature, as well as the high luminal pH towards the mixed segment and may also explain the accumulation of some hydrogen in this section. The Methanoplasmatales and Methanobacteriales, the only archaeal lineages recovered in this compartment comprise hydrogenotrophic lineages and are potentially responsible for maintaining low hydrogen levels in the midgut. *Methanobrevibacter* strains have been linked to hydrogen dependent oxygen reduction especially at the hind gut wall in lower termites (Leadbetter and Breznak, 1996).The low abundance of bacteria in the midgut (7% overall gut bacteria) may be due to elevated pH and oxygen stress. Lachnospiraceae, Ruminococcaceae and unclassified Clostridiales have been linked to fermentation of sugars in the gut and may be responsible for the hydrogen gradients detected in the midgut. The accumulation of hydrogen especially at the posterior end may be due to some hydrogen being used to reduce oxygen in the tubular anterior midgut. It is worth noting that a substantial proportion of bacterial population in the midgut (0.3% of the gut bacteria) belongs to unclassified bacteria whose role in the gut is still not clear.

**Alkaline P1 compartment**

The high luminal pH in the P1 gut compartment (Fig. 2B), which also accumulates high hydrogen partial pressures, suggest that hydrogen is not consumed as fast as it is formed

in this compartment or that it emanates from the neighboring compartments. The uncultured archaea loosely affiliated to Crenarchaeota, which were the most dominant group in this compartment are not known to be methanogenic and are probably utilizing substrates other than hydrogen in their metabolism. It may also be possible that the Methanoplasmatales, the second most dominant group in this compartment, is either utilizing other substrates for methanogenesis or they are not actively involved in methanogenesis probably due to the high pH conditions. There was very low abundance of archaea in the anterior gut with communities in these sections being different from those of the posterior gut. But the low density of bacteria in the P1 gut compartment (12% of overall gut bacteria) as compared to the neighboring the P3 compartment (44% of overall gut bacteria respectively) suggest that the high pH may also be inhibitive to bacteria, and that most of the hydrogen accumulating in the P1 compartment, which is most likely a product of fermentation reactions by gut microbiota, is probably emanating from the neighboring compartments. The most dominant bacteria in the P1 compartment, Lachnospiraceae and Ruminococcaceae, and other uncultured Clostridiales are lineages implicated in cellulose and protein degrading activities in the termite guts (Köhler *et al*., 2012 and references therein) and it is possible that some hydrogen and other methanogenic substrates are generated in this compartment. Highest abundance of Lachnospiraceae was localized here.

**Dilated anoxic P3 compartment**

The highest abundance of bacteria (44% of overall gut bacteria) observed in the highly dilated P3 compartment (Table 1 and Fig. 5) potentially suggest that most fermentation processes occur in this chamber, and is therefore a likely source of major methanogenic substrate gradients in the gut. Since the pH in this compartment is unusually uniformly high, the high abundance of clostridia particularly those in the family Ruminococcaceae and Lachnospiraceae (26% and 2% overall gut bacteria respectively), which have been implicated in cellulose degradation in termite guts suggest that these lineages may be adapted to alkaline conditions and may be involved in the substantial hydrogen detected in the anterior part of this compartment as well as in the neighboring P1 compartment.

The Spirochaetaceae and the termite cluster of Fibrobacteres, which also formed substantial proportion of the community in this compartment, have been associated with high activities of glycosyl hydrolases, which are key enzymes linked to degradation of sugars in termite guts. The P3 also had substantial proportions of other bacterial taxa including Synergistaceae and unclassified bacteria whose role is still not clear. Despite the anoxic nature of the P3 compartment especially at the gut centre, and the high abundance of fermenting bacteria, the low abundance of methanogenic archaea is probably due to influence of the unusually high alkalinity (pH 9.8) which was stable throughout the compartment, and may be inhibitive to most archaea. Unlike in *Cubitermes* sp., in which the P3a is more dilated than the P3b section, a phenomenon which clearly marks a gradual drop in its pH, such distinction is lacking in the P3 compartment of *Amitermes* sp. in which the compartment is uniformly dilated with no clear separation between the P3a and P3b sections (Fig. 1), a condition also well corroborated with the uniform pH observed almost throughout this compartment except at the junction with the P4compartment (Fig. 2B). It is thus tempting to speculate that gut morphology probably plays a role in influencing the *in situ* gut pH. Since only the anterior part of the P3 compartment (near the junction with P1 compartment), which is highly alkaline accumulated detectable levels of hydrogen, methanogenic activities will most likely be localized at the extreme posterior end of P3 gut where the pH may be less alkaline and in which hydrogen levels was below detection limit. But it is also possible that there are micro-habitats within this compartment where pH is moderate. Our investigation of abundances of cells in the gut of *Cubitermes* species have demonstrated almost one order of magnitude more bacterial cells in the P3b section than in the P3a portion suggesting that high alkalinity in soil-feeding termites limits bacterial colonization of specific gut regions (chapter 6). The low abundance of methanogenic archaea in this compartment suggests that the low hydrogen partial pressures witnessed is most likely as a result of bacterial processes. It is possible that the hydrogen is used for maintaining anoxic status in this compartment. Previously homoacetogenic Firmicute *Sporomusa aerivorans*, capable of oxygen reduction using hydrogen as major electron donor was isolated from the posterior gut of the soil-feeding termite *Thoracotermes*

*macrothorax* (Boga *et al*. 2003). The Spirochetes have also been implicated in reductive acetogenesis reducing $CO_2$ with $H_2$ in the guts of *Nasutitermes* sp. and lower termites and hence forming important hydrogen sink organisms in the gut (Köhler *et al*., 2012 and references therein). It is not clear whether members of Ruminococcaceae which were the majority in this compartment also contribute to the observed low hydrogen partial pressures or if they are involved in any oxygen reduction activities. The predominant colonization of this compartment by Methanoplasmatales which comprise methylotrophic lineages potentially suggests their ability to adapt to high gut pH, and points to potential role of substrates other than hydrogen in supporting methanogenesis in termite guts. Because Methanoplasmatales were also the dominant group in the midgut and the highly alkaline P1 compartment, it is possible that this group may comprise lineages which are well adapted to the alkaline pH conditions existing in the gut. Indeed, Methanoplasmatales were the most dominant methanogenic euryarchaeota distributed throughout the unusually alkaline gut of *Amitermes* sp. The presence of other methanogenic groups including Methanobacteriales, Methanomicrobiales and Methanosarcinales (Fig. 3), which comprise hydrogenotrophic lineages, may be responsible for the low hydrogen gradients in the P3 compartment especially at the posterior end in which most of them may be localized. However, unlike in *Cubitermes* sp., in which hydrogen accumulates mainly in the mixed segment and in the P3 compartment (Schmitt-Wagner and Brune, 1999), the accumulation of hydrogen mainly in the highly alkaline P1 compartment in *Amitermes* sp., potentially suggest that hydrogen diffuses into the P1 from the neighboring midgut compartment and not posterior gut where consuming activities may be high. In *Nasutitermes takasagoensis*, it was also observed that hydrogen mainly accumulates in the anterior part of the highly dilated P3 compartment (Köhler *et al*., 2012) potentially suggesting anterior gut origin. The complete anoxic conditions obtained at the center of the dilated gut compartments P1 and P3 may further suggest presence of oxygen consuming activities. Highest abundance of members of Sanguibacteraceae (0.1%) and Desulfovibrionaceae (0.2%) the lineages which have been implicated in oxygen reduction were localized in the P3 compartment. Previous studies on other soil-feeding termites using oxygen micro-sensors have

demonstrated oxygen consumption in most of the hindgut compartments with the gut centre of dilated compartments being completely anoxic (Brune *et al*., 1995; Brune and Friedrich, 2000).

**P4 compartment and rectum**

Unlike in *Cubitermes* sp. in which most archaea (60% of overall gut archaea) colonize the dilated P3 gut compartment (Nonoh *et al*., chapter 3), the situation is different in *Amitermes* sp. in which most of the archaea (87% of overall gut archaea) are localized in the tubular P4 gut compartment (Table 1 and Fig. 3). The high oxygen partial pressures in the less dilated P4 gut compartment suggest that the prokaryotes in this compartment have to device mechanisms of withstanding oxygen diffusing into the gut probably through consumption and oxygen reducing mechanisms. Surprisingly, at a pH above neutrality (7.7-7.9), the P4 compartment of *Amitermes* sp. is still more alkaline as compared to *Cubitermes* sp. in which the P4 is just about neutral (pH 7). Nevertheless, it is interesting to note that in *Cubitermes* sp. and *Amitermes* sp., the pH conditions in the P3 and the P4 compartments, which are respectively the most densely colonized compartments by archaea, seem to be just above neutrality, suggesting that most archaea are capable of withstanding mild alkaline conditions. But at the same time, one is not lost of the observation that in both termites, most bacteria are localized in the dilated P3 compartment in which most fermentation reactions likely occur resulting in availability of substrates, suggesting that the high abundance of archaea in the tubular P4 compartment may be influenced by availability of substrate gradients emanating from the adjacent P3 compartment either through diffusion or via the haemolymph. However, the P4 compartment was the second most colonized by bacteria (23% of overall gut bacteria) after the P3 compartment suggesting that a significant proportion of substrates may be generated within the compartment. The high abundance of bacterial taxa (Fig. 4 and 5) comprising the families Ruminococcaceae and Lachnospiraceae (7% and 3% of overall gut bacteria respectively) and Spirochaetacea (2% of gut bacteria), which have been implicated in degradation of sugars in the gut, suggest possible presence of fermentation intermediates in the P4 compartment. The presence of Acidobacteriaceae (1% of overall

gut bacteria) may suggest activities responsible for reduced alkalinity in this compartment, making it favorable for archaeal colonization. The highest abundance of Actinobacteria particularly Coriobacteriaceae (1%) in this compartment, are probably involved in oxygen reduction activities. Unlike in *Cubitermes* sp., in which most archaea are localized in the P3 compartment, the high abundance of archaea in the P4 compartment (84% of overall gut archaea) than in the P3 compartment is probably because of the unusually highly alkaline P3 compartment in which most archaea may not be tolerant. Considering the tubular nature of the P4 compartment, the more oxygen sensitive archaea will most likely be restricted to the dilated anoxic P3 compartment, where substrates are also likely to be accessible. But it is also possible that these archaea are capable of shuttling forth and back between the P4 and P3 compartments if only to temporarily access the substrates. The communities in both P3 and P4 compartments were largely similar. The predominant colonization of this compartment by Methanoplasmatales and Methanosarcinales, which comprise both hydrogenotrophic and methylotrophic lineages probably suggest the role of substrates other than hydrogen in driving gut methanogenesis. But the presence of members of Methanomicrobiales and Methanobacteriales, may suggest formicotrophic and hydrogenotrophic metabolism. Members of *Methanobrevibacter* has been implicated in oxygen consumption activities in the gut of the lower termite *Reticulitermes* flavipes (Leadbetter and Breznak 1996) and have also been speculated to perform similar role in higher termites because their genomes carry genes coding for oxygen catalyzing enzymes (Brune, 2010). The only neutral gut compartment, the rectum exhibits very low methanogenic archaeal abundance, with only Methanoplasmatales and Methanobacteriales being recovered, probably because of oxygen inhibition and may be due to substrate limitation. But the presence of substantial populations of Firmicutes belonging to Ruminococcaceae, Lachnospiraceae and unclassified clostridia potentially suggests availability of some fermentation products. The Coriobacteriaceae in this compartment is probably involved in oxygen reduction.

The predominant colonization of the gut of *Amitermes* sp. by members of an uncultured archaeal lineage loosely affiliated to the Crenarchaeota is an observation that seems to be

unique to this termite. This group of archaea previously also recovered in the gut of another soil-feeding termite *Cubitermes orthognathus* (Friedrich *et al*., 2001), in the gut of litter-feeding *Cornitermes cumulans* (Grieco *et al*., 2012), and in several other environments (chapter 2 and references therein) was then thought to be Crenarchaeota. But as every phylogenetic analysis would show, their association with the phylum Crenarchaeota is not supported by any treeing algorithm. They are distantly related (74-80% sequence similarity) to known sulfur metabolizing Crenarchaeota in the class Thermoprotei. Currently little is known about this group and it is not clear what role they play in the gut of this termite and in many other termites in which they are abundant. In *Amitermes* sp., this group is the most dominant (Table 1) accounting for 51% of overall gut archaea and colonizing all gut compartments except the anterior crop and midgut. In a related study, we observed that this group also forms significant archaeal populations in other soil-feeding termites (Nonoh *et al*., chapter 2). The presence of this deep-branching termite-specific group of unclassified archaea mainly in soil-feeding termites suggests that their activities in the gut are probably influenced by the host diet. Our current knowledge of this group of archaea is still very thin and therefore we cannot draw conclusions. However, their presence in soil-feeding species which are also among the highest methane emitters (Brauman *et al*., 1992), and particularly in the posterior gut sections which are not only more methanogenic, but are also colonized by high densities of methanogenic euryarchaeota is interesting and should therefore invoke further investigations. Besides employing culture independent approaches, having enrichment cultures of this group would stimulate efforts towards understanding their physiological roles in situ.

## Conclusions

The highly compartmented and unusually highly alkaline gut of *Amitermes* sp. is colonized by diverse assemblage of archaea. Unlike the soil-feeding members of *Cubitermes* group, *Amitermes* sp. is predominantly colonized by uncultured archaea loosely affiliated to Crenarchaeota. Most of the archaea are localized in the tubular P4 gut

compartment in which the pH was neutral. The structured archaeal and bacterial colonization of the highly compartmented gut seems to be influenced by the specific gut micro-environmental conditions, including pH, oxygen and hydrogen gradients and availability of alternative methanogenic substrates.

## Acknowledgments

This work was supported by grant from DFG. James Nonoh received a doctoral fellowship from DAAD. We are grateful to Kenya Wildlife Service (KWS) for giving us permission to collect termites from Kenya for this study.

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## Chapter Five

# ‘Methanoplasmatales’: *Thermoplasmatales*-related archaea in termite guts and other environments are the seventh order of methanogens

**Kristina Paul, James O. Nonoh, Lena Mikulski, Andreas Brune**

Author contributions:

K.P. designed experiments, made clone libraries, performed qPCR, performed phylogenetic analysis, enrichment and characterized strain MpT1, analyzed and visualized the data, interpreted the data and wrote the manuscript.

J.O.N. designed experiments, obtained the termite samples, prepared DNA, made clone libraries, analyzed data and visualized the data.

L.M. enriched strain MpM2.

A.B. conceived the study, designed the experiments, secured funding and wrote the manuscript.

## Abstract

The *Euryarchaeota* comprise both methanogenic and non-methanogenic orders and many lineages of uncultivated archaea with unknown properties. One of these deep-branching lineages, distantly related to the *Thermoplasmatales*, has been discovered in various environments, including marine habitats, soil, and also the intestinal tracts of termites and mammals. By comparative phylogenetic analysis, we connected this lineage of 16S rRNA genes to a large clade of unknown *mcrA* gene sequences, a functional marker for methanogenesis, obtained from the same habitats. The identical topology of 16S rRNA and *mcrA* gene trees and the perfect congruence of all branches, including several novel groups that we obtained from the guts of termites and cockroaches, strongly suggested that they stem from the same microorganisms. This was further corroborated by two highly enriched cultures of closely related methanogens from the gut of a higher termite (*Cubitermes ugandensis*) and a millipede (*Anadenobolus* sp.), which represented one of the arthropod-specific clusters in the respective trees. Numerous other pairs of habitat-specific sequence clusters were obtained from the guts of other termites and cockroaches, but were also found in previously published datasets from the intestinal tracts of mammals (e.g., Rumen Cluster C) and other environments. Together with the recently described *Methanomassiliicoccus luminyensis* isolated from human feces, which falls into Rice Cluster III, the results of our study strongly support that the entire clade of 'uncultured *Thermoplasmatales*' in fact represents the seventh order of methanogenic archaea, for which the provisional name 'Methanoplasmatales' is proposed.

# Introduction

Methanogenesis is an important process in the carbon cycle with a significant impact on global warming. Methane is produced exclusively by methanogenic archaea – strictly anaerobic microorganisms that occur in almost all anoxic habitats on earth, from the marine environment, to freshwater sediments, to soils, including hot springs and the deep subsurface, in sewage sludge, and in the digestive tracts of animals and humans (Liu and Whitman, 2008).

All methanogens belong to the phylum *Euryarchaeota*. They presently comprise members of six orders. The basal groups are *Methanopyrales*, *Methanococcales*, and *Methanobacteriales* (Class I), *Methanomicrobiales* (Class II) (Bapteste *et al*., 2005), and *Methanosarcinales* (Class III; Anderson *et al.*, 2009), with the recently recognized sister group *Methanocellales* (Sakai *et al*., 2008). It has been hypothesized that the genes for hydrogenotrophic methanogenesis were already present in a common ancestor and were vertically inherited in a broader monophyletic unit embedding all methanogens (Bapteste *et al*., 2005). Consequently, it has to be postulated that methanogenesis was lost in the *Archaeoglobales* (which fall among Class I methanogens), the *Thermoplasmatales*, and the *Halobacteriales* (which fall between Class I and Class II) (Bapteste *et al*., 2005).

In addition, there are many deep-branching lineages of archaea that are exclusively represented by their 16S rRNA genes (Gribaldo and Brochier-Armanet, 2006; Schleper *et al*., 2005; Ufnar *et al*., 2007), whose properties cannot be safely predicted for lack of any cultivated representatives. One of these lineages is a diverse clade of sequences distantly related to the *Thermoplasmatales*. Originally discovered in the marine environment (DeLong, 1992; Fuhrman *et al*., 1992) and the deep subsurface (Takai and Horikoshi, 1999), related clones were subsequently obtained from rice field soil (Grosskopf *et al*., 1998), the water column and sediment of freshwater lakes (Jurgens *et al*., 2000; Nüsslein *et al*., 2001), and soil and leachate of landfills (Huang *et al*., 2002; Luton *et al*., 2002). Other members of this clade were found in the guts of termites (Friedrich *et al*., 2001; Miyata *et al*., 2007; Shinzato *et al*., 1999), wood-feeding cockroaches (Hara *et al*., 2002),

and scarab beetle larvae (Egert *et al*., 2003). Also studies of the mammalian digestive tract reported sequences of uncultured archaea distantly related to the *Thermoplasmatales* in cattle (Denman *et al*., 2007; Janssen and Kirs, 2008; Tajima *et al*., 2001; Wright *et al*., 2007), sheep (Wright *et al*., 2004), wallabies (Evans *et al*., 2009), and in the gut and subgingival pockets of humans (Li *et al*., 2009; Mihajlovski *et al*., 2008; Mihajlovski *et al*., 2010; Scanlan *et al*., 2008). Although concrete evidence was lacking, several of these earlier reports had already suggested that such 'uncultured *Thermoplasmatales*' may represent a novel lineage of methanogens.

The *mcrA* gene, which encodes the α-subunit of methyl coenzyme-M reductase, has been established as a molecular marker for methanogenic archaea (Lueders *et al*., 2001). Studies of the diversity of methanogens in landfill soil yielded several novel *mcrA* gene sequences that formed a deep-branching cluster separate from those of the established orders of methanogens (Luton *et al*., 2002). Related sequences were soon discovered in a eutrophic lake (Earl *et al*., 2003) and in saltmarsh sediments (Castro *et al*., 2004). Later studies of vertebrate guts also revealed the presence of novel *mcrA* genes in the cow rumen (Denman *et al*., 2007), feces of pigs, chicken, and horses (Ufnar *et al*., 2007), the guts of humans (Mihajlovski *et al*., 2008; Scanlan *et al*., 2008), and the foregut of wallabies (Evans *et al*., 2009).

Kemnitz *et al*. (2005) observed a correlation between the abundance of Rice Cluster III (RC-III) archaea and the rate of methanogenesis in enrichment cultures. Mihajlovski *et al*. (2008) claimed that a new *mcrA* phylotype and a new 16S phylotype obtained from the same stool sample belonged to the same organism and subsequently postulated that they represent a putative new order of methanogens (Mihajlovski *et al*., 2010). Also Evans *et al*. (2009) had speculated that the unknown *mcrA* gene sequences in the foreguts of wallabies and ruminants belong to a lineage of uncultivated archaea encountered in these habitats. However, the final proof for this hypothesis is still lacking.

Previous studies have shown that 16S rRNA and *mcrA* genes in the established methanogenic lineages have the same phylogeny (Lueders *et al*., 2001; Luton *et al*., 2002). This allows correlating unknown *mcrA* sequences with the corresponding 16S

rRNA gene sequences – a strategy that has been successfully employed to predict the methanogenic nature of the uncultivated archaea in Rice Cluster I (Lueders *et al*., 2001), which eventually led to the enrichment and isolation of *Methanocella paludicola* (Sakai *et al*., 2008).

In this study, we comprehensively analyzed the phylogeny of all *Thermoplasmatales*-related 16S rRNA genes available to date and the unknown *mcrA* genes from the respective habitats. To further corroborate the hypothetical congruence of the resulting trees, we obtained additional sequence sets of archaeal 16S rRNA and *mcrA* genes from the hindguts of various higher termites and wood-feeding cockroaches, which are known to harbor abundant and diverse populations of 'uncultured *Thermoplasmatales*' (Brune, 2010). In addition, we initiated enrichment cultures from the hindgut of termites and millipedes to isolate a potentially methanogenic member of this novel lineage.

# Methods

## Termites and cockroaches

*Cubitermes ugandensis* and *Ophiotermes* sp. were collected in Kakamega Forest Reserve (Kenya) and *Macrotermes michaelseni* was collected near Kajiado (Kenya). *Trinervitermes* sp. and *Alyscotermes trestus* originated from the campus of the Jomo Kenyatta University of Agriculture and Technology, Gachororo (Kenya). Only worker caste termites were used for this work. The wood-feeding cockroaches *Salganea esakii* and *Panesthia angustipennis* were collected in the vicinity of the Keta Shrine in Ishikawa Prefecture, Japan by Dr. Kiyoto Maekawa, Toyama University. The millipede *Anadenobolus* sp. was obtained from a commercial breeder (b.t.b.e. Insektenzucht, Schnürpflingen, Germany). All animals were kept in plastic containers at room temperature in the dark.

## DNA extraction and purification

The hindguts of 10–20 termites were dissected with sterile, fine-tipped forceps and pooled in 2 ml tubes containing 750 µl sodium phosphate buffer (120 mM; pH 8.0), and homogenized. Homogenates of individual cockroach hindguts were prepared in a similar manner. DNA was prepared using a bead-beating protocol combined with phenol/chloroform extraction. The homogenate was transferred to a 2 ml bead-beating vial, and 250 µl sodium dodecyl sulfate (SDS) solution (10% SDS; 0.5 M Tris-HCl, pH 8.0; 0.1 M NaCl), and 0.7 g heat-sterilized zirconia-silica beads (0.1 mm diameter, Carl Roth, Karlsruhe, Germany) were added. Cells were lysed by shaking with a cell disruptor (FastPrep-24, MP Biomedicals, Ilkirch, Germany) for 45 s at a velocity of 6.5 m/s. Cell debris was sedimented by centrifugation at 20,000 × *g* for 4 min. The supernatant was extracted with 1 volume of phenol/chlorofom/isoamyl alcohol (24:24:1, by vol., pH 8.0). After a second centrifugation step, the supernatant was extracted with 1 volume of chloroform/isoamyl alcohol (24:1, vol/vol) and centrifuged again in a 2 ml phase lock gel heavy tube (Eppendorf, Hamburg, Germany). The DNA was precipitated by mixing the aqueous phase with 2 volumes of polyethylene glycol (PEG) solution (30% PEG 6000 in 1.6 M NaCl). After centrifugation for 30 min, the pellet was washed with 500 µl ice-cold ethanol (70%) and dried under vacuum. DNA was dissolved in 50 µl elution buffer (MinElute PCR Purification Kit, Qiagen, Hilden, Germany), checked photometrically for purity (Nanodrop, PeqLab, Erlangen, Germany), quantified fluorimetrically (Qubit, Invitrogen, Eugene, OR, USA), and stored at –20 °C.

## PCR amplification and cloning

16S rRNA genes were amplified using either the archaea-specific primer pair Ar109f (5'-AMDGCTCAGTAACACGT-3'; Imachi *et al*., 2006) and Ar912r (5'-CTCCCCCGCCAATTCCTTTA-3'; Lueders and Friedrich, 2000), or the archaea-specific primer Ar109f and the prokaryote-specific primer 1490R with the modification of Hatamoto *et al*. (2007) (5'-GGHTACCTTGTTACGACTT-3'), a combination that yields only archaeal 16S rRNA genes (Mochimaru *et al*., 2007). Each PCR reaction (50 µl)

contained reaction buffer, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase (all Invitrogen, Carlsbad, CA, USA), 50 µM deoxynucleoside triphosphate mix, 0.3 µM of each primer, 0.8 mg/ml bovine serum albumin, and 1 µL DNA extract. The PCR program consisted of initial denaturation step (94 °C for 3 min), followed by 32 cycles of denaturation (94 °C for 20 s), annealing (52 °C for 20 s), and extension (72 °C for 50 s), and a final extension step (72 °C for 7 min). For the amplification of the *mcrA* gene, the primer pair *mcrA*-f (5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3') and *mcrA*-r (5'-TTCATTGCRTAGTTWGGRTAGTT-3'; 37) was used; the reaction mix and the PCR protocol was the same as described above, except for the annealing temperature (53.5 °C) and the cycle number (Lueders and Friedrich, 2000), and a decreased ramp temperature rate of 1 °C/s. The PCR products were purified and cloned as described by Schauer *et al*. (2012).

## Sequences analysis

The 16S rRNA gene sequences obtained in this study were imported into the current Silva database (version 106; Pruesse *et al*., 2007; http://www.arb-silva.de) using the ARB software package (Ludwig *et al*., 2004). Sequences from other studies that were not included in Silva were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/). Sequences were automatically aligned, and the alignments were refined manually. A 30%-consensus filter was used to exclude highly variable positions. Phylogenetic trees of almost-full-length sequences (1,250 bp) were calculated using RAxML, a maximum-likelihood method (Stamatakis, 2006). Tree topology and node support (100 bootstraps) were tested using the maximum-parsimony method (DNAPARS) implemented in ARB. The *mcrA* gene sequences were imported into a seed alignment complemented with sequences of unknown origin that were retrieved from the NCBI database. Trees were calculated at the amino acid level (140 amino acids) using PhyML, a maximum-likelihood method (Guindon *et al*., 2010) implemented in ARB. Tree topology and node support (100 bootstraps) were tested using the maximum-parsimony method (PROTPARS) implemented in ARB.

## Cultivation

Enrichment cultures were set up in anoxic, bicarbonate-buffered AM5 medium under an atmosphere of $N_2$–$CO_2$ (80:20, vol/vol) (Boga and Brune, 2003) but dithiothreitol (DTT) was omitted. The basal medium was supplemented with casamino acids (2 g/l), coenzyme M (10 mg/l), cysteine (2 mM), and palladium on activated charcoal (10 ml/l), and (optionally) with yeast extract (2 g/l) or rumen fluid (10%). The medium (4.5 ml) was dispensed into 15-ml rubber-stoppered glass vials. Hydrogen gas (5 ml) was added to the headspace. Substrates were added from sterile stock solutions (final concentrations): formate (50 mM), methanol (50 mM), acetate (30 mM), or xylan (9 g/l). Tubes were inoculated (0.5 ml) with gut homogenates of *C.ugandensis* or *Anadenobolus* sp. prepared in basal medium (1 gut per ml), and the tubes were incubated at 30 °C in the dark. Methane content in the headspace was measured every week. The culture headspace (0.2 ml) was sampled with a gas-tight syringe, and the methane content was analyzed using a gas chromatograph with a flame ionization detector (McWilliam and Dewar, 1958).

## Quantitative PCR and pyrotag sequencing

DNA was extracted from the enrichment culture (2 ml, see above), and the copy numbers of archaeal 16S rRNA genes were determined by quantitative 'real-time' PCR (qPCR) as described by Kemnitz *et al*. (2005) using the primers A364aF (5'-CGGGGYGCASCAGGCGCGAA-3'; Burggraf *et al*., 1997) and A934b (5'-GTGCTCCCCCGCCAATTCCT-3'; Grosskopf *et al*., 1998). Bacterial 16S rRNA genes were quantified as described by Stubner (2002) using the primer pairs 519fc (5'-CAGCMGCCGCGGTAANWC-3') and 907r (5'-CCGTCAATTCMTTTRAGTT-3') (Lane, 1991). In addition, the community structure of the sample was determined by 454 pyrotag sequencing as described elsewhere (Köhler *et al*., 2012).

### Accession numbers

The sequences obtained in this study were submitted to Genbank. The accession numbers are JX266062–091 for 16S rRNA genes and JX266092–145 for *mcrA* genes from hindgut homogenates. Accession numbers JX266068, JX266097, JX648297 and JX648298 are for the corresponding genes of strains MpT1 and MpM2.

## Results

### Comparison of the 16S rRNA and *mcrA* clone frequency

Analysis of the archaeal 16S rRNA gene sequences from the hindgut of several higher termites revealed a diverse community of methanoarchaea, consisting of *Methanobacteriales*, *Methanosarcinales*, and *Methanomicrobiales*, although not all lineages were represented in each species (Table 1). In addition, each termite species yielded a substantial proportion of clones that clustered with a deep-branching lineage distantly related to *Thermoplasmatales* previously obtained from termite guts and other intestinal environments. A detailed analysis of the entire archaeal diversity in the different termite species will be published in a different context (J. O. Nonoh, K. Paul, D. K. Ngugi, and A. Brune, in preparation).

Clone libraries of the *mcrA* genes amplified from the same samples yielded not only the *mcrA* genes expected of the methanogens identified in the rRNA-based analysis, but each contained an additional cluster of *mcrA* genes of unknown origin (Table 1). In each termite, the distribution patterns of the different clone groups were in agreement with the assumption that the novel *mcrA* genes stemmed from the uncultured lineage of *Thermoplasmatales*.

Table 1. Clone frequencies in libraries of archaeal 16S rRNA genes and *mcrA* genes obtained from the hindgut of higher termites, documenting the co-occurrence of a novel lineage of *Thermoplasmatales*-related archaea and a cluster of novel *mcrA* genes.

| Termite species [a] | 16S rRNA genes (%) [b] | | | | | *mcrA* genes (%) [c] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Methano-microbiales* | *Methano-bacteriales* | *Methano-sarcinales* | Novel lineage | Number of clones | *Methano-microbiales* | *Methano-bacteriales* | *Methano-sarcinales* | Novel cluster | Number of clones |
| *Cubitermes ugandensis* | 27 | 26 | 20 | 28 | 66 | 19 | 33 | 14 | 35 | 34 |
| *Ophiotermes* sp. | 0 | 7 | 65 | 28 | 80 | 0 | 49 | 28 | 23 | 19 |
| *Trinervitermes* sp. | 50 | 31 | 0 | 19 | 42 | 18 | 64 | 0 | 18 | 11 |
| *Macrotermes michaelseni* | 0 | 52 | 45 | 2 | 44 | 0 | 74 | 16 | 11 | 19 |
| *Alyscotermes trestus* | 3 | 84 | 0 | 14 | 37 | 4 | 64 | 0 | 32 | 25 |

[a] Represent all major lineages of higher termites (*Termitidae*): *C. ugandensis* and *Ophiotermes* sp. (*Termitinae*); *Trinervitermes* sp. (*Nasutitermitinae*); *M. michaelseni* (*Macrotermitinae*); *A. trestus* (*Apicotermitinae*).

[b] Obtained with primer pair Ar109f and Ar912r.

[c] Obtained with primer pair *mcrA*-f and *mcrA*-r.

## Phylogenetic analysis of the 16S rRNA genes

Since the 16S rRNA gene sequences obtained in the first dataset were relatively short (800 bp), the phylogenetic resolution was not sufficient for the requirements of our study. Therefore, we also constructed smaller clone libraries with the primer pair Ar109f and Ar1490r for the termites *Cubitermes ugandensis* and *Ophiotermes* sp., and for the cockroaches *Salganea esakii* and *Panesthia angustipennis* to obtain longer sequences (1,380 bp) – together with those already present from previous studies – for all lineages of *Thermoplasmatales*-related archaea affiliated with termites and cockroaches. We included 16S rRNA gene sequences from all established lineages of *Euryarchaeota* from the Silva database and Genbank, including all sequences of 'uncultured *Thermoplasmatales*' obtained in previous studies. The resulting phylogenetic trees showed the same major lineages of methanoarchaea previously documented by others, with the *Thermoplasmatales* and their uncultured relatives clearly falling within the radiation of methanogens, confirming the paraphyletic character of methanoarchaea as a taxonomic group (Fig. 1).

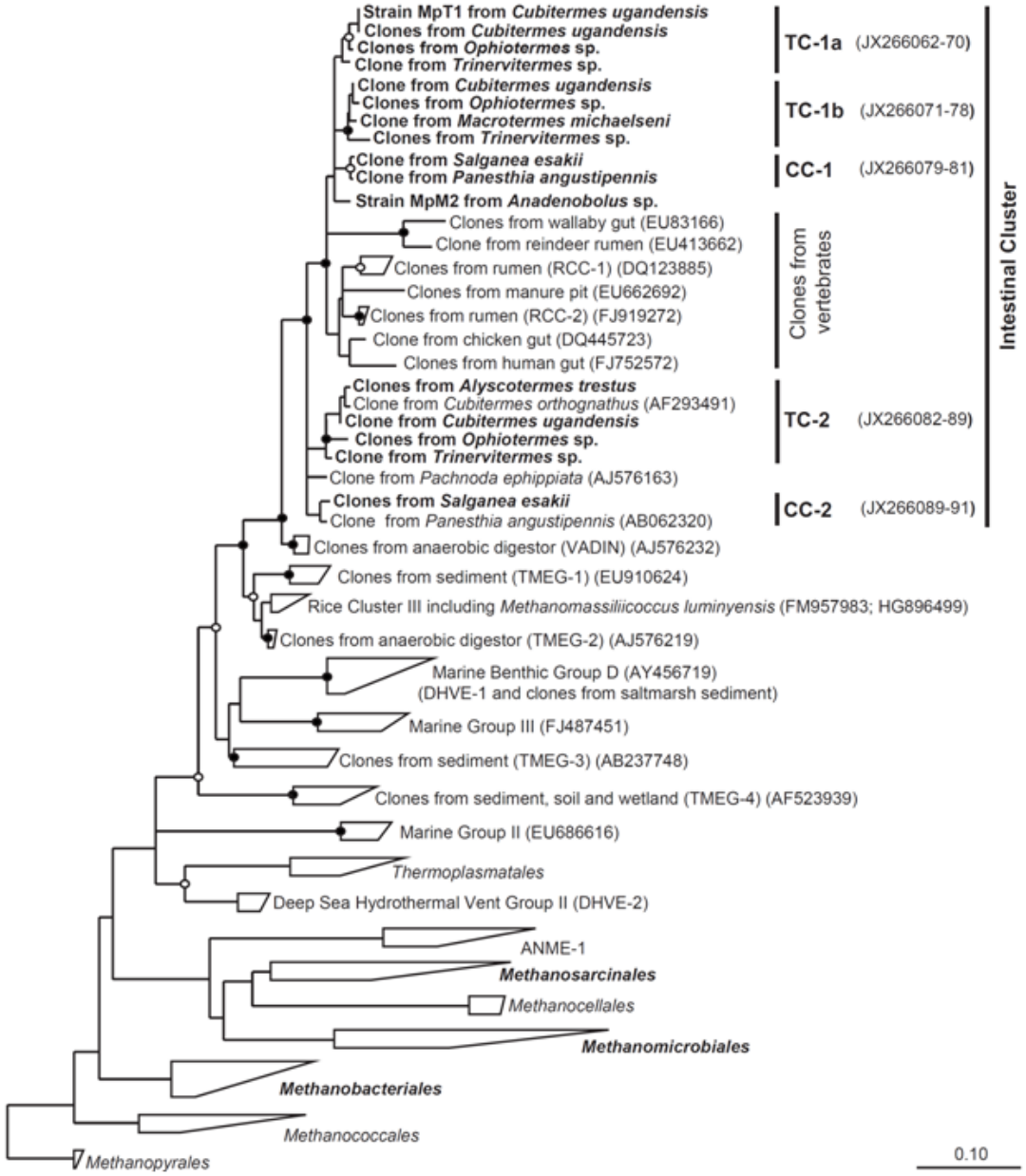


Fig. 1: Phylogenetic tree showing the relationships among uncultured archaea related to *Thermoplasmatales* and to representatives of all other orders of methanogenic archaea and the ANME-1 group. Clusters of clones from termite (TC) and cockroach (CC) gut are indicated. The tree is based on a maximum-likelihood analysis of an alignment of archaeal 16S rRNA genes (1250 bp) of archaea in public databases; sequences obtained in this study are marked in bold. Sequences of *Trinervitermes* sp., *M. michaelseni,* and *A. trestus* were

shorter and were added to the tree using the ARB parsimony tool. Bullets indicate bootstrap support (●, >95%; ○, >70%). Scale bar indicates substitutions per site.

The sequences of *Thermoplasmatales*-related archaea obtained from termites and cockroaches fell into a distinct clade of clones obtained exclusively from intestinal environments that was clearly separated from previously published clades containing sequences from diverse marine and freshwater habitats. Next relatives of this clade were clones previously obtained from an anaerobic digestor (VADIN Group; 18). Within the intestinal cluster, the sequences from insect guts formed two distinct lineages, each comprising both termite-specific and cockroach-specific lineages, with well-supported subclusters reflecting the phylogeny of their respective hosts. Other lineages in the intestinal cluster consisted of clones from vertebrate guts, which were previously obtained from the intestinal tracts of cattle, wallabies, chickens, and humans (see Introduction), and clones obtained from a manure pit.

## Phylogenetic analysis of the *mcrA* genes

To test the phylogenetic position of the novel *mcrA* genes obtained in this study, we added the sequences to a comprehensive set of *mcrA* sequences from public databases, comprising all major lineages of methanogens and including all *mcrA* genes of uncertain origin from environmental studies. Phylogenetic analysis confirmed the presence of *mcrA* genes in insect guts belonging to representatives of the orders *Methanosarcinales*, *Methanobacteriales*, and *Methanomicrobiales*, which was in agreement with the results of the 16S rRNA analysis (Fig. 2). The clones of unknown origin obtained from termite guts (Table 1) and from the guts of the cockroaches *Salganea esakii* and *Panesthia angustipennis* (this study) formed two distinct insect-specific lineages in a larger cluster of *mcrA* genes from intestinal habitats, including cows, wallabies, pigs, chickens, and humans. Also the *mcrA* genes from the intestinal tract of termites and cockroaches formed well-supported subclusters reflecting the phylogeny of their respective hosts.

As in case of the 16S rRNA gene sequences of *Thermoplasmatales*-related archaea, the novel *mcrA* genes from intestinal environments were most closely related to clones from

an anaerobic digestor and clearly separated from other, previously published clades containing sequences from diverse marine and freshwater habitats, including additional sequences of intestinal origin.

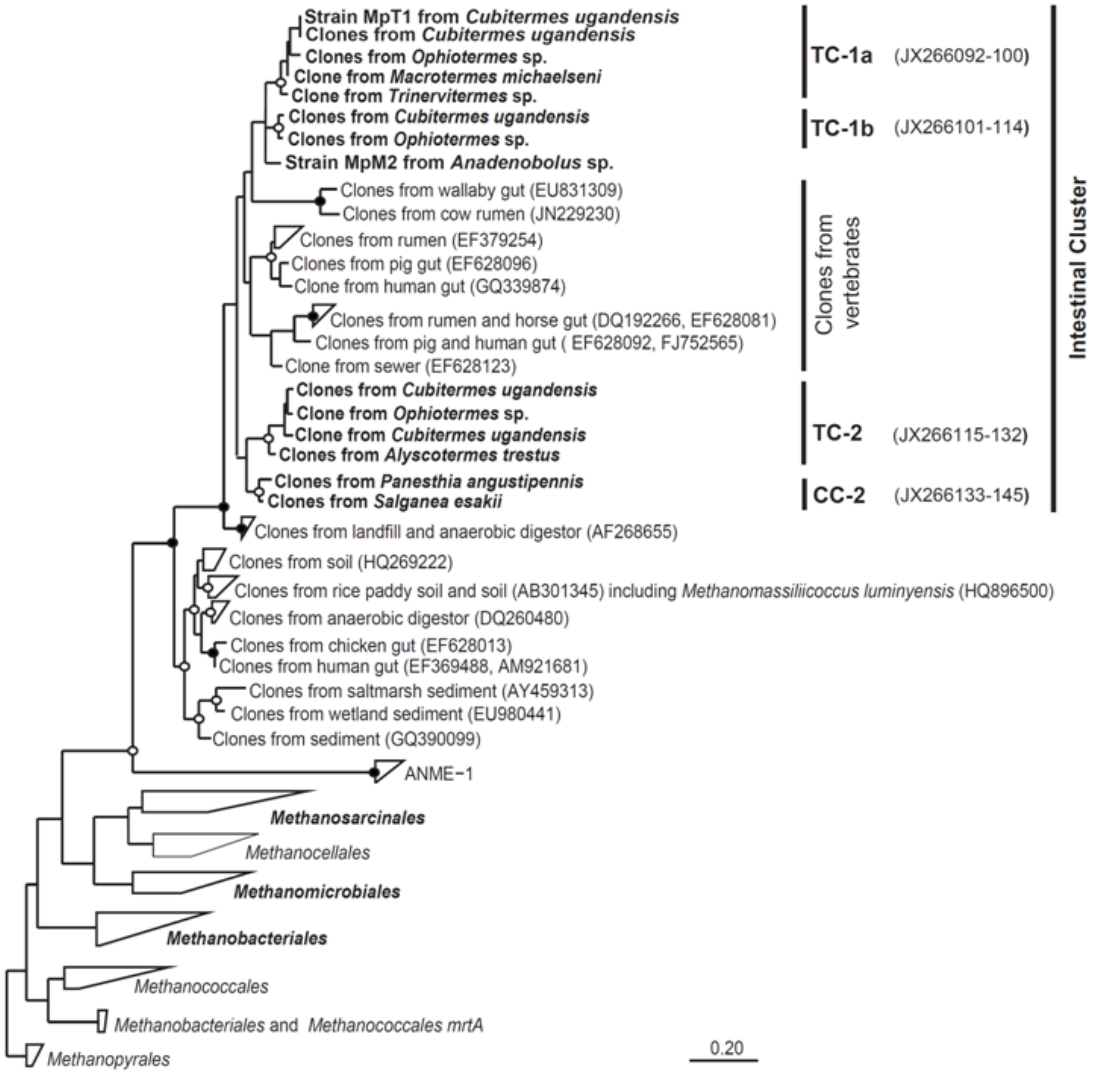


Fig. 2: Phylogenetic tree showing the relationships among the novel *mcrA* genes and to representative *mcrA* genes of all other orders of methanogenic archaea and the ANME-1 group. Clusters of clones from termite (TC) and cockroach (CC) gut are indicated. The tree is based on a maximum-likelihood analysis of an alignment of the *mcrA* genes (140 amino acids) of archaea in public databases; sequences obtained in this study are marked in bold. Bullets indicate bootstrap support (●, >95%; ○, >70%). Scale bar indicates substitutions per site.

## Enrichment of novel methanogens from arthropod guts

Hindgut homogenate of *Cubitermes ugandensis* was inoculated into basal medium with or without yeast extract with optional additions of methanol, formate, or xylan, and incubated under a headspace containing $H_2$ and $CO_2$. After a lag phase of several weeks, the culture containing methanol and yeast extract started to form $CH_4$. No methane formation was observed under any other condition even after 6 months of incubation, also not if rumen fluid was added to the cultures. Subsequent transfers of the culture on the same medium led to robust $CH_4$ formation (up to 17 kPa headspace partial pressure); rumen fluid was not required. Transfers of the enrichment culture to medium lacking methanol showed no methanogenesis; transfers to medium containing methanol in the absence of $H_2$ produced much less methane than with $H_2$. No methanogenesis occurred with acetate as the sole substrate (Fig. 3).

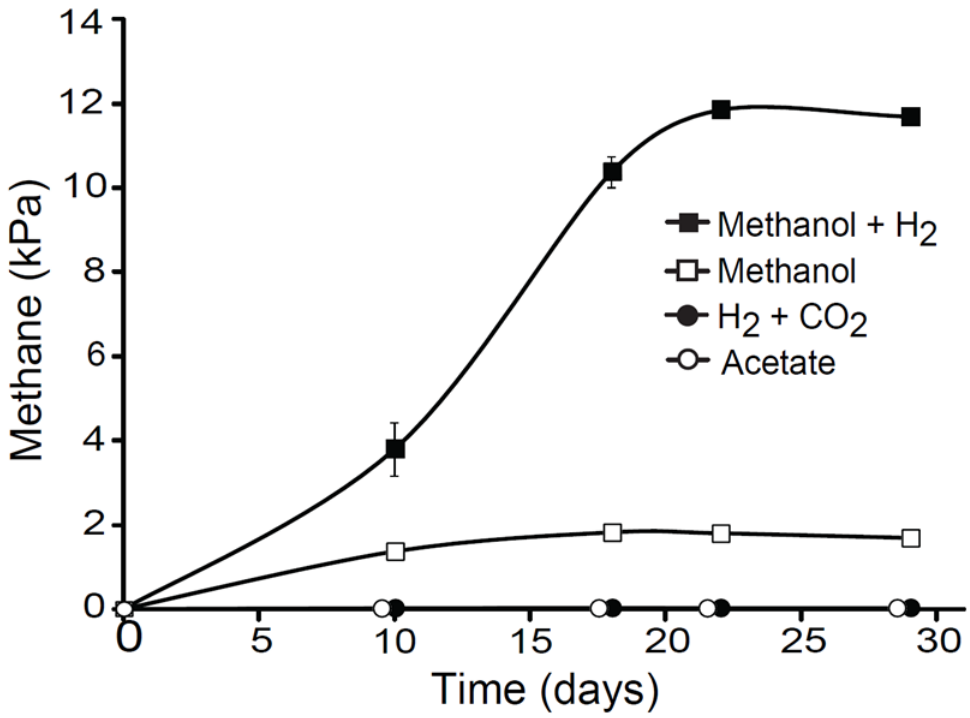

Fig. 3: Time course of methane partial pressure in the headspace of the enrichment culture MpT1 ($N_2$–$CO_2$; 80/20) inoculated from a methanol-starved preculture into basal medium supplemented with different substrates: $H_2$ (50 kPa in headspace), methanol (50 mM), or acetate (30 mM). Values are means of two cultures; mean deviations are shown only if they are larger than the symbols.

Already after the initial transfers, the culture consisted mostly of small, roundish cells (ca. 0.6–1 µm in diameter) (Fig. 4A). DNA was extracted from several subcultures, and the archaeal 16S rRNA genes were amplified using specific primers (Ar109f and 1490R). Each PCR product could be sequenced without cloning, and the sequencer traces indicated that in each case only a single phylotype of archaea was present. The sequences obtained from the different subcultures were identical. Phylogenetic analysis revealed that the archaeal 16S rRNA sequence (phylotype MpT1) fell into the apical cluster of putative methanogens consisting exclusively of clones from higher termites (TC-1a; Fig. 1). Also the *mcrA* genes amplified from the same samples yielded identical sequences, which fell into the corresponding cluster of novel *mcrA* genes in the phylogenetic tree (TC-1a; Fig. 2).

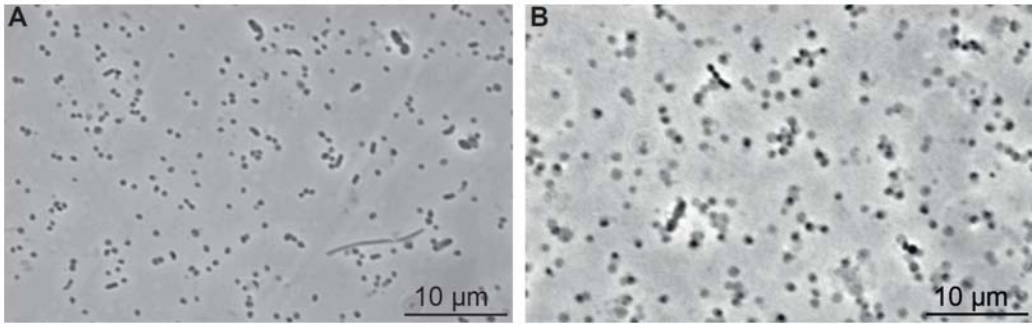


Fig. 4: Phase-contrast photomicrographs of the methanogenic enrichment cultures MpT1 (A) and MpM2 (B) after several transfers in basal medium supplemented with $H_2$ and methanol. Both cultures consisted mostly of small roundish cells (diameter 0.6–1.0 µm).

When the abundance of archaeal 16S rRNA genes in the enrichment culture had increased to 64% (based on the total copy numbers of archaeal and bacterial 16S rRNA, determined by qPCR), the bacterial contaminants remaining in the enrichment culture were determined by 454 pyrotag sequencing. Classification of the bacterial sequences

revealed that the bacteria remaining in the enrichment culture represent several lineages of so-far uncultivated *Clostridiales* (Fig. 5). All attempts to isolate strain MpT1 in pure culture were so far unsuccessful.

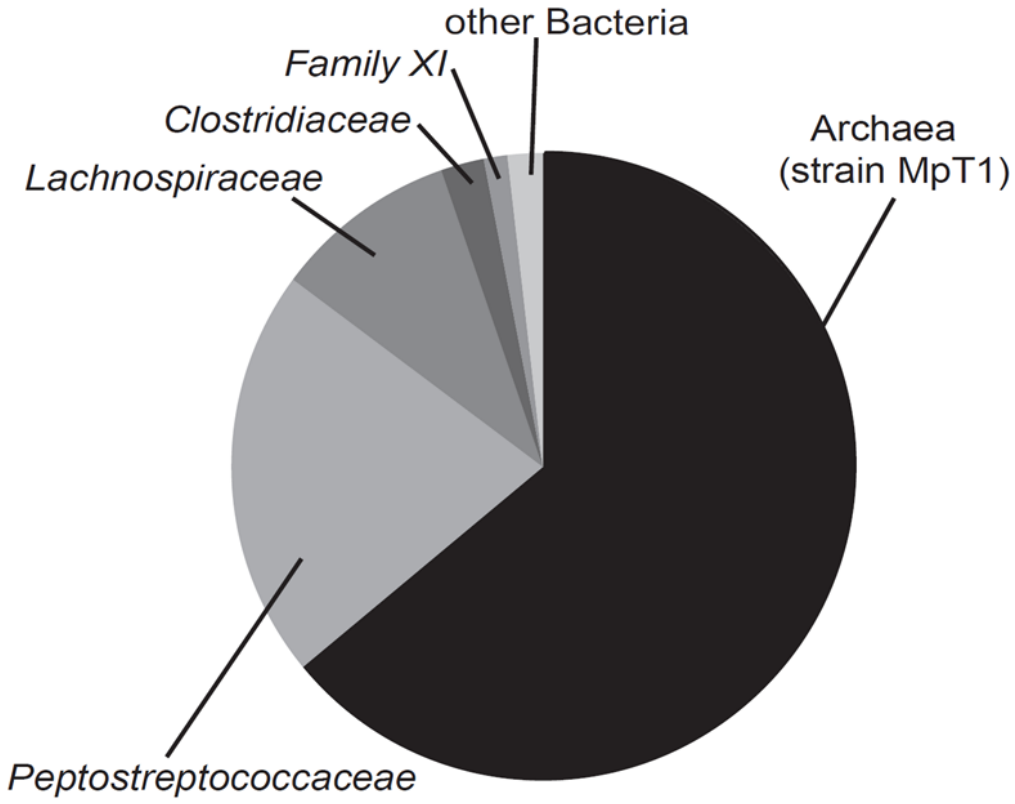


Fig. 5: Composition of the enrichment culture of strain MpT1, determined by quantitative real-time PCR of bacterial and archaeal 16S rRNA genes. 454 pyrotag sequencing revealed that the bacterial contaminants belonged almost exclusively to families of the order *Clostridiales*.

Meanwhile, we also obtained a second methanogenic enrichment culture from the hindgut homogenate of a millipede (*Anadenobolus* sp.), using the same medium and enrichment strategy as for strain MpT1. The culture accumulated even higher amounts of methane (45 kPa in the headspace) than strain MpT1. Again, the PCR products obtained with specific primers for archaeal 16S rRNA genes and *mcrA* genes could be sequenced without cloning, which indicated that also this enrichment culture was dominated by a single strain of methanogens. Strain MpM2 had the same coccoid morphology as strain MpT1 but were slightly larger (Fig. 4B); both strains did not show the typical $F_{420}$

autofluorescence of many methanogens. Phylogenetic analysis showed that the 16S rRNA sequence of strain MpM2 also fell into the intestinal cluster of the novel methanogens within the radiation of sequences from termites and cockroaches (TC-1 and CC-1; Fig. 1). The *mcrA* gene of strain MpM2 clustered with the corresponding *mcrA* genes of the TC-1 subcluster (Fig. 2).

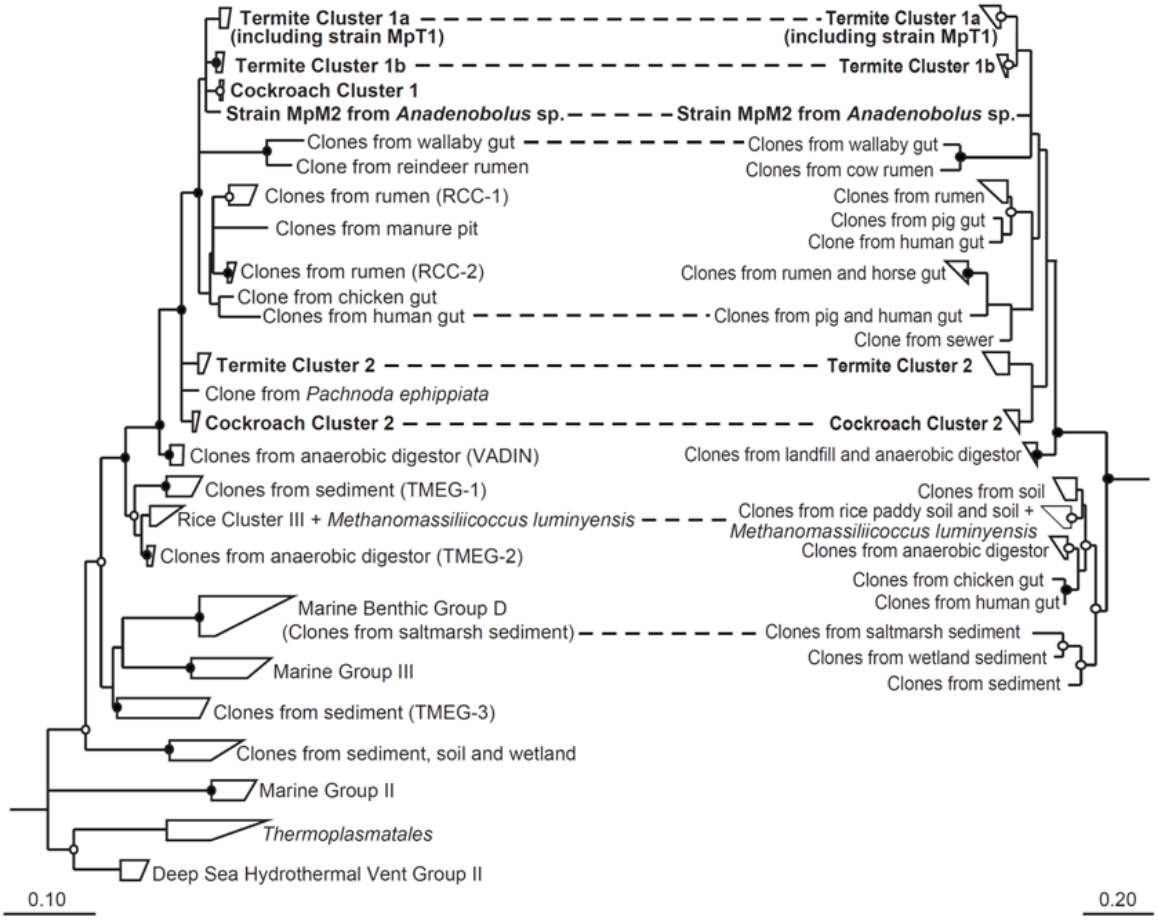


Fig. 6: Tanglegram illustrating the congruence of the phylogenies of *Thermoplasmatales*-related archaea (16S rRNA) and the *mcrA* genes of unknown origin (for details, see Figs. 1 and 2). Sequence pairs stemming from the same study are connected by dotted lines. Sequences obtained in this study are marked in bold. Bullets indicate bootstrap support (●, >95%; ○, >70%). Scale bars indicate substitutions per site.

# Discussion

The results of this study are the final proof that the deep-branching lineage of so far uncultured *Euryarchaeota* distantly related to the *Thermoplasmatales* represents the seventh order of methanogens. This is supported by the congruence of the phylogenies of 16S rRNA and *mcrA* genes, which indicates that the corresponding gene sets obtained from termite and cockroach gut (this study) and from mammalian guts and several other environments (previous studies) stem from the same organisms (Fig. 6). Further evidence for the methanogenic nature of the entire lineage comes from the highly enriched strains of methanogens from the hindgut of termites and millipedes and the isolate *Methanomassiliicoccus luminyensis* from human feces (11; see below).

**Novel archaea in the guts of termites and cockroaches**

Previous studies of archaeal diversity in the hindgut of *Cubitermes orthognathus* (subfamily *Termitinae*) and *Nasutitermes takasagoensis* (subfamily *Nasutitermitinae*) had revealed the presence of four major lineages of *Euryarchaeota* in higher termites: *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteriales* (for references, see 5) and a deep-branching clade distantly related to *Thermoplasmatales* (Friedrich *et al*., 2001; Miyata *et al*., 2007). Clones from the same lineages were recovered also from *Cubitermes ugandensis* and *Ophiotermes* sp., *Trinervitermes* sp., *Macrotermes michaelseni*, and *Alyscotermes trestus* (this study), which indicated that representatives of this clade are consistently present in all subfamilies of higher termites. In addition, clones of this lineage were obtained also from the wood-feeding cockroaches *Salganea esakii* and *Panesthia angustipennis* (Hara *et al*., 2002; this study), which are distantly related to termites.

Interestingly, the novel archaea from insect guts form two distinct lineages, each comprising clones from higher termites and wood-feeding cockroaches that seem to be specific for their respective hosts. The general absence of this group from lower termites is in agreement with previous studies reporting that these insects are exclusively colonized by members of the genus *Methanobrevibacter* (Ohkuma *et al*., 1995); the

single clone of *Thermoplasmatales*-related archaea obtained from *Reticulitermes speratus* (Shinzato *et al*., 1999) is affiliated with Cluster TC-1b (Fig. 1).

**Methanogenic nature of novel archaea**

The tree topologies of the 16S rRNA genes of novel archaea (Fig. 1) and the *mcrA* genes of unknown origin (Fig. 2) strongly resemble each other. A simplified tanglegram of the two trees illustrates that the phylogenetic positions of the major clusters of 16S rRNA and *mcrA* genes match perfectly (Fig. 6). This is true for all studies that reported both 16S rRNA and *mcrA* clones from the same environments: termite and cockroach guts (this study), wallaby gut (Evans *et al*., 2009), human gut (Mihajlovski *et al*., 2008; Mihajlovski *et al*., 2010), and saltmarsh sediment (Castro *et al*., 2004). In addition, other opposing clusters in the tree contain clones that originated from the same (e.g., the rumen) or related habitats (i.e., from the guts and the manure of farm animals). Also the internal topologies of the respective groups are highly coincident (Figs. 1 and 2), which provides strong support that the sequence pairs from different animals originated from the same archaeal lineages. This is corroborated further by the similar clone frequencies of 16S rRNA and *mcrA* genes in the corresponding libraries of different gut termite species (Table 1), although the results are probably affected by differences in copy numbers of the 16S rRNA gene in *Methanosarcinales* and *Methanobacteriales* (Acinas *et al*., 2004).

Further proof of the methanogenic nature of the new lineage came from the successful enrichment of strains MpT1 and MpM2 – the only archaea present in the highly methanogenic enrichment cultures from termite and millipede guts. The 16S rRNA and *mcrA* gene sequences of both strains cluster with corresponding clones obtained from the guts of termites and cockroaches (TC-1 and CC-1; Figs. 1 and 2). They are part of the 'Intestinal Cluster' of putative methanogens that comprises also clones from the rumen (RCC; Tajima *et al*., 2001) and the human gut (Mihajlovski *et al*., 2010). More distant relatives are found in anaerobic digestors (VADIN), rice field soil (RC-III), sediments, and other terrestrial environments (TMEG-1 und 2). Since matching *mcrA* genes were obtained from most of these habitats, it is safe to assume that all these lineages are methanogenic.

The final piece of evidence for the methanogenic nature of the new lineage was provided by the study of Dridi *et al*. (2012), which was published during the revision stage of the present study. They isolated and described a new genus and species of methanogens, *Methanomassiliicoccus luminyensis*, from human feces, and reported that its 16S rRNA gene sequence was most closely related to several clones of 'uncultured *Thermoplasmatales*' previously obtained from the digestive tracts of various mammals. They claimed that these clones and their isolate represent a new order of methanogens, but their phylogenetic analysis was superficial and comprised only a limited set of taxa. Our detailed phylogenetic analysis of both 16S rRNA and *mcrA* genes revealed that *M. luminyensis* is not a member of the vertebrate clones in the Intestinal Cluster (Figs. 1 and 2), which comprises most of the clones previously obtained from the digestive tracts of mammals. Instead, the isolate falls within the radiation of RC-III, where it clusters with clones from rice field soil (Grosskopf *et al*., 1998; Chin *et al*., 1999) and a single clone previously obtained from human gut (Mihajlovski *et al*., 2008).

The methanogenic character of euryarchaeota in RC-III had been suggested already by Kemnitz *et al*. (2005), who observed that the abundance of RC-III clones in a methanogenic enrichment culture from rice field soil was reduced by the addition of bromoethanesulfonate (BES), a specific inhibitor of methanogenesis. Considering the methanogenic character of *M. luminyensis* and the fact that *mcrA* sequences corresponding to RC-III have been obtained from rice paddies and other soils (Fig. 6), it is likely that all members of RC-III are methanogens.

The tanglegram (Fig. 6) shows that the most basal cluster in the new lineage of *mcrA* genes belongs to Marine Benthic Group D, based on the matching positions of two sets of 16S rRNA and *mcrA* genes (ARC-7 and MCR-2) obtained from the same saltmarsh samples (Castro *et al*., 2004). Although there are no *mcrA* genes matching the deeper-branching lineages, this may be due to the general lack of *mcrA*-based studies of methanogenic diversity particularly in marine habitats. Therefore, it is not possible to predict whether also the deeper-branching lineages are methanogenic.

Interestingly, we observed a consistent and moderately supported sister-group position of the novel *mcrA* genes and those of the ANME-1 group, an uncultivated lineage of methane-oxidizing archaea that may involve a methyl-CoM reductase in anaerobic methane oxidation (Knittel and Boetius, 2009). However, in view of the methanogenic properties of *M. luminyensis* (Dridi *et al*., 2012) and our enrichment cultures, and the cumulative evidence for the absence of methane oxidation in termite guts (Pester *et al*., 2007), a methanotrophic character of this novel lineage of archaea can be excluded.

**Physiological properties of the enrichment cultures**

In the highly enriched cultures of strains MpT1 and MpM2, methanogenesis was strongly stimulated by the simultaneous supply of both $H_2$ and methanol. The small amount of methane formation in the enrichment culture containing only methanol is most likely due to hydrogen formation by the clostridial members of the enrichment culture during fermentation of substrates stemming from yeast extract. Although a final statement on the substrate requirements will have to wait until these strains have been brought into pure culture, it seems that the metabolism of strains MpT1 and MpM2 ('Intestinal Cluster') resembles that of *M. luminyensis* (RC-III) and obligately $H_2$-requiring methylotrophic methanogens from other lineages, like *Methanosphaera stadtmanae* (*Methanobacteriales*) and *Methanomicrococcus blatticola* (*Methanosarcinales*). Interestingly, such organisms have so far been isolated exclusively from the intestinal tracts of humans (Dridi *et al*., 2012; Miller and Meyer, 1985) and cockroaches (Sprenger *et al*., 2000). It is likely that this mode of methanogenesis is an adaptation to the intestinal habitat.

In a study of the archaeal diversity in the hindgut of the termite *Nasutitermes takasagoensis*, the relative abundance of 'uncultured *Thermoplasmatales*' (Cluster TC-1a and TC-2; Fig. 1) increased when the animals were fed with xylan (Miyata *et al*., 2007), a substrate that contains substantial amounts of *O*-methylated glucuronic acid residues (Rosell and Svennson, 1974). It is possible that the apparent enrichment of these methanogens was an indirect effect caused by methanol formation during the fermentative breakdown of xylan.

**The seventh order of methanogens**

Although it has been repeatedly proposed that environmental clones distantly affiliated with the *Thermoplasmatales* represent a separate order of methanogens (e.g., 15, 39), none of these studies provided enough evidence to substantiate this claim. Analysis of the entire set of sequences available to date clearly documents the diversity of the new lineage, including numerous habitat-specific clades and its sister-group relationship to the *Thermoplasmatales,* and provides robust evidence for the presence of *mcrA* genes in all members. The methanogenic nature of the lineage is further corroborated by the isolation of *M. luminyensis* (Dridi *et al*., 2012) and the enrichment cultures of strains MpT1 and MpM2 (this study). Based on this evidence, we propose the provisional name 'Methanoplasmatales' for the entire deep-branching lineage of euryarchaeota outlined in Figure 6. Although a first representative of the lineage has been isolated and described (Dridi *et al*., 2012), we suggest postponing a formal description of any higher taxa until further representatives have been obtained in culture, their cell envelopes have been characterized, and the presence of *mcrA* genes in the basal lineages (particularly the marine groups) has been assessed.

Considering the apparently obligate hydrogen dependence of methanol reduction both in *M. luminyensis* and in the enrichment cultures, it may be promising to use such combinations of methanogenic substrates for enrichments also from other habitats. There are several other deep-branching lineage of euryarchaeota that may also be methanogenic, and even more diversity may be present because of a bias of commonly used PCR primers against hitherto undetected lineages (Teske and Sorensen, 2008). This is underlined by two studies of archaeal diversity in termite guts that had failed to detect clones affiliated with 'Methanoplasmatales'. In one case (Ohkuma *et al*., 1999), this was most likely due to a mismatch in the reverse primer to the consensus sequence of 'Methanoplasmatales', whereas in the other case (Donovan *et al*., 2004) the sequence of the forward primer slightly differed from the forward primer successfully used by Hara *et al*. (2002).

It is striking that the majority of the *mcrA* genes of the 'Methanoplasmatales' have so far been retrieved only from intestinal samples. That there are only few clones from other environments may simply be due to the lack of such studies, particularly in marine environments. More cultivation efforts are required to expand our knowledge about this novel group of methanogens, not at least to investigate their metabolic relationship to *Thermoplasmatales*, a clade of *Euryarchaeota* that may have experienced a secondary loss of their capacity for methanogenesis (Bapteste *et al.*, 2005).

## Acknowledgments

We are grateful to Michael Friedrich for providing a core database of *mcrA* genes, Kiyoto Maekawa for wood-feeding cockroaches, and Rudolf Scheffrahn for termite identification. We thank David Ngugi and Eugen Bauer for providing DNA samples, and Katja Meuser and Aram Mikaelyan for help with the clone libraries and the 454 analysis.

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**Chapter Six**

# Other relevant work

**James O. Nonoh**

# Enrichments from gut homogenates revealed potential role of methanol in driving methanogenesis in the guts of soil-feeding higher termites

## Materials and methods

### Termites

Worker castes of *Cubitermes ugandensis* (batches TD 107 and TD113) were used for methanogen enrichment experiments. The termites were collected in a grassland area in Kakamega forest, Kenya and were used while being maintained in the laboratory on soil collected from the vicinity of their mound as previously described (Odelson and Breznak, 1983).

### Preparation of gut homogenates

Termites were degutted in an anoxic glove box as described by Breznak and Pankratz (1977) to get the whole gut, including the crop and midgut. For *Cubitermes* sp. (batch TD107), the gut was then sectioned into P3 and P4 proctodeal compartments respectively (known to have methanogenic potential). Ten extracted guts each of P3 and P4 compartments were pooled in 5 ml of DTT and/or $Na_2S$-reduced buffered salts solution respectively (AM-5 media described below) at two guts per ml and then homogenized using a clean sterile pestle as described by Breznak and Switzer (1986).

## Media

Anoxic cultivation of posterior gut derived methanogens was regularly done by using modified mineral medium. The gut methanogens were cultivated using $CO_2$- and bicarbonate-buffered, Sodium sulfide ($Na_2S$) and/or dithiothreitol (DTT)-reduced AM-5 media (Boga and Brune, 2003) under anoxic atmosphere of $N_2/CO_2$ (80:20) as described by Breznak *et al*. (1988). Medium AM-5a contained (in grams per liter) NaCl, 1.0; KCl, 0.5; $MgCl_2$ .$6H_2O$, 0.4; $CaCl_2$ .$2H_2O$, 0.1; $NH_4Cl$, 0.3; $KH_2PO_4$, 0.2; $Na_2SO_4$, 0.15 (1 mM $Na_2SO_4$); $NaHCO_3$, 5.5 (30 mM $NaHCO_3$); trace element solution and selenite-tungstate (Se/W sln.) solution (Leadbetter and Breznak, 1996); and seven-vitamin solution as described by Leadbetter and Breznak, (1996). The trace elements, vitamins and any further supplements (where specifically noted) were added to the autoclaved medium from previously prepared sterile stock solutions as described by Widdel and Pfennig (1981). The pH was adjusted to 7.4, when necessary, with sterile 1 M solutions of either HCL or $Na_2CO_3$. For P3 gut homogenates, the pH of the media was adjusted to 10.4 with sterile 1 M $Na_2CO_3$. Prior to inoculation, the reducing agents, either DTT (1 mM final concentration) or $Na_2S$ (2 mM final concentration) was added to the medium. Where indicated, the medium was also supplemented with 0.01% (wt/vol) yeast extract and 0.05% (wt/vol) Casamino Acids (Difco) autoclaved separately. For enrichment transfers, the medium was identical to AM-5a but was also supplemented with 20 % clarified bovine rumen fluid and 50 µM $Na_2S_2O_4$ to supplement further as reducing agents. For solid media, agar (Difco; washed 3 times with water before use) was incorporated at a final concentration of 1%. The media was always supplemented with 2.5 mM acetate. During the experimental, anoxic conditions were maintained throughout the procedures and all bottles and tubes used in this study were kept under $N_2/CO_2$ atmosphere and sterile syringes were routinely flushed with either of the gases or gas mixture before and during every use. After each preparation, the media was dispensed into 20 ml serum bottles and 15-20 ml tubes and made anoxic as described by Leadbetter and Breznak 1996.

## Enrichments

Hydrogen $H_2/CO_2$ (80:20); Methanol (2.5 mM) plus $H_2$ (80%); Formate (15 mM); butyrate (15 mM); and propionate (15 mM) were used as substrates for the enrichment of methanogens. 200 µl of P3 and P4 gut homogenates (2 guts/ml) and whole gut homogenate (3 guts/ml) of *Cubitermes* species were grown in 60 ml serum bottles tubes containing 20 ml of AM-5a liquid media. Two sets of control enrichments were appropriately set up as above, one without gut homogenate inoculums and the other without substrates. All culture vessels were closed with gas tight black butyl rubber stoppers and crimped with aluminum seals, and were then incubated in the dark at 30 ℃ without shaking unless indicated otherwise. To check for growth, liquid cultures were checked periodically for visible turbidity and methane concentration in the headspace monitored using gas chromatography. After and during the incubation periods, the bottles were scored positive for the presence of methanogens when methane accumulated in the headspace gas to detectable levels, a negative pressure developed in the tube, and $F_{420}$ auto-fluorescent cells were observed by UV epifluorescence microscopy. For positive cultures, transfers were then made onto fresh media only for the enrichments showing relatively steady methane concentration in the headspace and observation of the presence of $F_{420}$ fluorescing cells in the enrichment media.

## Microscopy and $F_{420}$ auto-fluorescence from enrichment cultures

For the enrichments that showed methane production in the gas headspace, $F_{420}$ auto-fluorescence microscopy was done to check the occurrence of methanogenic cells. To concentrate the cells, 300 µl of enrichment cultures showing detectable methane production in the headspace volume was aliquoted into a sterile eppendorf tube and centrifuged at 2000 rpm for two minutes. Half the volume was then removed and the cells re-suspended in the remaining volume. Phase contrast micrographs and factor $F_{420}$ auto-fluorescence were observed by using wet mounts (100 µl) of enrichments on a glass slide and allowed enough time to oxidize in air before covering with cover slip. The cells were then viewed with a fluorescent microscope (Zeiss Axiophot, Germany) equipped with UV light source. The excitation and emission spectra of coenzyme $F_{420}$ in the AM-5

media enrichment were recorded at corresponding emission and excitation maxima at 470 and 420 nm respectively (Zeiss filters 395–440; FT460; LP470). The same microscope was used for phase contrast microscopy.

## Microscopic examination of individual compartments

Live worker caste termites were used in this study. With the aid of a stereomicroscope (Zeiss, Jena, Germany) the entire whole gut was pooled out and then sectioned into individual compartments crop (C), midgut (M), and proctodeal compartments P1–5 (P) using fine tipped forceps. The P3 gut compartment was then sectioned into P3a (more dilated) and P3b (more tubular) sections at the constriction at the center. Each gut section was slit open and either directly placed on the microscope slide together with the gut tissues or the gut contents was washed off with PBS solution before the gut tissues was placed on the clean slide for microscopy. The preparation was then examined under both phase contrast and fluorescence microscope as described above in order to observe the density and morphotypes of cells in each compartment.

## Quantitative analysis of archaea and bacteria in the dilated P3 compartment

In order to investigated which section of the P3 gut compartment was most colonized by archaeal and bacterial cells, 10 pooled guts each of P3a and P3b sections were homogenized using sterile pestle (Eppendorf, Hamburg, Germany). The DNA was extracted following the Zirconium bead-beating method combined with phenol-chloroform method as described in chapter two. Quantification of archaeal and bacterial 16S rRNA gene copy numbers in each section was carried out using archaeal and bacterial primers as described in chapter two.

# Results

## Enrichments

Detectable methane production was only observed in $CH_3COOH$ + $H_2$ and $H_2/CO_2$ enrichments (Fig. 1). There was higher methane production in the enrichments from the P4 gut section which were cultured at a pH of 7.4 than from the P3 gut sections cultured at a pH of 10.4. In some enrichment, it took several weeks before even trace amounts of methane production could be observed in the gas headspace. No methane emission was observed in the control enrichments without gut homogenate inoculums, while minimal methane production was observed in the first few days of incubation for the control with only gut inoculum but with no substrate added in the culture media.

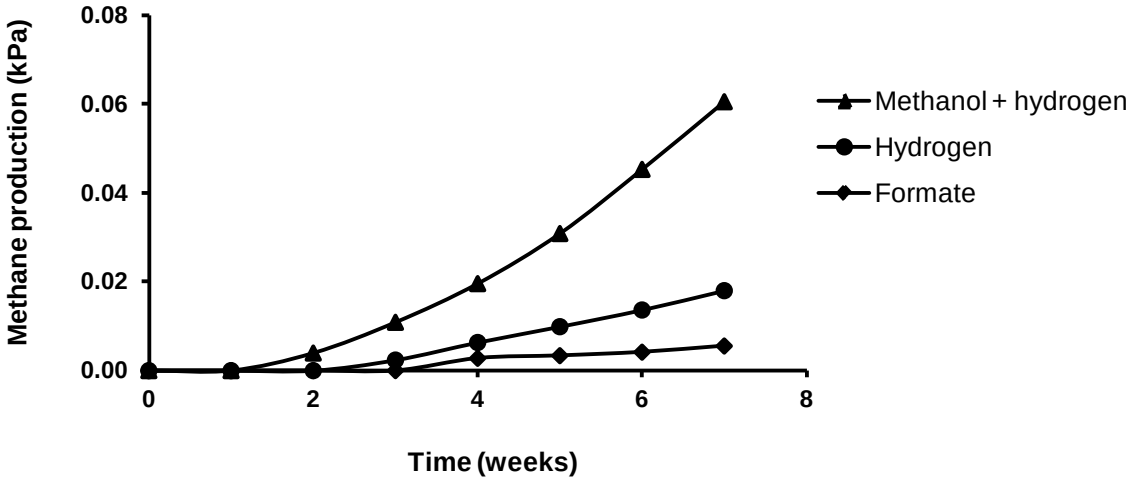


Figure 1: Time course for partial pressure of $CH_4$ in the headspace of cultures enriched with different substrates from P4 gut homogenates of *Cubitermes* sp. The media was reduced with 2 mM $Na_2S$. No rumen fluid supplemented was made in a static liquid AM-5 culture media at 30 °C. Substrates are: methanol (2.5 mM), formate (15 mM) and $H_2$:$CO_2$ (80:20%).

Transfers were only made for the P4 gut homogenate enrichments which had initially showed promising prospects. When the enrichments were transferred in the same media as described above but without rumen fluid, no methane production was detected in any

of the enrichment transfers even after 8 weeks of incubation. However, when the enrichments were transferred into a fresh AM-5 media as above (pH 7.4), supplemented with 20% (vol/vol.) clarified rumen fluid, all the enrichments started to show methane production, with the methanol enrichment supplemented with hydrogen substrate showing almost 10 kPa of methane in the headspace gas (Fig. 2). Rumen fluid was observed to be important for stabilizing the enrichments, and was therefore included in subsequent transfers.

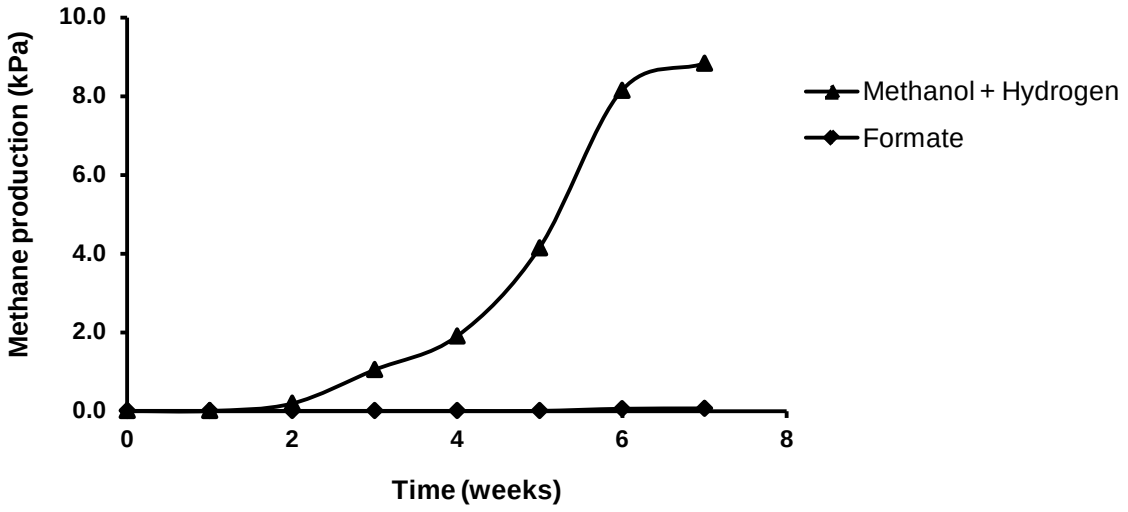


Figure 2: Time course for partial pressure of $CH_4$ in the headspace of transfer cultures of P4 gut compartment homogenates of *Cubitermes ugandensis* enriched with methanol plus hydrogen and formate substrates. The media was reduced with 2 mM $Na_2S$ and supplemented with 20% rumen fluid in a static liquid AM-5 culture media at 30 °C. Substrates are methanol (10 mM), formate (15 mM) and $H_2$ (80%).

Enrichments set up with whole gut homogenates cultured at a pH of 7.4 showed similar patterns as those with gut compartment P4, with enrichments set up with methanol plus hydrogen mixture showing more methane production than the other enrichments (data not shown). To test whether only methanol, hydrogen or a combination of both was responsible for methane production in the enrichments, two sets of experiments were set up using the same AM-5 medium as described above. In one, the enrichment was

transferred to a medium in which methanol-hydrogen mixture was added as substrate, while in the second set, only hydrogen + $CO_2$ was added. Enrichments transferred with only hydrogen without methanol showed decreased methane production.

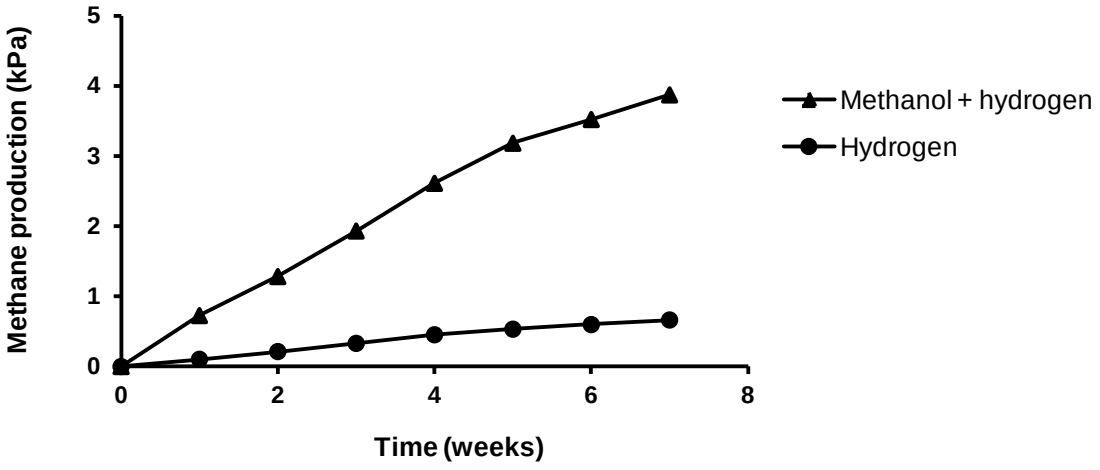


Figure 3: Time course for partial pressure of $CH_4$ in the headspace of cultures of whole gut homogenate of *Cubitermes ugandensis* transferred with or without methanol. The media was reduced with 2 mM $Na_2S$ and supplemented with 20% rumen fluid in a static liquid AM-5 culture media at 30 °C. Substrates are methanol (20 mM) and $H_2$:$CO_2$ (80:20%).

## Microscopy of the enrichment cultures

DAPI staining of the methanol-hydrogen enrichment cultures from the whole gut homogenate of *Cubitermes ugandensis* showed mostly coccoid cells in the culture (Fig. 4A). However, Phase contrast microscopy of the same culture showed presence of long rods, short rods and spherical cells (Fig. 4B). Some rods appeared attached to each other in pairs or in chains while others occurred in singles.

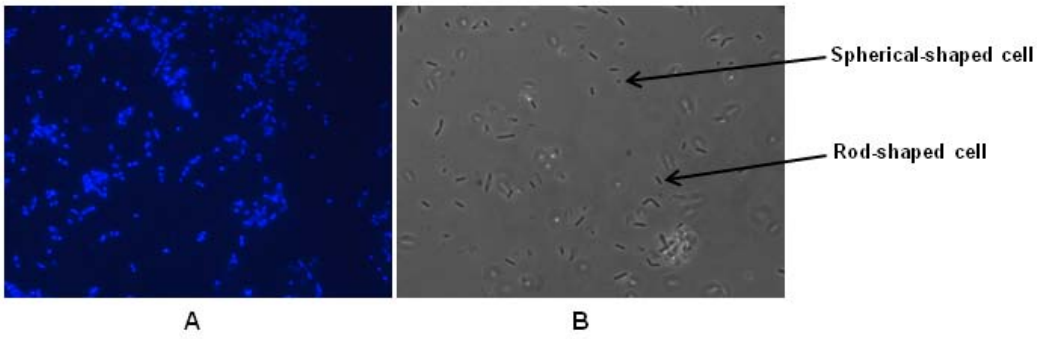


Figure 4: DAPI staining (A) and Phase contrast microscopy of culture from whole gut homogenate of *Cubitermes ugandensis* enriched with methanol + $H_2$ (1000×).

## Microscopy of the compartment homogenates and gut tissues

$F_{420}$ auto-fluorescence microscopy of the whole gut and gut compartments of *Cubitermes ugandensis* revealed diverse morphotypes of microbial cells. In the whole gut, rod-shaped, spherical and filamentous cells were observed. Most of the cells occurred in chains. In the anterior crop and midgut, there was hardly any cell observed. In the P1 compartment very few rod-shaped and coccoid cells were observed. Most the long filaments were observed in the more dilated P1 and P3a compartments. Majority of the cells were observed in the P3b and P4 compartments, most of which were rod-shaped both long and short ones, as well as coccoid cells. Filamentous cells were also abundant in the P3 compartment (Fig. 5a). Most of the rod-shaped cells were also attached at the gut wall and this was observed even after washing out the gut contents with PBS solution (Fig 5b). Most of the cells in the P4 compartment were spherical and rod-shaped in morphology. Only a few filaments were observed in this compartment (Fig. 6).

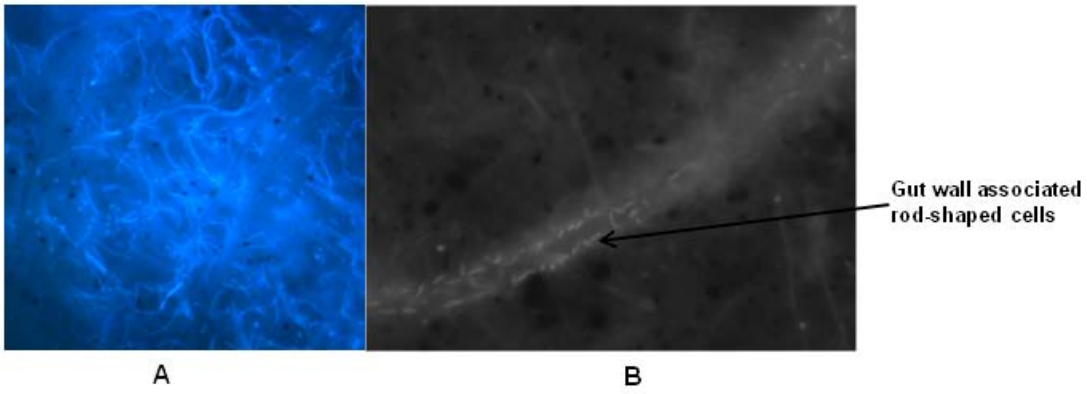


Figure 5: $F_{420}$ fluorescent microscopy of the gut of *Cubitermes ugandensis* showing fluorescent morphotypes (A) in the gut content of P3 compartment and (B) associated with the gut wall of the P3b section slit open and washed with PBS to get rid of gut contents (1000 ×). The background in Fig. 5B was changed to clearly visualize the $F_{420}$ auto-fluorescing cells.

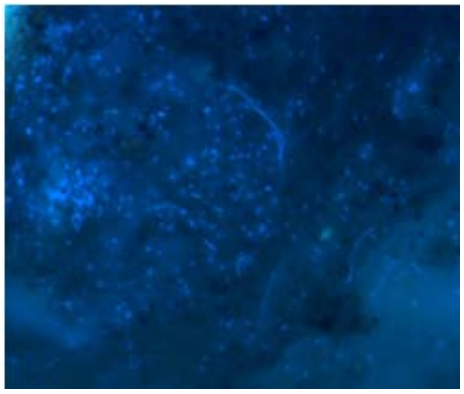

Figure 6: $F_{420}$ auto fluorescing cells in the P4 gut compartment of *Cubitermes ugandensis* slit open and washed with PBS to get rid of gut contents (1000 ×).

## qPCR analysis of the P3 compartment

Results showed that most archaea and bacteria were localized in the tubular P3b section in which the pH is less alkaline as compared to the more dilated P3a section. The abundance of archaea in the P3b section was more than one order of magnitude higher than in the P3a section. The same was true for bacteria. The percentage of archaea over that of prokaryotes in the P3a section was 10% while in the P3b section, it was 2%.

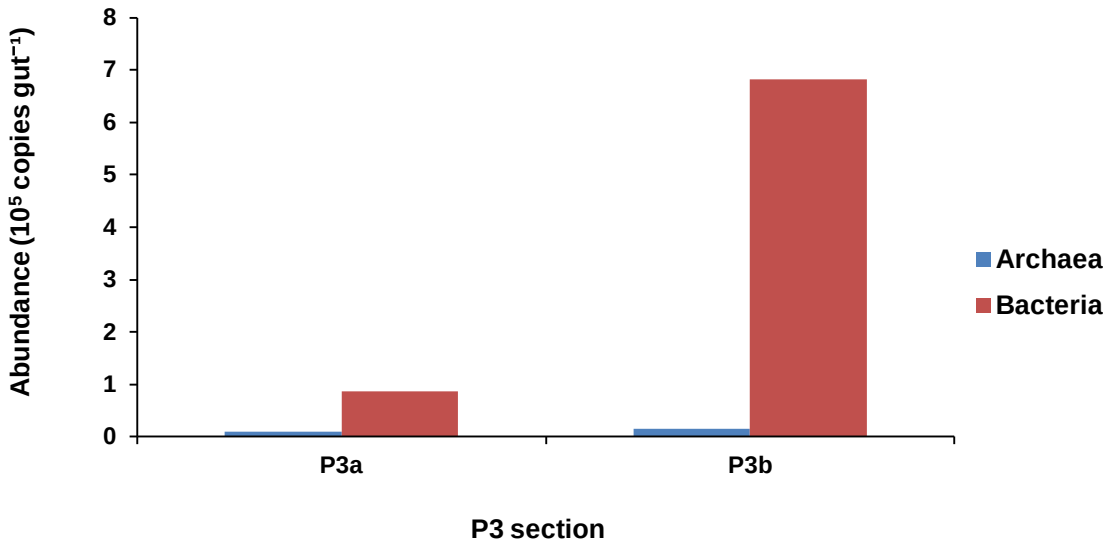


Figure 8: Density of archaea and bacterial 16S rRNA gene copy numbers in the P3a and P3b sections of the dilated P3 compartment of *Cubitermes ugandensis*.

## Discussion

The better performance of the P4 enrichments over P3 enrichments may be explained by the differences in the culture pH and potentially indicates that the highly alkaline pH used in the P3 enrichments is inhibitive to most methanogens and this may suggest that gut methanogens preferentially colonize less alkaline micro-habitats. While the former, enriched at pH of 7.4 showed high methane production rates, the latter, incubated at pH of 10.4 did not show any stable methane production in the enrichments, and further points that methanogens may be sensitive to more alkaline conditions in the gut. This observation was important because it gave us a hint that in the P3 compartment of soil-feeding termites, methanogens may be confined to the more posterior sections in which the pH is favorable. This observation informed our decision to use neutral buffer in the stimulation experiments (chapter 3). Although many termite gut methanogens utilize hydrogen and $CO_2$ as major substrates for methanogenesis, alternative substrates seem to be important in driving methanogenesis in the guts of higher termites. Methanol appears to be one such substrate driving methanogenesis in the gut of higher termites. Methylotrophic methanogens seems to be present in the hindgut of *Cubitermes*

*ugandensis*, and their activity was greatly improved when $H_2$ was added to the enrichment cultures suggesting hydrogenotrophic nature of gut methylotrophic methanogens. This was later on supported by our potential rate measurements in which methanol stimulated methane production in whole gut and in all major hindgut compartments with increased activities when it was supplemented with $H_2$ (chapter 3). This potentially suggests that most of the hydrogenotrophic methanogens in the gut are highly dependent on other substrates like methanol. The high methane emission in the methanol enrichments and the strong stimulation of methane emission by methanol supplemented with hydrogen, particularly in the posterior gut sections suggest that methylotrophic methanogens may be widespread in the termite guts. This was later supported by detection of methanol concentrations in almost all compartments (chapter 3). Our culture independent studies showed higher abundances of lineages of Methanomicrobiales, Methanobacteriales, Methanoplasmatales, and even Methanosarcinales in the posterior gut, in which methanol showed very strong stimulation, suggesting that many methanogenic lineages may be methylotrophic. Methanosarcinales and Methanoplasmatales comprise methylotrophic lineages, but it is possible that this characteristic is widespread among gut methanogens. $F_{420}$ autofluorescence microscopy of methanol cultures revealed mainly spherical cells, which can now be linked to Methanoplasmatales (see chapter 5), but other rod-shaped cells were also observed in these enrichments. Although our P3 homogenates enriched with methanol-hydrogen substrate mixtures did not grow, possible because of the elevated pH conditions in the culture media, the highest abundance of Methanomicrobiales, the dominant group in the P3 compartment in which exogenous methanol-hydrogen substrates also showed strong stimulation may suggest that some lineages of this group may be methylotrophic. The enrichment of methylotrophic methanogens and stimulation of methanogenesis by substrate mixtures suggest that in guts of termites feeding on highly humified soil diet, methanol, and possibly other substrates may intermediates, resulting from degradation of recalcitrant polyaromatic compounds including lignin, pectin and xylan by the gut microbiota. Substrate combination may be a promising approach for isolation of methylotrophic and many other metabolic groups of gut

methanogens and informed our strategy to test for potential rates of methanogenesis in the gut with exogenous methanol (chapter 3).

The observation of diverse morphotypes in the enrichments and in the gut suggests that gut methanogens may have diverse substrate range than is presently known. Recently, *Methanomassiliicoccus luminyensis*, a methylotrophic methanogen which grow on methanol in presence of hydrogen was isolated from the human stool (Dridi *et al*., 2012). Although this isolate clusters distantly with termite derived lineages, it is the first cultivated representative of the new methanogenic order of Methanoplasmatales in which many termite clones also belong. Paul *et al*. (2012) managed to get a highly enriched methylotrophic culture from the gut of a soil-feeding termite, confirming potential role of methanol in gut methanogenesis. The minimal methane production from our posterior gut homogenates enrichment cultures using formate substrate even after weeks of incubation may be because of competition for the substrate by archaea as well as gut bacterial symbionts.

Microscopic examination of individual gut compartments showed that highest diversity of archaeal morphotypes are localized in the posterior gut (Fig. 6 and 7), and observation that was also confirmed by our culture independent studies (chapter 3). The low diversity observed in the anterior gut may be due to extreme physicochemical conditions in these sections. In the dilated P3 compartment, in which most archaea are localized, the methanogens seems to be localized in the less alkaline P3b section. Our microscopic examination of the P3 compartment revealed high diversity of morphotypes of $F_{420}$ auto-fluorescing cells in the P3b than P3a sections. This was corroborated by our qPCR analysis which showed more than one order of magnitude more archaeal and bacterial 16S rRNA gene copy numbers in the P3b than P3a sections (Fig 8), suggesting the influence of gut alkalinity on microbial colonization of the gut.

It appears that availability of substrates resulting from degradation of termite diet, and the physicochemical conditions in the gut influence colonization of specific gut compartments by methanogenic archaea. Depending on the diet, alternative substrates are potentially responsible for high methane emission rates in soil-feeding than wood-feeding termites. A more optimized enrichment culture conditions, including the strategy of

substrate mixtures to isolate representative gut methanogens may give insights on the physiological characteristics and metabolic roles of higher termite archaeal symbionts.

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## Chapter Seven

# General discussion and future prospects

**James O. Nonoh**

## Diversity of archaeal populations in higher termites

Despite the diversity in taxonomy and feeding behavior, both phylogenetically lower and higher termites emit methane, with current estimates ranging between 2–4% to the global budget of this greenhouse gas (Sugimoto *et al*., 2000). Higher termites (family Termitidae), which have a diverse feeding behavior, generally emit more methane than wood-feeding lower termites (Brauman *et al*., 1992; Bignell *et al*, 1997; Sugimoto *et al*., 1998). It has been demonstrated, that among the higher termites, members of the soil-feeding taxa emit high amounts of methane as compared to members of the other feeding guilds. Nevertheless, all termites investigated to date harbor methanogenic archaea in their hindguts (Brauman *et al*., 1992; Brauman *et al*., 2001) despite the variations in methane emission rates. The reason for the dynamics in methane emission rates is still not clearly understood. The variations suggest differences in archaeal community structure among higher termites. However, only little is known about the diversity and community structure of archaea in higher termites. By using 16S rRNA-based sequencing and qPCR analysis for a comparative study of archaeal communities in the guts of higher termites from different feeding groups, we could show that unlike lower termites, which mainly host members of Methanobacteriales in their hindguts, higher termites host a diverse assemblage of methanogenic Euryarchaeota comprising four orders: Methanobacteriales, Methanosarcinales, Methanomicrobiales and the recently discovered Methanoplasmatales (chapter 2). Highest diversity of archaea was observed in members of the soil-feeding taxa (*Cubitermes*, *Ophiotermes*, *Amitermes* and *Alyscotermes* species), which have a highly compartmented gut and are also among the highest methane emitting

species. Our results agree with previous studies on the soil-feeding termites *Cubitermes orthognathus* (Friedrich *et al*., 2001) and *Cubitermes fungifaber* (Donovan *et al*., 2004) who also observed high archaeal diversity in members of the soil feeding taxa. Our comparative investigation of representatives of the higher termite sub-families, demonstrate that higher termites harbor an assemblage of methanogenic lineages including higher-termite specific phylotypes. In soil-feeding termites, the presence of host-specific lineages suggest co-evolve with the host termite and this may be influenced by the host diet and by the micro-environmental conditions in specific gut micro-habitats. The highly distended and compartmented gut, together with the extreme gut alkalinity in soil-feeding termites may result in compartment specific communities whose colonization of the gut may be influenced by potential alternative substrate intermediates emanating from microbial degradation of recalcitrant polysaccharides present in their complex organic-poor soil diet. This may result in the high diversity of methanogenic archaea in the gut and may influence methane emission rates in soil-feeding taxa than their wood-feeding counterparts. The soil-feeding termites not only harbor methanogenic Euryarchaeota, but they also host uncultured members of other archaeal phyla comprising lineages closely related to aerobic ammonia-oxidizing Thaumarchaeota in the *Nitrososphaera* cluster, which includes *Nitrososphaera gargensis* from thermophilic spring (Hatzenpichler *et al*., 2008) and *Nitrososphaera viennensis* isolated from soil (Tourna *et al*., 2011). The anterior guts of soil-feeding termites which is colonized by Thaumarchaeota experiences high oxygen partial pressures (Brune *et al*., 1995) and low ammonia concentrations, in the lower mili-molar ranges (Pester *et al*., 2007), and therefore offers an ideal habitat for ammonia oxidation. Based on these micro-environmental conditions, it seems reasonable to speculate that these Thaumarchaeota may be involved in ammonia oxidizing activities in termite guts. It would be important to investigate their potential activities by profiling for *amoA* gene transcripts in the gut and by getting cultures to study their physiology. Considering their speculated role in the gut, it would also be important to get some insight on their morphology and to establish whether they are associated with the gut wall or to any structures or are free living in the lumen of the compartments they colonize. The close relationship of termite gut

Thaumarchaeota with lineages from other environments suggests that this group is not specific to the termite gut environment, but may have been horizontally transferred during termite foraging activities. This may also be corroborated by their relatively low abundance (1% of the overall gut archaea) in the gut of *Cubitermes ugandensis* in which they are most abundant, potentially suggesting that they are not yet fully established in the gut. Other than Thaumarchaeota, the higher termite specific group of unclassified archaea loosely affiliated to Crenarchaeota exclusively colonizing the posterior gut compartments (companion paper) of soil feeding termites are very distantly related (less than 80% sequence similarity) to sulfur metabolizing Crenarchaeota in the class Thermoprotei. This group was only recovered in the soil-feeding termites *Cubitermes ugandensis*, *Ophiotermes* sp, *Amitermes* sp. and *Alyscotermes trestus* where they are very abundant (20%, 8%, 51%, and 15% of overall gut archaea respectively). Their role in the termite gut is presently not known, but their high abundance in soil-feeding termites, particularly in *Cubitermes ugandensis* (20% of overall gut archaea) and *Amitermes* sp. (51% of overall gut archaea) suggests a yet to be recognized ecological role in the gut. This uncultured archaeal group was previously recovered in the guts of soil-feeding termite *Cubitermes orthognathus* (Friedrich *et al.*, 2001) and in the litter-feeding *Cornitermes cumulans* (Grieco *et al.*, 2012). The presence of this group only in soil-feeding termites suggests that the host diet greatly influences their establishment in the gut. Termite-derived sequences of this group formed a deep-branching termite-specific cluster distinct from those from other environments, suggesting co-evolution with the host termite. Cultivation of representatives of this group will provide an insight into their functional roles in the gut.

Except for *Microtermes* sp., archaeal diversity in the fungus-cultivating *Macrotermes michaelseni*, the grass-feeding *Trinervitermes* sp., and in the wood-feeding *Microcerotermes* sp., which emit relatively less methane than their soil-feeding counterparts, was much lower. Wood-feeding *Microcerotermes* sp. (Termitinae) were dominated by Methanobacteriales (93% of overall gut archaea) and Methanoplasmatales (7% of overall gut archaea), while grass-feeding *Trinervitermes* sp. (Nasutitermitinae) were dominantly colonized by Methanomicrobiales (55% of overall gut archaea),

Methanobacteriales (26%) and Methanoplasmatales (18%). The fungus cultivating *Macrotermes michaelseni* and *Microtermes* sp. (both Macrotermitinae) were dominantly colonized by members of Methanosarcinales, a phenomenon that was also observed by Brauman *et al*., (2001).

Our analysis revealed four orders of methanogenic Euryarchaeota in higher termites. The presence of Methanoplasmatales, a recently discovered order of methanogenic archaea (Paul *et al*., 2012) in all the higher termites investigated in this study corroborates high diversity of methanogens in higher termites than in lower termites in which the group has not been reported. This group, initially thought to be the non-methanogenic Thermoplasmatales, and previously also recovered in the gut of soil-feeding *Cubitermes orthognathus* (Friedrich *et al*., 2001) comprises methylotrophic lineages and includes the human gut isolate *Methanomassiliicoccus luminyensis* (Dridi *et al*., 2012), the only available cultivated representative of this order, growing on methanol in presence of hydrogen. Members of this order seems to be highly established (3–30% of the overall archaea) in the hindgut of higher termites investigated in this study, with highest abundances in soil-feeding *Cubitermes ugandensis* and *Amitermes* sp. (23% and 30% of overall gut archaea respectively), suggesting it forms an important archaeal microbiota in higher termites. In *Amitermes* sp., Methanoplasmatales were the dominant methanogenic group accounting for 30% of the overall gut archaea, suggesting that they may be the major methanogenic Euryarchaeota in this termite, leading to speculations that they may have a wider substrate range than merely methylotrophic. Egert *et al*. (2003) also reported that lineages of this novel order were highly abundant in *Pachnoda ephippiata* hindgut forming 25% of archaeal 16S rRNA genes in the gut. Since only two clones representing this group have been reported in the wood-feeding lower termite *Reticulitermes speratus* (Shinzato *et al*., 1999), the discovery of methanogenic nature of lineages of the new order of methanogenic Euryarchaeota and their colonization of the guts of all higher termites investigated in this study and in other previous studies helps to explain the high methane emission rates in this species.

The presence of specific cluster of higher termite Euryarchaeal Methanomicrobiales consisting of only clones from higher termites, especially those from soil-feeding *Cubitermes* spp. and grass-feeding *Trinervitermes* sp., suggest their co-evolution with the host termite probably based on evolution in feeding behavior. There was some overlap in higher termite phylotypes of this group, with sequences from soil-feeding *Cubitermes* clustering with those of grass-feeding *Trinervitermes* sp., suggesting similarities in the communities. However, the presence of soil-feeding and Nasutitermitinae-specific sub-clusters suggests a separate co-evolution with the individual host termites. The compartment specific colonization of the posterior gut of higher termites by this group, particularly the P3 compartment of soil-feeding *Cubitermes ugandensis* in which highest densities were detected (companion paper) potentially indicates that the availability of substrates and other micro-environmental conditions creates specialized compartment specific communities. This group seems to be a core archaeal microbiota in the sub-family Nasutitermitinae comprising about 55% of overall gut archaea in *Trinervitermes* sp. Previous investigation in *Nasutitermes takasagoensis* also observed high proportional abundance of Methanomicrobiales representing almost half of the clones from this termite (Ohkuma *et al*., 1999). The loose phylogenetic relationship (88–91% sequence similarity) of this higher termite-specific cluster of Methanomicrobiales to their closest cultivated relative *Methanospirillum hungatei* potentially suggests a novel higher termite-specific family of the order Methanomicrobiales. Currently there is no culture or isolate representing this group from higher termites and their physiology is poorly understood. However, their abundance in the gut of many higher termites particularly in the posterior gut of grass-feeding and soil-feeding termites may lead to speculation of their role in metabolism of substrates resulting from degradation of lignocellulosic diet. It would be important to understand the physiological relevance of this group in the termite gut, particularly in members of Nasutitermitinae in which it is more abundant. However, Methanomicrobiales was not recovered in the wood-feeding *Microcerotermes* sp., the soil-feeding *Ophiotermes* sp., and in the fungus-cultivating *Macrotermes michaelseni*, a phenomenon that seems to be influenced by the nature of host diet rather than the termite taxonomy of feeding guild.

Members of Methanobacteriales seem to be a core archaeal microbiota in termites being present in all higher termites investigated in this study and in previous studies. So far, Methanobacteriales is the only most abundant archaeal group known in lower termites and they may have been transferred vertically to the higher termites from their lower termite counterparts. However, Methanobacteriales lineages from higher termites form a distinct group (93–95% sequence similarity) from that of lower termites demonstrating phylogenetic differences, probably due to separate co-evolution with the higher termite host, a phenomenon that may also be exhibited in their methanogenic potential and may explain the differences in methane emission rates between these two groups of termites. It is possible that the higher termite lineages are adapted to the highly compartmented gut morphology together with its pre-existing physicochemical conditions, including specialization to utilize alternative substrates resulting from metabolism of their complex diet. In most phylotypes, Methanobacteriales lineages from soil-feeding termites in the sub-family Termitinae, clustered together and they formed distinct cluster from that of the soil-feeding Apicotermitinae. However, within Termitinae, clones from *Cubitermes* sp. formed distinct sub-clusters from those of *Ophiotermes* sp. as well as those of *Amitermes* sp., demonstrating that higher termites co-evolve with their archaeal symbionts irrespective of their phylogeny, a phenomenon that may be influenced by the nature of the host diet. However, archaeal communities from wood-feeding Termitinae were different from those of soil-feeding Termitinae suggesting separate host-based co-evolution. Clones from grass-feeding *Trinervitermes* clustered with those of wood-feeding *Nasutitermes* spp. and in some cases, they overlapped with those of soil-feeding Termitinae suggesting a shared origin. Those of fungus-cultivating termites also formed Macrotermitinae-based phylotypes and in most cases they clustered with clones from xylophagous cockroaches, suggesting some form of genetic origin. In the insect clusters, Methanobacteriales lineages from termites overlapped with those of cockroaches, suggesting that they are probably vertically acquired from the ancestors and then co-evolved separately with the host in the process of termite evolution. The higher termite-specific lineages of Methanobacteriales, particularly those in the posterior gut of soil-feeders and the fungus-cultivating termite-specific clusters, may help to explain high

methane emission rates by these termites. The animal rumen phylotype consisting of novel Methanobacteriales lineages from fungus-cultivating *Microtermes* sp. clustering only with sequences from animal rumen, may be a case of horizontal transfer during foraging activities of the termite.

Methanosarcinales recovered only in soil-feeding and fungus cultivating termites, a group of termites associated with high methane emission rates seems to be one of the major methanogenic archaeal microbiota of termites feeding on litter or humified diet. This group has not been clearly documented in lower termites. However, using family level probes targeting the 16S rRNA genes, Brauman *et al.*, (2001) detected members of Methanosarcinaceae in only one species of wood-feeding lower termite *Coptotermes formosanus*. In our study, highest densities of this group were found in the fungus-cultivating termites (81–87% of overall gut archaea) and in soil-feeding termites (26–67% of overall gut archaea) particularly in the alkaline gut compartments P1 and P3 of *Cubitermes* sp. and *Ophiotermes* sp., suggesting that this methanogenic group potentially influences high methane emission rates in these termites. These results agree with those of Brauman *et al.*, (2001), who also detected members of this group mainly in soil-feeding and fungus-cultivating termites. The niche differentiation in the Methanosarcinales colonization of the gut is probably responsible for the distinct phylotypes (95–97% similarity) between those in the alkaline anterior gut and those in the posterior neutrophilic gut compartments. Currently, there is no cultivated representative of this group from the termite gut and therefore their physiology is poorly understood. However, their close relationship with the only cultivated member of this group from insects *Methanomicrococcus blaticola*, a methylotrophic isolate from the cockroach *Periplaneta americana*, potentially suggests methylotrophic nature of the termite derived Methanosarcinales.

Termites host unique lineages of archaea in their guts which are specific to the gut environment and are different from those in their food soil and nest material, and from other methanogenic environments. The variations in the diversity and community composition of archaea in the gut is reflected in the differences in methane emission rates

in higher termites, with the taxa harboring high diversity and density of methanogenic archaea being among the highest methane emitters. The presence of non-methanogenic lineages of Thaumarchaeota and unclassified group loosely affiliated to Crenarchaeota only in the high methane emitting soil-feeding termite species seems to have a yet to be recognized relevance and is a phenomenon worth further investigating. One can only speculate if they are not involved in activities that complement the methanogenic activities in the gut. This is a subject worth further investigating.

## Methanogenic potentials of the gut of soil-feeding termites

Soil-feeding termites, which have the highest rates of methane emission, have a highly compartmented structure with varied morphological and physicochemical conditions. In these termites, methane emission is mainly detected in the posterior gut (Schmitt-Wagner and Brune 1999). These differences suggest that the specific gut micro-habitats colonized by the methanogens shape the methanogenic archaeal communities structure and hence influencing the methane emission potential of the gut and of the host termite. Little is still known about the distribution, diversity and population size variations of archaea in the highly compartmented gut of soil-feeding termites. Also, little is still known about the methanogenic potential, and the exact localization of methanogenic activities in the gut compartments. Our 16S rRNA clone sequencing and qPCR-based comparative analysis of the density and distribution of archaeal communities in the specific gut compartments of soil-feeding *Cubitermes ugandensis* and *Ophiotermes* sp. demonstrated a heterogeneous pattern of archaeal distribution along the gut axis. Our results show that all gut compartments harbor methanogenic archaea albeit in different abundances. Whereas the anterior gut sections harbor low abundance of archaea, most archaea colonize the posterior gut sections in which highest densities are localized. Nevertheless, diverse archaeal groups colonize even the anterior gut sections including the crop. There seems to be a niche differentiation in the archaeal colonization of the major gut compartments, a phenomenon that seems to be influenced not only by the highly dynamic physicochemical characteristics of the gut, but also by availability of substrate gradients

resulting from degradation of diet. In both *Cubitermes ugandensis* and *Ophiotermes* sp., Methanosarcinales preferentially colonize the anterior alkaline gut sections with highest abundances observed in the highly alkaline P1 and P3 gut compartments (7% and 6% of overall gut archaea respectively). These results generally agree with those previously reported in a related soil-feeding *Cubitermes orthognathus* (Friedrich *et al.*, 2001), suggesting that Methanosarcinales are the dominant methanogenic group in the alkaline anterior gut sections, a phenomenon that may lead one to carefully speculate their adaptation to more alkaline conditions in the gut. However, In *Ophiotermes* sp., other than being the only archaeal group recovered in the anterior midgut and P1 compartments (1% and 21% of overall gut archaea), highest density of Methanosarcinales (46% of overall gut archaea) were observed in the posterior P3 compartment in which it was also the most dominant archaeal group. Based on these results, it seems that even members of Methanosarcinales preferentially colonize the comparatively less alkaline P3 compartment, potentially suggesting that Methanosarcinales may also be inhibited by highly alkaline conditions in the gut. But it could also mean that availability of substrate gradients in the P3 compartment may strongly influence its colonization by gut archaea. Our P3 homogenate enrichment cultures set up at a pH of 10.4 (chapter six) produced only minimal amount of methane in the headspace gas, suggesting that the high pH inhibited their growth. This may indicate potential existence of less alkaline micro-habitats even in the highly alkaline P1 gut compartment. The Methanosarcinales in the anterior crop, midgut and P1 compartments, formed distinct phylotypes from those of the posterior gut, demonstrating community differences between the two sections, a phenomenon that may be reflected in the different micro-environmental conditions existing in the two sections. Methanobacteriales and members of the recently discovered order of Methanoplasmatales were heterogeneously distributed in the gut, with highest densities in the posterior P3 and P4 gut compartments. Whereas highest abundance of Methanobacteriales (11% of overall gut archaea) was localized in the P3 compartment, Methanoplasmatales preferentially colonized the P4 gut compartment (14% of overall gut archaea), in which it was also the most dominant group. Methanomicrobiales preferentially colonized the dilated P3 gut compartment (18% of overall gut archaea) in

which it was the most dominant methanogenic Euryarchaeota. Members of the phylum Thaumarchaeota, closely related to ammonia-oxidizing lineages in the genus *Nitrososphaera*, colonized mainly the crop, midgut and rectum of *Cubitermes ugandensis*. Because of their close relationship with known ammonia-oxidizing lineages, and the fact that the anterior gut and rectum in which they colonize also experiences high oxygen partial pressures (Brune *et al*., 1995) as well as lower concentrations of ammonia (Pester *et al*., 2007), one cannot help but speculate if these termite gut Thaumarchaeota are not catalyzing ammonia-oxidizing activities in the gut. Since Thaumarchaeota were the most dominant archaea in the crop, it would be interesting to investigate their exact localization, whether they are attached to tissues at the periphery or are localize in the gut center. Currently, there is no isolate representative of the gut associated Thaumarchaeota and it would be important to get a culture of this group to understand their physiological relevance in the gut. A deep-branching termite specific cluster of unclassified archaea loosely affiliated to Crenarchaeota, whose role in the gut is still unclear, preferentially colonized the posterior gut sections with highest densities (18% of overall gut archaea) in the P3 gut compartment. Previously, this unclassified group was also recovered in the guts of soil-feeding termite *Cubitermes orthognathus* (Friedrich *et al*., 2001) and in the litter-feeding *Cornitermes cumulans* (Grieco *et al*., 2012). Termite derived lineages of this group form a deep-branching termite-specific cluster distinct from those from other environments, suggesting co-evolution with the host termite and may represent a novel group with some yet to be recognized relevance in termite guts. Since the posterior gut, which is preferentially colonized by this group experiences not only high methane concentrations (Schmitt-Wagner and Brune, 1999), but also high ammonia concentrations in the upper milimolar ranges (Pester *et al*., 2007), it is tempting to speculate if this group does not represent extant lineages of Thaumarchaeota with high ammonia concentration threshold. This would make them to be among the gut microbiota actively involved in maintaining the anoxic status of the posterior gut, particularly the P3 compartment in which they are dominant. But it is also possible that this group comprise a yet to be recognized methane-oxidizing lineages in the gut. It would be interesting to get a cultured representative of this group to study their morphology and physiological significance in

the gut as well as the exact compartment micro-habitats where they are localized. Most archaea colonize the P3 gut compartment of *C. ugandensis* in which highest abundance of archaea (60% of overall gut archaea) are localized. This was also the case in the related soil-feeding *Ophiotermes* sp. in which 77% of the overall gut archaea were localized in the highly dilated P3 compartment. Based on the highly alkaline nature of the P3 compartment of soil-feeding termites, particularly in the anterior dilated sections (Brune and Kühl, 1995), and on the fact that our P3 homogenate enrichment cultures grown at elevated pH did not show any sign of growth (chapter six), it seems reasonable to consider that the preferential colonization of the P3 compartment by archaea is a phenomenon probably influenced more by the availability of substrate gradients than gut alkalinity. But it is also likely that most of these archaea may be localized in the less alkaline P3 compartment micro-habitats where the pH is favorable. Interestingly, highest density of bacterial 16S rRNA gene copies ($3.53 \times 10^6$ copies $gut^{-1}$) was also detected in the P3 compartment, potentially suggesting that most fermentation reactions, and thus fermentation intermediates may be localized in this compartment. This may be the reason why most archaea are localized in P3 than in P4 compartment and may explain the strong stimulation of methanogenesis in the latter by all the exogenously supplied substrates. Archaeal lineages in the P3 compartment formed distinct compartment specific communities unique to this specific compartment, with only very little overlap with other posterior compartments, suggesting compartment-based co-evolution. Those of the P4 were also specific to that compartment but showed some little overlap with the P5 compartment, suggesting possibility of shuttling to the neighboring P4 compartment to access substrates or to avoid inhibition by unfavorable physicochemical conditions such as oxygen stress. The communities in the anterior gut were largely similar with overlap only with those of the rectum, reflecting the almost similar micro-environmental conditions in those compartments. Micro-environmental conditions in the specific gut compartments likely influence co-evolution of the archaeal gut microbiota with the host termite.

Our rate measurements in gut compartments showed that community structure is influenced by differences in micro-environmental conditions in the gut, including oxygen

status and availability of methanogenic substrates. By using exogenous substrates to stimulate methanogenesis in different gut compartments, we could show that methanogens in all gut compartments are highly substrate limited. Methanogenic archaea are heterogeneously distributed in the gut, even in compartments previously thought to be non-methanogenic, and this distribution seems to be influenced by the availability of utilizable substrates in gut compartments. The exogenous formate and methanol substrates stimulated methanogenesis in all gut compartments, particularly when the substrates were supplemented with hydrogen, demonstrating the hydrogenotrophic nature of most gut methanogenic archaea. Formate and methanol stimulated methanogenesis even in the anterior gut sections in which exogenous hydrogen showed only minimal effect, suggesting that degradation of organic matter in the soil diet results in availability of alternative intermediates in the gut. Formate seems to be an important substrate for methanogenesis in most gut compartments, particularly in the posterior gut sections in which Methanomicrobiales, Methanobacteriales and Methanoplasmatales were abundant. The high stimulation of methanogenesis by formate in the P3 compartment of the soil-feeding *Cubitermes ugandensis* in which highest density of Methanomicrobiales ($1.30 \times 10^4$ copies $gut^{-1}$) were localized may lead to a speculation of their role in formate metabolism in the gut. Cultured relatives of Methanomicrobiales are hydrogenotrophic methanogens which require formate in their growth media, and they have been isolated from various environments including anaerobic digestors and sediments in which syntrophic bacteria have also been isolated (Sakai *et al.*, 2009). They potentially form important hydrogen sink methanogens in the posterior gut. It is however not clear, whether the activities of Methanomicrobiales contributes to maintaining the anoxic status of the P3 compartment. Given the high abundance of other members of methanogenic archaea in the P3 compartment, it is possible that many methanogenic lineages may be capable of formate oxidation. The high abundance of Methanobacteriales in the P3 compartment in which highest abundance of bacteria are also localized may be a strategic phenomenon to access the substrates either directly or through interspecies substrate transfer. But they may also comprise lineages involved in maintaining the P3 anoxia, in which case they would also form important hydrogen sink organisms in this

compartment. *Methanobrevibacter*-related lineages of Methanobacteriales from termite guts have been shown to be involved in oxygen reduction activities especially at the hindgut periphery of *Reticulitermes flavipes* (Leadbetter and Breznak, 1996). The high abundance of bacterial symbionts in the P3 compartment may be responsible for the detectable hydrogen concentrations in this compartment, but they may also be involved in hydrogen consumption via oxygen reduction activities. Boga *et al.* (2003) isolated a homoacetogenic oxygen-reducing Firmicute *Sporomusa aerivorans* from the posterior P3 compartment of the soil-feeding *Thoracotermes macrothorax*. The strong stimulation by methanol in the P3 compartment may be attributed to the Methanoplasmatales and Methanosarcinales which are also abundant in this compartment and whose members comprise methylotrophic lineages. But it is also possible that some Methanomicrobiales and *Methanobrevibacter* lineages posses yet to be recognized methylotrophic capabilities. The strong potential rates of methanogenesis with formate than any other substrate in all gut compartments even without exogenous hydrogen supplement potentially projects formate as an important intermediate in the fermentation reactions in the gut, but which is in limiting supply due to its utilization by most gut archaea, and that some methanogens may be capable of oxidizing formate for growth. Pester and Brune (2007) observed that when labeled formate was injected to the hindgut of the lower wood-feeding termite *Cryptotermes secundus* (Kalotermitidae), it was rapidly converted to acetate and $CO_2$ with half of it oxidized to $CO_2$ while 30% to acetate. Their finding suggested that not all formate is converted to acetate and $CO_2$, and gives room to speculate that some methanogens potentially derive their energy by oxidizing formate to $CO_2$ and methane. Methanogens would probably oxidize formate if they have formate dehydrogenases but lack hydrogenases. Such methanogens may then use the reducing equivalents from formate oxidation to reduce $CO_2$ to methane, but may also create avenues for hydrogenotrophic methanogens to reduce the formed $CO_2$ to methane in the presence of hydrogen. Currently, there is no isolate known to grow purely on formate, as even all the available *Methanobrevibacter* isolates from the lower termite grows only poorly on the substrate. It would be important to isolate termite gut related formate utilizing methanogens in order to study their physiological characteristics and to establish their

functional roles in the gut. Methanol, an apparently important substrate for gut methanogens, stimulated methanogenesis in all gut compartments including the anterior sections. However, its utilization in most gut compartments is highly dependent on hydrogen, demonstrating hydrogenotrophic nature of methanol utilizers. The strong stimulation of methanogenesis by methanol in the posterior gut, particularly in the P4 compartments in which Methanoplasmatales was the most dominant group demonstrates methylotrophic nature of lineages in the new order of methanogenic Euryarchaeota. Their requirement for hydrogen for reduction of methanol places them as potential hydrogen sink organisms in the gut. But the strong stimulation by exogenous formate in the same compartment may also suggest that Methanoplasmatales comprise some formate utilizing lineages. Interestingly, the only isolated representative of Methanoplasmatales *Methanomassiliicoccus luminyensis* isolated from human feces (Dridi *et al*., 2012) is methylotrophic, growing on methanol only in presence of hydrogen and it requires formate in its growth media. The high abundance of Methanobacteriales in the P4 compartment in which no known methanol utilizer other than Methanoplasmatales was recovered may indicate that unidentified novel methylotrophic lineages of *Methanobrevibacter*, the only genus representative of Methanobacteriales recovered from termites, potentially colonize the gut of soil-feeding termites. Our results provide a direct link of methanol as a potential substrate for gut methanogenesis and agree with previous speculations that it may be an intermediate in fermentation reactions in the gut of termites (Schmitt-Wagner and Brune, 1999) as well as humivorous arthropods (Lemke *et al*., 2003; Egert *et al*., 2003). Because soil-feeding termites consume humified diet consisting of decaying plant debris, methanol may be derived from recalcitrant methoxylated aromatics that may still find its way into the soil diet. A possible sources of methanol in the termite gut may be pectin, a polysaccharide in form of α (1-4) linked D-galacturonic acid which forms a substantial portion of middle lamella of plant cell walls, and whose de-esterification with pectin methyl esterases yield methanol (Harholt *et al*., 2010). Such methyl esterases may be produced by microbial symbionts easily acquired from the soil diet. Because hemicelluloses are difficult to degrade than celluloses, xylan, major hemicelluloses in the cell wall of plants may also still be present in the soil diet, and

provides another potential source of methanol in the gut. Most gut compartments seem to host hydrogenotrophic methanogens which are highly dependent on methanol and formate. The phylotype separation between the archaeal lineages in the anterior gut and the posterior gut demonstrate community differences between these two sections, a phenomenon that may be influenced by the prevailing micro-environmental conditions in the specific gut compartments, creating specialist methanogenic groups and this may explain the high methanogenic potential in the posterior gut as compared to the anterior gut sections. Highest rates in the soil-feeding taxa suggest that differences in the diet may influence methane emission rates.

## Methanoplasmatales is a new order of methanogens

A deep branching lineage of Euryarchaeota, then loosely associated with uncultured Thermoplasmatales, was previously recovered in the gut of soil-feeding termite *Cubitermes orthognathus* (Friedrich *et al*., 2001), in which high abundance of this group was mainly found in the posterior gut sections. The uncultured group was later recovered in the guts of xylophagous cockroaches *Panesthia angustipennis* and *Salganea taiwanensis* (Hara *et al*., 2002) and in many other insects. In the gut of the humus-feeding beetle larva *Pachnoda ephippiata*, Egert *et al*. (2003) observed that this deep-branching group was highly abundant, forming about 20% of archaea in the gut. Members of this archaeal lineage were also recovered from the whole gut of grass-feeding *Nasutitermes takasagoensis* (Miyata *et al*., 2007). Termite gut derived lineages of this uncultured group formed a deep-branching clade which comprised sequences from guts of other insects, as well as clones from cow rumen (Wright *et al*., 2007), sheep rumen (Wright *et al*., 2004), rumen of Tammar wallaby *Macropus eugenii* (Evans *et al*., 2009) and human stool clones (Mihajlovski *et al*., 2010). This clade also comprised clones form other methanogenic environments like sludge digesters (Hatamoto *et al*., 2007), landfills (Huang *et al*., 2002), rice paddy soils (Chin *et al*., 1999), among others. This deep-branching group of archaea was initially thought to represent the non-methanogenic Thermoplasmatales. However, phylogenetic analysis constantly showed that this group formed a distinct cluster of

Euryarchaeota radiating away from the non-methanogenic Thermoplasmatales. Lack of cultures representing this deep-branching clade of euryarchaeota, continued to limit the understanding of their physiology and it was difficult to predict their functional position in the gut. Furthermore, previous investigations of their presence in termites covered only a few termites species and therefore it could not be generalized that the group formed a common microbiota in the guts of higher termites. Also, their population density in the gut of higher termites remained uninvestigated and therefore it could not be elucidated whether they exist in large enough numbers to influence any ecological function *in situ*. In our study, this deep-branching clade of Euryarchaeota, present in the gut of higher termites (3–32% of overall gut archaea), and other environments, has been demonstrated to represent a new order of methanogens for which the name 'Methanoplasmatales' has been proposed. Our 16S rRNA gene sequencing combined with qPCR analysis, together with *mcrA* signatures of archaeal communities in representative species of higher termite sub-families demonstrate that this group of archaea is well established in the guts of termites, being present in all higher termites investigated by us, and in previous investigations. Both 16SrRNA and mcrA trees showed a lot of congruence in major lineages of the group, demonstrating common evolutionary patterns. Except for two clones recovered from a colony of the lower wood-feeding termite *Reticulitermes speratus* (Shinzato *et al.*, 1999), this new order of methanogenic Euryarchaeota, which is highly abundant in some termites, especially in soil-feeding *Cubitermes ugandensis* and *Amitermes* sp. (22% and 32% of overall gut archaea respectively), has so far not been reported in lower termites. The first evidence on the methanogenic nature of this group was provided by the first pure cultured representative of this group *Methanomassiliicoccus luminyensis*, a methylotrophic methanogen which grows on methanol in presence of hydrogen, isolated from the human feces (Dridi *et al.*, 2012). Paul *et al.*, (2012) also successfully managed to bring to culture the first termite gut representative of this new methanogenic group strain MpT1, a methylotrophic methanogen growing on methanol in presence of hydrogen, demonstrating that lineages of this group of archaea are methanogenic and representing a new order of methanogenic Euryarchaeota. The availability of methanogenic cultured representatives of the deep-

branching group of Euryarchaeota and phylogenetic analysis of sequence representatives from the guts of termites and other insects as well as sequences from animal rumen and various other environments provides proof that the group represents a new order of methanogenic Euryarchaeota for which the order 'Methanoplasmatales,' has been proposed (Paul *et al*., 2012). The discovery of this new order of methanogenic archaea, with high abundance in higher termites may help to explain the high methane emission rates observed in higher termites than in their lower termite counterparts. In *Cubitermes ugandensis*, Methanoplasmatales was recovered in all the gut compartments, further providing indication that the methanogenic nature by almost all gut compartments may be due to presence of novel methanogenic groups. In *Amitermes* sp. (Termitinae), Methanoplasmatales was the most dominant methanogenic Euryarchaeota, with presence in all the gut compartments, suggesting that members of this group may be very diverse in terms of substrate range, besides also being able to adapt to varying physicochemical conditions in the gut. Phylogenetic analysis revealed two major groups of Methanoplasmatales from the termite gut; one consisting mainly of clones from the posterior gut while the other consisting of clones from both anterior and posterior gut. Understanding the methanogenic potential of each of these groups will help in estimating their physiological relevance to the host termite and their methanogenic potential. Description of more representatives of this novel group of Methanoplasmatales will be critical to understanding their functional roles in termite guts and in other environments where they are a common microbiota. A termite gut isolate would provide a major milestone in understanding metabolic capacities of this group. In view of the fact that the strongest potential rates of methanogenesis with exogenous formate was observed in the posterior P4 gut compartment of *Cubitermes ugandensis* in which highest abundance of Methanoplasmatales (12% of overall gut archaea) was localized, and in which they form the most dominant archaeal group, it is possible that some lineages of Methanoplasmatales potentially posses formate oxidizing capabilities. Formate oxidizing methanogenic isolates are currently unknown and our understanding of this aspect is still primarily speculative. Bringing such methanogens to culture is of interest if we are to understand gut microbiota dependent metabolisms. Knowledge of the physiology of this

new order of methanogens will give new insights in their metabolism and hence functional roles in the gut.

## Prokaryotic community structure in *Amitermes* sp.

Previously, little was known about the prokaryotic community composition of members of the *Amitermes* group (Termitinae). Other than previous studies on the wood-feeding *Microcerotermes* sp. (Deevong *et al*., 2004) and the litter-feeding *Cornitermes cumulans* (Grieco *et al*., 2012), the soil-feeding members of this group have been largely uninvestigated. Information on the physicochemical conditions in the gut environment was completely lacking, yet this is important for understanding the functional ecology of the gut microbiota. In this study, we collected a soil-feeding *Amitermes* sp., whose colony had a highly reduced number of soldiers, from subterranean tunnels near the grass-feeding *Trinervitermes* sp. mound and investigated the prokaryotic community composition and their distribution relative to the *in situ* physicochemical characteristics of the gut. The unusually highly alkaline compartmented gut of *Amitermes* sp. with no gut section recording pH values below six seems to greatly influence the structure of the prokaryotic community and their distribution along the gut axis. Like in the other soil-feeding Termitinae, the gut hosts high diversity of methanogenic Euryarchaeota comprising Methanosarcinales, Methanomicrobiales, Methanobacteriales and the recently discovered order of Methanoplasmatales, besides also hosting members of other archaeal phyla including lineages closely related to ammonia-oxidizing Thaumarchaeota and deep-branching archaeal group loosely affiliated to Crenarchaeota. There was high diversity of bacteria recovered in the gut of this termite with major phyla comprising Firmicutes, Spirochetes, Bacteroidetes, Actinobacteria and Proteobacteria being represented. Firmicutes were the dominant phylum comprising over 70% of the bacteria in the gut. However, a significant proportion of bacteria (8% of the overall gut bacteria) remained unrepresented because they were below the 0.1% relative abundance threshold in any of the compartments of *Amitermes* sp. There was also a significant proportion of unclassified bacteria (4% of overall gut bacteria) distributed throughout the gut, with

highest abundances in the posterior P3 compartment. This unclassified group potentially represents uncultured bacteria. The size variation patterns along the gut axis suggest that distribution and localization of prokaryotic communities in the gut is influenced by the environmental conditions *in situ*.

Unlike *Cubitermes* sp., all the gut compartments of this termite, including the more tubular oxygen diffusing midgut and posterior gut sections as well as the highly alkaline P1 compartments are predominantly colonized by members of archaeal lineage loosely affiliated to Crenarchaeota (51% of overall gut archaea) and Methanoplasmatales with (30% of overall gut archaea). This suggests that the unclassified archaea may be a robust group whose role in the gut may be widespread in most compartments. Only the crop was exclusively colonized by lineages closely related to ammonia-oxidizing Thaumarchaeota. While members of Methanoplasmatales are Euryarchaeotic methanogens which comprise methylotrophic lineages highly dependent on hydrogen for their metabolism (Dridi *et al*., 2012; Paul *et al*., 2012), little is known about the unclassified archaeal group loosely affiliated to Crenarchaeota. However, since the group form the most dominant archaea in this termite including tubular sections in which oxygen partial pressures are high, it is possible that the unclassified group of archaea may have aerobic form of metabolism, thereby keeping oxygen levels under control in the gut, or they have mechanisms for withstanding the oxygen diffusing into the gut, probably by oxygen detoxifying mechanisms, a phenomenon that was previously observed in *Reticulitermes flavipes* with the members of *Methanobrevibacter* sp. (Leadbetter and Breznak, 1996). The predominance of Methanoplasmatales and the unclassified archaeal group also in the highly alkaline anterior gut sections, potentially suggest their capacity to withstand the highly alkaline conditions in the gut, a phenomenon that was previously observed with Methanosarcinales in soil-feeding *Cubitermes* spp. (Friedrich *et al*., 2001, Nonoh *et al*., companion paper). However, unlike in *Cubitermes ugandensis* in which most archaea are localized in the highly dilated P3 compartment (Nonoh *et al*., companion paper), majority of the archaeal populations in *Amitermes* sp. seems to colonize the posterior P4 gut compartment which has a mildly alkaline pH conditions, and in which highest archaeal density (87% of overall gut archaea) were localized, suggesting that most archaea may be

sensitive to highly alkaline conditions. On the contrary, low archaeal abundance was detected in the highly dilated posterior P3 gut section (3% of overall gut archaea) in which the pH was uniformly highly alkaline (pH 10). Methanoplasmatales was the most abundant methanogenic archaea in these two neighboring compartments forming 2% and 23% of the overall gut archaea in the P3 and P4 compartments respectively. The high abundance of archaea in the tubular P4 than in the P3 compartment may be attributed to the unusually very high alkalinity in the latter, which remained uniformly high throughout the compartment, and which may be inhibitive to archaeal colonization. This suggests that in this termite, gut alkalinity seems to play a central role in shaping archaeal distribution along the gut axis. However, highest abundance of bacteria in the dilated alkaline P3 compartment (44% of the overall gut bacteria) may be an indication that most fermentation reactions likely occur in the dilated P3 compartment and that bacteria are more adapted to the highly alkaline conditions than their archaeal counterparts. The high abundance of Firmicutes in the P3 compartment especially members of the family Ruminococcaceae and Lachnospiraceae (26% and 2% of overall gut bacteria) which have been implicated in degradation of sugars in termite guts, suggest that these lineages are potentially involved in fermentation of polysaccharides in this compartment and may be responsible for the hydrogen concentrations detected in the P3 compartment. The Spirochetes and termite cluster of Fibrobacteres which formed substantial proportion of bacteria in the P3 compartment, have been associated with high activities of glycosyl hydrolases, key enzymes in degradation of sugars in termite guts (Köhler *et al*., 2012). Owing to the low archaeal abundance in the P3 compartment, the low hydrogen levels are most likely maintained by bacterial metabolism. The Spirochetes have previously been implicated in reductive acetogenesis reducing $CO_2$ with hydrogen thus forming important hydrogen sink in *Nasutitermes* sp. (Köhler *et al*., 2012). Because the dilated P3 compartments is potentially the fermentation chamber, one would expect then, that most archaeal cells would align themselves more towards the P3 compartment where they may access the substrates with ease, but at the same time avoid the inhibitive effects of the high alkalinity and oxygen stress. Nevertheless, in the P4 compartment in which most archaea were localized, there was also high proportion of bacteria with density of 1.99 ×

$10^6$ copies $gut^{-1}$ (23% of overall gut bacteria), with members of the family Ruminococcaceae and Lachnospiraceae being the most dominant followed by Spirochetes. This suggests that members of these families may be responsible for some fermentation reactions in this tubular compartment, the products of which may potentially support the high archaeal abundance. Since some members of Actinobacteria are known to be gut wall associated where they play a role in oxygen reduction activities, it is possible that members of the family Coriobacteriaceae (1% of overall gut bacteria) present in the tubular P4 compartment are probably also involved in oxygen reduction, making it favorable for the archaeal inhabitants. It is also possible that some lineages of Methanoplasmatales and Methanosarcinales, which are dominant in the P4 compartment, as well as members of Methanomicrobiales and Methanobacteriales may possess oxygen removing mechanisms. This role has previously been linked only to gut wall-associated members of *Methanobrevibacter* sp. in the wood-feeding lower termite *Reticulitermes flavipes* (Leadbetter and Breznak, 1996). In previous investigations on related soil-feeding termites *Cubitermes orthognathus* and *Cubitermes ugandensis* (both *Cubitermes* group-Termitinae), the posterior gut were demonstrated to be more methanogenic than the anterior gut (Schmitt-Wagner and Brune, 1999; Nonoh *et al*, in preparation). If that pattern was to be assumed for *Amitermes* sp., then it would follow that Methanoplasmatales, which is the most dominant methanogenic Euryarchaeota (32% of overall gut archaea) in the both the anterior and posterior gut with highest abundance (23% of the overall gut archaea) in the P4 compartment, may be a core methanogenic Euryarchaeota in the gut and together with Methanosarcinales (10% of overall gut archaea), Methanomicrobiales (4%) and Methanobacteriales (3%) are responsible for most methanogenic activity, especially in the posterior gut compartments in which they are most abundant. Although Methanoplasmatales were also the major euryarchaeote in the alkaline P1 compartment in which highest hydrogen partial pressures was detected, their high abundance in the posterior gut compartments in which hydrogen was not detected may suggest that they are either actively involved in hydrogen consumption or utilize substrates other than hydrogen. Considering that close relatives of *Amitermes* gut Methanoplasmatales including an enrichment culture MpT1 (Paul *et al*., 2012) and a pure

isolate *Methanomassiliicoccus luminyensis* (Dridi *et al*., 2012) have been demonstrated to be methylotrophic, growing on methanol only in presence of hydrogen, it seems reasonable to speculate the role of methanol in driving methanogenesis in the gut of this termite as well. The predominant colonization of almost all gut compartments, including the highly alkaline P1 compartment in *Amitermes* sp by unclassified members of archaea loosely affiliated to the Crenarchaeota is a very striking observation in this termite given that the functional roles of this group of archaea which have so far been detected only in soil feeding termites, still remains largely unclear. This is further limited by the fact that currently no representative of this unclassified termite gut archaea has been brought to culture. In spite of this unusual pattern, it will be important to get cultures of representatives of the dominant archaeal members in order to understand their physiology in the gut. It will also be necessary to investigate methane emission rates in this termite in order to estimate the potential contribution to methanogenesis by the most dominant methanogenic euryarchaeota in the gut. In view of the high abundance of archaea in the P4 compartment, it would be important to localize methanogenic potential of the gut compartments. A large proportion of bacteria in the gut of this termite (4% of the overall gut bacteria) remains unclassified, suggesting that they belong to unrecognized group. This is because they are uncultured and therefore their role in the gut remains unclear. It would be important to get representatives of this group to culture, particularly in the posterior P3 compartment where they are more dominant, so that their physiology can be understood.

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## Summary

Phylogenetically higher termites emit higher amounts of methane than lower termites, but the reason for this phenomenon has not been clear. Our comparative study based on 16S rRNA gene sequencing and qPCR analysis of archaeal communities in the guts of higher termites revealed that unlike the lower termites, which host mainly members of Methanobacteriales in their guts, higher termites host a diverse assemblage of methanogenic euryarchaeota comprising representatives of four major orders: Methanobacteriales, Methanosarcinales, Methanomicrobiales and the recently discovered Methanoplasmatales. 16S rRNA-based diversity of archaea was highest in soil-feeding taxa, where nearly all major archaeal groups were represented. Besides the euryarchaeotal lineages, the gut contained also lineages closely related to ammonia-oxidizing Thaumarchaeota and a deep-branching termite specific group of uncultured archaea loosely affiliated to Crenarchaeota. Archaeal diversity in the fungus-cultivating *Macrotermes* species, in the grass-feeding *Trinervitermes* sp., and in the wood-feeding *Microcerotermes* sp., which show low methane emission rates, was much lower. These results show the high methane emission rates in higher termites is reflected in the high diversity, density and complex community structure of archaea in the termite hindguts. Higher termites host gut-specific archaeal communities different from those of lower termites and from other environments and these communities seem to co-evolve with the host termite probably with shift in feeding behavior. Higher termites harbor archaeal lineages which are specific to their gut environment and are different from communities from lower termites and from other environments.

Methanogenic archaea are heterogeneously distributed in the highly compartmented gut. Density and diversity of archaea was highest in posterior gut compartments, which also harbored most of the methanogenic activities. The highly alkaline anterior gut compartments were preferentially colonized by Methanosarcinales. Archaeal community structure differed strongly among gut compartments, with communities in the more methanogenic posterior gut sections being distinct from those of the anterior sections, a

phenomenon that is reflected in the different micro-environmental conditions in the compartments. Experimental stimulation of methanogenesis in isolated gut sections of soil-feeding termites revealed significant activities of hydrogenotrophic methanogens that are obligately dependent on methanol and formate. Our results suggest that community structure in the different microhabitats is shaped by exogenous factors, such as pH, oxygen status and the availability of methanogenic substrates.

The recently discovered Methanoplasmatales are the seventh order of methanogenic euryarchaeota comprising methylotrophic lineages which colonizes higher termite guts and various other environments, and helps to explain the high methane emission rates in higher termites. The methylotrophic nature of termite-derived lineage demonstrates that substrates other than hydrogen drive methanogenesis in higher termites.

# Zusammenfassung

Höhere Termiten emittieren größere Mengen an Methan als niedere Termiten, die Ursache für dieses Phänomen ist jedoch noch unklar. Unsere Vergleichsstudie zeigte, basierend auf der Sequenzierungen des 16S-rRNA-Gens und qPCR-Analysen von archeallen Gemeinschaften im Verdauungstrakt höherer Termiten, dass im Gegensatz zu den niederen Termiten, welche in ihrem Verdauungstrakt hauptsächlich Methanobacteriales beherbergen, der Darm von höheren Termiten durch eine größere Diversität von methanogenen Euryarchaeota besiedelt ist, die durch Vertretern von folgenden vier Ordnungen dominiert wird: Methanobacteriales, Methanosarcinales, Methanomicrobiales, sowie die kürzlich entdeckten Methanoplasmatales. Die 16S-rRNA-basierte Diversität von Archaeen war in Humus-fressenden Taxa am höchsten, hier waren fast alle archaellen Hauptgruppen vertreten. Neben den euryarchaeotischen Abstammungslinien beinhaltete der Darm auch nahe Verwandte der Ammonium-oxidierenden Thaumarchaeota, sowie eine weit verzweigte, Termiten-spezifische Gruppe bisher unkultivierter, entfernt mit Crenarchaeota verwandter Archaeen. Die archaeelle Diversität in den Pilz-kultivierenden *Macrotermes* sp., den Gras-fressenden *Trinervitermes sp.* und den Holz-fressenden *Microcerotermes* sp., welche eine niedrigere Methanemissionsraten aufweisen, war deutlich geringer. Die hohe Diversität, Dichte und komplexe Gemeinschaftsstruktur der Archaeen im Termitendarm spiegelt die hohen Methan-Emissionsraten in den höheren Termiten wider. Höhere Termiten beherbergen Darm-spezifische archaelle Gemeinschaften, die sich von denen in niederen Termiten und anderen Ökosystemen unterscheiden; diese Gemeinschaften scheinen mit der Wirts-Termite co-evolviert zu sein, abhängig von einem veränderten Fressverhalten. Höhere Termiten besitzen Abstammungslinien von Archaeen, die spezifisch für ihr Darm-Milieu sind, und sich von denen von niederen Termiten und anderen Milieus unterscheiden.

Methanogene Archaeen sind in dem stark unterteilten Darm unterschiedlich verteilt. Dichte und Diversität der Archaeen war in den hinteren Darmabschnitten am höchsten, hier war auch die höchste methanogene Aktivität zu verzeichnen. Die stark alkalischen

vorderen Darmabschnitte waren vor allem von Methanosarcinales besiedelt. Die archaeelle Gemeinschaftsstruktur unterschied sich sehr deutlich zwischen den einzelnen Darmabschnitten. Hierbei unterschieden sich die Gemeinschaften in den stärker methanogenen hinteren Abschnitten deutlich von denen der vorderen Abschnitte. Ein Phänomen welches die verschiedenen Mikro-Umwelt-Bedingungen der Darmabschnitte reflektiert. Experimentelle Stimulation der Methanogenese in isolierten Darmbereichen von Humus-fressenden Termiten zeigte signifikante Aktivitäten von hydrogenotrophen Methanogenen, welche Methanol und Formiat abhängig sind. Unsere Ergebnisse legen nahe, dass die Gemeinschaftsstruktur in den verschiedenen Mikrohabitaten von exogenen Faktoren wie pH, Sauerstoffversorgung und der Versorgung mit methanogenen Substraten abhängt.

Die kürzlich entdeckten Methanoplasmatales stellen die siebte Ordnung von methanogenen Euryarchaeota dar. Alle bisher charakterisierten Vertreter dieser Abstammungslinie wachsen methylotroph und kolonisieren den Darm höherer Termiten, sowie verschiedener anderer Umgebungen. Dies demonstriert, dass höhere Termiten eine diverse Sammlung von Methanogenen beherbergen. Der methylotrophe Charakter der aus Termiten angereicherten Linie dieser Gruppe zeigt, dass die Methanogenese in Termiten neben Wasserstoff auch durch andere Substrate gefördert wird.

# Publications by the author

### Published

Paul, K., **Nonoh, J.O.,** Mikulski, L., Brune, A. (2012). "*Methanoplasmatales*," Thermoplasmatales-related archaea in termite guts and other environments, are the seventh order of methanogens. Appl. Environ. Microbiol. **78:** 8245–8253.

**Nonoh J.O**., Lwande, W., Masiga, D., Herrmann, R., Presnail, J.K., Schepers, E., Okech, M.A., Mungai, P., Nyende, A.B. and Boga, H.I. (2010). Isolation and characterization of Streptomyces species with antifungal activity from selected National Parks in Kenya. *Afric. J. Microbiol. Res*. **4:** 856–864.

### In preparation

**Nonoh, J.O.,** Paul, K., Boga, H.I., Köhler, T., Meuser, K., Kamanda, D.K. and Brune, A. Diversity and community structure of archaeal populations in the gut of higher termites (Blattodea: Termitidae).

**Nonoh, J.O.,** Dietrich, C., and Brune, A. Distribution of archaeal populations and methanogenic potentials in the highly compartmented gut of soil-feeding termites (*Cubitermes ugandensis* and *Ophiotermes* sp.).

**Nonoh, J.O.,** Dietrich, C., and Brune, A. Microenvironment and community structure of Archaea and Bacteria in the highly compartmented gut of *Amitermes* sp. (Isoptera: Termitidae).

# Curriculum vitae

**Personal data**

| | |
|---|---|
| *Name* | James Oluoch Nonoh |
| *Sex* | Male |
| *Date of birth* | 22nd August, 1976 |
| *Place of birth* | Gem District, Siaya County, Kenya |
| *Marital status* | Married, with two sons |

**Education**

| | |
|---|---|
| *2009–2013* | Doctorate (Microbial Ecology), Philipps University, Marburg, Germany |
| *2005–2008* | Master of Science (Microbiology), Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya |
| *1997–2000* | Bachelor of Science (Biological Sciences), Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya |
| *1991–1995* | Kenya Certificate of Secondary Education (KCSE), Ndiru Secondary School, Kisumu County, Kenya |
| *1983–1991* | Kenya Certificate of Primary Education (KCPE), Malele Primary School, Siaya County, Kenya |

**Fellowships**

| | |
|---|---|
| *2009–2013* | Deutscher Akademischer Austausch Dienst (DAAD) |
| *2005–2008* | Dissertation Research Internship Programme (DRIP-ICIPE) |