Diversity, ultrastructure, and comparative genomics of "*Methanoplasmatales*", the seventh order of methanogens

Dissertation

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Summary

Methanogenic archaea are strict anaerobes that occur in diverse environments like marine and freshwater sediments, soils, hot springs, sewage sludge and the digestive tracts of animals and humans. Methanogens belong to the phylum *Euryarchaeota*, which comprises both methanogenic and non-methanogenic orders and many lineages of uncultivated archaea with unknown properties. By a comprehensive phylogenetic analysis, we connected the 16S rRNA gene sequences of one of these deep-branching lineages, distantly related to *Thermoplasmatales*, to a large clade of unknown *mcrA* gene sequences, a functional marker for methanogenesis. The analysis suggested that both genes stem from the same organism, indicating the methanogenic nature of this group. This was further confirmed by our two highly enriched cultures of methanogenic archaea, *Candidatus* Methanoplasma termitum strain MpT1 from a higher termite and strain MpM2 from the millipede gut, which had 16S rRNA genes that fell within in this lineage. Together with the recent isolation of *Methanomassiliicoccus luminyensis* from human feces, the results of our study supported that the entire lineage, distantly related to the *Thermoplasmatales*, represents the seventh order of methanogens, the "*Methanoplasmatales*" (now referred to as *Methanomassiliicoccales*).

To gain deeper insight into this novel order of methanogens, we sequenced and analyzed the genome of *Ca.* Mp. termitum strain MpT1, and compared it to the three other genomes of the order *Methanomassiliicoccales* available to date. Our results confirmed that all members of the lineage are obligately hydrogen-dependent methylotrophs that perform methanogenesis by the hydrogen-dependent reduction of methanol or methylamines and lack the entire C1 pathway for reduction CO_2 to CH_4. However, this raises questions concerning the mechanism of energy conservation that had so far escaped attention. Our comparative analysis revealed that energy conversion in *Methanomassiliicoccales* differs from those of other obligately hydrogen-dependent methylotrophs. We identified a complex encoded by all four genomes that is related to the membrane-bound F_{420}:methanophenazine oxidoreductase (Fpo) of *Methanosarcinales*, but lacks the F_{420}-oxidzing module, as in the apparently ferredoxin-dependent Fpo-like homolog in *Methanosaeta thermophila*. We suggests that this Fpo-like complex of the *Methanomassiliicoccales* uses the

present D subunit of the heterodisulfide reductase as an electron acceptor to form an energy-converting ferredoxin:heterodisulfide oxidoreductase. This suggests that in *Methanomassiliicoccales,* the heterodisulfide serves two functions: the production of reduced ferredoxin during electron bifurcation at the cytoplasmic MvhADG/HdrABC complex, and the generation of a membrane portential during the reoxidation of ferredoxin via a membrane-bound electron transport chain. This dual function of heterodisulfide may be a unique characteristic of the entire order. Furthermore, we identified an unusual two-membrane system in *Ca.* Mp. termitum and strain MpM2 by transmission electron micrographs that might be typical for the complete order.

While methanogenesis in insect guts has been investigated by numerous authors, almost nothing is known about methanogenesis and the methanogenic community structure in millipedes, the only other group of arthropods that emit methane. Our analysis of the phylogenetic diversity of archaea associated with tropical millipedes documented that most methanogens in their guts fall into the orders *Methanobacteriales*, *Methanosarcinales, Methanomicrobiales* and *Methano-massiliicoccales*. Their close relatedness to methanogens from the guts of termites, cockroaches and scarab beetle larvae suggests that methanogenic community structure in methane-emitting arthropods is not necessarily shaped by cospeciation. Recently, it has been shown that bacterial communities mirror major events in the evolutionary history of the termites and cockroaches, which leads to the speculation if this is also case for the archaeal community. Here, we present a study that consists of both clone libraries and high-throughput sequencing which concludes that the archaeal community structure and phylogeny is shaped more by the major host groups than by coevolution and diet. This indicates that the host habitat is the major driving force for the selection of the archaeal community.

Zusammenfassung

Methanogene Archaeen sind strikte Anaerobier, die in unterschiedlichen Umgebungen vorkommen, wie zum Beispiel in marinen und süßwasser Sedimenten, in Boden, in heißen Quellen, im Klärschlamm und im Intestinaltrakt von Tieren und Menschen. Methanogene gehören zum Phylum der *Euryarchaeota*, diese umfassen unter anderem methanogene und nicht-methanogene Ordnungen, sowie viele Abstammungslinien von unkultivierten Archaeen mit unbekannten Eigenschaften. Durch eine umfassende phylogenetische Analyse konnten wir 16S-rRNA-Sequenzen einen dieser tiefabzweigenden Ordnungen, welche weitläufig mit den *Thermoplasmatales* verwandt ist („unkultivierte *Thermoplasmtales*“), einer großen Gruppe von unbekannten *mcrA*-Sequenzen zuordnen. Das *mcrA*-Gen wird als funktioneller Marker für Methanogenese verwendet. Unsere Analyse deutete an, dass beide Gene vom selben Organismus stammen, was darauf schließen lässt, dass es sich bei der Gruppe um methanogene Archaeen handelt. Dieses Ergebnis wurde durch zwei hoch angereicherte methanogene Kulturen unterstützt, *Candidatus* Methanoplasma termitum Stamm MpT1 aus dem Darm einer höheren Termite und Stamm MpM2 aus dem Tausendfüßlerdarm, deren 16S-rRNA-Sequenzen in die selbe Abstammungslinie fielen. Die vor kurzem durchgeführte Isolierung von *Methanomassiliicoccus luminyensis* aus menschlichen Fäzes unterstützt unser Ergebnis, dass die gesamte Abstammungsline der „unkultivierte *Thermoplasmtales*“, die siebte Ordnung von Methanogenen repräsentiert, für die wir den vorläufigen Namen „*Methanoplasmatales*“ vorgeschlagen haben (mittlerweile wurde diese Gruppe in *Methanomassiliicoccales* unbenannt).

Um einen tieferen Einblick in diese neue Ordnung von Methanogenen zu erhalten, wurde das Genom von *Ca.* Mp. termitum sequenziert und analysiert. Anschließend wurden die Ergebnisse mit drei weiteren Genomen der Ordnung *Methanomassiliicoccales* verglichen, welche bisher zur Verfügung stehen. Unsere Analyse bestätigte, dass es sich bei allen Mitgliedern dieser Abstammungslinie, um obligat Wasserstoff-abhängige methylotrophe Methanogene handelt. Diese Organismen betreiben Methanogenese durch die Wasserstoff-abhängige Reduktion von Methanol oder Methylaminen. Des Weiteren fehlt ihnen der vollständige C1-Weg für die Reduktion von CO_2 zu CH_4. Dies warf jedoch doch Frage auf, wie diese Organismen Energie konservieren, eine Frage, die in bisherigen Genomannotationen

keine Beachtung gefunden hat. Auf Grund der in dieser Arbeit durchgeführten Untersuchungen schlagen wir einen neuen Weg der Energiekonservierung für die Gruppe der *Methanomassiliicoccales* vor, der sich von dem anderer Wasserstoff-abhängiger methylotropher Organismen unterscheidet. Wir konnten einen Komplex identifizieren, der von allen vier Genomen kodiert wird und nahe verwandt ist mit der membrangebundenen F_{420}:Methanophenazin-Oxidoreduktase (Fpo) von *Methanosarcinales*. Dem gefundenen Komplex fehlt jedoch das Modul für die Oxidation von F_{420}. Ein Fpo-ähnliches Homolog konnte kürzlich auch in *Methanosaeta thermophila* identifiziert werden, welches scheinbar mit Ferredoxin interagiert. Auf Grund unserer Untersuchungen schlagen wir vor, dass in *Methanomassiliicoccales* dieser Fpo-ähnliche Komplex die D-Untereinheit der Heterodisulfidreduktase als Elektronenakzeptor verwendet und so eine Energie-konvertierende Ferredoxin:Heterodisulfid Oxidoreduktase formt. Dies lässt darauf schließen, dass das Heterodisulfid zwei Funktionen hat: zum einen die Produktion von reduziertem Ferredoxin während der Elektronenbifurkation am zytoplasmatischen MvhADG/HdrABC Komplex und zum anderen die Erzeugung eines Membranpotentials während der Reoxidierung von Ferredoxin durch eine membrangebundene Elektronentransportkette. Diese duale Funktion des Heterodisulfides könnte eine einzigartige Eigenschaft dieser neuen Ordnung sein. Weiterhin gelang es im Rahmen dieser Arbeit durch Transmissionselektronenmikroskopie ein ungewöhnliches Zwei-Membranen-System in *Ca.* Mp. termitum und im Stamm MpM2 zu identifizieren, welches wahrscheinlich typisch für die gesamte Ordnung ist.

Während die Methanbildung im Insektendarm bereits von verschiedenen Autoren untersucht wurde, ist nur sehr wenig über Methanogenese und die Struktur der methanogenen Gemeinschaft im Tausendfüßlerdarm bekannt. Tausendfüßler gehören zu einer der wenigen Ordnungen von Arthropoden, welche Methan bilden. Unsere phylogenetische Analyse der Diversität von Archaeen in tropischen Tausendfüßlern zeigte, dass die Methanogenen in den Därmen den Ordnungen *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales* und *Methano-massiliicoccales* zugeordnet werden können. Die große Verwandtschaft der erhaltenen Sequenzen zu Methanogenen in den Därmen von Termiten, Schaben und den Larven von Blatthornkäfern legt nahe, dass die methanogene Gemeinschaft in Methan-emittierenden Arthropoden nicht unbedingt durch Kospeziation geformt wird.

Des Weiteren konnte kürzlich gezeigt werden, dass die bakterielle Gemeinschaft wichtige Ereignisse in der evolutionären Geschichte von Termiten und Schaben widerspiegelt. Dies führte zu der Spekulation, ob dieser Effekt auch für die archaellen Gemeinschaft nachgewiesen wereden kann. Unsere hier durchgeführte Studie, bestehend aus Klonbibliotheken und Hochdurchsatz-Sequenzierung, lässt darauf schließen, dass die archaelle Gemeinschaft sowie ihre Phylogenie mehr durch die Wirtsgruppen als durch Koevolution und Nahrung geformt wird. Hieraus resultiert, dass das Wirtshabitat die hauptsächlich Selektion der archaellen Gemeinschaft bestimmt.

Chapter 1

General Introduction

Kristina Lang

1.1 Methane

When the physicist Alessandro Volta in the late 18th century measured methane (CH_4) for the first time, he could not suspect the importance of his observation. Today, methane is known to have a significant impact on global warming. Every year 600 million tons of methane are released into the atmosphere. Although the atmospheric concentration of carbon dioxide (CO_2) is much higher than that of methane, over a 100 year period methane has a 25-fold higher global warming potential (Forster *et al.*, 2007). Methanogenic archaea are important source of biogenic methane. These strictly anaerobic microorganisms occur in almost all anoxic environments like marine or freshwater sediments, wetlands, soils, hot springs, sewage sludge and in the digestive tracts of animals and humans (Liu and Whitman, 2008). However, not only biogenic methane sources are of importance, also the human population contributes a considerable amount to the global methane budget by energy production, waste water treatment, biomass burning, landfills and increased amounts of cattle livestock and rice agriculture (Denman, 2007). This strong increase in the atmospheric methane concentration over the last decades and its strong impact on global warming, shows the importance of investigating methanogenic archaea and understanding the process of methanogenesis.

1.2 Methanogenic archaea

Methanogens belong to the phylum *Euryarchaeota* and are comprised of the orders *Methanopyrales*, *Methanobacteriales*, *Methanococcales*, *Methanosarcinales*, *Methanomicrobiales* (Bapteste *et al*., 2005) and *Methanocellales* (Sakai *et al*., 2008). All of these orders are known to contain members that perform hydrogenotrophic methanogenesis, meaning the reduction of H_2 and CO_2 to CH_4 (Fig. 1). Because of this, and the presence of genes involved in the central steps of methanogenesis in non-methanogenic *Euryarchaeota,* it has been hypothesized that the genes for the hydrogenotrophic methanogenesis were already present in a common ancestor and were subsequently lost in the *Archaeoglobales*, *Thermoplasmatales* and *Halobacteriales* (Bapteste *et al*., 2005). Methanogens can be divided into two distinct groups: the *Methanosarcinales*, the only order of methanogens known to possess cytochromes (Kühn *et al*., 1979, 1983) and the remaining five orders of methanogens, which are devoid of cytochromes. Methanogens without cytochromes are restricted to growth on H_2 and CO_2 and/or formate. *Methanosphaera stadtmanae*

is so far the only identified methanogen without cytochromes that is able to reduce methanol to CH_4 with H_2 as external electron donor (Miller and Meyer, 1985). However, it is not completely clear how this organism conserves its energy. Within the *Methanosarcinales*, only some species of the genus *Methanosarcina* are able to perform hydrogenotrophic methanogenesis (Kendall and Boone, 2006), but most strains are able to grow on acetate, methanol and methylamines (for more details see Thauer, 1998).

1.3 Methanogenic pathways

Hydrogenotrophic methanogenesis is the most commonly used pathway among the methanogens and it is assume that it is the central pathway of methanogenesis. Other methanogenic pathways seem to be variations of this pathway. In principle, there are four different pathways described: the hydrogenotrophic, the aceticlastic, the methylotrophic and the methyl-reducing pathway.

In the hydrogenotrophic pathway CO_2 is sequentially reduced to CH_4 using H_2 as an external electron donor (Fig 1). CO_2 is transferred via methanofuran (MF) and tetrahydromethanopterin (H_4MPT) to coenzyme M (CoM) and is then reduced by methyl-coenzyme M reductase (MCR) to CH_4. This last step is common to all methanogens. Energy is conserved by the membrane-bound methyl-H_4MPT:CoM methyltransferase (Mtr) complex, which builds up an electrochemical sodium ion potential for synthesis of ATP during the transfer of methyl group to coenzyme M (Gottschalk and Thauer, 2001). To conduct the initial endergonic reduction of CO_2 to formylmethanofuran (CHO-MF), the reaction is energetically coupled to the last step of methanogenesis, the reduction of methyl-coenzyme M to CH_4 via the cytoplasmic methyl viologen hydrogenase/heterodisulfide reductase (MvhADG/HdrABC) complex (Costa *et al*., 2010; Kaster *et al*., 2011). Electrons derived from H_2 are bifurcated by this complex. Two electrons are used for the reduction of the heterodisulfide and the residual electrons are used for the formation of reduced ferredoxin (Costa *et al*., 2010; Kaster *et al*., 2011). The reduced ferredoxin then initiates the endergonic reduction of CO_2 to CHO-MF. The energy converting hydrogenase Eha has an anaplerotic function and replenishes the pool of reduced ferredoxin, when the intermediates are not otherwise replenished (Lie *et al*., 2012).

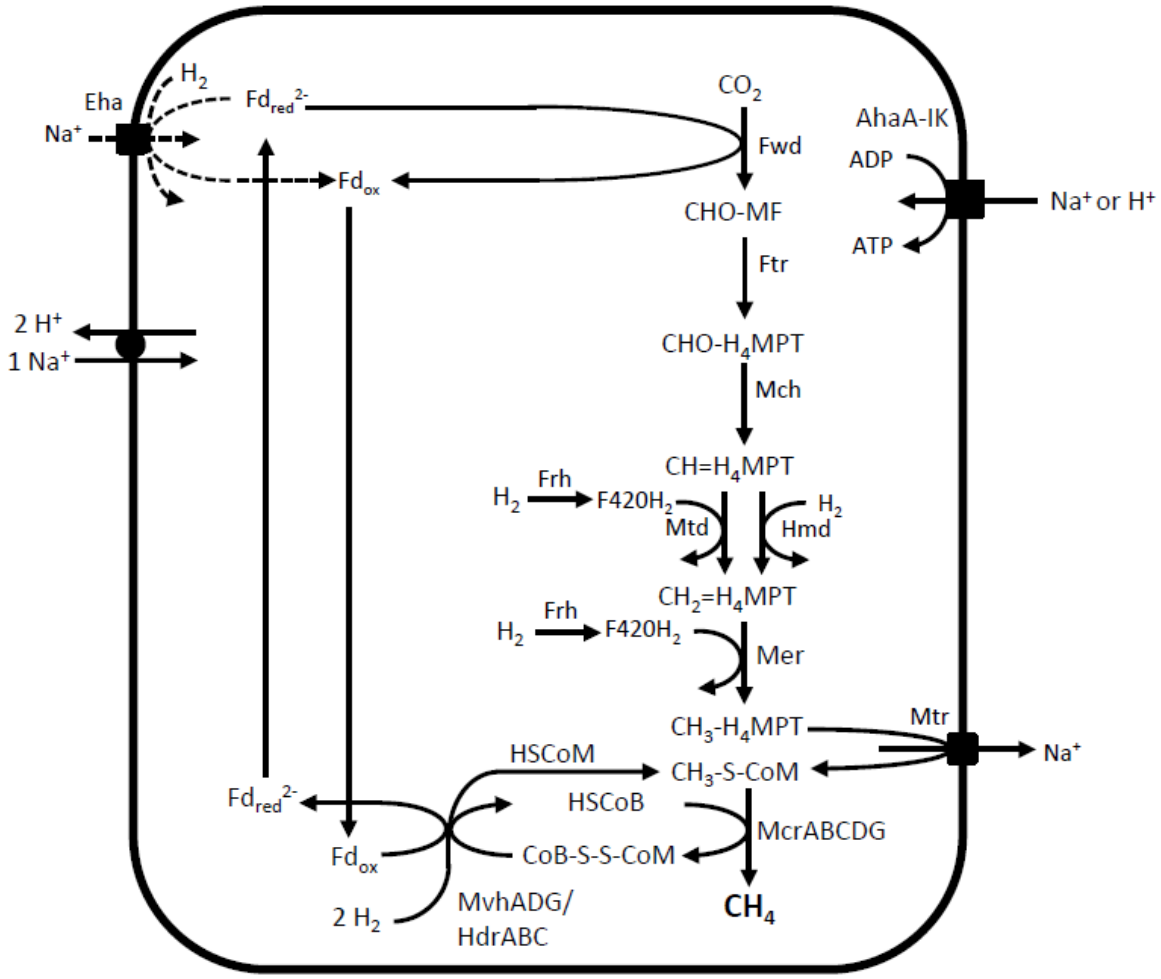

Figure 1. Hydrogenotrophic methanogenesis in methanogens without cytochromes. CO_2 is reduced to CH_4 using H_2 as external electron donor. The exergonic reduction of methyl-CoM (CH_3-S-CoM) to CH_4 is coupled via electron bifurcation to the endergonic reduction of CO_2 to formylmethanofuran (CHO-MF). Abbreviations: Eha, energy-converting hydrogenase, Fwd, formylmethanofuran dehydrogenase; Ftr, formylmethanofuran:H_4MPT formyltransferase; Mch, methenyl-H_4MPT cyclohydrolase; Hmd, coenzyme F_{420}-dependent N(5),N(10)-methenyl-tetrahydromethanopterin reductase, Mtd, methylene-H_4MPT dehydrogenase; Frh, F_{420} hydrogenase, Mer, methylene-H_4MPT reductase; Mtr, methyl-H_4MPT:CoM methyltransferase; Mvh, methyl viologen hydrogenase; Hdr, heterodisulfide reductase; Mcr, methyl-CoM reductase; Aha, A_1A_0-ATPases.

Hydrogenotrophic methanogenesis in methanogens with cytochromes functions in a similar way. However, in addition to the energy-conserving transfer of a methyl group to coenzyme M by Mtr, they possess an electron transport chain in the membrane (Fig 2). Here, the heterodisulfide reductase forms not a cytoplasmic complex with the methyl-viologen hydrogenase, but is anchored via a cytochrome *b* dependent subunit (HdrE) in the membrane. This subunit is linked through methanophenazine (Abken *et*

al., 1998) to a cytochrome *b*-dependent methanophenazine-reducing hydrogenase (VhoACG) (Deppenmeier, 2004).

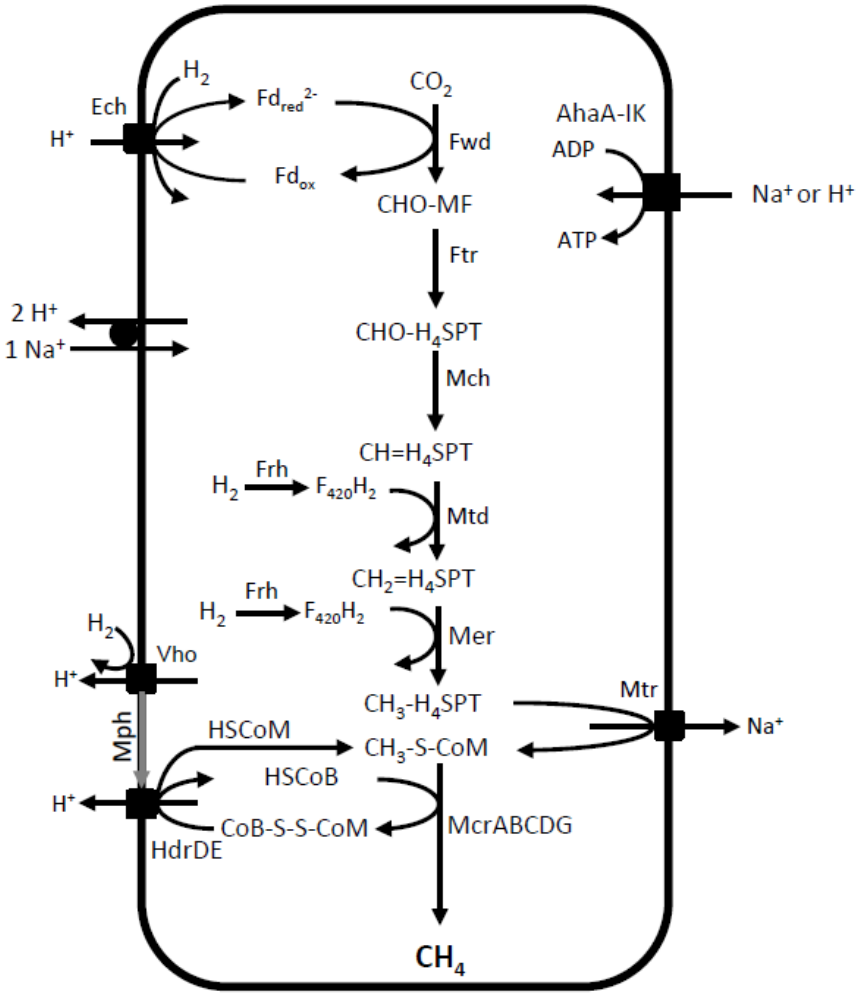

Figure 2. Hydrogenotrophic methanogenesis in methanogens with cytochromes. CO_2 is reduced to CH_4 using H_2 as an external electron donor. In addition to the methyltransferase (Mtr) transfer of methyl group to coenzyme M, energy is conserved via an electron transport chain in the membrane by methanophenazine (Mph). Abbreviations: Ech, energy-converting hydrogenase, Fwd, formylmethanofuran dehydrogenase; Ftr, formylmethanofuran:H_4SPT formyltransferase; Mch, methenyl-H_4SPT cyclohydrolase; Mtd, methylene-H_4SPT dehydrogenase; Frh, F_{420} hydrogenase, Mer, methylene-H_4MPT reductase; Mtr, methyl-H_4MPT:CoM methyltransferase; Mvh, non-F_{420}-reducing hydrogenase; Hdr, heterodisulfide reductase; Mcr, methyl-CoM reductase; Vho, viologen hydrogenase ; Mph, methanophenazine; Aha, A_1A_0-ATPases.

In some strains of genus *Methanosarcina*, which are growing on methanol, methanophenazine is linked with a $F_{420}H_2$ dehydrogenase (Fpo) (Bäumer *et al*., 2000) in addition to the Vho hydrogenase. The reduction of $F_{420}H_2$ or H_2 as an

electron donor for the reduction of methanophenazine, as well as the reduction of the heterodisulfide at the heterodisulfide reductase (HdrDE) complex, is coupled to an electrochemical proton gradient (Ide *et al*., 1999; Bäumer *et al*., 2000). For this reason methanogens that harbor cytochromes have a higher growth yield on H_2 and CO_2 than methanogens that lack cytochromes (Thauer *et al*., 2008). Because of the differences in energy conservation the *Methanosarcinales* are able to use acetate, methanol, and other methylated compounds as methanogenic substrates. In the aceticlastic pathway acetate is first activated to acetyl-CoA and subsequently the carbonyl group of acetyl-CoA is oxidized to CO_2. This step provides electrons for reducing of the remaining methyl group to CH_4 using the last two steps of the hydrogenotrophic pathway (Ferry, 1992). In the methylotrophic pathway, four methyl groups from methanol or other methylated compounds are transferred to coenzyme M (CoM) via methanol-CoM methyltransferase (Keltjens and Vogels, 1993). Methyl-CoM is then disproportionated in a 3:1 ratio. The oxidation of one mole of methyl-CoM to CO_2 provides the electrons for the reduction of the three residual moles of methyl-CoM to CH_4 (Keltjens and Vogels, 1993). For the oxidation of methyl-CoM to CO_2 the enzymes of the hydrogenotrophic pathway (reduction of CO_2 to CH_4) are used in the opposite direction. The fourth methanogenic pathway, the methyl reduction pathway, is similar to the methylotrophic pathway, but electrons needed for the reduction of methyl-CoM to CH_4 are not provided by the oxidation of methyl-CoM to CO_2. Instead, after the transfer of the methyl group to coenzyme M, methyl-CoM is reduced to CH_4 using H_2 as the external electron donor (Keltjens and Vogels, 1993).

1.4 Methanogenesis in arthropods

Methanogens occur in diverse environments, including the intestinal tracts of animals. A comprehensive analysis investigating CH_4 emission of insects revealed four different taxa of arthropods that emit CH_4. These arthropods are termites, cockroaches, millipedes, and scarab beetle (Hackstein and Stumm, 1994). Methane formation by these arthropods is widespread, but it is not present in all lineages. The ability to form CH_4 appears to have a geographical connection; most CH_4-emitting animals are from tropical environments (Hackstein and Stumm, 1994), but investigations revealed that some European millipedes are also able to form CH_4 (Sustr and Simek, 2009; Sustr *et al*., 2014). So far methanogenic archaea are shown to exclusively colonize the hindgut of these animals, as indicated by the localization

of the typical F420 autofluorescence of the methanogens (Hackstein and Stumm, 1994) and by the fact that only isolated hindguts of termites (Schmitt-Wagner and Brune, 1999) and scarab beetle larvae (Lemke *et al*., 2003) emit CH_4.

The hindguts of lower termites are mostly colonized by strains of the genus *Methanobrevibacter* (*Methanobacteriales*) (Ohkuma *et al*., 1995; Ohkuma and Kudo, 1998; Ohkuma *et al*., 1999; Shinzato *et al*., 1999, 2001), whereas higher termites have a more complex methanogenic community, composed of *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales* (Friedrich *et al*., 2001; Miyata *et al*., 2007). Comprehensive phylogenetic analyses showed that these groups are also present in wood-feeding cockroaches (Hara *et al*., 2002) and scarab beetle larvae (Egert *et al*., 2003). A DGGE analysis of tropical and European millipedes revealed sequences belonging to these same orders, but a phylogenetic analysis was not conducted (Sustr *et al*., 2014). So far aceticlastic methanogenesis could not be verified in any of these animals. It is suspected that the short retention times in the intestinal tracts do not favor the colonization of slow-growing aceticlastic methanogens (Liu and Whitman, 2008).

Except higher termites, most of these methane forming arthropods are known to harbor anaerobic protists that are typically associated with methanogens (see van Hoek *et al*., 2000; Hackstein *et al*., 2001). These protistsa possess hydrogenosomes that form H_2 as major fermentation product during the fermentation of pyruvate (Lindmark and Müller, 1973). Because H_2 is one of the major substrates of methanogenesis, it does not astonish that methanogens, mostly of the genus *Methanobrevibacter* (Tokura *et al*., 2000; Hara *et al*., 2004; Inoue *et al*., 2007), are associated with protozoa in the majority of these arthropods (Gijzen *et al*., 1991; Hackstein and Stumm, 1994; Radek 1994, 1997; Shinzato *et al*., 1992).

In addition to the methanogens, several of the before mentioned studies identified numerous sequences distantly related to the non-methanogenic *Thermoplasmatales*, representing a novel deeply branching lineage of archaea (Brune, 2010).

1.5. The uncultured *Thermoplasmatales*

The exact properties of many deeply branching archaeal lineages cannot reliable predicted because cultivated representatives are missing and they are exclusively known by their 16S rRNA genes (Schleper *et al*., 2005; Gribaldo and Brochier-

Armanet, 2006; Teske and Sorensen, 2008). One of these deeply branching lineages of uncultivated archaea is distantly related to cultivated members of the *Thermoplasmatales*. These "uncultured *Thermoplasmatales*" were originally discovered in marine environment (DeLong, 1992; Furman *et al*., 1992) and in the deep subsurface (Takai and Horikoshi, 1999). Further clones were obtained from rice paddy soil (Grosskopf *et al*., 1998), lakes (Jurgens *et al*., 2000; Nüsslein *et al*., 2001) and landfills (Huang *et al*., 2002; Luton *et al*., 2002). Sequences were also reported from intestinal tracts of cattle (Tajima *et al*., 2001; Denman *et al*., 2007; Wright *et al*., 2007; Janssen and Kirs, 2008), sheep (Wright *et al.*, 2004), wallabies (Evans *et al*., 2009) and the human gut (Mihajlovski *et al*., 2008, Scanlan *et al*., 2008; Mihajlovski *et al*., 2010) as well as from subgingival pockets (Li *et al.*, 2009; Horz *et al*., 2012). Additionally, in soil-feeding and wood-feeding termites (Shinzato *et al.*, 1999; Friedrich *et al*., 2001; Miyata *et al*., 2007), wood-feeding cockroaches (Hara *et al*., 2002) and humivorous beetle larvae (Egert *et al*., 2003) a substantial amount of this uncultured archaea distantly related to the *Thermoplasmatales* was present. Although concrete evidence is lacking, several of these reports speculated about the methanogenic nature of this lineage.

Diverse studies identified a novel group of deep branching *mcrA* genes, distantly related to known orders of methanogens. The *mcrA* gene encodes the alpha-subunit of the methyl coenzyme M reductase and has been established as a functional marker for methanogens (Lueders *et al*., 2001). Interestingly, these deep branching *mcrA* genes were present in the same environments as the "uncultured *Thermoplasmatales*", like in landfills (Luton *et al*., 2002), sediments (Castro *et al*., 2004), lakes (Earl *et al*., 2003) and the intestinal tracts of cattle (Denman *et al*., 2007), wallabies (Evans *et al*., 2009) and humans (Mihajlovski *et al*., 2008; Scanlan *et al*., 2008) and subgingival pockets (Horz *et al*., 2012).

Some of these research groups suggested that the new lineage of *mcrA* genes and the 16S rRNA genes distantly related to the *Thermoplasmatales* belong to the same organism (Mihajlovski *et al*., 2008; Evans *et al*., 2009; Horz *et al*., 2012) and may represent a new order of methanogens (Mihajlovski *et al*., 2010). However, the final proof for this hypothesis is still lacking.

1.6. Aims

The aim of this work was first to determine if the deep-branching lineage distantly related to the *Thermoplasmatales* represents a novel order of methanogenic archaea (Chapter 2). Previous studies revealed that 16S rRNA genes and *mcrA* genes have the same phylogeny, which allows the correlation of unknown *mcrA* genes with known 16S rRNA genes (Lueders *et al*., 2001; Luton *et al*., 2002). Taking advantage of this knowledge, I did a comprehensive phylogenetic analysis of all 16S rRNA genes distantly related to the *Thermoplasmatales* available to date and all unknown *mcrA* genes from the same habitats. To facilitate this work, additional 16S rRNA and *mcrA* sequences were obtained from hindguts of higher termites and wood-feeding cockroaches. In addition, enrichment cultures from the hindgut of termites and millipedes were established with the aim of isolating a member this putative novel order (Chapter 2).

The successful enrichment of *Ca.* Methanoplasma termitum strain MpT1 from the termite gut, belonging to the novel order of methanogens (see Chapter 2), allowed the sequencing of its genome (Chapter 3). I deeply analyzed the genome in order to resolve the problem of energy conservation of the novel order because further genomes of strains isolated or enriched from novel order (Borrel *et al*., 2012, Gorlas *et al*., 2012, Borrel *et al*., 2013) were only superficially analyzed.

In contrast to other CH_4-emitting arthropods, the methanogenic community of millipedes is rarely investigated. Therefore, I comprehensively analyzed the phylogeny of the archaeal 16S rRNA genes in tropical millipedes (Chapter 4). In addition, the bacterial composition of the millipede guts was investigated by high-throughput sequencing to expand the knowledge in this field. To identify the localization of methanogenic archaea in the millipede gut, the production rates of CH_4 were measured in isolated gut sections of the millipede *Anadenobolus monilicornis*. Furthermore, I conducted quantitative PCR of archaeal and bacterial 16S rRNA to get an overview about the cell numbers in the millipede guts.

In cooperation with Carsten Dietrich and James Nonoh it was tried to analyze the mechanisms that select archaeal lineages and shape the archaeal community structure in CH_4-emitting arthropods (Chaper 5). Therefore, we conducted clone libraries of the archaeal 16S rRNA gene from diverse arthropods. The achieved

sequenences were used for a phylogenetic analysis to identify host-specific groups. In addition, high-throughput sequencing was perfomed to gain deeper insides into the community structure.

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Chapter 2

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

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Running title: *The seventh order of methanogens*

Contributions:

K.P. designed the study, created the clone libraries of the 16S rRNA full length sequences and the *mcrA* sequences, performed the qPCR analysis, conducted the phylogenetic analysis, enriched and characterized strain MpT1, evaluated and visualized the data and wrote the manuscript.[#]

J.O.N. obtained the termite samples, prepared the DNA, and created the clone libraries of the short 16S rRNA sequences.

L.M. enriched strain MpM2.

A.B. conceived the study, supervised the experiments, discussed the results and wrote the manuscript.

[#]Parts of this work have been obtained during the master thesis of Kristina Paul: Identifizierung einer neuartigen Linie methanogener Thermoplasmatales in Termitendärmen

2.1 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.

2.2 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 (Schleper *et al.*, 2005; Gribaldo and Brochier-Armanet, 2006; 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 (Shinzato *et al.*, 1999; Friedrich *et al*., 2001;

Miyata *et al*., 2007), 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 (Tajima *et al*., 2001; Denman *et al*., 2007; Wright *et al*., 2007; Janssen and Kirs, 2008), sheep (Wright *et al*., 2004), wallabies (Evans *et al*., 2009), and in the gut and subgingival pockets of humans (Mihajlovski *et al*., 2008; Scanlan *et al*., 2008; Li *et al.*, 2009; Mihajlovski *et al*., 2010;). 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; Mihajlovski *et al*., 2010), 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.

2.3 Material and 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 (35), 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.

2.4 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).

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.

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*.

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).

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.

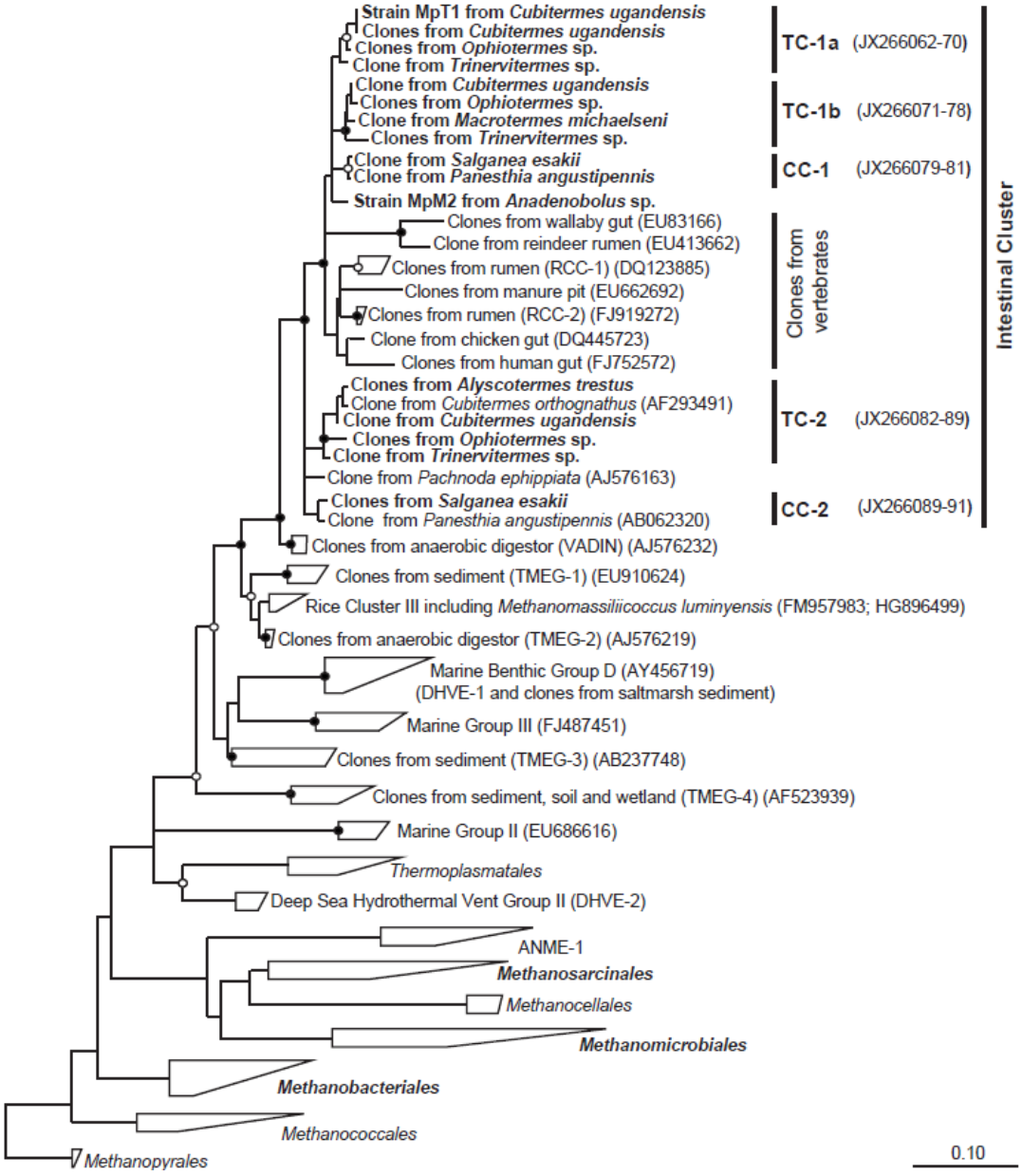

Figure 1. Phylogenetic tree showing the relationships among uncultured archaea related to *Thermoplasmatales* and to representatives of all other orders of methanogenic archaea and the ANME-1 group. Clusters of clones from termite (TC) and cockroach (CC) gut are indicated. The tree is based on a maximum-likelihood 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.

Next relatives of this clade were clones previously obtained from an anaerobic digestor (VADIN Group; Godon *et al*., 1997). 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.

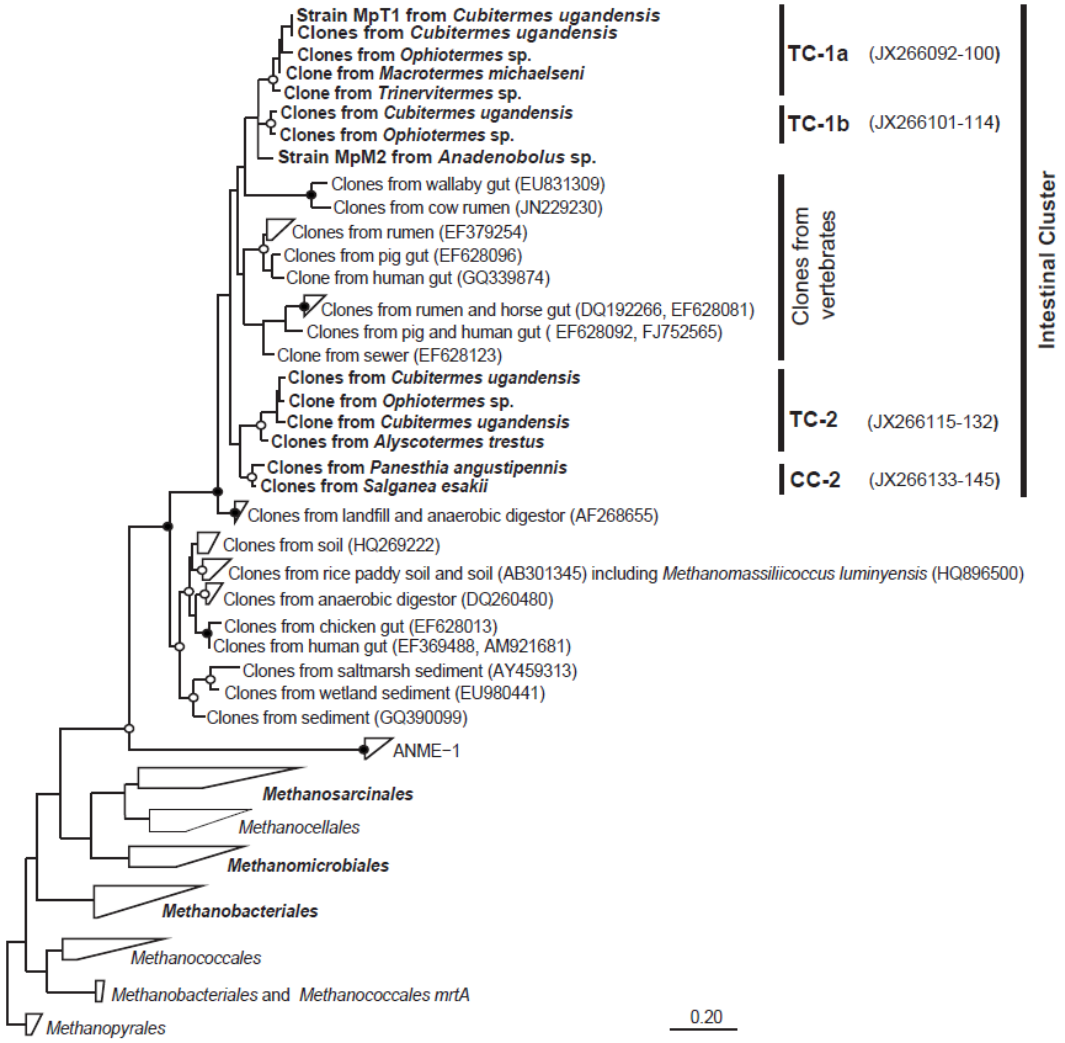

Figure 2. Phylogenetic tree showing the relationships among the novel *mcrA* genes and to representative *mcrA* genes of all other orders of methanogenic archaea and the ANME-1 group. Clusters of clones from termite (TC) and cockroach (CC) gut are indicated. The tree is based on a maximum-likelihood 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).

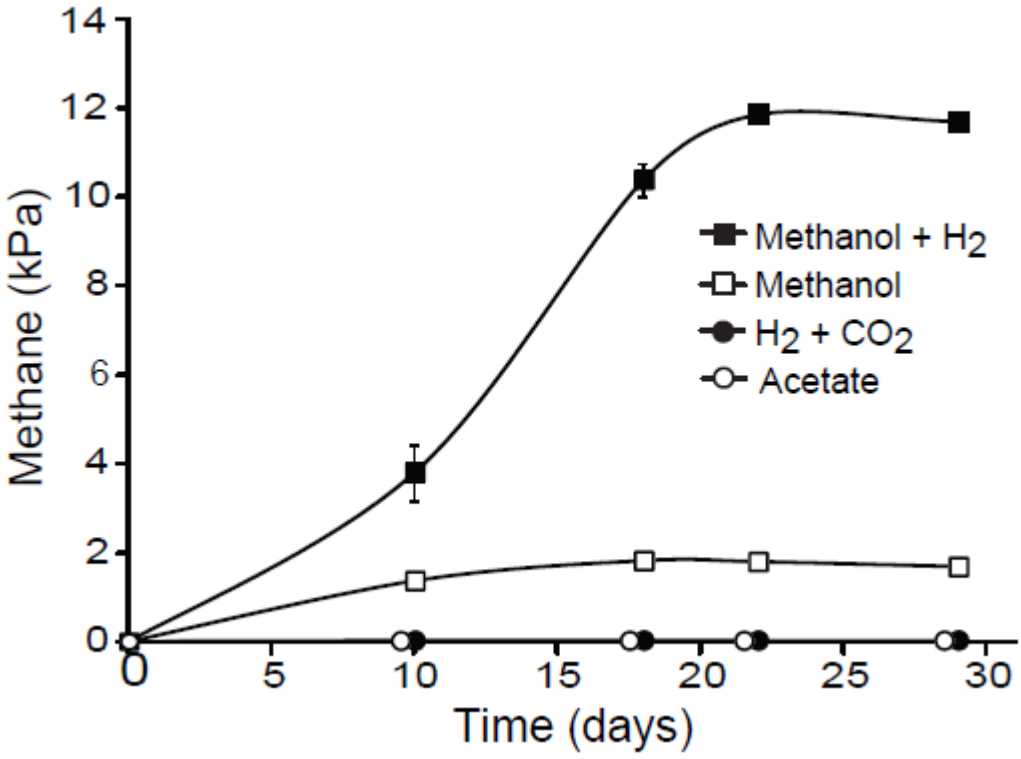

Figure 3. Time course of methane partial pressure in the headspace of the enrichment culture MpT1 (N_2–CO_2; 80/20) inoculated from a methanol-starved preculture into basal medium supplemented with different substrates: H_2 (50 kPa in headspace), methanol (50 mM), or acetate (30 mM). Values are means 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).

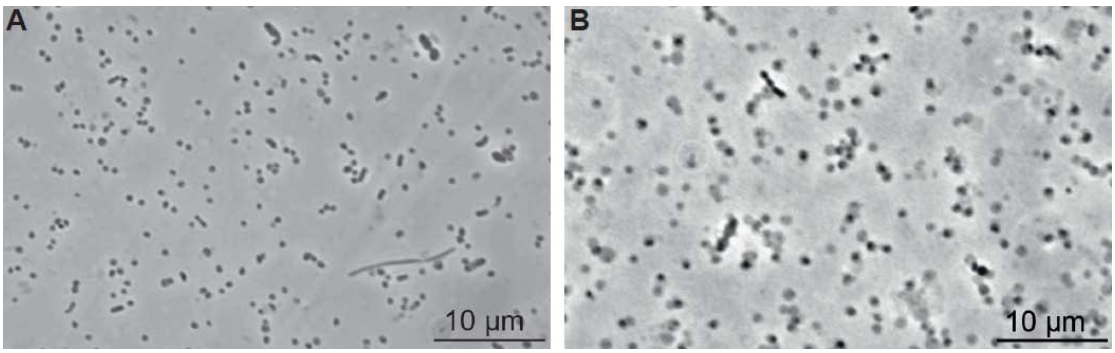

Figure 4. Phase-contrast photomicrographs of the methanogenic enrichment cultures MpT1 (A) and MpM2 (B) after several transfers in basal medium supplemented with H_2 and methanol. Both cultures consisted mostly of small roundish cells (diameter 0.6–1.0 µm).

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. 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.

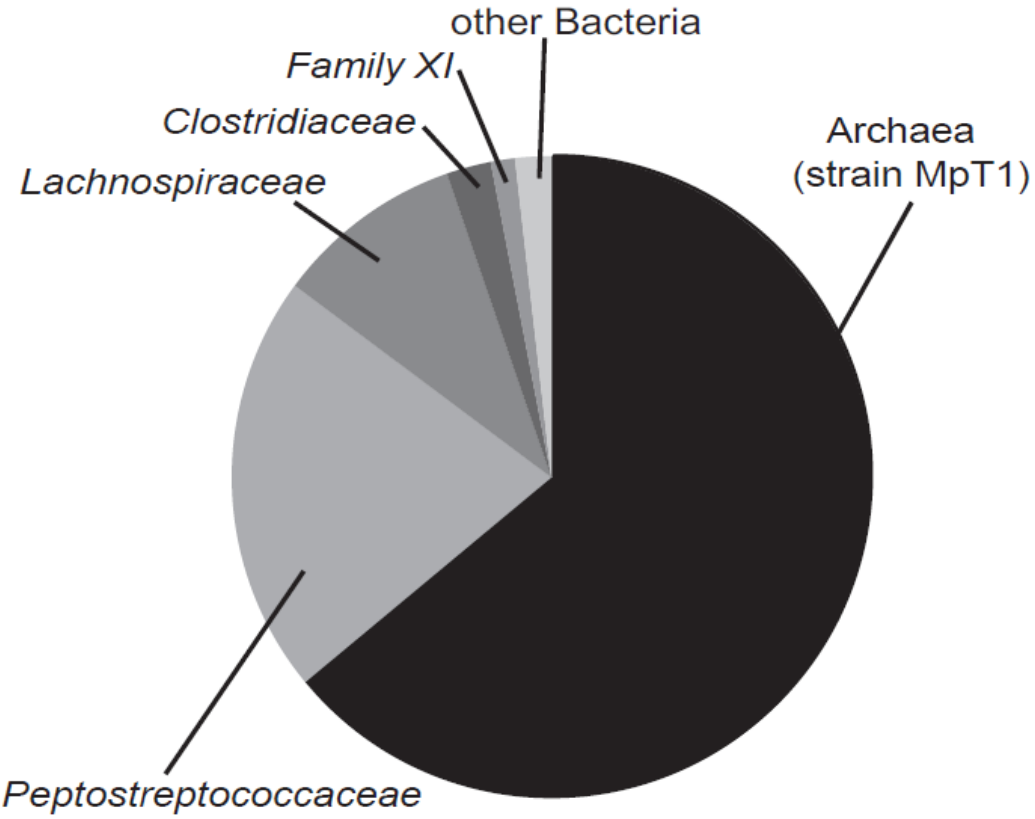

Figure 5. Composition of the enrichment culture of strain MpT1, determined by quantitative real-time PCR of bacterial and archaeal 16S rRNA genes. 454 pyrotag sequencing revealed that the bacterial contaminants belonged almost exclusively to families of the order *Clostridiales*.

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).

2.5 Discussion

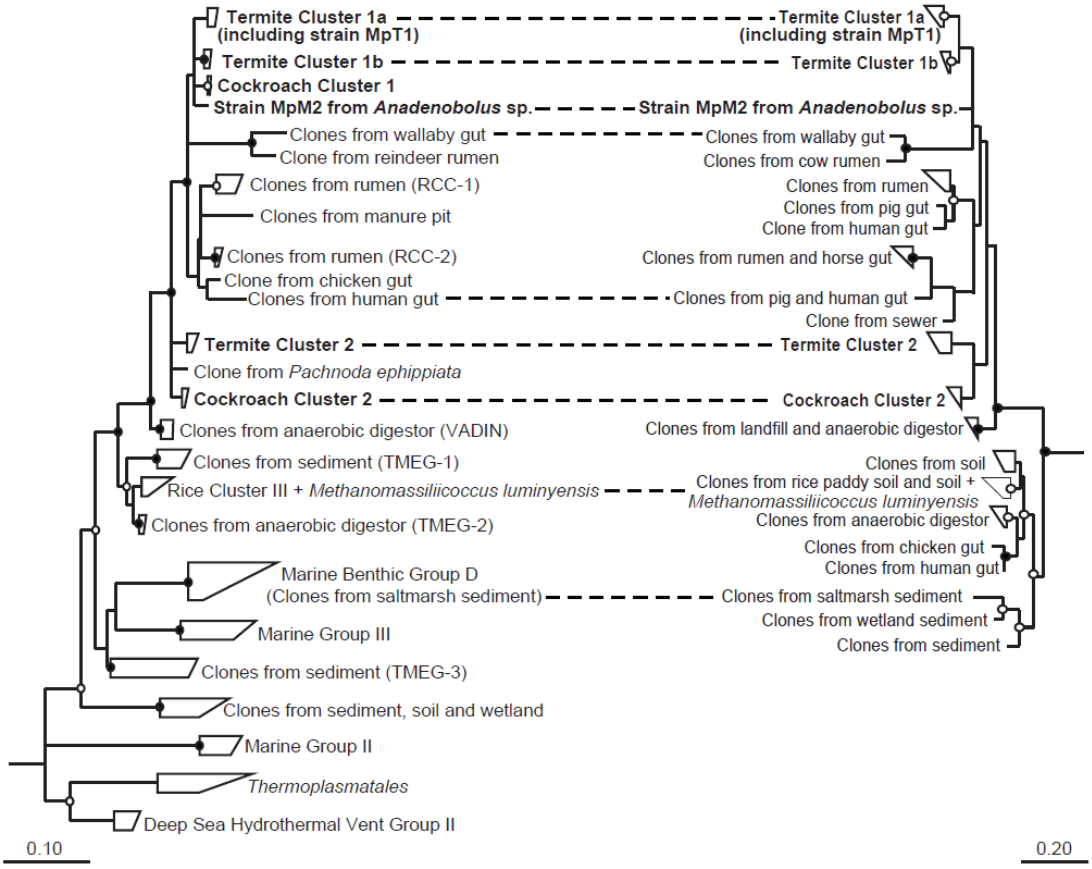

Figure 6. Tanglegram illustrating the congruence of the phylogenies of *Thermoplasmatales*-related archaea (16S rRNA) and the *mcrA* genes of unknown origin (for details, see Figs. 1 and 2). Sequence pairs stemming from the same study are connected by dotted lines. Sequences obtained in this study are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

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 (Dridi *et al*., 2012; 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 Brune, 2010) 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., Evans, *et al*. 2005; Mihajlovski *et al*., 2010), 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).

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Chapter 3

Submitted

Comparative genome analysis of "*Candidatus* Methanoplasma termitum" indicates a new mode of energy metabolism in the seventh order of methanogens

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Contributions:

K.L. designed the study, analyzed and annotated the genome, performed the time course of the methane accumulation, conducted the phylogenetic analyses, evaluated and visualized the data and wrote the manuscript.

J.S. and R.D. sequenced and assembled the genome

A.K. performed the electron microscopic analysis

A.B. conceived the study, supervised the experiments, discussed the results and wrote the manuscript.

3.1 Abstract

We sequenced the complete genome of "*Candidatus* Methanoplasma termitum", a member of the recently discovered seventh order of methanogens. With 1.49 Mbp, it is among the smallest genomes of methanogens. Comparative analysis of this genome with that of three other species of *Methanomassiliicoccales* (previously referred to as "*Methanoplasmatales*") sequenced to date confirmed that all members of this lineage are obligate methylotrophs that lack the entire pathway for CO_2 reduction to methyl coenzyme M and produce methane by hydrogen-dependent reduction of methanol and methylamines, which is consistent with additional physiological data. However, the absence in the entire group of both cytochromes and an obvious mechanism for recycling ferredoxin produced by the soluble heterodisulfide reductase poses a problem for energy conservation that had so far escaped attention. We document that *Methanomassiliicoccales* cannot employ a ferredoxin-dependent energy-converting hydrogenase as proposed for the obligately methylotrophic *Methanosphaera stadtmanae*. Instead, we identified a complex encoded in all genomes that is related to the F_{420}:methanophenazine oxidoreductase (Fpo) of *Methanosarcinales* but that lacks an F_{420}-oxidizing module, as in the apparently ferredoxin-dependent Fpo-like homolog in *Methanosaeta thermophila*. Since *Methanomassiliicoccales* lack the E subunit of the membrane-bound heterodisulfide reductase (HdrDE), we propose that their Fpo-like complex interacts directly with the D subunit, forming a second, energy-converting ferredoxin:heterodisulfide oxidoreductase. This dual function of heterodisulfide in *Methanomassiliicoccales*, which serves both in electron bifurcation and as terminal acceptor in a membrane-bound electron transport chain, may be a unique characteristic of the novel order.

3.2 Introduction

Methanogenesis is catalyzed exclusively by members of the archaeal domain. Methanogenic archaea occur only in the phylum *Euryarchaeota* and are phylogenetically diverse. The species described to date fall into seven orders that differ both in the biochemistry of their catabolic pathways and their ecological niches (Liu and Whitman, 2008; Thauer *et al*., 2008).

Methanogens from all basal orders (*Methanopyrales*, *Methanococcales*, and *Methanobacteriales*) are hydrogenotrophs. They reduce CO_2 to CH_4 via the C1 pathway, using H_2 or sometimes formate as electron donor (Liu and Whitman, 2008; Thauer *et al*., 2008). The hydrogenotrophic pathway is found also in most of the derived lineages of methanogens (*Methanomicrobiales* and *Methanocellales*) and was most probably present already in the common ancestor of the *Euryarchaeota* (Bapteste *et al*., 2005). Hydrogenotrophic methanogens typically lack cytochromes and conserve energy with the methyl-H_4MPT:coenzyme M methyltransferase complex (Mtr), which uses the free energy of methyl transfer to establish a Na^+-motive force across the membrane (Schlegel and Müller, 2013). The low-potential reducing equivalents for CO_2 reduction are provided by electron bifurcation at the cytoplasmic heterodisulfide reductase complex (HdrABC) (Costa *et al*., 2010; Kaster *et al*., 2011).

Members of the order *Methanosarcinales* are the only methanogens that possess cytochromes (Thauer *et al*., 2008). They have an entirely different mode of energy conservation, which involves a membrane-bound electron transport chain that couples heterodisulfide reduction to the generation of an electrochemical proton gradient (Blaut and Gottschalk, 1984), which is more efficient than electron bifurcation and allows a higher growth yield. In this way, they can grow (i) on H_2 and CO_2; (ii) on the methyl groups of methanol or methylamines, which are partially oxidized to CO_2 in order to provide reducing equivalents for methyl reduction; and (iii) by disproportionation of acetate, in which methyl groups are reduced to methane with electrons derived from the oxidation of the carbonyl group to CO_2 (Liu and Whitman, 2008; Thauer *et al*., 2008).

A third group of methanogens is restricted to growth on methanol and methylamines but lacks the ability to oxidize the latter to CO_2, which makes methanogenesis obligately dependent on molecular hydrogen. The group is phylogenetically and biochemically heterogeneous, comprising *Methanosphaera stadtmanae* (*Methanobacteriales*; Fricke *et al*., 2006), *Methanomicrococcus blatticola* (*Methanosarcinales*; Sprenger *et al*., 2000; Sprenger *et al*., 2005), and members of the recently discovered seventh order of methanogens (Dridi *et al*., 2012; Paul *et al*., 2012; Borrel *et al*., 2013a), for which we had suggested the provisional name "*Methanoplasmatales*" (Paul *et al*., 2012). The name was inspired by their roundish cell form (Dridi *et al*., 2012; Paul *et al*., 2012) and their close relationship to the non-methanogenic *Thermoplasmatales*. However, the bacteriological code (Lapage *et al*., 1992) dictates that the taxonomic name of an order, no matter how unwieldy, must be derived from the genus name of the first isolate, *Methanomassiliicoccus* (*Mmc.*) *luminyensis* (Dridi *et al*., 2012). Therefore, we will adopt the name "*Methanomassiliicoccales*" for the seventh order of methanogens.

So far, the *Methanomassiliicoccales* comprise only the type species, *Mmc. luminyensis*, and several, in part highly enriched cultures from the intestinal tract of termites (Paul *et al*., 2012), humans (Borrel *et al*., 2012; Borrel *et al*., 2013a), and an anaerobic digester (Iino *et al*., 2013). The consistent presence of the *mcrA* gene (encoding the alpha-subunit of methyl-CoM reductase) indicates that also the lineages without cultured representatives are methanogenic (Paul *et al*., 2012; Fig. 1).

Meanwhile, genome sequences of three members of the *Methanomassiliicoccales* have been reported. While the genome sequences of the enrichment cultures of *Candidatus* Methanomethylophilus (Mm.) alvus (Borrel *et al*., 2012) and *Candidatus* Methanomassiliicoccus intestinalis (Borrel *et al*., 2013a) are complete and annotated, that of the type strain, *Mmc. luminyensis* (Gorlas *et al*., 2012), remains to be finished. All strains lack the genes encoding the entire C1 pathway for the reduction of CO_2 to methyl coenzyme M (methyl-CoM), but possess the complete gene sets for the utilization of methanol and methylamines (Borrel *et al*., 2013b). This explains the strict dependence of methanogenesis on the simultaneous presence of hydrogen and methanol or trimethylamine documented for *Mmc. luminyensis* (Brugère *et al*., 2013).

However, the fundamental consequences of the absence of the C1 pathway for the energy metabolism of the *Methanomassiliicoccales* have so far escaped attention. Without formylmethanofuran dehydrogenase and an energy-converting Mtr complex, the reoxidation of reduced ferredoxin formed during heterodisulfide reduction and the strategy for energy conversion in *Methanomassiliicoccales* must differ fundamentally from that in other methanogens.

In this study, we comprehensively analyzed the genome of *Ca.* Methanoplasma (Mp.) termitum strain MpT1, which we previously enriched from a termite gut, and compared it to the genomes of its three distant relatives of the order *Methanomassiliicoccales* that stem from the human intestinal tract. Our results document that the energy metabolism of *Methanomassiliicoccales* differs fundamentally from that of the other orders and are supported by physiological data on the substrate requirements for methanogenesis of strain MpT1 and *Mmc. luminyensis*. Moreover, we present ultrastructural data of strain MpT1 that provide new information on the unusual cell envelope of *Methanomassiliicoccales*.

3.3 Material and Methods

Strains. The highly enriched cultures of *Ca.* Mp. termitum strain MpT1 and the closely related strain MpM2 were obtained in a previous study (Paul *et al*., 2012). *Mmc. luminyensis* (DSMZ **25720**) was purchased from the German Collection of Microorganism and Cell Cultures (http://www.dsmz.de/).

Cultivation. Cultures were grown in anoxic, bicarbonate-buffered mineral medium (AM5; Boga and Brune, 2003) under an atmosphere of N_2-CO_2 (80:20 [vol/vol]) with dithiothreitol (1 mM) as reducing agent (Paul *et al*., 2012). The basal medium was supplemented with casamino acids (2 g/l), yeast extract (2 g/l), coenzyme M (10 mg/l), acetate (1 mM), and formate (0.5 mM). The medium (4.5 ml) was dispensed into 15-ml rubber-stoppered glass vials. Substrates were added from sterile stock solutions; hydrogen gas (5 ml) was added to the headspace. Tubes were inoculated (0.3 ml) with methanol-starved precultures and incubated at 30 °C in the dark. At regular time intervals, aliquots of the headspace (0.2 ml) were sampled with a gas-tight syringe, and the methane content was analyzed using a gas chromatograph equipped with a packed column (Porapak Q, 80/100 mesh; 274 cm × 3.18 mm I.D.) and a flame ionization detector.

Light microscopy. Cells of 300 µl cultures were concentrated by centrifugation at 10,000 × *g* for 10 min and routinely inspected by phase-contrast microscopy using an Axiophot epifluorescence microscope (Zeiss, Wetzlar, Germany). Autofluorescence of cofactor F_{420} was tested using an HC filter set (F36-544, AHF Analysentechnik, Tübingen, Germany) with bandpass filters (wavelength/bandwith: excitation, 438/24 nm; beam splitter, 458 nm; emission, 483/32 nm).

Electron microscopy. For negative stains, fresh cultures were chemically fixed with 1.25% glutaraldehyde and concentrated by centrifugation (see above). Aliquots (5 µl) were applied to carbon-coated copper grids and stained as previously described (Bubendorfer *et al*., 2012). For ultrastructural characterization, 2 µl of concentrated but unfixed cells were frozen under high pressure, freeze substituted, embedded in Epon resin, ultrathin sectioned, and post-stained as described previously (Peschke *et al*., 2013). Freeze substitution was performed with acetone containing 0.2% OsO_4, 0.25% uranyl acetate, and 5% water. Transmission electron microscopy was carried out on a JEOL JEM2100 (JEOL, Tokyo, Japan) equipped with a LaB_6-cathode and operated at 120 kV. Images were recorded using a 2k × 2k fast scan CCD camera F214 in combination with the EM-Menu4 software package (TVIPS, Gauting, Germany).

Phylogenetic analysis. 16S rRNA gene sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) and imported into the current *Silva* database (version 115) (Quast *et al*., 2013; http://www.arb-silva.de) using the *ARB* software package (Ludwig *et al*., 2004). The automatic alignment was manually refined, and a 30% consensus filter was used to exclude highly variable positions. Phylogenetic trees of near-full-length sequences (>1,250 bp) were calculated using *PhyML* (Guindon *et al*., 2010), a maximum-likelihood method implemented in *ARB*. Tree topology and node support (100 bootstraps) were tested using the maximum-parsimony method (*DNAPARS*) implemented in *ARB*.

For phylogenetic analysis of the large subunit of the 11-subunit complex, complex 1 of the respiratory chain, $F_{420}H_2$ dehydrogenases, and [NiFe] hydrogenases, sequences were retrieved from the *IMG* database (https://img.jgi.doe.gov/cgi-bin/w/main.cgi) and analyzed using the *Mega5* software package (http://www.megasoftware.net/). Sequences were automatically aligned with the *ClustalW* function implemented in *Mega5*. The alignment was manually refined in

ARB. Trees were calculated based on the deduced amino acid sequence using *PhyML*. Tree topology and node support (100 bootstraps) were tested using the maximum-parsimony method (*PROTPARS*) implemented in *ARB*.

Genome sequencing. The genome of *Ca.* Mp. termitum was sequenced using a combined 454 pyrosequencing and Sanger sequencing approach. DNA was isolated from the enrichment culture by detergent extraction (CTAB method; Winnepenninckx *et al*., 1993) and used to generate a 454 shotgun library according to the GS Rapid Library protocol, which was sequenced with the Genome Sequencer FLX+ system (454 Life Sciences, Roche Applied Science, Branford, CT, USA) using titanium chemistry. In total, 107,475 shotgun reads were generated and assembled de novo into 72 large contigs (>500 bp) using Roche *Newbler* assembler software 2.6 FLX. Sequences were edited and final gaps were closed as described by Vollmers *et al*. (2013).

Sequence annotation. All genome sequences were uploaded to the Integrated Microbial Genomes Expert Review (IMG/ER) platform (Markowitz *et al*., 2009, https://img.jgi.doe.gov/cgi-bin/er/main.cgi). In the case of *Ca.* Mmc. intestinalis and *Ca.* Mm. alvus, the original RAST annotations in the GenBank entry were preserved. In the case of *Ca.* Mp. termitum and *Mmc. luminyensis*, coding sequences were predicted and annotated using the automated pipeline of IMG/ER. Briefly, protein-coding genes were identified with *GeneMark*, and candidate homolog genes of the genomes were computed using *BLASTp*. Automated annotations of coding sequences were verified and curated by comparing various annotations based on functional resources, such as COG clusters (Tatusov *et al*., 2003), Pfam (Punta *et al*., 2012), TIGRfam (Selengut *et al*., 2007), and Gene Ontology (The Gene Ontology Consortium, 2010). In addition, genes were associated with gene product names in the SwissProt database (Gattiker *et al*., 2003), EC numbers (Fleischmann *et al*., 2004), KEGG orthology terms (Kanehisa *et al*., 2014), COG functional categories, KEGG categories (Kanehisa *et al*., 2014), and MetaCyc pathway collections (Caspi *et al*., 2012). The annotated genome sequences of *Ca.* Mp. termitum (Gi21292) and *Mmc. luminyensis* (Gi17673) are available in the Genomes Online database (http://www.genomesonline.org/). The annotated genome of *Ca.* Mp. termitum was deposited also in GenBank.

3.4 Results and Discussion

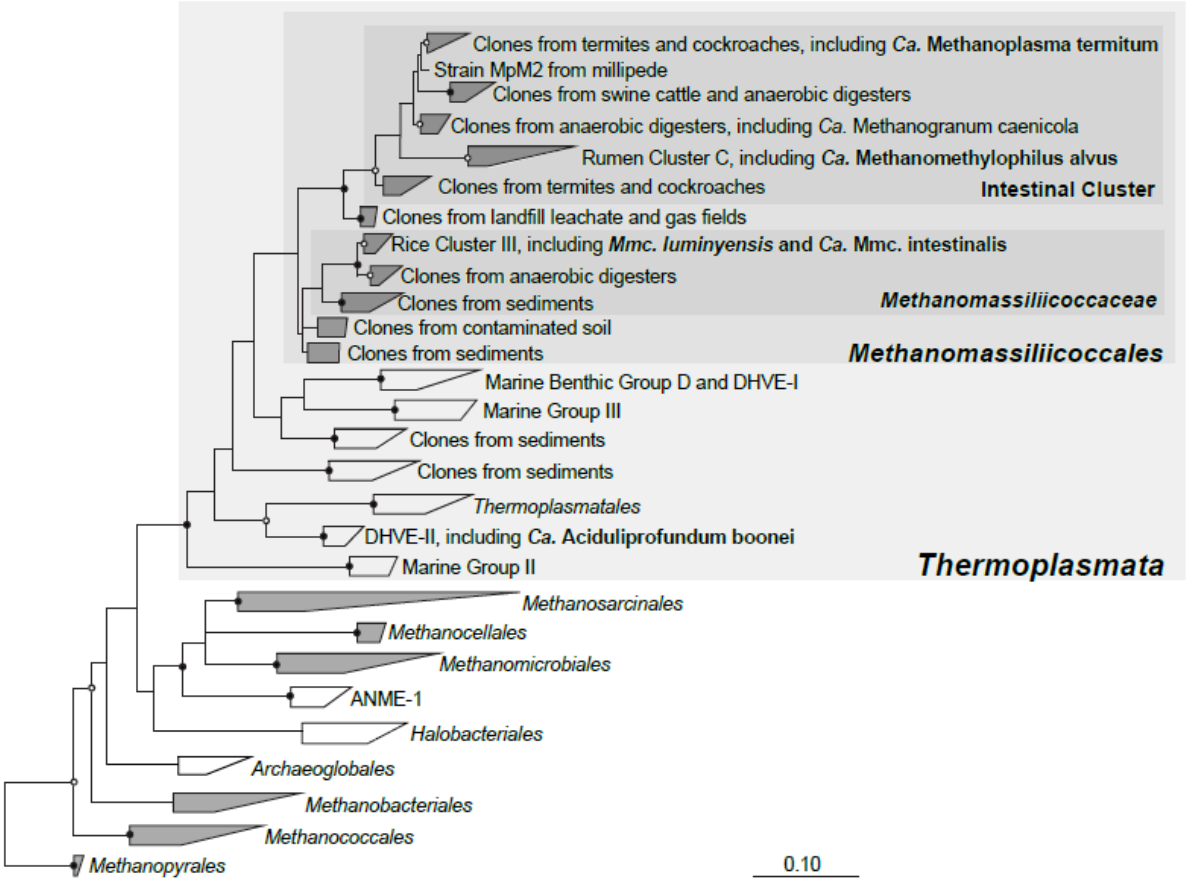

Figure 1. Phylogenetic tree of the major lineages in the class *Thermoplasmata*, illustrating the relationship among the mostly uncultivated members of the order *Methanomassiliicoccales* and to other lineages of the *Euryarchaeota*. Methanogenic lineages are shaded in gray. Strains with published genomes or draft genomes are in bold. The original tree is based on an unambiguous alignment of more than 300 16S rRNA genes (>1,250 nucleotide positions) and was reconstructed using a maximum-likelihood algorithm (RAxML). The tree was rooted using representatives of other methanogenic lineages. Nodes not supported by neighbor-joining and maximum-parsimony (MP) analyses are shown as multifurcations; highly supported nodes (100 bootstraps, MP) are marked ●, (>95%; ○, >70%). Scale bar indicates 0.1 substitutions per site.

Genome characteristics. The genome of Candidatus Methanoplasma termitum strain MpT1 is the fourth genome sequence reported for a member of the *Methanomassiliicoccales*. Pyrosequencing analysis of the DNA extracted from a

highly enriched culture of strain MpT1 yielded 107,475 shotgun reads, which were initially assembled into 72 contigs with an average coverage of 37-fold. Subsequent gap closure yielded a single circular DNA sequence; no evidence for the presence of plasmids was found.

With a size of 1,488,669 bp, the genome of *Ca.* Mp. termitum is even smaller than that of *Ca.* Mm. alvus (1.66 Mbp), a close relative in the intestinal cluster (Fig. 1). Genome sizes of the distantly related members of the genus *Methanomassiliicoccus* are considerably larger (1.93 Mbp in *Ca.* Mmc. intestinalis and >2.62 Mbp in the unfinished genome of *Mmc. luminyensis*). The coding density of all genomes is similar, with about 1,000 bp per gene. The G+C content of the strains differs strongly, but does not correlate with phylogenetic distance. Details are presented in Table 1.

rRNA operon structure. *Ca*. Mp. termitum has a single set of ribosomal RNA genes, which are located in different regions of the chromosome. The rRNA genes in most other methanogens are organized in an operon, but a separation of 5S, 16S, and 23S rRNA genes has been reported also for other members of the *Methanomassiliicoccales* (Borrel *et al*., 2012; Gorlas *et al*., 2012; Borrel *et al*., 2013a). Since the same feature is present also in *Thermoplasma acidophilum* (Tu and Zillig, 1982) and the deep-branching "*Candidatus* Aciduliprofundum boonei" strain T469 (GenBank ID: CP001941.1), it may be a trait shared by all members of the class *Thermoplasmata* (Fig. 1). A second copy of the 5S rRNA gene, which is encountered in all other *Methanomassiliicoccales*, is absent in *Ca.* Mp. termitum.

tRNAs. The genome of *Ca.* Mp. termitum encodes 46 tRNAs (Table S1). The same number of tRNAs are present in *Ca.* Mmc. intestinalis (Borrel *et al*., 2013a). *Ca.* Mm. alvus and *Mmc. luminyensis* have two additional tRNA genes, but several tRNA genes are pseudogenized (Table 1). Like the three other strains, *Ca.* Mp. termitum possesses a tRNA for pyrrolysine, the corresponding $tRNA_{Pyl}$ synthetase, and all enzymes required for pyrrolysine biosynthesis. Pyrrolysine operon structure and position of the tRNA gene are the same as in *Mmc. luminyensis* and *Ca*. Mmc. intestinalis (Borrel *et al*., 2014). A tRNA gene for selenocysteine is not present in any of the genomes.

Table 1. Comparison of the genome features of *Ca.* Methanoplasma termitum and other methanogens of the order *Methanomassiliicoccales*.

	Intestinal cluster		*Methanomassiliicoccaceae*	
	Ca. Mp. termitum strain MpT1	*Ca.* Mm. alvus strain Mx1201	*Mmc. luminyensis* strain B10	*Ca.* Mmc. intestinalis strain Mx1
Isolation source	Termite gut	Human feces	Human feces	Human feces
Enrichment/isolation	Paul et al. 2012	Borrel et al. 2012	Didri et al. 2012/Gorlas et al. 2012	Borrel et al. 2012
Genome annotation	This study	Borrel et al. 2012	This study[a]	Borrel et al. 2012
(GOLD ID[b]	Gi21292	Gc0042696	Gi17673	Gc0050196
GenBank accession number	########	CP004049	CAJE01000001–26	CP005934
Genome size (bp)	1,488,669	1,666,795	2,620,233 (26 contigs)	1,931,561
G+C content (mol%)	49.2	55.6	60.5	41.3
Protein-coding genes	1,415	1,646	2,625	1,826
rRNA genes	3	4[c]	4[c]	4[c]
tRNA genes	46	48[d]	48[e]	46

[a] The original genome announcement (13) contains no gene list and the GenBank submission lacks annotation.

[b] http://genomesonline.org/index

[c] Genome contains two copies of the 5S rRNA gene.

[d] Three of the 48 tRNA genes are pseudogenized.

[e] Five of the 48 tRNA genes are pseudogenized. The original genome announcement (13) reported 42 tRNAs

The methyl-reducing pathway. Like all other members of *Methanomassiliicoccales*, *Ca*. Mp. termitum possesses the complete gene sets encoding methanol transferase (MtaABC) and methyl-CoM reductase (McrABCDG), the key enzymes of the methyl reduction pathway (Thauer, 1998). Also a methyl-viologen-dependent hydrogenase/heterodisulfide reductase complex (MvhADG/HdrABC), which is required for the regeneration of CoM, is encoded by the genome (Fig. 2). The genes required for the reduction of CO_2 to the methyl level, however, are lacking in all strains.

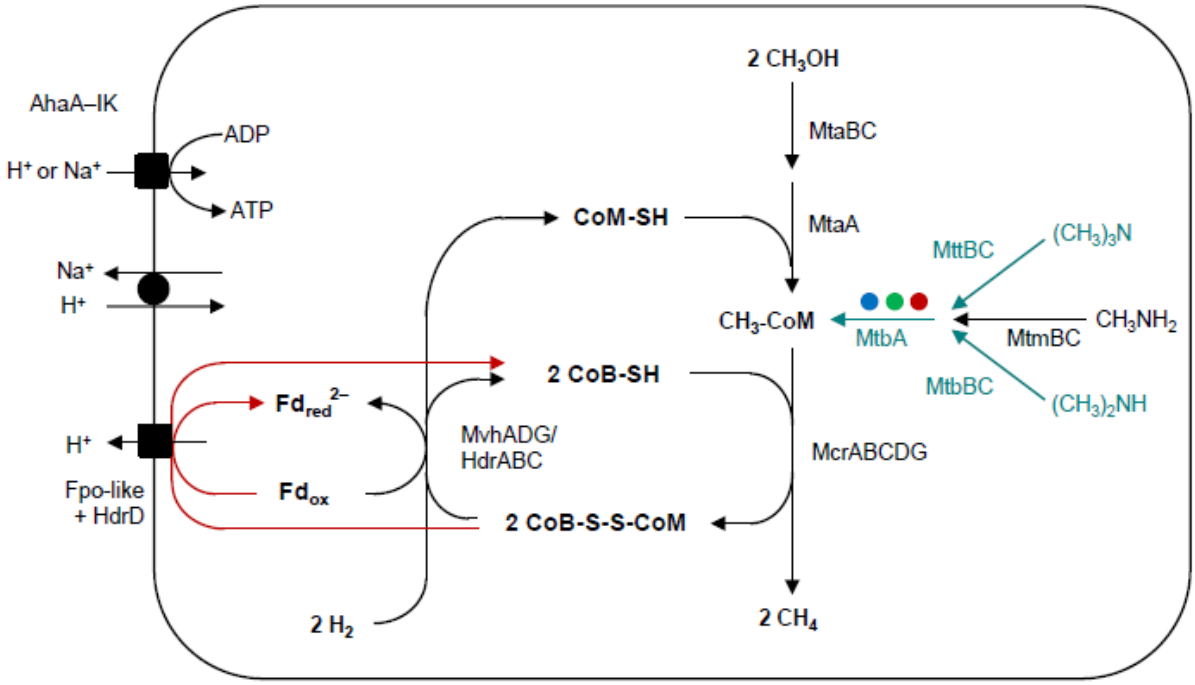

Figure 2. Energy metabolism of *Ca.* Methanoplasma termitum and other members of the order *Methanomassiliicoccales*. Black arrows indicate reactions whose enzymes are encoded in all genomes. Blue-green arrows indicate that the enzymes are not present in *Ca.* Mp. termitum but are present in the genomes indicated by colored dots (blue, *Methanomassiliicoccus luminyensis;* green, *Ca.* Methanomassiliicoccus intestinalis; red, *Ca.* Methanomethylophilus alvus). Gray arrows indicate enzymes of the hydrogenotrophic pathway that are not encoded by any of the four genomes, including the anaplerotic reaction (dotted lines). Abbreviations: Mta, methanol:CoM methyltransferase; Mvh, non-F_{420}-reducing hydrogenase; Hdr, heterodisulfide reductase; Mcr, methyl-CoM reductase; Fpo-like, $F_{420}H_2$-dehydrogenase-like complex MtbA, methylcobamide:CoM methyltransferase; Mtm, mono-methylamine methyltransferase; Mtb, di-methylamine methyltransferase; Mtt, tri-methylamine methyltransferase; Aha, A_1A_O-ATP synthase.

Comparative genome analysis of *Ca.* Methanoplasma termitum

The conspicuous absence of the CO_2 reduction pathway in all *Methanomassiliicoccales* (Borrel *et al*., 2013b; this study) is in agreement with the obligate dependence of methanogenesis on methanol, which has been experimentally confirmed for *Mmc. luminyensis* (Brugère *et al*., 2013; Fig. 3a) and *Ca.* Mp. termitum (Fig. 3b).

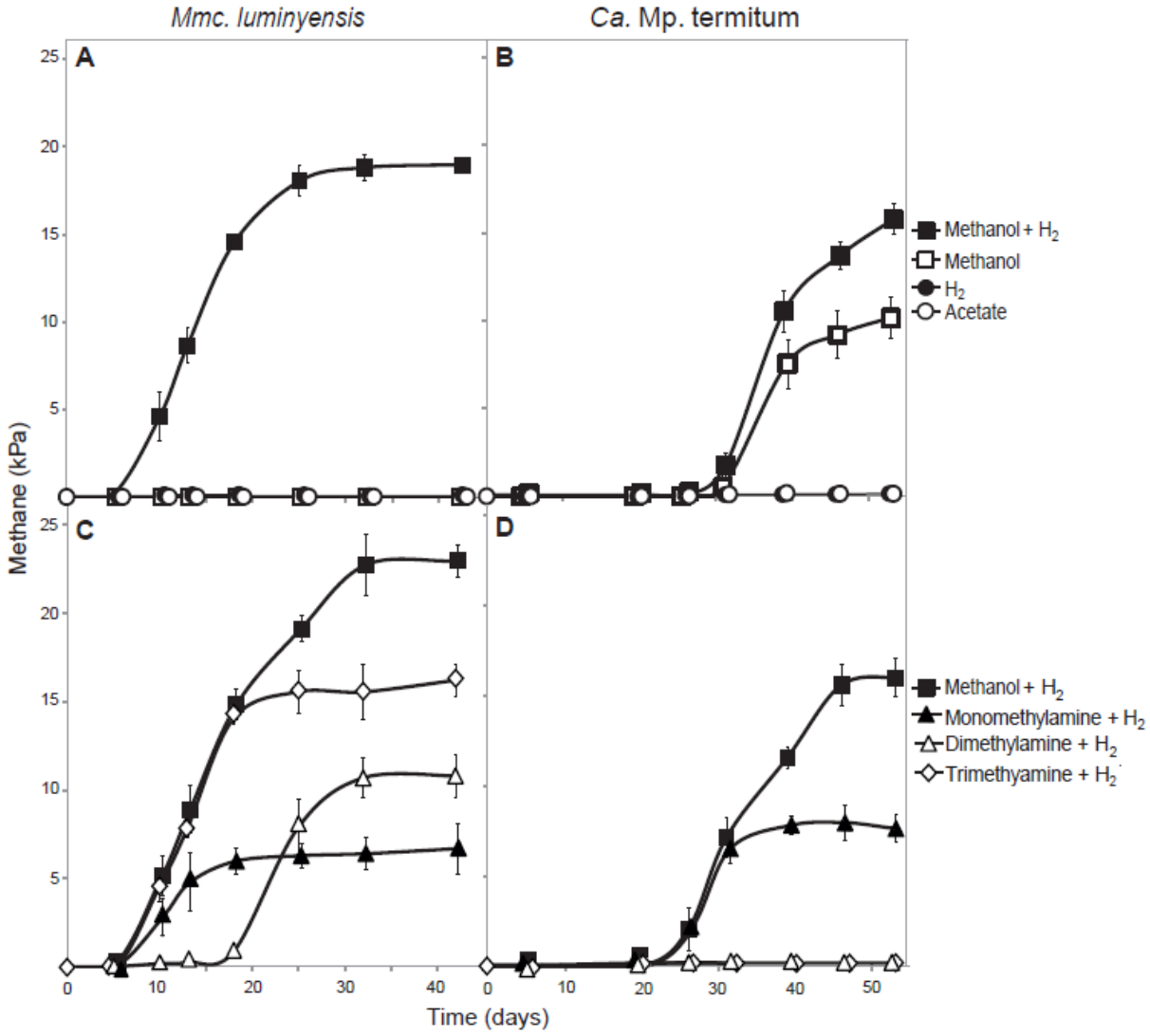

Figure 3. Time course of methane accumulation in the culture headspace of *Methanomassiliicoccus luminyensis* (a,c) and *Ca.* Methanoplasma termitum (b,d) incubated in bicarbonate-buffered medium supplemented with H_2 (ca. 50 kPa), methanol (50 mM), or acetate (30 mM) (a,b) or H_2 combined with different methylamines (10 mM) (c,d). To avoid a transfer of residual methanol with the inoculum, the precultures were grown under methanol limitation. The values are means of three replicate cultures; standard deviations are shown only if they are larger than the symbols.

The absence of this pathway also precludes the oxidation of methyl groups to CO_2, which explains the obligate hydrogen requirement of methanogenesis in *Mmc. luminyensis*, a so far undocumented trait (Dridi *et al*., 2012) that is validated by the present study (Fig. 3a). Moreover, it substantiates the proposal that methane formation from methanol alone by *Ca.* Mp. termitum (Fig. 3b) is driven by an internal hydrogen production of the clostridia present in the enrichment culture (Paul *et al*., 2012).The inability to disproportionate methanol is found also in *Methanosphaera stadtmanae* (*Methanobacteriales*). In contrast to *Methanomassiliicoccales*, *M. stadtmanae* possesses all genes required for the reduction of CO_2 to methane and for the oxidation of methanol to CO_2 (Fricke *et al*., 2006), but the activities of the corresponding enzymes in cell extracts are either low or absent (Schwörer and Thauer, 1991;Van de Wijngaard *et al*., 1991). It has been speculated that the absence of Mtr activity indicates that the enzyme is not required for methanogenesis from methanol and H_2, and the low specific activities of formylmethanofuran dehydrogenase, together with the apparent molybdopterin auxotrophy of *M. stadtmanae*, may be related to an exclusively anabolic function of this enzyme (Fricke *et al*., 2006). Also in the obligately methylotrophic *Methanomicrococcus blatticola* (*Methanosarcinales*), the low activities of F_{420}-dependent enzymes indicate an inability to oxidize methyl groups (Sprenger *et al*., 2005). Further insights into the pathway will be possible when a genome sequence is available also for this strain.

Growth on methylamines. The previously reported presence of the complete gene sets for the utilization of mono-, di-, and tri-methylamine in all genomes of *Methanomassiliicoccales* (Borrel *et al*., 2012; Borrel *et al*., 2013a; Borrel *et al*., 2014; Fig. 2) suggested that methylamines can be used as substrates by all strains investigated so far. However, growth on tri-methylamine had been documented only for *Mmc. luminyensis* (Brugère *et al*., 2013), correcting a contradictory statement in the original species description (Dridi *et al*., 2012). Our results provide experimental evidence that *Mmc. luminyensis* utilizes also mono- and di-methylamine for methanogenesis (Fig. 3b).

The genome of *Ca.* Mp. termitum, however, contains only homologs encoding the substrate-specific mono-methylamine methyltransferase (MtmB) and the mono-methylamine corrinoid protein (MtmC). As in all other *Methanomassiliicoccales* (Borrel *et al*., 2014), the *mtmB* gene of *Ca.* Mp. termitum is interrupted by an in-frame

amber codon, which indicates that the enzyme contains pyrrolysine, a common feature of all methylamine methyltransferases (Paul *et al*., 2000; Hao *et al*., 2002). Although a homolog of *mtbA*, which encodes the methylcobamide:CoM methyltransferase (MtbA) present in all methylamine utilization complexes (Burke and Krzycki, 1997), is missing in the genome (Fig. 2), the capacity to produce methane from mono-methylamine (Fig. 3c) indicates that *Ca.* Mp. termitum produces a functional methyltransferase complex, in which MtbA is probably replaced by MtaA, its homolog in the methanol methyltransferase complex.

The lack of capacity of di- and tri-methylamine utilization and the putative loss of the *mtbA* gene may be related to streamlining of the *Ca*. Mp. termitum genome, which is even smaller than that of the closely related *Ca.* Mm. alvus. Since also their relatives in the bovine rumen (Rumen cluster C; Janssen and Kirs, 2008) can utilize all three methylamines (Poulsen *et al*., 2013), the capacity to metabolize methylamines may not provide an advantage in termite guts.

Energy metabolism. It is has remained entirely obscure how members of the Methanomassiliicoccales reoxidize reduced ferredoxin formed by electron bifurcation at the soluble heterodisulfide reductase (HdrABC) and how they couple this process with the generation of an electrochemical membrane potential. In the case of the obligately methyl-reducing *M. stadtmanae*, it has been speculated that both tasks are accomplished by the energy-converting [NiFe] hydrogenase Ehb (Thauer *et al*., 2008), a homolog of the anaplerotic Eha complex of hydrogenotrophic methanogens (Tersteegen and Hedderich, 1999; Lie *et al*., 2012). However, homologs of the Eha and Ehb gene clusters are entirely absent in all *Methanomassiliicoccales*. Also ferredoxin-dependent hydrogenases of the Ech type (Meuer *et al*., 1999; Welte *et al*., 2010), which are involved in energy conversion in most *Methanosarcina* species (Thauer *et al*., 2010; Welte and Deppenmeier, 2014), are absent in *Ca.* Mp. termitum and *Ca.* Mm. alvus, members of the intestinal cluster. The two complete gene sets in the genomes of *Mmc. luminyensis* and *Ca.* Mmc. intestinalis (Table S2), which have highest sequence similarity to the Ech genes of *Methanosarcina barkeri* (Fig. 4) and the canonical NiFe-binding motif of [NiFe] hydrogenases (Fig. 5), are probably involved in the redox cycling of ferredoxin produced/consumed by the CO dehydrogenase/acetyl-CoA synthetase complex present only in the *Methanomassiliicoccaceae* (see below).

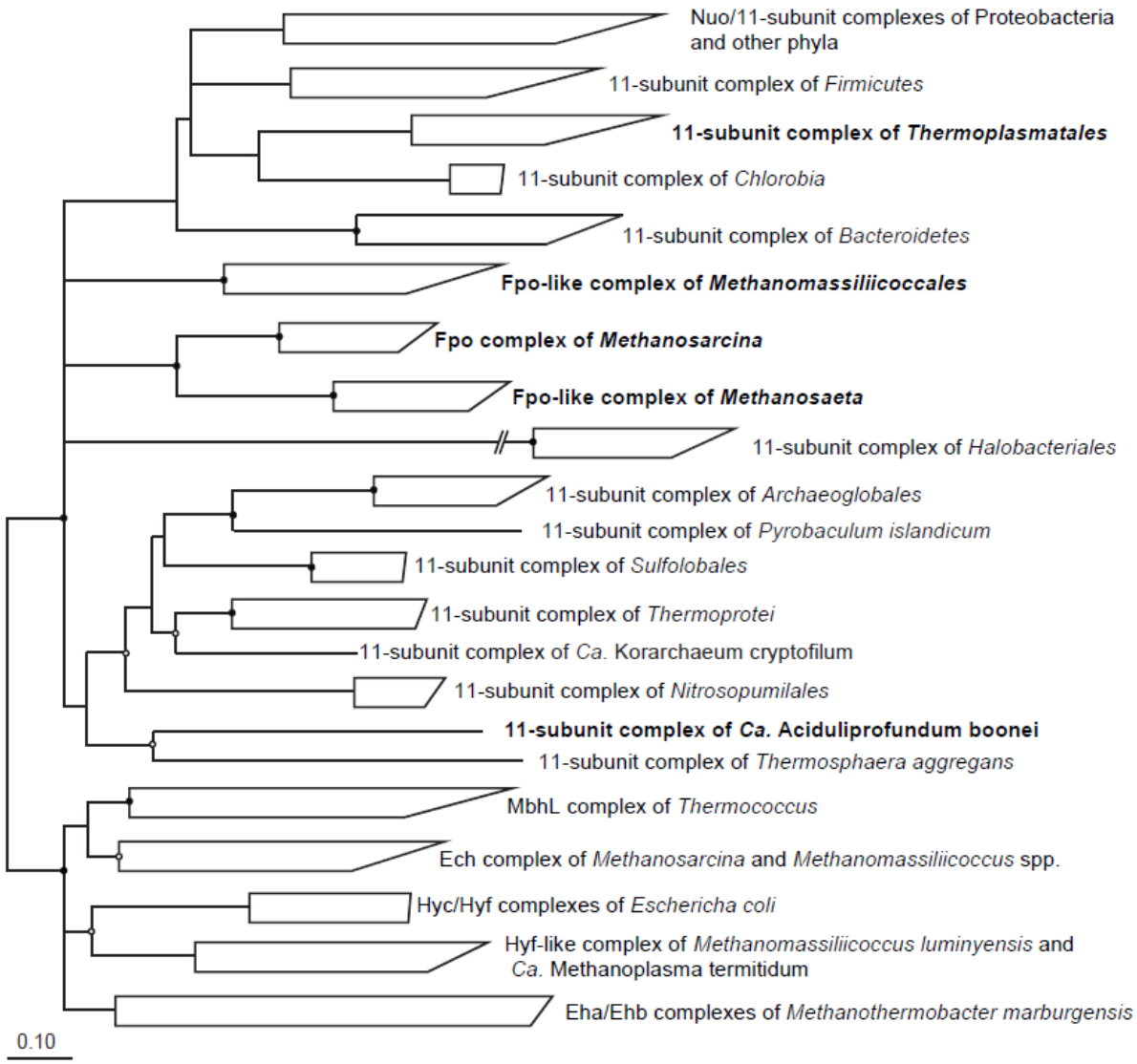

Figure 4. Phylogenetic tree of Fpo, Nuo, and related 11-subunit complexes and [NiFe] hydrogenases of bacteria and archaea. The Fpo-like complexes of *Methanomassiliicoccales* do not cluster with the 11-subunit complexes of their closest relatives, *Thermoplasmatales* and *Ca.* Aciduliprofundum boonei but share high sequence similarity with the Fpo and Fpo-like complexes of *Methanosarcinales* (strains indicated in bold). The tree is based on a translated amino acid alignment of the homologs encoding the large subunit of the respective complex and was reconstructed using a maximum-likelihood algorithm (PhyML). Nodes that were not supported by neighbor-joining and maximum-parsimony (MP) analyses are shown as multifurcations; highly supported nodes (1,000 bootstraps, MP) are marked (●,> 95%; ○, > 70%). Scale bar indicates 0.1 substitutions per site.

The only other putative hydrogenases in *Methanomassiliicoccales* are the Hyf-like complexes encoded in the genomes of *Ca.* Mp. termitum and *Mmc. luminyensis* (Table S3). Their large subunits are most closely related to the [NiFe] hydrogenases

Hyc and Hyf of *Eschericia coli* (Fig. 4). However, genes for several subunits of Hyc and Hyf are missing (Table S3), and the large subunit of the Hyf-like complex (HyfG) shows several deviations from the canonical [NiFe]-binding motif (Fig. 5). Even if the Hyf-like complexes were functional energy-converting hydrogenases, their absence in *Ca.* Mm. alvus and *Ca.* Mmc. intestinalis make them unlikely candidates for energy metabolism, which should be conserved among all *Methanomassiliicoccales*. It is also not possible that Ech and Hyf-like complexes substitute for each other, because none of the complexes are present in *Ca.* Mm. alvus. Therefore, it seems safe to conclude that the reoxidation of ferredoxin in *Methanomassiliicoccales* does not involve an energy-converting hydrogenase.

The Fpo-like complex of *Methanomassiliicoccales*. The genomes of all *Methanomassiliicoccales* have a gene cluster that encodes homologs of the 11 core subunits shared by the membrane-bound F_{420}-methanophenazine oxidoreductase complex (Fpo) of methanogens and the NADH-ubiquinone oxidoreductase complex (Nuo) and its homologs in many bacteria. However, homologs of the subunits responsible for binding and oxidation of F_{420} (FpoFO) or NADH (NuoEFG) are lacking (Table 2). Phylogenetic analysis of the amino acid sequences of the large subunit revealed that the 11-subunit complex of *Methanomassiliicoccales* is more closely related to the Fpo and Fpo-like complexes of *Methanosarcina* and *Methanosaeta* spp. than to bacterial 11-subunit complexes (including Nuo) or the [NiFe] hydrogenases of methanogens (Fig. 4). The numerous deviations from the canonical [NiFe]-binding motifs in the large subunit (Fig. 5) make it unlikely that the new complex is a [NiFe] hydrogenase. It has been proposed that 11-subunit complexes are derived from [NiFe] hydrogenases that lost their [NiFe] cluster and gained new functions by association with additional electron-transferring subunits, such as NuoEFG or FpoFO (Moparthi and Hägerhäll, 2011). Although 11-subunit complexes are present in many bacteria and archaea, their interacting partner proteins or the redox process catalyzed by the respective complex are often unclear (Moparthi and Hägerhäll, 2011).

Table 2. Genes encoding the different subunits of Fpo in *Methanosarcina mazei* and their homologs in the 11-subunit complex present in all *Methanomassiliicoccales*. The homologs in the Nuo complex of *Escherichia coli* are shown for comparison.

	Fpo	11-subunit complex				Nuo
	Ms. mazei	*Ca*. Mp. termitum	*Ca*. Mm. alvus	*Mmc. luminyensis*	*Ca*. Mmc. intestinalis	*E. coli*
Large subunit	*fpoD*	Mpt1_01267	MMALV_01980	WP_019176180	MMINT_02020	*nuoD*
Small subunit	*fpoB*	Mpt1_01269	MMALV_01960	WP_019176182	MMINT_02000	*nuoB*
4Fe/4S - Fd	*fpoI*	Mpt1_01265	MMALV_02000	WP_019176178	MMINT_02040	*nuoI*
Small protein	*fpoC*	Mpt1_01268	MMALV_01970	WP_019176181	MMINT_02010	*nuoC*
Transmembrane proteins	*fpoL*	Mpt1_01261	MMALV_02040	WP_019176174	MMINT_02080	*nuoL*
	fpoM	Mpt1_01260	MMALV_02050	WP_019176173	MMINT_02090	*nuoM*
	fpoN	Mpt1_01259	MMALV_02060	WP_019176172	MMINT_02100	*nuoN*
	fpoH	Mpt1_01266	MMALV_01990	WP_019176179	MMINT_02030	*nuoH*
	fpoK	Mpt1_01262	MMALV_02030	WP_019176175	MMINT_02070	*nuoK*
	fpoJ	Mpt1_01264	MMALV_02010	WP_019176177	MMINT_02050	*nuoJ*
	fpoA	Mpt1_01270	MMALV_01955	WP_019176183	MMINT_01985	*nuoA*
F_{420} and phenazine binding module	*fpoFO*	–[a]	–	–	–	–
NADH-oxidizing module	–	–	–	–	–	*nuoEFG*

[a] Not present

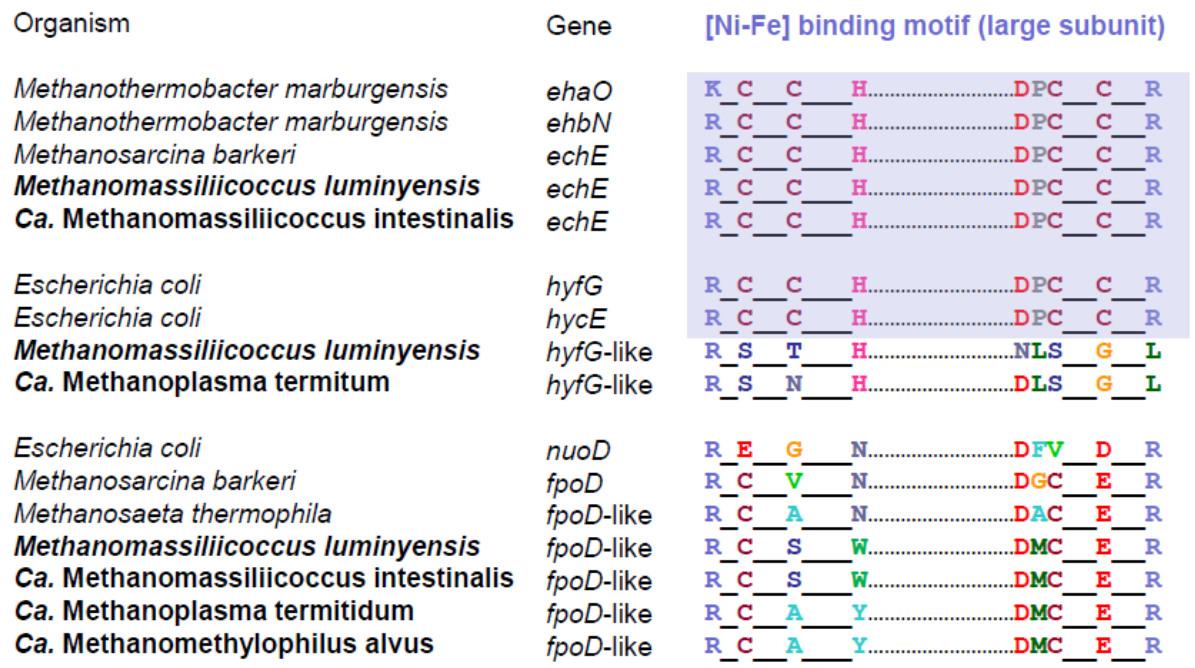

Figure 5. Comparison of the [NiFe]-binding motif in the large subunit of selected [NiFe] hydrogenases with the corresponding amino acid residues in the homologous complexes encoded in the genomes of *Methanomassiliicoccales* (in bold). Blue shading indicates the typical motif of bona-fide hydrogenases. Like the homologous subunits in Fpo and Nuo, which do not contain a [NiFe] cofactor, both the Hyf-like and Fpo-like complexes of *Methanomassiliicoccales* deviate strongly from this consensus.

A novel mechanism of energy conversion. Recently, Welte and Deppenmeier (2011) provided strong evidence that the Fpo-like complex in the obligately aceticlastic *Methanosaeta* (*Mt.*) *thermophila* does not oxidize cofactor F_{420} but catalyzes the ferredoxin-dependent reduction of methanophenazine (Fig. 6). Unlike the aceticlastic *Methanosarcina* species, where the ferredoxin produced during the cleavage of acetyl-CoA is reoxidized either by an Ech hydrogenase or an Rnf complex, *Mt. thermophila* directly channels the electrons of ferredoxin into a membrane-bound electron transport chain consisting of a ferredoxin:methanophenazine oxidoreductase (the Fpo-like complex) and the canonical methanophenazine-dependent heterodisulfide reductase (HdrDE) (Welte and Deppenmeier, 2014). The assumption that the “headless” Fpo-like 11-subunit complex (lacking FpoF) does not interact with $F_{420}H_2$ but accepts electrons directly from Fd_{red} is consistent with the absence of F_{420}-dependent activities and the

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presence of ferredoxin-dependent heterodisulfide reductase activities in the membrane fraction of *Mt. thermophila* (Welte and Deppenmeier, 2011).

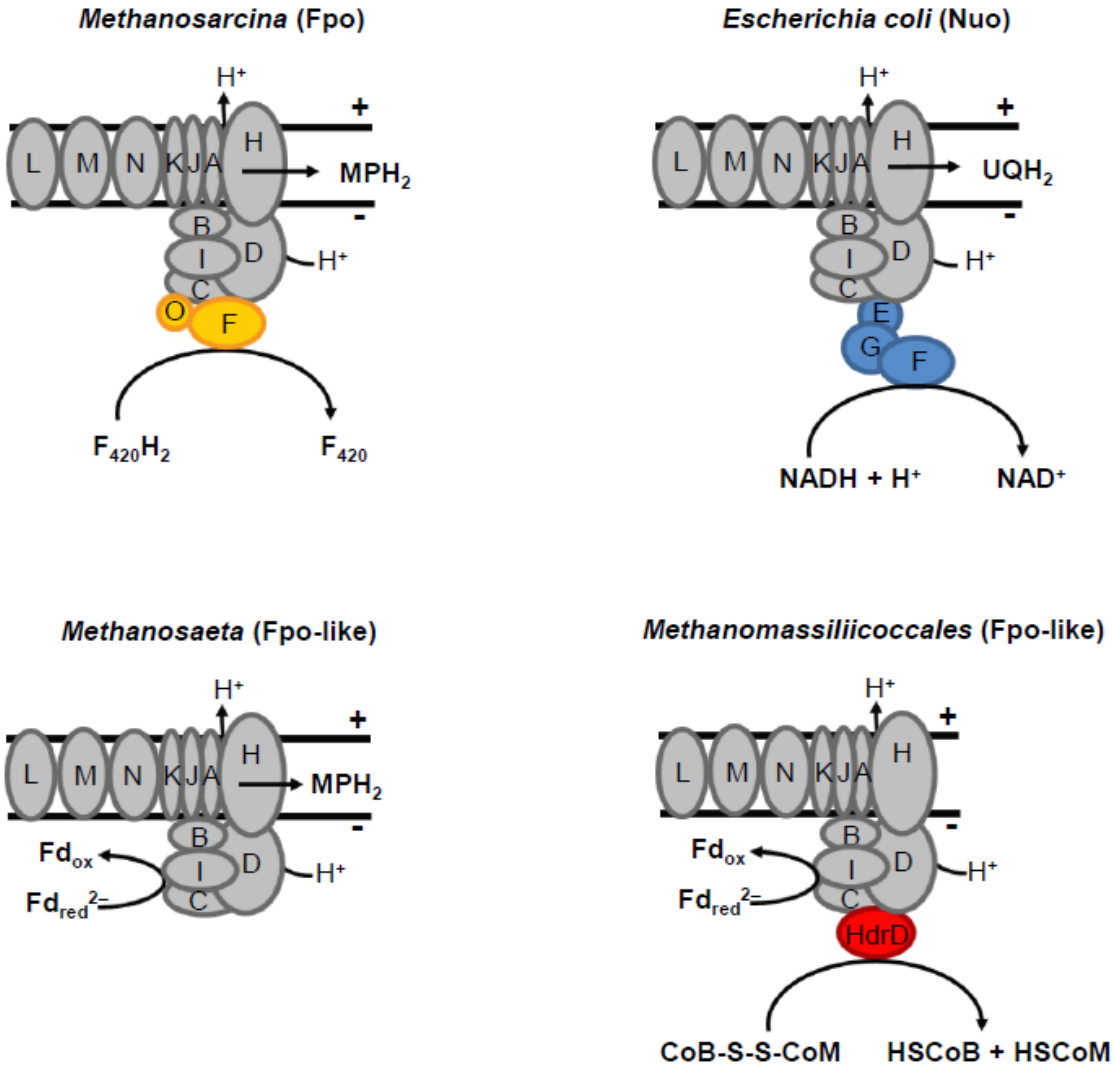

Figure 6. Redox processes catalyzed by the 11-subunit core complexes and their specific electron-transferring modules in *Methanosarcina mazei* (Fpo) and *Escherichia coli* (Nuo) and hypothetical processes and potential interaction partners of the Fpo-like complexes in *Methanosaeta thermophila* (Welte and Deppenmeier, 2011) and *Methanomassiliicoccales* (this study). The common core complex of 11 subunits is shown in gray; specific subunits of the different complexes are indicated by different colors. In all cases, the complex serves as a redox-driven proton pump. For further explanations, see text. F_{420}, coenzyme F_{420}; Fd, ferredoxin; MP, methanophenazine; UQ, ubiquinone.

There is also no evidence for the presence of F_{420}-dependent enzymes in any of the four *Methanomassiliicoccales* genomes, and although autofluorescence at 420 nm is mentioned in the species description of *Mmc. luminyensis* (Dridi *et al.*, 2012), we

could not detect the characteristic autofluorescence of cofactor F_{420} in *Mmc. luminyensis* or *Ca.* Mp. termitum (Paul *et al*., 2012; this study). Therefore, we assume that also the Fpo-like 11-subunit complex of *Methanomassiliicoccales* must interact directly with ferredoxin.

It is striking that all *Methanomassiliicoccales* genomes encode a homolog of HdrD, the heterodisulfide-reducing subunit of the membrane-bound heterodisulfide reductase complex (HdrDE) in *Methanosarcinales*, but lack the *hdrE* gene, which encodes the cytochrome-*b*-containing membrane anchor of the complex that accepts electrons from methanophenazine (Buan and Metcalf, 2010). Since there is also no evidence for other enzymes with cytochromes or cytochrome biosynthesis in any of the genomes (see below), the Fpo-like complex of *Methanomassiliicoccales* cannot couple ferredoxin oxidation to heterodisulfide reduction the same way as proposed for *Mt. thermophila*, i.e., via methanophenazine and a canonical heterodisulfide reductase (HdrDE) (Welte and Deppenmeier, 2011). Instead, we propose that it interacts directly with HdrD, imparting the entire complex with the function of an energy-converting ferredoxin:heterodisulfide oxidoreductase (Fig. 6).

The subunit of the Fpo-like complex responsible for ferredoxin oxidation remains to be identified. It has been suggested that the unusual density of lysine at the extended C-terminus of FpoI in *Mt. thermophila* (FIG S1) may serve for interaction with the acidic ferredoxin (Welte and Deppenmeier, 2014). Although also the C-terminus of the FpoI subunit of *Methanomassiliicoccales* species is extended and rich in lysine, it is noteworthy that these features are not present in the homologous subunits of the ferredoxin-oxidizing [NiFe] hydrogenases (HycF, HyfH) (FIG S1).

Energetic aspects. Welte and Deppenmeier (Welte and Deppenmeier, 2014) have pointed out that the more negative redox potential of ferredoxin ($E_0' = –500$ mV) compared to that of cofactor F_{420} (–360 mV) renders the reaction with methanophenazine catalyzed by the Fpo-like complex of *Mt. thermophila* more exergonic than that of the canonical F_{420}-dependent Fpo of *Methanosarcina* species. In the case of the *Methanomassiliicoccales*, the direct reduction of the heterodisulfide via HdrD should be even more favorable, since the midpoint potential of the heterodisulfide (–140 mV) is slightly more positive than that of methanophenazine (–165 mV; Welte and Deppenmeier, 2014). It would be premature to speculate on the number of protons translocated by the Fpo-like complex of *Methanomassiliicoccales*,

but we want to point out that electron bifurcation at the soluble heterodisulfide reductase dictates that only the electrons of every second hydrogen oxidized by the Mvh/HdrABC complex will feed into the energy-converting ferredoxin:heterodisulfide oxidoreductase (Fpo-like/HdrD) complex (Fig. 2). This should negatively affect growth yield but may also increase the competiveness by decreasing the threshold for hydrogen.

All genomes of *Methanomassiliicoccales* encode an H^+/Na^+ antiporter and the typical A_1A_0 ATP synthase of archaea. The C subunit of the ATP synthase (AhaC) has the same conserved Na^+-binding motif as in *Methanosarcina acetivorans* and *Methanosarcina mazei*, but also in those organisms the ion specificity of ATP synthase is not fully resolved (Schlegel and Müller, 2013). Since all 11-subunit complexes are considered to be proton pumps (Moparthi *et al*., 2014) and since the Fpo-like complex is the only energy-converting complex in *Methanomassiliicoccales*, it is likely that methanogenesis is coupled to ATP synthesis via a proton-motive force.

Acetyl-CoA synthesis. Like the other *Methanomassiliicoccales, Ca.* Mp. termitum possesses a homolog of the *acsA* gene encoding an ADP-forming acetyl-CoA synthetase, which allows heterotrophic growth on acetate (Fig. 7). The presence of gene clusters encoding a CO dehydrogenase/acetyl-CoA synthase complex, two Ech hydrogenases, and a 5,10-methylenetetrahydrofolate reductase (MetF) in *Mmc. luminyensis* and *Ca.* Mmc. intestinalis suggests that the members of the *Methanomassiliicoccaceae* may be able to synthesize acetyl-CoA also from formate and CO_2 (Fig. 7). However, it should be noted that the structure of the gene cluster encoding CO dehydrogenase/acetyl-CoA synthase differs from that in other methanogens (FIG S2). It lacks the gene encoding the epsilon subunit (CdhB) typical of methanogens, and the genes encoding the alpha and beta subunits (CdhA/C) seem to be fused and truncated as compared to the *cdhA* gene of other methanogens. The fused gene shows highest sequence similarity to a homolog in the homoacetogenic *Acetonema longum*, although the latter possesses also a second, non-truncated *cdhA* gene. In addition, the beta-part of the *cdhA/C* gene of *Mmc. luminyensis* is interrupted by several insertions, which suggests that the gene may no longer encode a functional enzyme.

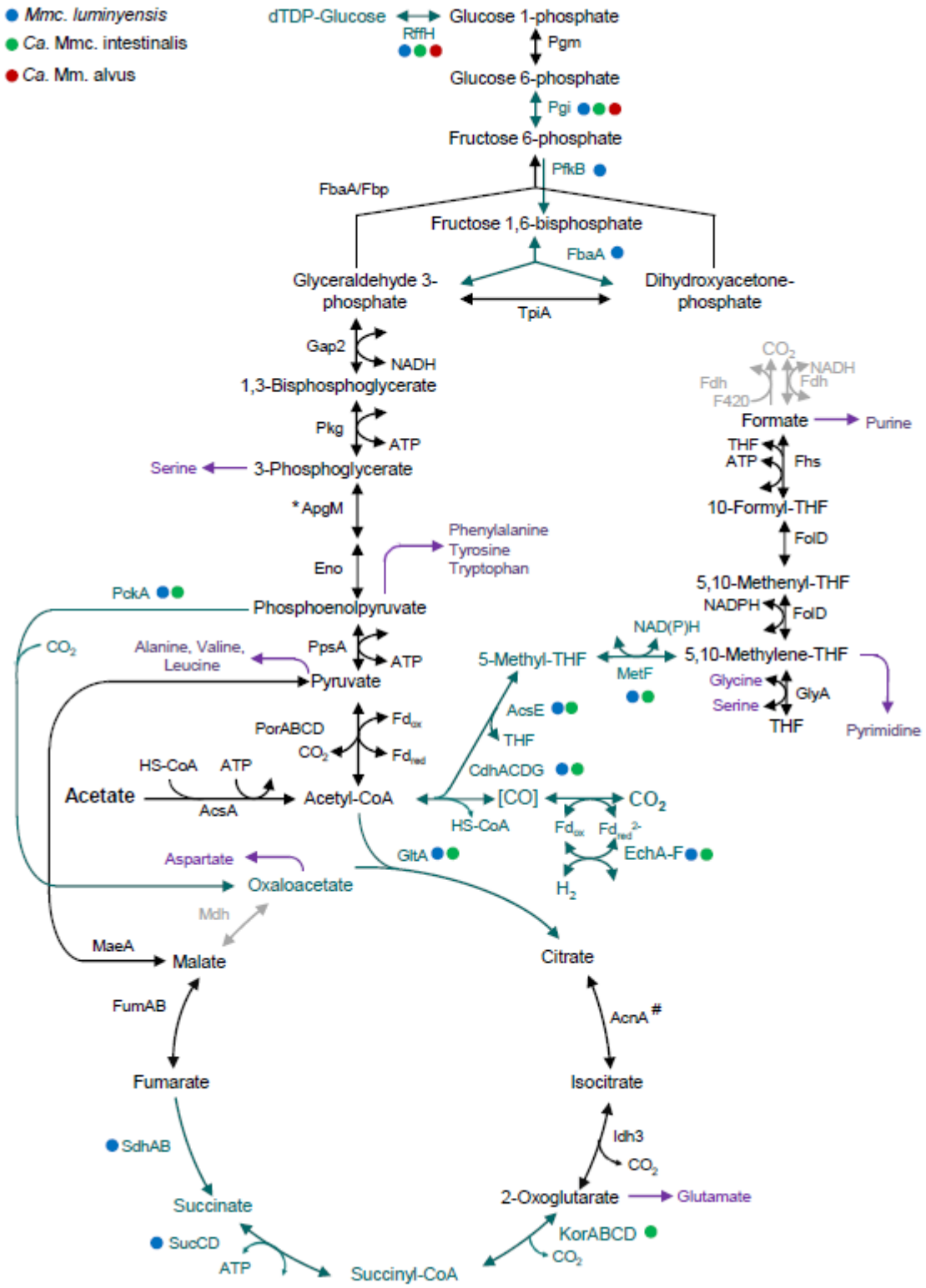

Figure 7. Intermediary metabolism and glycolysis/gluconeogenesis of *Ca*. Mp. termitum and other *Methanomassiliicoccales*. Black arrows indicate reactions whose enzymes are encoded by all genomes. Blue-green arrows indicate that the enzymes are not present in *Ca*. Mp. termitum but are present in the genomes indicated by the colored dots. Gray arrows indicate important enzymes not encoded by any of the four genomes. *, *Ca.* Mm. alvus has *gpmA* instead of *apgM*. #, *acnA* was not found in *Mmc. luminyensis*.

One-carbon metabolism. All Methanomassiliicoccales possess the genes required to generate 5,10-methylenetetrahydrofolate from formate. However, the absence of formate dehydrogenase suggests that the pathway operates in the reverse direction, generating both 5,10-methylenetetrahydrofolate (for pyrimidine biosynthesis) and formate (for purine biosynthesis and as cosubstrate of the ribonucleotide reductase) from serine (Fig. 7). The same anabolic role of the C1 pathway has been postulated for M. stadtmanae (Fricke et al., 2006). Also the CO dehydrogenase/acetyl-CoA synthase of Methanomassiliicoccales (if at all functional) may serve to generate C1 compounds from acetyl-CoA.

Gluconeogenesis and glycolysis. All *Methanomassiliicoccales* possess the genes required for gluconeogenesis via pyruvate-ferredoxin oxidoreductase and a 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (ApgM), which is characteristic of archaea (van der Oost *et al*., 2002). Only *Ca.* Mm. alvus possesses the bacterial, bisphosphoglycerate-dependent variant (GpmA). Also the bifunctional fructose-1,6-bisphosphate aldolase/phosphatase present in all strains is typical for archaeal gluconeogenesis (Say and Fuchs, 2010). The genome of *Ca.* Mp. termitum lacks a homolog encoding phosphoglucoisomerase (Pgi), but since the pathway of gluconeogenesis is otherwise complete, this step may involve an unknown enzyme. Genes for glycogen biosynthesis or degradation are not present in any of the strains. Only *Mmc. luminyensis*, the strain with the largest genome, may be capable of glycolysis because it possesses a phosphofructokinase (PfkB) and an archaeal class-I fructose-bisphosphate aldolase (FbaA) (Fig. 7).

Intermediary metabolism. As in all methanogens, the TCA cycle of *Methanomassiliicoccales* is incomplete. *Mmc. luminyensis* and *Ca.* Mmc. intestinalis both possess a phosphoenolpyruvate carboxykinase (PckA) for providing oxaloacetate, a Si-citrate synthase (GltA), and the remaining enzymes of the oxidative branch (a homolog encoding aconitase (AcnA) is missing in the draft genome of *Mmc. luminyensis*). Both *Methanomassiliicoccus* species should also have the capacity to synthesize succinyl-CoA, either via 2-oxoglutarate (*Ca.* Mmc. intestinalis) or via the reductive branch (*Mmc. luminyensis*), involving a cytochrome-free succinate dehydrogenase (ShdAB) and succinyl-CoA synthetase (SucCD). All four strains should be able to synthesize malate from pyruvate via malic enzyme (MaeA) but lack a malate dehydrogenase, which should cause aspartate auxotrophy

in members of the intestinal cluster. The latter should also be unable to synthesize 2-oxoglutarate and succinyl CoA, resulting in a requirement for glutamate and methionine.

Amino acid and nucleotide synthesis. Although all strains may be unable to form glutamate and/or aspartate *de novo*, the pathways for the biosynthesis of other amino acids are mostly complete (FIG S). The absence of genes encoding threonine aldolase (ItaE) and homoserine O-acetyltransferase (MetX) in all strains except *Mmc. luminyensis* suggests methionine auxotrophy in the former strains. *Ca*. Mmc. intestinalis is the only strain that lacks genes required for tryptophan synthesis from serine and chorismate. As in all methanogens, the gene coding for histidinol phosphatase (HisJ) remains to be identified. The genes required to operate the pentose phosphate pathway and for biosynthesis of phosphoribosyl pyrophosphate (PRPP) and nucleic acids are present in all strains (Table S1).

Biosynthesis of porphyrinoids. All methanogens can synthesize cofactor F_{430}, a Ni porphyrinoid that functions as the prosthetic group of Mcr and is essential for methanogenesis (Diekert *et al*., 1980; Whitman and Wolfe, 1980). All *Methanomassiliicoccales* possess the genes for the entire pathway of corrinoid biosynthesis via glutamyl-tRNA reductase up to precorrin-2 (HemABCDL, CobA; Thauer and Bonacker, 1994). Like all other methanogens, they lack the typical pathway for heme biosynthesis via coproporphyrinogen III, but also the genes for the alternative pathway for heme biosynthesis via precorrin-2 (Kühner *et al*., 2014) are absent in the genomes, underscoring the inability of *Methanomassiliicoccales* to synthesize cytochromes. Since none of the strains has the capacity to synthesize methionine, the methyl group donor in the biosynthesis of factor F_{430} (Jaenchen *et al*., 1981), neither via the methionine biosynthesis pathway I (which would require succinyl-CoA) nor via one of the other pathways (FIG S3), they must depend on an external source of this amino acid.

Ultrastructure. Negative stains of Ca. Mp. termitum strain MpT1 and the closely related *Ca*. Mp. millipedum strain MpM2 showed coccoid cells with diameters between 500 and 800 nm (Fig. 8). No obvious dividing cells were observed. A small number of cells carried appendages, but generally not more than one per cell. Although the diameter (12 nm) of the appendage matches the typical size of an archaellum, none of the *Methanomassiliicoccales* genomes contain the typical

archaellum operon present in other archaea (Jarrell and Albers, 2012). Only *Ca*. Mp. termitum, *Mmc. luminyensis,* and *Ca*. Mm. alvus possess genes that may represent homologs of the archaellum biosynthesis pathway, such as prearchaellin peptidase (FlaK; all three strains), secretion ATPase (FlaI; only *Mmc. luminyensis*), and a polytopic membrane protein (FlaJ; only *Mmc. luminyensis*) that interacts with ATPase (Jarrell and Albers, 2012). However, genes encoding archaellin (FlaB), the major filament component of the archaellum, are absent in all strains. Since the same is true also for all genes potentially involved in pilus biosynthesis, the nature of the cell appendages observed in the negative stains of *Ca*. Mp. termitum and strain MpM2 remains obscure.

In ultrathin sections, both strains showed a homogenous cytoplasm surrounded by a cytoplasmic membrane and an outermost layer that resembled a second membrane (Fig. 8C,F). Although great care was taken to preserve the structure during preparation, the outermost layer was often not present or seemed to be detached from the cells (Fig. 8E). The distance between the two membranes ranged from 10 and 300 nm, often even within the same cell. Since the integrity of its structure was affected by centrifugation, fixation, and freeze substitution, the possibility of artifacts cannot be excluded.

Interestingly, also the species description of *Mmc. luminyensis* (Dridi *et al*., 2012) contains evidence for a second membrane system. The transmission electron micrograph of an ultrathin section shows a single cell surrounded by two electron-dense layers, one enclosing the cytoplasm and the other separated from the former by a wide electron-lucent ring. Although this interpretation differs from that of the authors (Dridi *et al*., 2012), and despite obvious differences to our preparations in structure and contrast (unfortunately, no experimental procedures were given), we are confident that the cell envelope of both *Ca.* Mp. termitum and *Mmc. luminyensis* does not consist of a single lipid membrane covered by a proteinaceous S-layer, as in most other archaea (König *et al*., 2007; Kling *et al*., 2013), but that the cells have a two-membrane system. Dual membranes in archaea have so far been restricted to *Ignicoccus* species (Rachel *et al*., 2002) and the ultrasmall ARMAN cells (Comolli *et al*., 2009). However, in view of the sensitivity to manipulation of the outermost membrane of *Ca.* Mp. termitum, it is possible that this structure is more widespread than it appears.

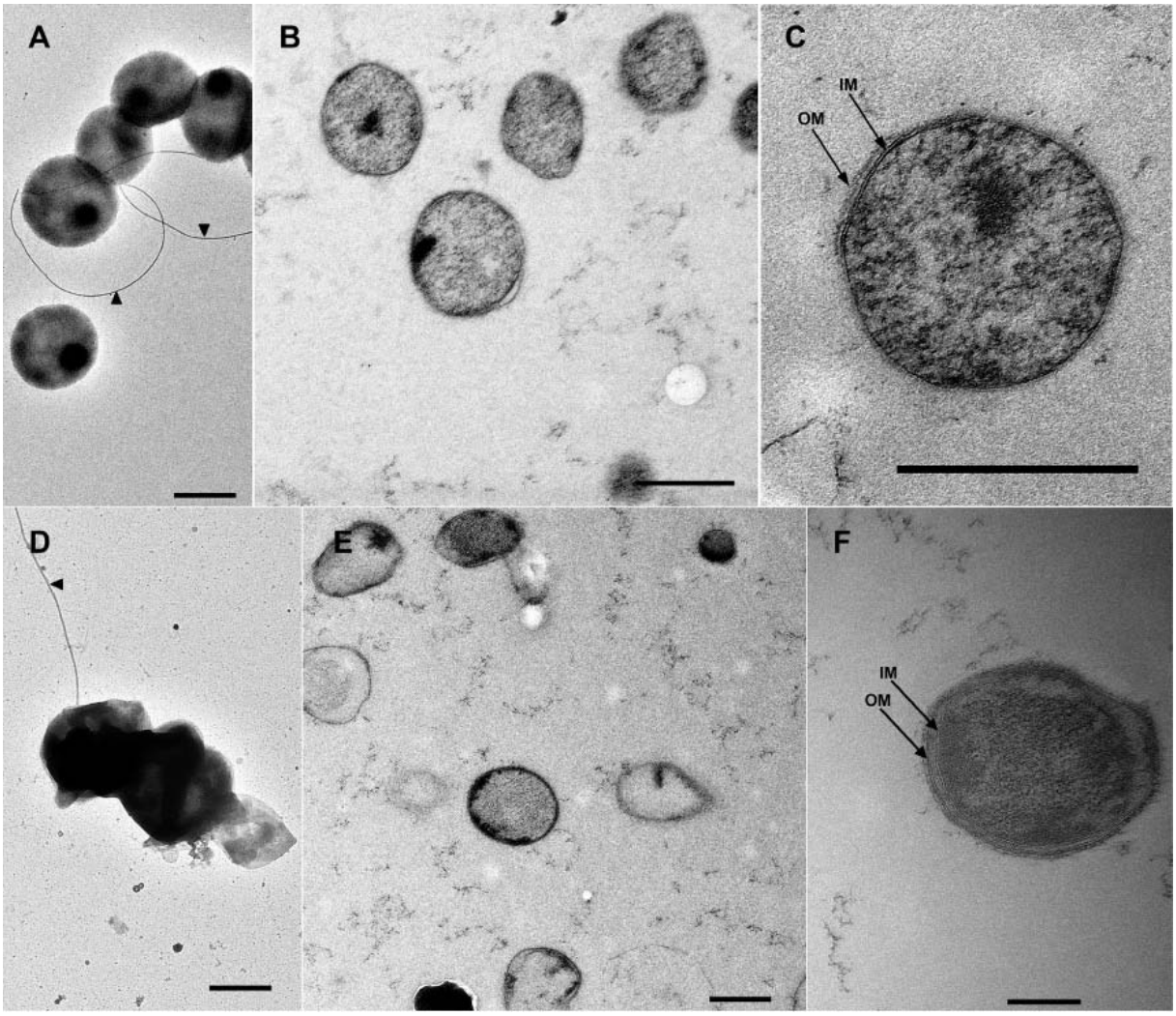

Figure 8. Ultrastructure of *Ca.* Methanoplasma termitum strain MpT1 (A–C) and the closely related Strain MpM2 from a millipede (D–F). Panels A and D show cells negatively stained with uranyl acetate, illustrating the coccoid shape and occasional cell appendages (arrowheads). Ultrathin sections of high-pressure frozen cells at intermediate (B and E) and high magnification (C and F), showing the homogenous cytoplasm surrounded by a cytoplasmic membrane (IM) and an additional outermost membrane (OM) that occasionally showed the characteristics of a lipid bilayer (F). Scale bars: 500 nm (A–E) and 200 nm (F).

Neither the ultrathin sections of *Ca.* Mp. termitum (this study) nor the image of Mmc. luminyensis (Dridi *et al*., 2012) shows indications of a proper cell wall. This is in agreement with the absence of most genes involved in the synthesis of UDP-*N*-acetyl-D-glucosamine, the precursor of pseudomurein (Kandler and König, 1978), from the genomes of *Ca*. Mp. termitum and *Ca.* Mm. alvus. Interestingly, both *Mmc. luminyensis* and *Ca*. Mmc. intestinalis retain all genes required to synthesize this compound.

Evolution. Although the Euryarchaeota comprise several non-methanogenic lineages, the apparent co-cladogenesis of phylogenetic (16S rRNA genes) and functional marker genes (*mcrA*) suggests that methanogens and anaerobic methane oxidizers are a monophyletic group (Bapteste *et al*., 2005; Knittel and Boetius, 2009). Also a recent phylogenomic analysis supports the hypothesis that the *Methanomassiliicoccales* are derived from methanogenic ancestors (Borrel *et al*., 2013b).

Other lineages in the *Thermoplasmata* obviously lost the capacity for methanogenesis and acquired other modes of energy metabolism. The *Thermoplasmatales* are facultative anaerobes (Huber and Stetter, 2006), whereas their closest relatives from deep sea hydrothermal vent group II (which includes *Ca.* A. boonei, whose complete genome is now available) possess a sulfur-based energy metabolism (Reysenbach *et al*., 2006). There is also no evidence for the presence of *mcr* genes for other deep-branching lineages of *Thermoplasmata* found in marine sediments or the deep subsurface (Paul *et al*., 2012; Borrel *et al*., 2013b; Lloyd *et al*., 2013; Ragon *et al*., 2013; see Fig. 1).

While the soluble heterodisulfide reductase (HdrABC) is a common feature of all methanogens, its membrane-bound analogue is present only in the apical lineages. Interestingly, *Methanocellales* and *Methanomassiliicoccales* possess only a homolog of the subunit carrying the catalytic domain (HdrD), whereas the cytochrome-containing membrane anchor (HdrE) must have been acquired at a later stage, since HdrDE is present only in the *Methanosarcinales*. It is not clear whether the homologs in *Archaeoglobales* (HmeDC) are derived from their methanogenic ancestor or the result of lateral gene transfer, which would also explain the presence of HdrD in *Methanosphaerula palustris* (*Methanomicrobiales*).

Homologs of the 11-subunit complex are present only in a few euryarchaeotal lineages. They are entirely absent from all basal Euryarchaeota, but present in the Thermoplasmata (*Thermoplasmatales*, *Methanomassiliicoccales*) and the euryarchaeotal crown groups (*Archaeoglobales*, *Methanosarcinales,* and *Halobacteriales*). The phylogeny of the large subunit of the Fpo-like complex of *Methanomassiliicoccales* is more similar to the homologous subunits in the Fpo and Fpo-like complexes of *Methanosarcina* and *Methanosaeta* spp. than to those in their closer, non-methanogenic relatives, the strictly anaerobic *Ca.* Aciduliprofundum

boonei (Reysenbach *et al*., 2006) and the facultatively anaerobic *Thermoplasmatales* (Segerer *et al*., 1988), which suggests that some of them have acquired the complex by lateral gene transfer (Fig. 4). However, also the function of related complexes may change by interaction with different electron-accepting modules. This is nicely illustrated by the Fpo-like complexes of *Methanomassiliicoccales* and *Methanosarcinales*, which may be of common origin but interact with different electron donors (ferredoxin of cofactor F_{420}) or electron acceptors (HdrD or methanophenazine).

Ecological considerations. It is striking that obligately methyl-reducing methanogens have so far been isolated only from intestinal tracts, but at least in the case of *Methanomassiliicoccales*, seem to occur in diverse habitats. At standard conditions, the hydrogen-dependent reduction of methanol to methane ($H_2 + CH_3OH \rightarrow CH_4 + H_2O$; $\Delta G°' = -112.5$ kJ per mol CH_4) is thermodynamically more favorable than its disproportionation to methane and CO_2 ($4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$; $\Delta G°' = -103.7$ kJ per mol CH_4; calculated after Thauer *et al*., (1977). However, the difference becomes smaller with decreasing hydrogen concentrations, and methanol disproportionation would be energetically superior already at moderate hydrogen partial pressures ($P_{H_2} < 10^{-2}$ bar).

Nevertheless, it is likely that the hydrogen thresholds of methyl-reducing methanogens differ between different lineages. In *Methanosphaera stadtmanae*, which lacks both an Fpo-like complex and HdrD and probably grows by H_2 coupling via an energy-converting (Ehb) and an uptake hydrogenase (Mvh) (Thauer *et al*., 2008), these enzymes may not be able to operate at extreme hydrogen partial pressures. Little is known about *Methanomicrococcus blatticola* except that it has F_{420} and, being a member of *Methanosarcinales,* probably also HdrDE; its affinity to H_2 when growing on methanol is slightly higher than that of *Ms. barkeri* growing on H_2 and CO_2 (Sprenger *et al*., 2007). In *Methanomassiliicoccales*, however, the bifunctional role of heterodisulfide in the production of reduced ferredoxin and its subsequent oxidation would allow only one event of energy conservation per two CH_4 produced, resulting in a decreased energy yield but increased hydrogen affinity of the methyl-reducing pathway (see above). This would be similar to the trade-offs between affinity and growth yield encountered in aceticlastic methanogens.

The many variations in the biochemistry of the methanogenic pathways may represent adaptations to cope with special environmental conditions. Understanding these strategies in detail will require physiological and biochemical studies of the groups in question.

Description of "*Candidatus* Methanoplasma termitum".

Me.tha.no.plas'ma. N.L. n. *methanum* [from French n. *meth*(*yle*) and chemical suffix -*ane*], methane; N.L. pref. *methano*-, pertaining to methane; Gr. neut. n. *plasma*, something formed or molded, a form; N.L. neut. n. *Methanoplasma*, a methane-producing form.

ter'mi.tum. L. masc. n. *termes, termitis* (variant of *tarmes*), a woodworm, a termite; L. masc. n. gen. pl. *termitum*, of termites, referring to the habitat of the organism.

Short description: roundish cells, 0.5 to 0.8 µm in diameter, without apparent cell wall, surrounded by two membranes, possess archaellum-like cell appendages. Obligate anaerobe. Methanogenic metabolism; obligately methylotrophic; methyl donors: methanol, mono-methylamine, but not di- or tri-methylamine. Obligately hydrogen dependent. Form a monophyletic group within the radiation of the "Intestinal cluster" of *Methanomassiliicoccales*. Habitat: intestinal tract of termites and cockroaches. Basis of assignment: strain MpT1 from *Cubitermes ugandensis* (16S rRNA gene sequence JX266068; complete genome sequence ########), and 16S rRNA gene sequences of so far uncultured representatives (accession numbers JX266062 to JX266070).

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3.7 Supplementary Information

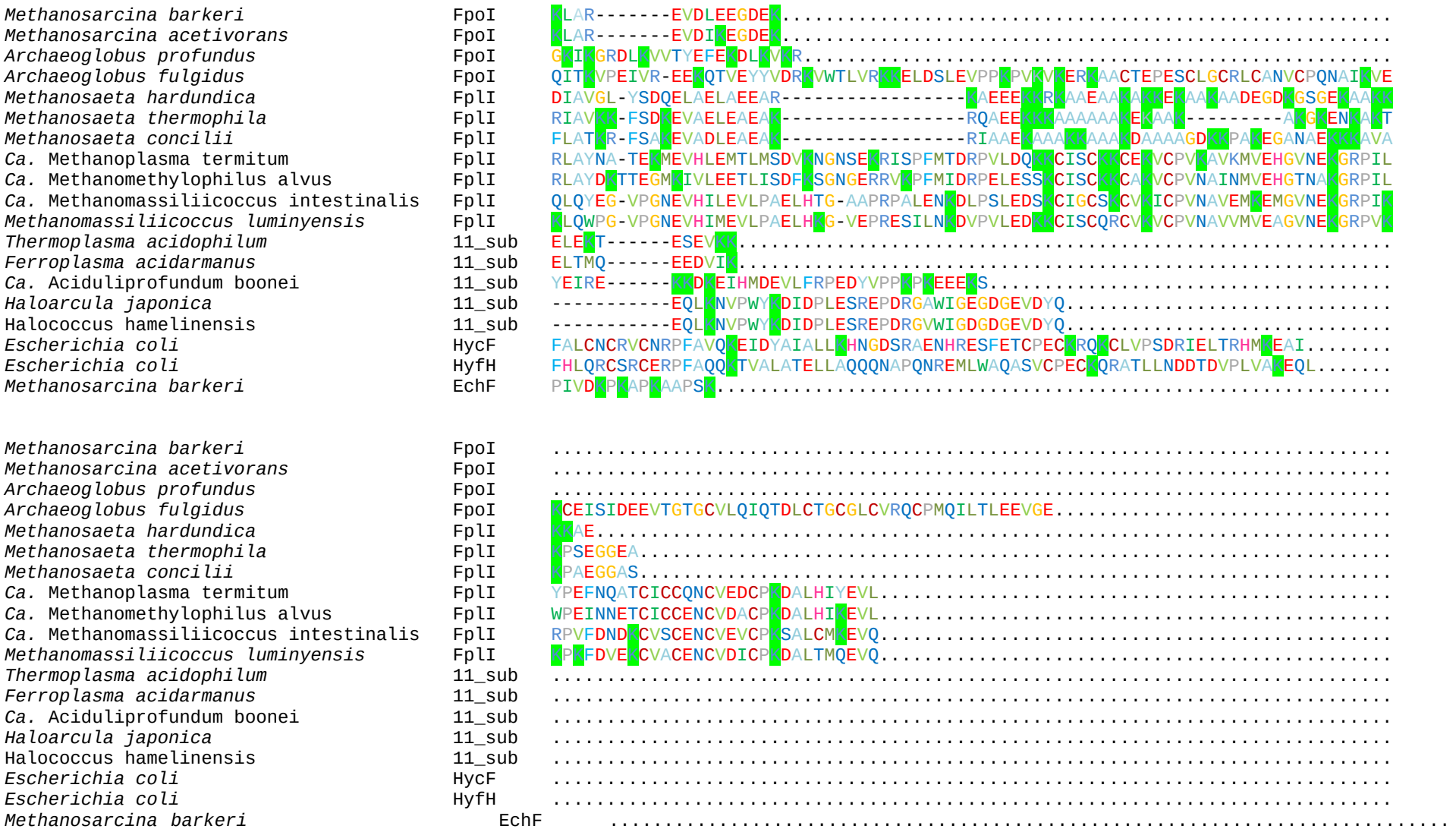


Figure S1. Alignment of the C-terminus of FpoI of *Methanosarcina* and *Archaeoglobales* species; FpoI-like (FpII) of *Methanosaeta* and *Methanomassiliicoccales* species; the I subunit of the 11-subunit complex (11_sub) of *Thermoplasmatales* species, *Ca.* Aciduliprofundum boonei, and *Halobacteriales* species; the ferredoxin-reducing subunits of the hydrogenases Hyc and Hyf of *Escherichia coli;* and the Ech hydrogenase of *Methanosarcina barkeri*. Amino acids of the same families have the same color; lysine is highlighted in green.

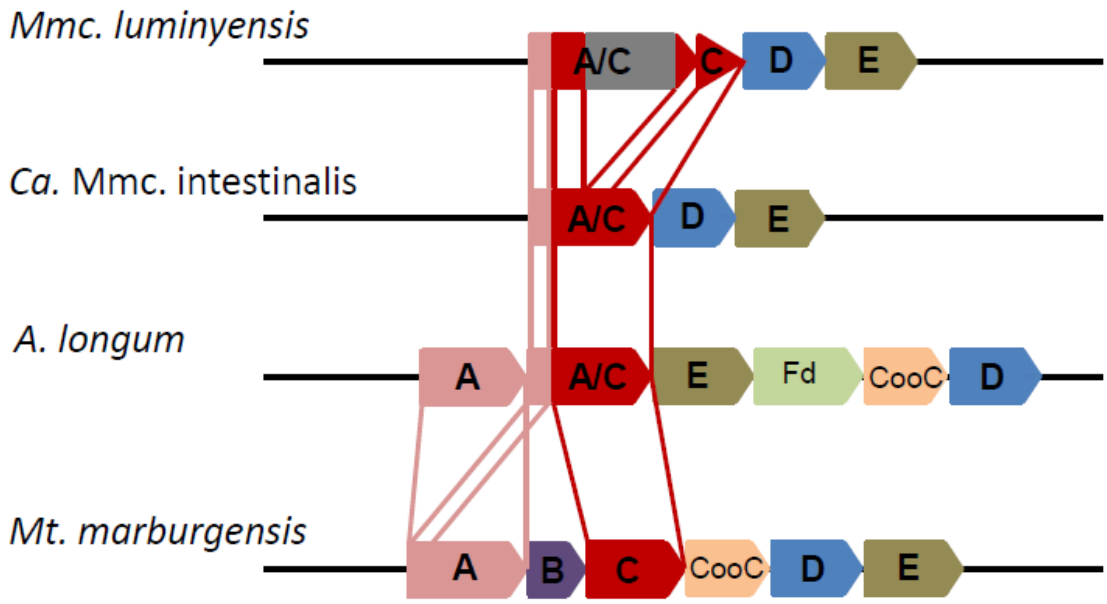
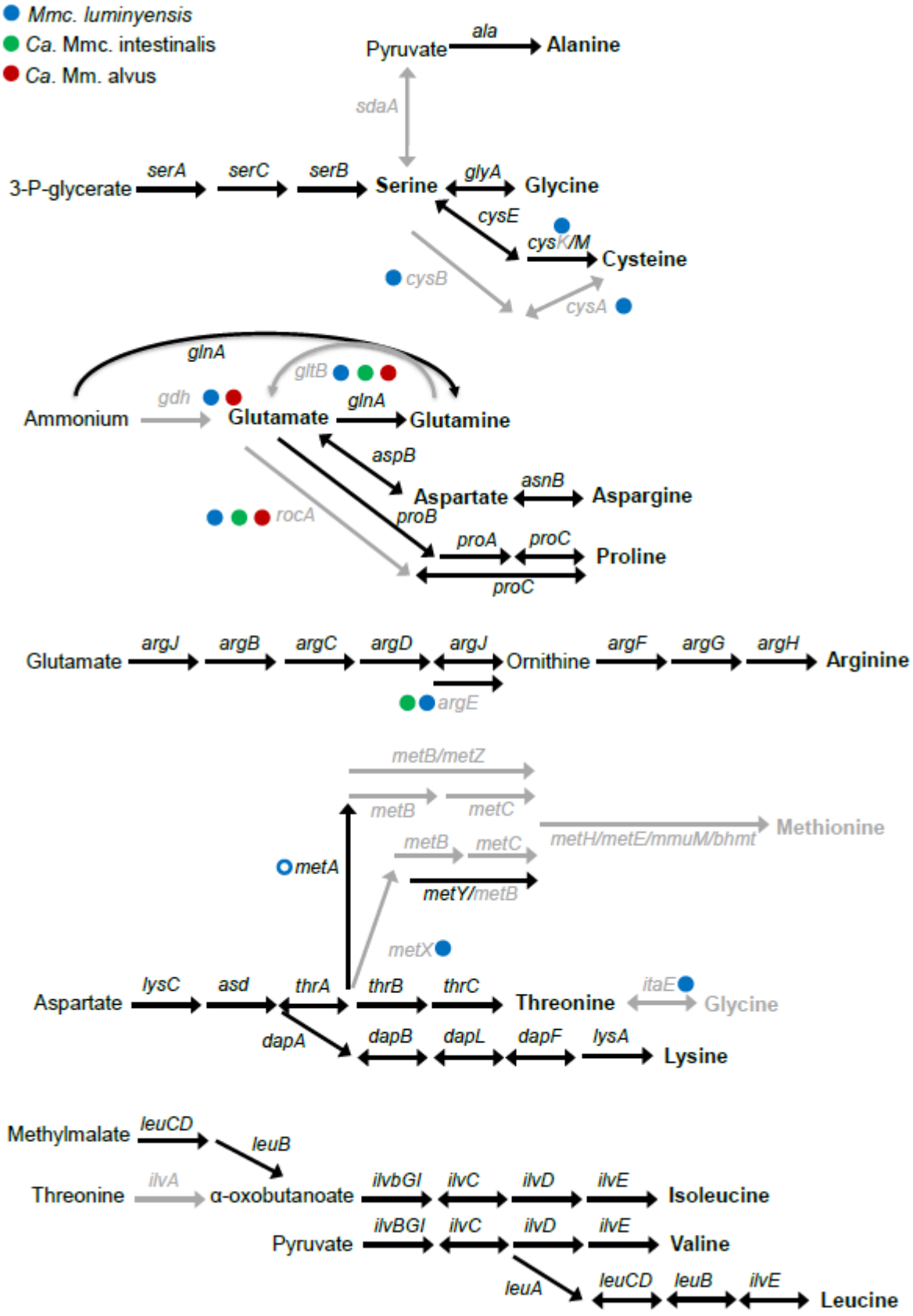


Figure S2. CO dehydrogenase/acetyl-CoA synthase operon struc**ture** of *Methanomassiliicoccus luminyensis*, *Ca. Methanomassiliicoccus intestinalis*, *Acetonema longum*, and *Methanothermobacter marburgensis*. Abbreviations: A, alpha subunit; C, beta subunit; A/C, fused alpha/beta subunit; D, delta subunit; E, gamma subunit; Fd, ferredoxin; CooC, CO dehydrogenase maturation factor; B, epsilon subunit; Fd, Fe-S-cluster-containing protein.


Mmc. luminyensis
Ca. Mmc. intestinalis
Ca. Mm. alvus
Pyruvate
ala
Alanine
sdaA
3-P-glycerate
serA
serC
serB
Serine
glyA
Glycine
cysE
cysK/M
Cysteine
cysB
cysA
glnA
gltB
gdh
Ammonium
Glutamate
glnA
Glutamine
aspB
Aspartate
asnB
Aspargine
rocA
proB
proA
proC
Proline
proC
Glutamate
argJ
argB
argC
argD
argJ
Ornithine
argF
argG
argH
Arginine
argE
metB/metZ
metB
metC
metH/metE/mmuM/bhmt
Methionine
metB
metC
metA
metY/metB
metX
Aspartate
lysC
asd
thrA
thrB
thrC
Threonine
itaE
Glycine
dapA
dapB
dapL
dapF
lysA
Lysine
Methylmalate
leuCD
leuB
Threonine
ilvA
α-oxobutanoate
ilvbGI
ilvC
ilvD
ilvE
Isoleucine
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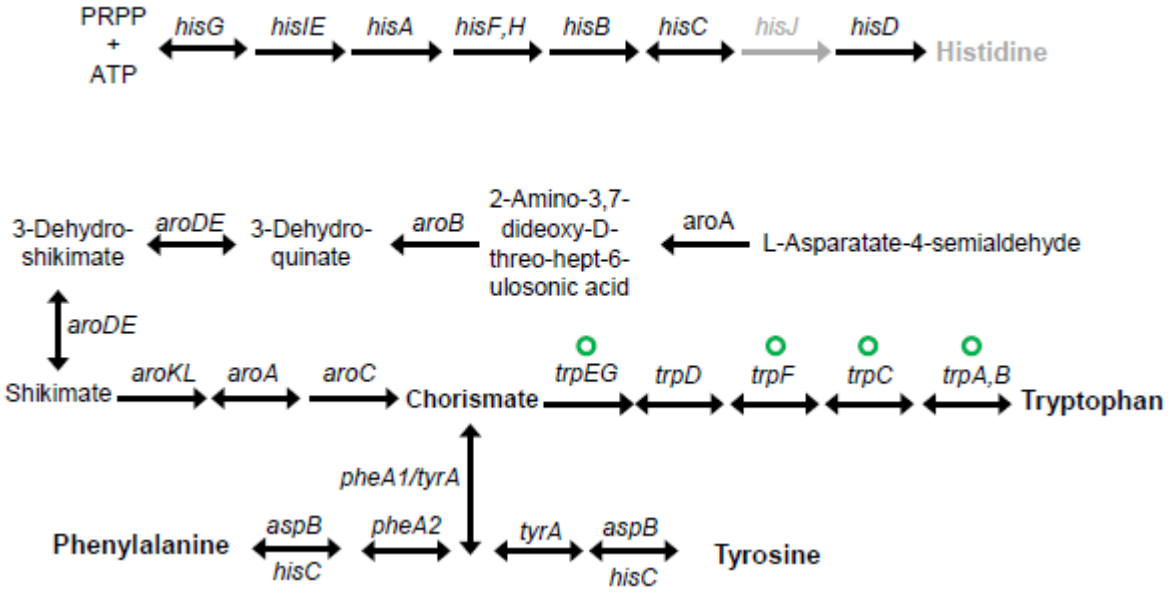

Figure S3. Schematic overview of the amino acid anabolism of *Ca*. Methanoplasma termitum and other species of *Methanomassiliicoccales*. Genes present in the genome of *Ca*. Mp. termitum are shown in black; an open circle indicates that the gene is absent in the other genomes. Amino acids that cannot be synthesized by *Ca*. Mp. termitum and the corresponding missing genes are shown in gray; colored, filled circles indicate that the genes are present in the other genomes.

TableS1. Annotated list of genes in the genome of *Ca*. Methanoplasma termitum. The gene product names and the predicted functions are manually curated. The genes are listed by gene ID but can be sorted as desired. In addition to the enzyme, the COG, Pfam, Tigrfam, KO, and IMG assignment of each gene product are given. Indicated are the number of nucleotides and deduced amino acids, start and stop coordinates, strand, locus type, and presence of signal peptides and transmembrane helices. Please download Table S1 from http://www.termites.de/brune/publ/suppl/Lang_Diss_Chap3_Table_S1.html**.**

Table S2. Genes encoding the different subunits of Ech hydrogenases in *Methanosarcina barkeri* and their homologs in *Methanomassiliicoccus luminyensis* and *Ca.* Methanomassiliicoccus intestinalis (*Methanomassiliicoccaceae*). Ech hydrogenases are absent in members of the intestinal cluster.

	Ech	Ech-1	Ech-1	Ech-2	Ech-2
	Ms. barkeri	*Mmc. luminyensis*	*Ca.* Mmc. intestinalis	*Mmc. luminyensis*	*Ca.* Mmc. intestinalis
Large subunit	*echE*	WP_019178475	MMINT_01070	WP_019176382	MMINT_17010
Small subunit	*echC*	WP_019178473	MMINT_01050	WP_019176384	MMINT_17030
4Fe/4S - Fd	*echF*	WP_019178476	MMINT_01080	–[a]	MMINT_17000
Small protein	*echD*	WP_019178474	MMINT_01060	WP_019176383	MMINT_17020
Transmembrane proteins	*echA*	WP_019178471	MMINT_01030	WP_019176386	MMINT_17050
	echB	WP_019178472	MMINT_01040	WP_019176385	MMINT_17040

[a] Not present

Table S3. Genes encoding the different subunits of Hyc and Hyf hydrogenases in *Escherichia coli* and their homologs in *Ca*. Methanoplasma termitum and *Methanomassiliicoccus luminyensis*. Hyf-like hydrogenases are absent in *Ca.* Methanomethylophilus alvus and *Ca.* Methanomassiliicoccus intestinalis.

	Hyc	Hyf	Hyf-like	Hyf-like
	E. coli	*E. coli*	*Ca*. Mp. termitum	*Mmc. luminyensis*
Large subunit	*hycE*	*hyfG*	Mpt1_00881	WP_019178467
Small subunit	*hycG*	*hyfI*	Mpt1_00880	WP_019178469
4Fe/4S - Fd	*hycF*	*hyfH*	Mpt1_00880	WP_019178468
Small protein	*hycE*	*hyfG*	Mpt1_00880	WP_019178467
Transmembrane proteins	*hycC*	*hyfD*	Mpt1_00882/85	WP_019178466/63
	hycD	*hyfC*	Mpt1_00884	WP_019178464
	– [a]	*hyfB,F*	Mpt1_00885/82	WP_019178463/66
	–	*hyfE*	Mpt1_00883	WP_019178465
Other subunits	*hycBH*	*hyfAJR*	–	–

[a] Not present

Chapter 4

Manuscript in preparation

Methanogenic community structure in the digestive tracts of tropical millipedes

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Running title: *Methanogenic community structure in millipedes*

Contributions:

K.L. designed the study, supervised the bachelor thesis of Lena Mikulski, measured CH_4 emission, conducted the phylogenetic analysis, performed qPCR analysis, evaluated the data, and prepared the draft manuscript.

L.M. measured CH_4 emission, dissected the millipedes, extracted DNA, created clone libraries, and evaluated the data.

C.D. supported the evaluation of the CH_4 emission rates, set up the required bioinformatic pipelines for processing, and analyzed the pyrosequencing data.

K.M. prepared libraries for the high-throughput sequencing analysis

A.B. conceived the study, supervised the experiments, and discussed the results.

4.1 Abstract

Millipedes belong to one of the rare taxa of arthropods that emit CH_4. However, almost nothing is known about methanogenesis and the methanogenic community structure in their guts. Measurements of CH_4 production rates by isolated hindgut sections of the tropical millipede *Anadenobolus monilicornis*, together with the high abundance of archaeal 16S rRNA genes measured by quantitative PCR, strongly supports the theory that methanogens are exclusively located in the hindgut of millipedes. So far phylogenetic analyses of the methanogenic community structure of millipedes are lacking. Therefore, we conducted the first comprehensive phylogenetic analysis of the guts of the tropical millipedes *Anadenobolus monilicornis*, *Aphistogoniulus corallipes*, *Microtrullius uncinatus*, *Harpagophoridae sp.*, documenting that methanogens from the genera *Methanobrevibacter* (*Methanobacteriales*), *Methanomicrococcus* (*Methanosarcinales*) and *Methanocorpusculum* (*Methanomicrobiales*) are the most abundant methanogenic archaea in the guts of these millipedes, as well as methanogens from the recently discovered seventh order of methanogens, the *Methanomassiliicoccales*. Most of these strains perform hydrogentrophic methanogenesis or use H_2 as the electron donor for the reduction of methylated compounds, indicating the importance of H_2-emitting bacteria or ciliates in the millipedes. An additional high-throughput sequencing analysis of the bacterial communities in the guts of *A. monilicornis* and *A. corallipes* revealed a high number of the *Enterobacteriaceae, Desulfovibronaceae* (*Protobacteria*), *Lachnospiraceae* and *Ruminococcaceae* (*Firmicutes*). These bacteria are known to produce H_2 as a major fermentation product. Clones obtained from the methanogenic community structure analysis were affiliated with sequences from the guts of termites, cockroaches and scarab beetle larvae. This result suggests that millipedes have a similar methanogenic community to other CH_4 emitting arthropods.

4.2 Introduction

Millipedes are of scientific interest because of their role in organic matter decomposition and nutrient cycling. In some habitats millipedes are responsible for the uptake of 5-10% of the leaf litter fall, however, when earthworms are absent, millipedes may occur at densities of several hundred/m^2 and consume 25% of the litter fall (Hopkin and Read, 1992).

Although millipedes are known for organic matter decomposition, it is the gut microbiota of the millipede that is most important for the breakdown of leaf litter material. As early as 1849 Joseph Leidy indentified long filamentous bacteria of the genus *Arthromitus* and the ciliate *Nyctotherus sp*. in the gut of millipedes (Leidy, 1849). The foregut of millipedes is sparsely populated by microorganisms (Byzov, 2006). However, at the junction point between the midgut and the hindgut the Malphigian tubules provide a nutrient broth, containing mineral compounds, urea and uric acid, which is an effective buffer solution. For this reason the hindgut offers a suitable site for microbial colonization (Bignell, 1849). Investigations by scanning electron microscopy confirmed that this region is densely colonized by diverse microorganism (Bignell, 1849).

A DAPI staining of bacteria in gut preparations of tropical millipede *Chicobolus sp.* revealed high numbers of microorganisms in the hindgut (15 x 10^9 cells/ml gut) and showed less densely populated fore- and midguts (1.7 x 10^9 cells/ml gut and 1.4 x 10^9 cells/ml gut) (Cazemier *et al.*, 1997). So far most of the bacteria isolated from millipede guts belong to the *Gammproteobacteria*, specifically to the family of the *Enterobacteriaceae* (genera: *Klebsiella, Enterobacter, Plesiomonas, Pseudomonas, Salmonella, Erwinia, Escherichia*) (Márialigeti *et al*., 1985; Contreras, 1990; Byzov *et al*., 1996; Tretyakova *et al*.; 1996; Knapp *et al*., 2010). The second most abundant group of bacteria isolated from millipede guts are *Actinobacteria,* belonging to the families *Promicromonosporacaeae, Cellulomonadaceae, Streptomycetaceae* (Dzingov *et al*., 1982; Jáger *et al*.; 1983; Szabó *et al*., 1983; Márialigeti *et al*., 1985; Byzov *et al*., 1996; Tretyakova *et al*.; 1996; Jarosz and Kania, 2000; Oravecz *et al*., 2002). However, these investigations were limited by the constraints of classical cultivation methods and reflect

only a small portion of the true diversity of the microbial community in the millipede gut. The first molecular analysis of the gut of the European millipede *Cylindroiulus fulviceps* by the molecular fingerprinting technique DGGE revealed that the intestinal tracts were dominated by *Gamma*- and *Deltaprotobacteria* (72%), as well as *Firmicutes* (17%) and *Bacteriodetes* (6%) (Knapp *et al*., 2009). An additional bacterial clone library analysis of the gut from the same millipede indicated a diverse bacterial community dominated by *Bacteroides* and *Proteobacteria* which were closely related to sequences from the intestinal tract of termites and beetles (Knapp *et al*., 2010).

Little is known about the bacterial composition in the guts of millipedes, and even less is known about the methanogenic community structure. Millipedes belong to one of the rare taxa of arthropods that emit methane (CH_4); other CH_4 producing arthropods are termites, cockroaches and scarab beetle larvae (Hackstein and Stumm, 1994). CH_4 is produced exclusively by methanogenic archaea that colonize the gut of these arthropods. The strictly anaerobic methanogens are widely distributed and occur in environments from the marine to freshwater sediments, soils, hot springs, sewage sludge, and in the digestive tracts of animals and humans (Liu and Whitman, 2008). Methanogens belong to the phylum of the *Euryarchaeota* that presently comprises seven orders of methanogens, the *Methanopyrales*, *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales* (Bapteste *et al*., 2005), *Methanocellales* (Sakai *et al*., 2008) and the recently discovered *Methanomassiliicoccales* (Dridi *et al*., 2012; Paul *et al*., 2012).

Hackstein and Stumm (1994) were the first to identify millipedes as a source of biogenic CH_4. However, CH_4 emission could only be measured for tropical species, while methanogens seemed to be absent from European millipedes. Interestingly, the European millipede *Glomeris sp.* emitted CH_4 after cocultivation with African diplopods (Hackstein and Stumm, 1994). An additional study on methanogenesis in millipedes documented that a few European millipedes are also able to emit methane (Sustr and Simek, 2009; Sustr *et al*., 2014). The characteristic F_{420} autofluorescence of methanogens revealed that they are exclusively localized in the hindgut of the animals, where they are free living, attached to food particles or the gut wall, or associated with anaerobic ciliates (Hackstein and Stumm, 1994). Methanogens colonizing these

anaerobic ciliates convert the intracellularly formed H_2 into CH_4 within the ciliate (Hackstein *et al*., 2006). Knapp *et al*., (2009) were the first to amplify 16S rRNA gene sequences of archaea by PCR-DGGE from the gut of European millipede *Cylindroiulus fulviceps*, but did not identify the bands. A subsequent study of the methanogenic community in the digestive tracts of millipedes identified sequences retrieved by PCR-DGGE related to the *Methanosarcinales*, *Methanobacteriales* and *Methanomicrobiales* and some uncultured archaea (Sustr *et al*., 2014). However, the study lacked a phylogenetic analysis; instead the classification of the sequences was carried out by a BLAST search (Sustr *et al*., 2014).

In this study, we comprehensively analyzed the phylogengy of methanogenic archaea in the guts of different tropical millipedes, investigated the bacterial composition by high-throughput sequencing and measured the abundance of 16S rRNA genes of archaea and bacteria by quantitative PCR. Furthermore, we analyzed the CH_4 production rate of the millipede *Anadenobolus monilicornis* and investigated the localization of the methanogens in its gut. The methanogenic clones fell within the orders *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales* and *Methano-massiliicoccus* and clustered with sequences from termites, cockroaches and scarab beetle larvae, suggesting a similar methanogenic community in these CH_4-emitting arthropods.

4.3 Material and Method

Millipedes. The tropical millipedes *Anadenobolus monilicornis*, *Aphistogoniulus corallipes*, *Microtrullius uncinatus*, and an unknown species of *Harpagophoridae* were obtained from commercial breeders (b.t.b.t. Insektenzucht, Schürpflingen,Germany; Wirbellosen Welt, Rödinghausen, Germany). The European millipedes *Julus scandinavius*, *Glomeris marginata*, and *Tachypodoiulus niger* were collected in the forest around the Max Planck Institute in Marburg. All animals were kept in plastic containers at room temperature and were fed on leaf litter and fruit.

Gut preparation. The millipedes were euthanized by freezing or by exposure to N_2-CO_2 (80/20, vol/vol) gas atmosphere for around 10 min. For the dissection, the animals were decapitated with scissors in a preparation dish filled with insect Ringer's solution (Brune

et al., 1995). *A. monilicornis* was the only millipede where it was possible to differentiate between the mid- and the hindgut. The midgut section included the foregut, and the hindgut section included the ileum, colon, and rectum. Reported data are based on the fresh weight of the animals.

CH_4 production rates. CH_4 production rates were measured for the tropical millipede *A. monilicornis* and the European millipedes *J. scandinavius*, *G. marginata*, and *T. niger*. Therefore, animals were placed into a 14 ml glass vials, which were closed (under air) with a rubber stopper. Animals were incubated for several hours at room temperature in the dark. The CH_4 production rates of the individual gut compartments of *A. monilicornis* were tested in a 10 ml glass vial as described by Schmitt-Wagner and Brune, 1999. CH_4 measurements were taken hourly and analyzed by gas chromatography following the procedure of Schmitt-Wagner and Brune, 1999.

DNA extraction and purification. The prepared whole guts and gut sections were homogenized in 2 ml (whole gut) or 1 ml (gut sections) sodium phosphate buffer (120 mM, pH 8.0) and DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals; Illkirch, Germany). DNA was checked photometrically for purity (Nanodrop; PeqLab, Erlangen, Germany), quantified fluorimetrically (Qubit; Invitrogen, Eugene, OR), and stored at -20°C.

Pyrotag sequencing. Amplification**,** sequencing, classification, and statistical analyses of the 454 pyrotag sequences from different samples were performed as described by Dietrich *et al*., 2014.

PCR amplification and cloning. 16S rRNA gene sequences were amplified using the archaeal-specific primer pair AR109f (5'-AMDGCTCAGTAACACGT-3') (Imachi *et al*., 2006) and 1490R (5'-GGHTACCTTGTTACGACTT-3') with the modification described in Hatamoto *et al*., 2007. Each PCR reaction included reaction buffer (10x), 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase (all Invitrogen, Carlsbad, CA), 50 µM deoxynucleoside triphosphate mixture, 0.3 µM each primer, 0.8 mg/ml bovine serum albumin, and 1 µl of the extracted DNA to a final volume of 50 µL. The PCR program consisted of an 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). PCR products were purified and cloned as described by Schauer *et al*., 2012.

Sequence analysis. 16S rRNA gene sequences obtained by cloning were imported into the version 115 of the Silva database (Quast *et al*., 2013) using the software package ARB (Ludwig *et al*., 2004). Sequences from other studies that were not included in the Silva database were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). The sequences were automatically aligned, and the alignments were refined manually. To exclude variable positions 30% consensus filters were used. Phylogenetic trees of nearly full-length sequences (1,250 bp) were calculated using PhyML, a maximum-likelihood method (Guindon *et al*., 2010) implemented in ARB. DNAPARS, a maximum-parsimony method also implemented in ARB, was used to test tree topology and node support (100 bootstraps).

Quantitative PCR. For the determination of the copy numbers of the bacterial 16S rRNA genes by quantitative real-time PCR, DNA was extracted as described above and the PCR was performed as described by Stubner, 2002 using the primers 519f (5`-CAGCMGCCGCGGTAANWC-3`) (Lane, 1991) and 907r (5`-CCGTCAATTCMTTTRAGTT-3`). The archaeal 16S rRNA genes were quantified as described by Kemnitz *et al*., 2005, using the primer pair A364aF (5`-CGGGGYGCASCAGGCGAA-3`) (Burggraf *et al*., 1997) and A934r (5`-GTGCTCCCCCGCCAATTCCT-3`) (Grosskopf *et al*., 1998).

4.4 Results

CH_4 production rates. No CH_4 production could be measured for the European millipedes *Julus scandinavius*, *Glomeris marginata* and *Tachypodoiulus niger*. However, all tested individuals of tropical millipede *Anadenobolus monilicornis* formed CH_4, with strong individual variation of rates ranging between 5 and 95 nmol g^{-1} h^{-1} (Table 1). Stimulation of these animals with 25% H_2 increased the CH_4 production rate to 49 – 176 nmol g^{-1} h^{-1}. The other tropical millipedes *Aphistogoniulus corallipes*, *Microtrullius uncinatus*, and the unknown species of *Harpagophoridae* also showed methane production, however because of short survival time in captivity, it was not to possible obtain replicates of the production rates.

Table 1. Methane production rates by the tropical millipede *Anadenobolus monilicornis* with and without stimulation of 25% H_2. Values are given in nmol $g^{-1}h^{-1}$ and are based on fresh weight of the animal.

	Methane production (range)	Methane production (average)	Number tested animals
Without stimulation	5–95	45±28	9
Stimulation with 25% H_2	49–176	98±68	3

A. monilicornis was the only millipede where it was possible to differentiate between the midgut and the hindgut section. Therefore, we used this millipede to analyze CH_4 emission from isolated gut sections. When the midgut and the hindgut were incubated separately from each other, CH_4 was produced exclusively by the hindgut with an average emission of 16–186 nmol g^{-1} h^{-1} (Table 2)

Table 2. Methane production rates by the isolated midgut and the hindgut of the tropical millipede *Anadenobolus monilicornis*. Values are given in nmol $g^{-1}h^{-1}$ and are based on fresh weight of the animal.

	Methane production (range)	Methane production (average)	Number tested animals
Midgut[a]	0	0	3
Hindgut[b]	16–186	95±85	3

qPCR analysis of bacterial and archaeal 16S rRNA genes. To determine the bacterial and archaeal cell numbers present in the gut of the different tropical millipedes, quantitative PCR of the 16S rRNA genes was performed (Table 3). Around 10^8 to 10^9 bacterial 16S rRNA genes were present in the different guts. Interestingly, the number of bacteria available in the midgut sample of *A*. *monilicornis* was four times lower ($8{,}25\times10^8$) than in the hindgut ($3{,}19\times10^9$). The abundance of archaeal 16S rRNA genes, depending on the sample, was 100–100000 times lower than in the bacterial samples, 10^4 to 10^6 archaeal 16S rRNA genes. In the midgut section of *A*. *monilicornis* the copy number of the archaeal 16S rRNA genes was 200 times lower ($3{,}46\times10^4$) than in the hindgut sample ($6{,}68\times10^6$). The qPCR results of the millipedes *M. uncinatus* and *Harpagophoridae sp.* may be unreliable because the millipede died of natural causes a

few hours before DNA extraction. Furthermore, it was not possible to amplify archaeal 16S rRNA genes from the gut sample of *A. corallipes.*

Table 3. Determination of bacterial and archaeal 16S rRNA genes by quantitative real-time PCR in the millipedes *Anadenobolus monilicornis* (midgut and hindgut), *Aphistogoniulus corralipes* (whole gut), *Microtrullius uncinatus* (whole gut) and an unknown species of *Harpagophoridae*. Values are given in 16S rRNA gene copies per gut.

Millipede	Bacteria	Archaea
***A. monilicornis* (midgut)**	$8{,}25\times10^{8} \pm 1{,}93\times10^{8}$	$3{,}46\times10^{4} \pm 2{,}89\times10^{3}$
***A. monilicornis* (hindgut)**	$3{,}19\times10^{9} \pm 4{,}70\times10^{8}$	$6{,}68\times10^{6} \pm 1{,}31\times10^{6}$
A. corallipes	$3{,}07\times10^{8} \pm 5{,}14\times10^{7}$	– [a]
M. uncinatus [b]	$5{,}62\times10^{8} \pm 1{,}26\times10^{8}$	$3{,}38\times10^{5} \pm 1{,}05\times10^{5}$
Harpagophoridae sp. [b]	$2{,}60\times10^{9} \pm 3{,}26\times10^{9}$	$5{,}51\times10^{5} \pm 7{,}84\times10^{4}$

[a] amplification failed

[b] natural death a few hours before the DNA extraction

High-throughput sequencing of bacteria. A high-throughput sequencing analysis was performed for the whole gut of the tropical millipede *A. corallipes* and for the midgut and hindgut sections of the tropical millipede *A. monilicornis* to deeply analyze the bacterial composition in these two millipedes (Fig. 1). The analysis was not conducted for the millipedes *M. uncinatus* and *Harpagophoridae sp.* because the animals died of natural causes before the DNA extraction, which could influence the bacterial composition in the gut.

The most abundant groups available in *A. corallipes* belong to the phyla *Bacteroidetes*, *Proteobacteria* and *Firmicutes*, specifically the families *Enterobacteriaceae* (*Proteobacteria*) and *Porphyromonadaceae* (*Bacteroidetes*) (Fig. 1).

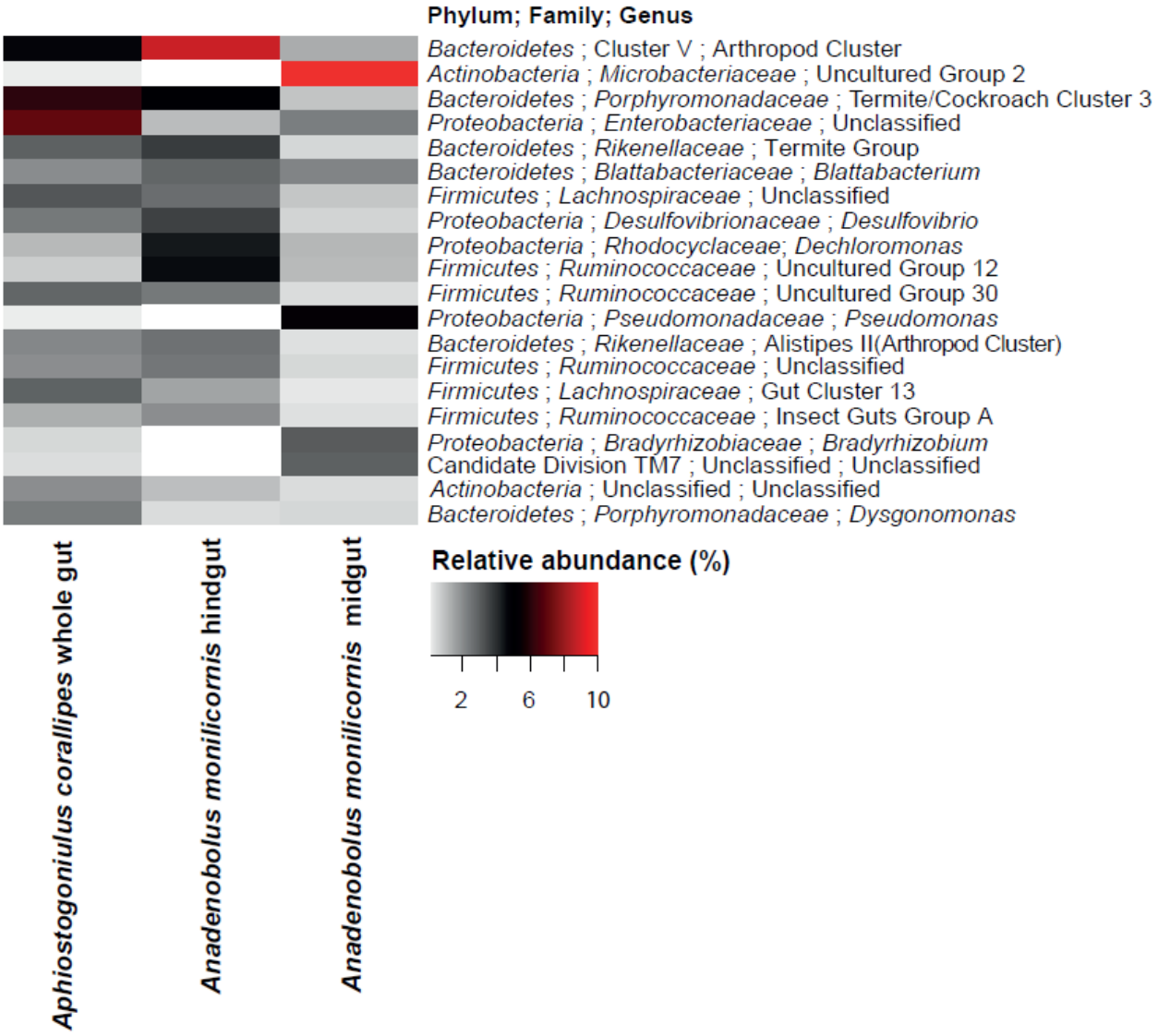

Figure 1. Relative abundance of the bacterial lineages in the millipedes *Aphistogoniulus corallipes* (whole gut), *Anadenobolus monilicornis* (fore-/midgut; hindgut) revealed a high abundance of bacteria of the phyla *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Firmicutes*.

The analysis of the hindgut of *A. monilicornis* revealed similar results as for *A. corallipes* (Fig. 1). In the hindgut of *A. monilicornis* sequences from the phyla *Bacteroidetes*, *Proteobacteria* and *Firmicutes* were the most abundant groups. However, the distribution of bacterial sequences at the family level differed between the two millipedes. In *A. monilocornis,* the majority of the sequences fall within the cluster V of the *Bacteroidetes.* Other abundant groups were *Ruminococcaceae*, *Rhodocyclaceae* (both *Firmicutes*) and, like in *A. corallipes, Porphyromonadaceae* (*Bacteroidetes*).

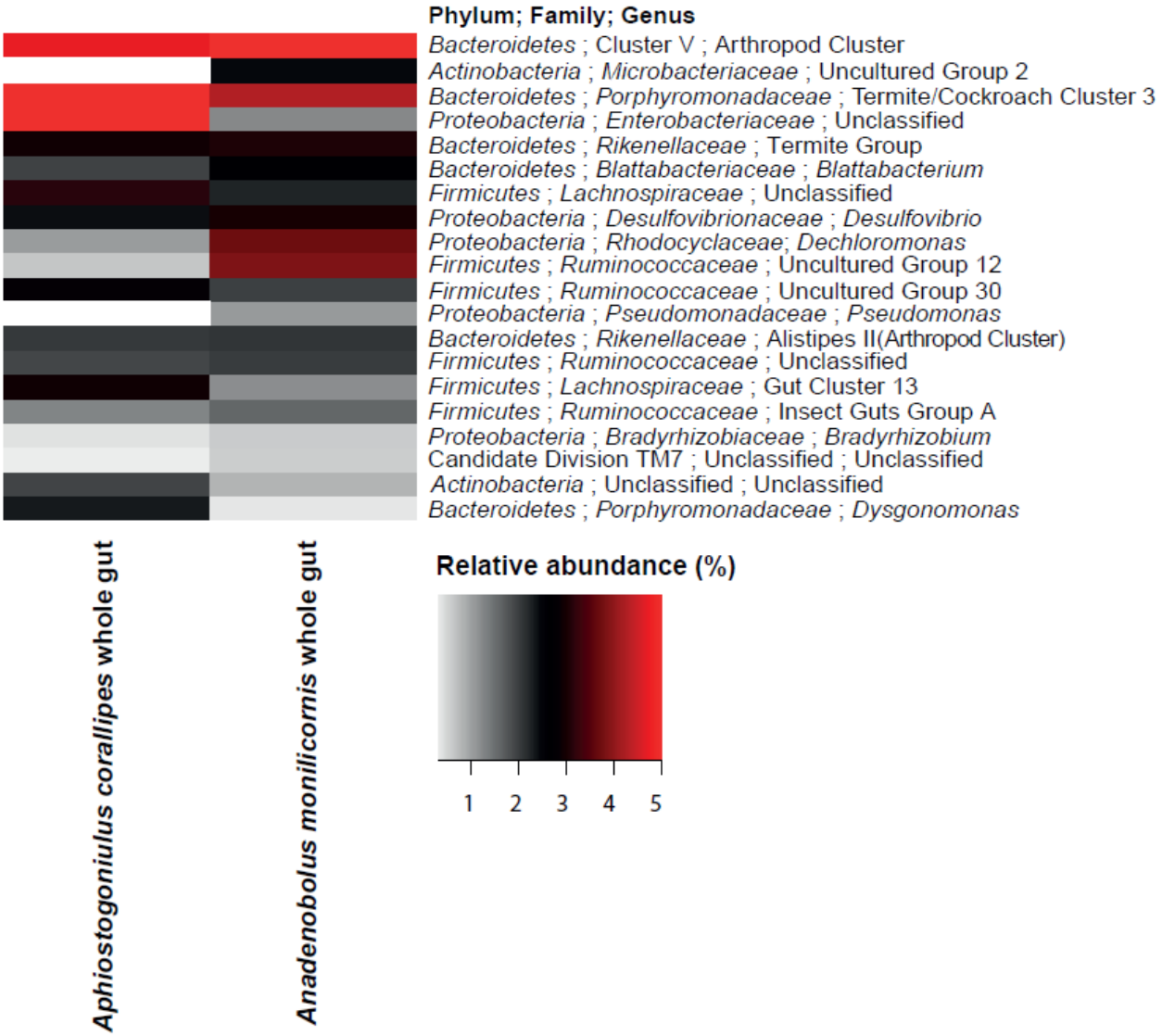

Figure 2. Comparison of the whole gut samples of *Anadenobolus monilicornis* and *Aphistogoniulus corallipes* revealed by high-throughput sequencing. The whole gut sample of *Anadenobolus monilicornis* was calculated from the results of the high-throughput analysis of the separated midgut and hindgut section (see Fig. 1) and taking the relative abundance of bacterial 16S rRNA genes into account (hindgut/midgut 4:1).

Interestingly, groups that were most abundant in the hindgut sample of *A. monilicornis* were less abundant in the midgut section (Fig. 1). Here, the most abundant groups were sequences from the families *Microbacteriaceae* (*Actinobacteria*), *Pseudomonadaceae*, *Bradyrhizobiaceae* (both *Proteobacteria*) and Candidate division TM7. The *Blattabacteriaceae* was the only family present in considerable amounts in both

samples. Some of the amplified sequences in the samples were similar to sequences from termites, cockroaches and beetle larvae.

Taking the 4:1 ration (measured by qPCR, see Table 3) in abundance of bacterial 16S rRNA genes between the midgut and the hingut sample of *A. monilicornis* into account, a “whole gut” sample was calculated and compared to the sample of *A. corallipes* (Fig. 2). The analysis revealed that both samples have a similar bacterial community. However, in the sample of *A. corallipes* more bacteria of the genus *Enterobacteriaceae* were present whereas *A. monilicornis* possessed more *Rhodocyclaceae* and *Ruminococcaceae* (group: uncultured 12).

Archaeal diversity in the guts of different millipedes. Five separate clone libraries were generated from nearly full-length 16S rRNA gene sequences (1,380 bp) amplified from the guts of different millipedes. For *A. monilicornis*, clone libraries were conducted for the midgut and hindgut section. However, cloning of the 16S rRNA gene sequences from the midgut section was difficult and only a small number of clones (10) were obtained (Table 4).

Table 4. Clone frequencies in libraries of archaeal 16S rRNA genes obtained from the gut of different millipedes. For the millipede *Anadenobolus monilicornis* the gut was separated into a midgut and a hindgut section. Sequences were obtained with the archaeal primer pair Ar109f and 1490R. Abbreviations: Mmc: *Methanomassiliicoccales*, Ms: *Methanosarcinales*, Mmb: *Methanomicrobiales*, Mb: *Methanobacteriales*.

Millipede species	Mmc (%)	Ms (%)	Mmb (%)	Mb (%)	Clones
Anadenobolus monilicornis	*14*	*27*	*6*	*53*	*71*
Fore- and midgut	*30*	*10*	*0*	*60*	*10*
Hindgut	*12*	*29*	*7*	*52*	*61*
Aphistogonoiulus corallipes	*0*	*5*	*30*	*65*	*43*
Microtrullius uncinatus	*11*	*6*	*0*	*83*	*18*
Harpagophorida sp.	*14*	*29*	*36*	*21*	*28*

From the archaeal clone libraries, randomly selected clones were sequenced. The phylogenetic analyses revealed that all clones were affiliated with the phylum *Euryarchaeota*, specifically with the orders *Methanomassiliicoccales*, *Methanosarcinales*, *Methanomicrobiales* and *Methanobacteriales*. In *A. monilicornis* and the unknown species of *Harpagophoridae* clones from all of these orders were obtained.

No sequences of the order *Methanomassiliicoccales* could be amplified from the gut of *A. corallipes*. In addition, clones from the *Methanomicrobiales* were absent from of gut *M. uncinatus*.

50–80% of the obtained clones in all millipedes belonged to the order *Methanobacteriales* (Table 4), forming the most abundant group of methanogens in the millipede guts. However, in the unkown species of *Harpagophoridae* only 29% of the clones could assigned to *Methanobacteriales*. In this millipede most of the clones were affiliated with the group of the *Methanomicrobiales* (36%).The abundances of the other orders were dependent on the host and showed no specific pattern.

Clones related to the *Methanomassiliicoccales* (Fig. 3) formed a cluster with sequences from the humus-feeding larva of *Pachnoda ephippiata* (97–99% sequence similarity) and the soil-feeding termite *Cubitermes ugandensis* (96–97% sequence similarity). This cluster was related to additional sequences from different higher termites, cockroaches and scarab beetle larvae (*Pachnoda ephippiata*) (95–97% sequences similarity). One sequence obtained from the hindgut of *A. monilicornis* clustered with the 16S rRNA sequences of the enrichment culture *Ca.* Methanoplasma millipedium strain MpM2, which was also enriched from the hindgut of *A. monilicornis* (96% sequence similarity). The only isolate of this order, *Methanomassiliicoccus luminyensis* (Dridi et al., 2012) was isolated from the human gut and showed only a distant relationship to the clones from the millipedes (87-90% sequence similarity).

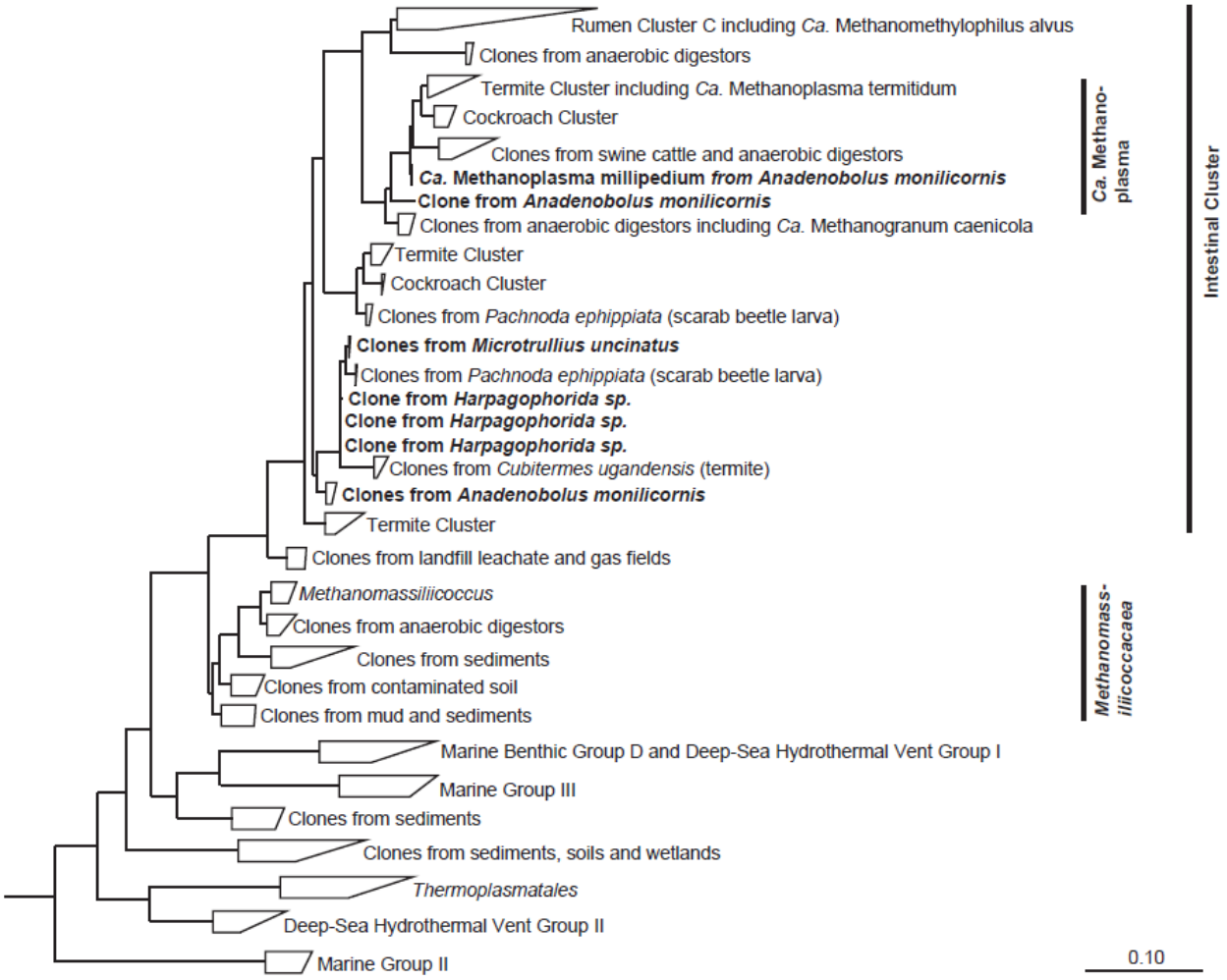

Figure 3. Phylogenetic tree showing the positions of the 16S rRNA genes (1,380 bp) recovered from the guts of the millipedes *Anadenobolus monilicornis*, *Microtrullius uncinatus* and *Harpagophoridae sp.* relative to the members of the *Methanomassiliicoccales*. Tree is based on a maximum likelihood analysis and rooted with the most basal group of the methanogens, the *Methanopyrales*. Sequences obtained from this study are marked in bold. The scale bar indicates substitutions per site.

Clones that were obtained from the order *Methanosarcinales* (Fig. 4) grouped with sequences from different higher termites (93–95% sequence similarity). This cluster was closely related to the genus *Methanomicrococcus*. Additional sequences from higher termites, cockroaches and scarab beetle larvae showed a similarity to the only isolate of this genus, *Methanomicroccocus blatticola,* from the hindgut of the cockroach *Periplaneta americana* (Sprenger *et al*., 2000). *M. blatticola* revealed a sequence similarity of 94–95% to the clones isolated from the millipede guts.

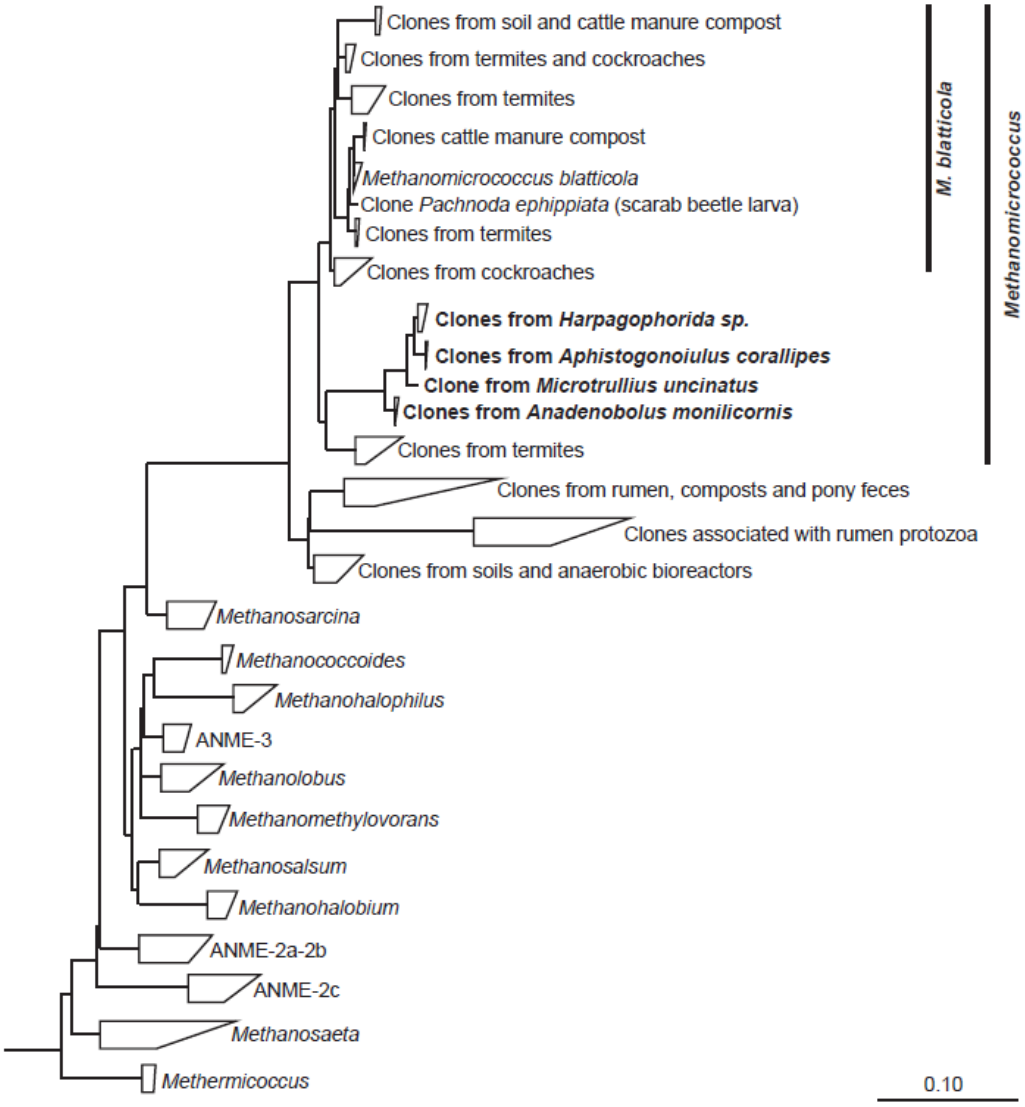

Figure 4. Phylogenetic tree of the16S rRNA genes (1,380 bp) of the *Methanosarcinales* outlining the position of clones from the millipedes *Anadenobolus monilicornis*, *Aphistogoniulus corallipes*, *Microtrullius uncinatus* and *Harpagophoridae sp.*. Tree is based on a maximum likelihood analysis and rooted with the most basal group of the methanogens, the *Methanopyrales*. Sequences obtained from this study are marked in bold. The scale bar indicates substitutions per site.

Sequences affiliated with the order *Methanomicrobiales* (Fig. 5) were closely related to clones from the gut of cockroaches (97–98% sequence similarity). Interestingly, the sequence similarity of clones obtained from the millipede *Harpagophoridae* sp. compared to sequences from the cockroach gut was slightly higher (97–98%) than the similarity of these clones to sequences from the other millipedes *A. monilicornis* and *A. corallipes* (96–97%).

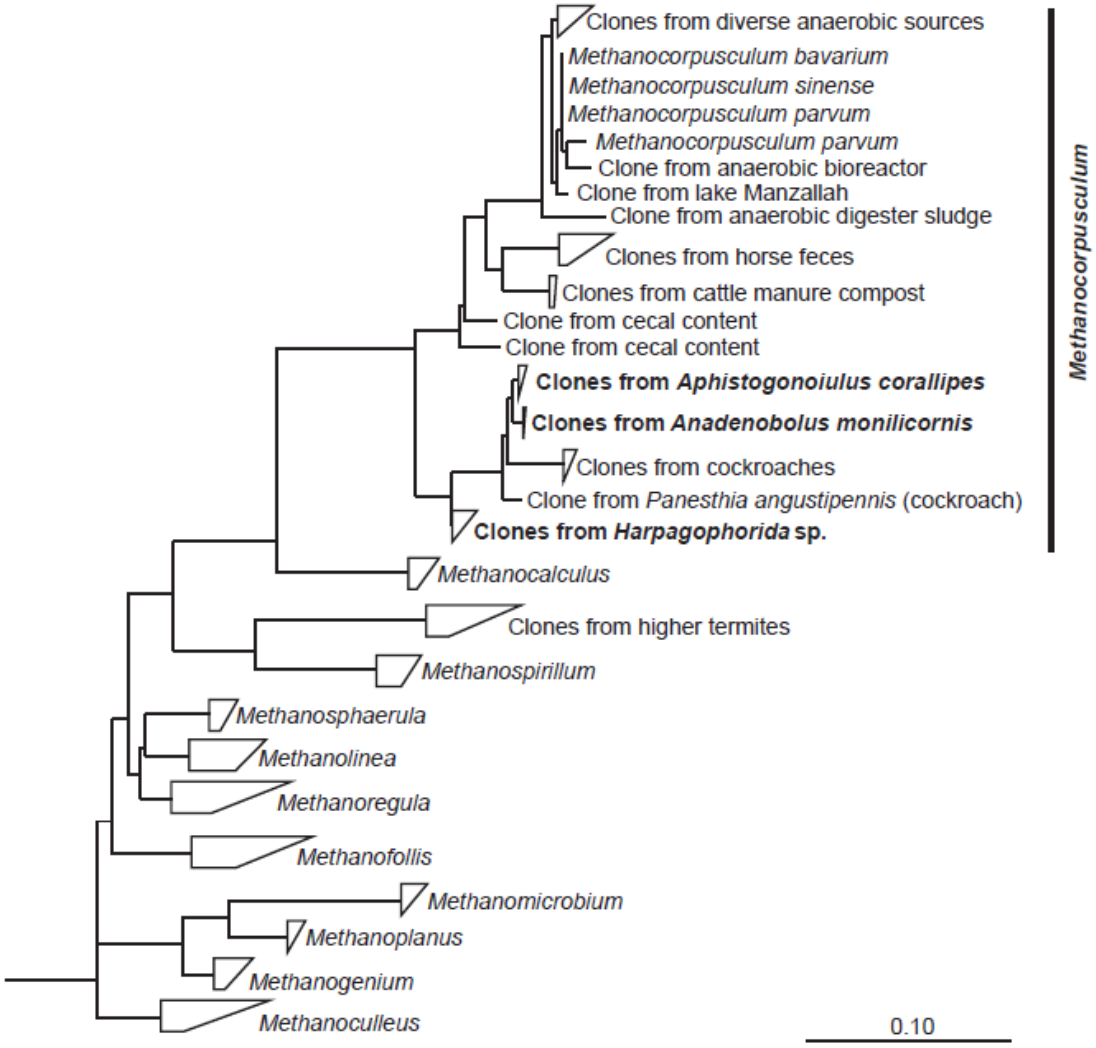

Figure 5. Phylogenetic tree indicating the positions of the 16S rRNA genes (1,380 bp) recovered from the millipede guts of *Anadenobolus monilicornis*, *Aphistogoniulus corallipes*, and *Harpagophoridae sp.* outling the positions of the sequences within the *Methanomicrobiales.* Tree is based on a maximum likelihood analysis and rooted with the most basal group of the methanogens, the *Methanopyrales*. Sequences obtained from this study are marked in bold. The scale bar indicates substitutions per site.

The millipede/cockroach cluster was associated with clones from the genus *Methanocorpusculum*, in particular with clones from intestinal tracts of poultry, cattle and horses (93-97% sequence similarity). The sequence similarity between the clones from millipedes and diverse isolates of the genus *Methanocorpusculum* was equal to the similarity of the clones from the intestinal tracts. Although termites belong to the few groups of arthropods that emit CH_4, sequences from these animals were closely associated with the genus *Methanospirillium* and not with the millipede and cockroach sequences.

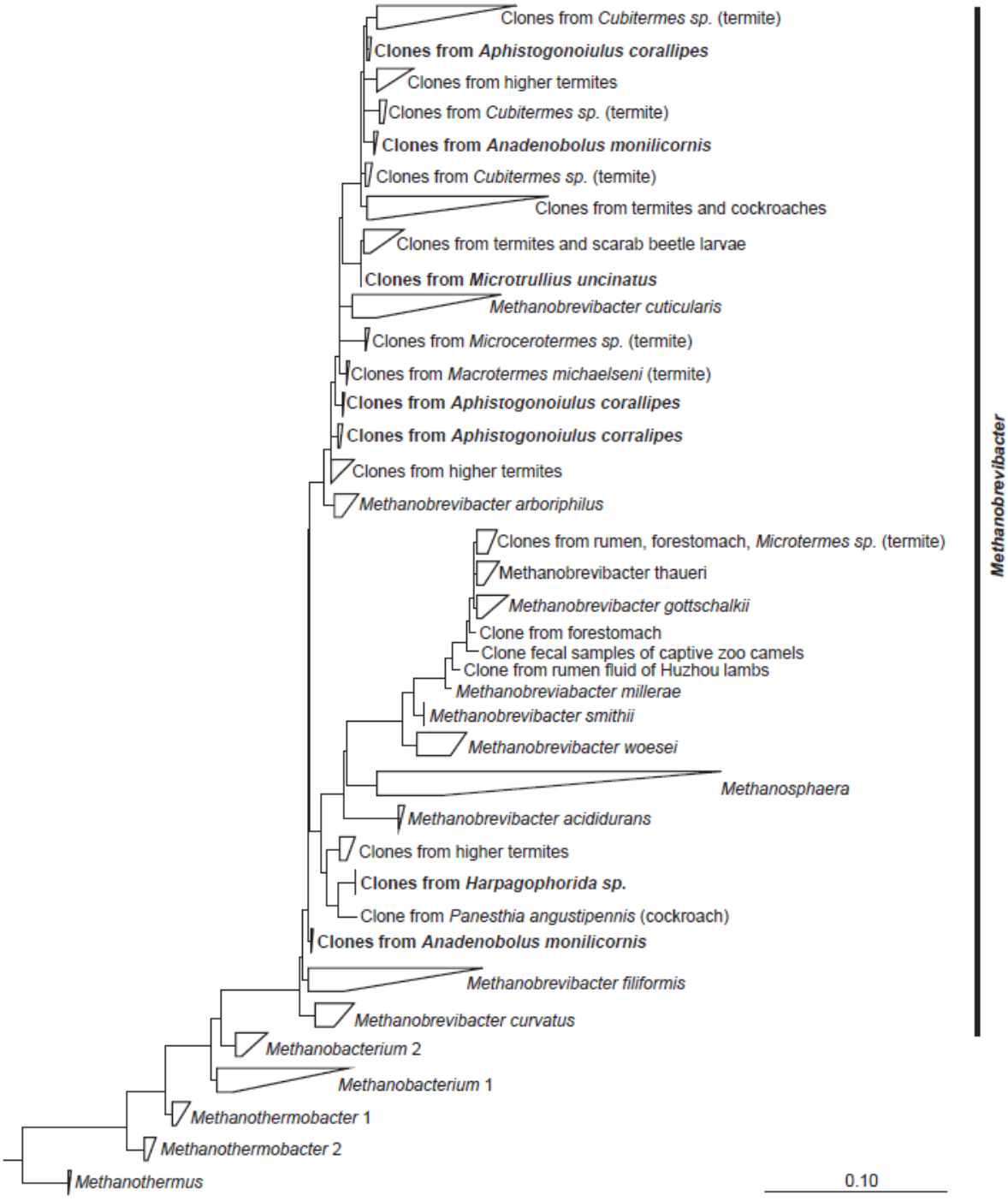

Figure 6. Phylgenetic tree of the 16S rRNA genes showing the relationship of sequences revealed from guts of the millipedes *Anadenobolus monilicornis*, *Aphistogoniulus corallipes*, *Microtrullius uncinatus* and *Harpagophoridae sp.* to the *Methanobacteriales*. Tree is based on a maximum likelihood analysis and rooted with the most basal group of the methanogens, the *Methanopyrales*. Sequences obtained from this study are marked in bold. The scale bar indicates substitutions per site.

The majority of the clones were affiliated with the order *Methanobacteriales* (Fig. 6), specifically clustering with clones from the genus *Methanobrevibacter*. All millipede clones revealed a sequence similarity of about 97–100%, but did not form a coherent cluster. The different millipede clusters were mostly affiliated with sequences from the gut of higher termites, cockroaches, and scarab beetle larvae (94–99% sequence similarity). Some clones from the millipede *A. monilicornis* grouped closely together with *Methanobrevibacter cuticularis*, an isolate from the lower termite *Reticulitermes flavipes* (Leadbetter and Breznak, 1996) (97% sequence similarity). None of the remaining sequences clustered with one of the other isolates (*Methanobrevibacter filiformis* or *Methanobreviabacter curvatus*) from *R. flavipes* (Leadbetter and Breznak, 1996). Although, *Methanosphaera* is known to forms its own genus, in all calculated trees using different algorithm and filters the group falls within the genus *Methanobrevibacter.*

4.5 Discussion

The impact of millipedes on global warming was first described by Hackstein and Stumm (1994), who identified these animals as one of the rare taxa of arthropods that emit methane. Since this time several studies have investigated the methanogenic community of termites (e.g. Ohkuma *et al*., 1995; Leadbetter and Breznak, 1996; Friedrich *et al*., 2001; Miyata *et al*., 2007), cockroaches (Hara *et al*., 2002) and scarab beetle larvae (Egert *et al*., 2003, 2005), but almost nothing is known about the methanogenic community in the guts of millipedes. A superficial study of methanogenesis in millipedes by Sustr *et al*., (2014) amplified 16S rRNA sequences by PCR-DGGE and used only a BLAST search for their classification. This is the first comprehensive phylogenetic analysis of the methanogenic community structure within the guts of different millipedes. Furthermore, we provide quantitative data on presence of archaeal and bacterial 16S rRNA genes in the guts of these millipedes. Bacterial community analyses from millipedes are also rare; therefore, we performed a high-throughput analysis from the millipede guts of *Anadenobolus monilicornis* and *Aphistogoniulus corallipes* to show the association between bacterial and archaeal communities

Methanogenesis in millipedes. CH_4 emission of millipedes has been mostly the documented in tropical species (Hackstein and Stumm, 1994). However, Sustr colleagues measured CH_4 emission also for some European millipedes of the familiy *Julidae* (Sustr and Simek, 2009; Sustr *et al*., 2014). One of the millipedes was *Julus scandinavius,* which is described as a "facultative methane producing organism" (Sustr *et al*., 2014). Several animals of this species collected from a forest in Marburg showed no CH_4 emission at all. In addition, the European millipedes *Glomeris marginata* and *Tachypodoiulus niger* did not emit methane, confirming previus studies (Hackstein and Stumm, 1994; Sustr *et al*., 2014). Furthermore, it was not possible to amplify the alpha-subunit of methyl coenzyme M reductase, a molecular marker for methanogenesis, from any of the animals.

A comprehensive analysis of CH_4 emission rates of millipedes showed strong variations between the different animals, ranging from 0 to 415 nmol/g/h (Hackstein and Stumm, 1994). Interestingly, emission rates vary not only strongly between the different millipedes but also within one species. For example, the CH_4 emission rates of *A. monilicornis,* is between 5–95 nmol/g/h (Table 1). However, the average CH_4 emission rate of *A. monilicornis* (45 nmol/g/h) is similar to the average emission rate of different millipedes measured by Hackstein and Stumm, 1994 (58 nmol/g/h). Furthermore, the average production rate of cockroaches with 46 nmol/g/h is in the same range. Lower termites (0–1300 nmol/g/h; Brauman *et al*., 1992; Shinzato *et al*., 1992, Sugimoto *et al*., 1998b) and higher termites (20–1090 nmol/g/h; Brauman *et al*., 1992; Shinzato *et al*., 1992; Bignell *et al*., 1997; Sugimoto *et al*., 1998) as well as scarab beetle larvae (0–751 nmol/g/h; Hackstein and Stumm, 1994) emit significantly more CH_4 than millipedes. The exact reason for this remains unclear; but correlation between the size of the animals and methane production can be excluded since termites are definitely smaller than millipedes. It can be speculated that termites and scarab beetle larvae possess higher numbers of methanogenic archaea in their gut, which could form more CH_4.

Methanogens are able to grow on the wide range of substrates, including H_2 + CO_2 as well as methanol + H_2. Stimulation experiments of *A. monilicornis* with H_2 resulted in an increased emission of CH_4, suggesting that H_2 is an important substrate for

methanogenesis in this millipede. In *A. monilicornis* methanogenesis occurs exclusively in the hindgut, because this was the only gut section that emitted CH_4 (Table 2). The production rates by the isolated hindgut were in the same range as the production rates from animals stimulated with H_2. However, as in the live millipedes, the isolated hindgut showed strong individual variations in the emission rate, which makes it difficult to compare the values. The observation that the methanogens are located in the hindgut is consistent with the detection of the typical F420 autofluorescence of methanogens only in the hindguts of millipedes (Hackstein and Stumm, 1994). Furthermore, the abundance of archaeal 16S rRNA genes in the hindgut of *A. monilicornis* was 200 times higher than in the midgut (Table 3). The presence of archaeal 16S rRNA genes in the midgut section can be attributed to failures in the preparation process. For example, methanogenic archaea could leak from the hindgut to the midgut during the dissection or the gut was cut at the wrong position. The low abundance of methanogenic archaea in the midgut explains why it was difficult to clone archaeal 16S rRNA genes from this section. Furthermore, in other CH_4 emitting arthropods, like termites (Schmitt-Wagner and Brune, 1999) and scarab beetle larvae (Lemke *et al*., 2003), measurements of isolated guts show that methanogenesis is restricted to the hindgut. This is not surprising because methanogenesis is an oxygen-sensitive process and the hindgut is the only gut compartment in insect guts with a negative redox potential (Bayon, 1980; Ebert and Brune, 1997, Kappler and Brune, 2002). However, the digestive tract of millipedes is relatively simple and most millipedes lack an enlarged pouch like in other arthropods (Hackstein and Stumm, 1994) but the inner surface of the millipede hindgut is strongly developed and harbors both flat cuticular surfaces and ornaments. These sites may facilitate colonization (Byzov, 2006).

Microbial community structure. The abundance of bacterial 16S rRNA genes (10^8–10^9 copies/gut; Table 3) in the millipede guts was higher than the abundance of archaeal 16S rRNA genes (10^5–10^6 copies/gut). However, it is important to remember that most bacteria possess multiple copies of the 16S rRNA gene, whereas in archaea this is the case for only a few species (Acinas *et al*., 2004). Interestingly, gut preparations of the tropical millipede *Chicobolus sp.* contained $1.4–15\times10^9$ bacterial cells per ml gut and not per whole gut as in our millipedes. Like in *Chicobolus sp.* (Cazemier *et al*., 1997), the

hindgut of *A. monilicornis* contained more cells than the fore- and midgut. The general difference in the cell numbers measured for our millipedes and *Chicobolus sp.* can be explained by primer bias and/or the differences in the gut size of the millipedes, because *Chicobolus sp.* belongs to the larger millipedes. Furthermore, it has to be taken into account that the millipedes *M. uncinatus* and *Harpagophoridae sp.* died of natural cause several hours before the DNA extraction, which could have an influence on the cell numbers.

The bacterial high-throughput sequencing analysis of the millipedes *A. monilicornis* and *A. corallipes* revealed the presence of the same major bacteria phyla (*Proteobacteria*, *Actinobacteria, Bacteroidetes* and *Firmicutes;* Fig 1) that were identified in DGGE profiles and clone libraries of the millipede *Cylindroiulus fulviceps* (Knapp *et al*., 2009, 2010). The high abundance of the *Proteobacteria* and *Actinobacteria* in the guts of *A. monilicornis* and *A. corallipes* may be why only these bacteria have been isolated from the guts of diverse millipedes (Dzingov *et al*., 1982; Jáger *et al*.; 1983; Szabó *et al*., 1983; Márialigeti *et al*., 1985; Contreras, 1990; Byzov *et al*., 1996; Tretyakova *et al*.; 1996; Jarosz and Kania, 2000; Oravecz *et al*., 2002; Knapp *et al*., 2010). Interestingly, bacterial groups that are highly abundant in the hindgut of *A. monilicornis* are reduced in the midgut (Fig. 1), showing a different bacterial composition in both sections. A calculated whole gut sample for *A.monilicornis* reveals a similar bacterial pattern than the whole gut sample of *A. corallipes* (Fig. 2), suggesting that the bacterial community in the midgut and hindgut section of *A. corallipes* is also different like in gut sections of *A. monilicornis*. The sequences from the high-throughput analysis like the sequences from the clone libraries (Knapp *et al*., 2010), were similar to sequences from millipedes, cockroaches and scarab beetle larvae.

Studies of the termites *Zootermopsis angusticollis* (Wenzel *et al*., 2003), *Neotermes cubanus* (Stingl *et al*., 2004) and other dry wood termites (Desai *et al*., 2010) document the association of *Bacteroidetes* with different gut protozoa, most likely providing amino acids or cofactors for its hosts. This group of microorganism is also highly abundant in the guts of the investigated millipedes (Fig. 1), suggesting the presence of protozoa in their guts. An early study by Joseph Leidy indentified the ciliate *Nyctotherus sp*. in millipede guts (Leidy, 1849), which was later found to live in symbiosis with

methanogenic archaea (Gijzen *et al*., 1991). Like the *Enterobacteriaceae, Desulfovibrionaceae* (both belonging to the phyla of the *Proteobacteria*, Fig. 1), *Lachnospiraceae* and *Ruminococcaceae* (both belonging to the phyla of the *Firmicutes*; Fig. 1), ciliates produce H_2 as a fermentation product by hydrogenosomes during the fermentation of pyruvate (Lindmark and Müller, 1973). Interestingly, most millipedes that perform methanogenesis also emit H_2 (Hackstein and Stumm, 1994).

Methanogenic archaea in tropical millipedes. H_2 is one of the major substrates for hydrogenotrophic and methyl-reducing methanogenesis. For this reason it is not uncommon that methanogenic archaea are associated with H_2–producing protozoa (Gijzen *et al*., 1991; Shinzato *et al*., 1992; Hackstein and Stumm, 1994, Radek, 1994, 1997). In lower termites the protozoa are exclusively affiliated with the hydrogenotrophic methanogens of the genus *Methanobrevibacter* (Tokura *et al*., 2000; Hara *et al*., 2004; Inoue *et al*., 2007). Most of the sequences found in our clone libraries also clustered with clones of the order *Methanobacteriales*, in particular with clones of the genus *Methanobrevibacter* (Fig. 6). This suggests that in the tested millipedes some of the *Methanobrevibacter* species may affiliate with ciliates. The presence of the ciliates in the analyzed millipedes is only speculative since the guts have not been investigated for the presence or absence of protozoa. However, some of the sequences from *A. monilicornis* clustered with *Methanobrevibacter cuticularis*, an isolate from the lower termite *Reticultiermes flavipes* (Leadbetter and Breznak, 1996), which is known to harbor flagellates. None of the remaining clones were affiliated with one of the other isolates from *Reticultiermes flavipes*. Further sequences were mostly associated with clones from higher termites, like *Cubitermes* sp. and *Macrotermes* sp. or clones from scarab beetle larvae (Fig., 6), which are other taxa of arthropods that emit CH_4 (Hackstein and Stumm, 1994). DGGE patterns from different tropical and European millipedes suggest that clones from these animals are also affiliated with *Methanobrevibacter* (Sustr *et al*., 2014). However, this study is missing a phylogenetic analysis.

The remaining clones were associated with the orders *Methanomassiliicoccales* (Fig. 3), *Methanosarcinales* (Fig. 4) and *Methanomicrobiales* (Fig. 5). Interestingly, strains of these orders are also present in higher termites (Friedrich *et al*. 2001; Miyata *et al*., 2007), wood-feeding cockroaches (Hara *et al*., 2002) and scarab beetle larvae (Egert *et*

al., 2003). However, not all of these orders were present in every investigated millipede (Table 4). Because the amplified sequences of the different millipedes are in most cases closely related, we can exclude that the amplification failed because of primer bias. One possible explanation for the absence could be that they are low in abundance and were not captured by the clone libraries or that these orders are indeed absent from some millipedes.

Sustr *et al*., (2014) mentioned that clones affiliated with the order *Methanomassiliicoccales* were completely absent from their DGGE patterns, but their BLAST analysis revealed that some of the clones were 99–100% identical to Rice Cluster III archaea. It was recently shown that this group is a member of the *Methanomassiliicoccales* (Paul *et al*., 2012). Our analysis identified several clones that were associated with this group. All sequences fell within the intestinal cluster of the *Methanomassiliicoccales* and were closely related to clones from scarab beetle larvae, higher termites and cockroaches. One sequence from the gut of *A. monilicornis* was associated with the 16S rRNA sequences *Ca*. Methanoplasma millipedium strain MpM2 (Paul *et al*., 2012), which was enriched from the same species. The fact that only one clone was associated with *Ca*. Methanoplasma millipedium and this clone was isolated from the same host indicates that strains of *Ca.* Methanoplasma may be not highly abundant in millipedes. A further explanation could be a bias of the commonly used PCR primer against these strains in millipedes; however this reason is unlikely because it was possible to amplify sequences from this genus in termites and cockroaches with this primer set. So far all enriched or isolated strain of the *Methanomassiliicoccales* are obligately methyl-reducing methanogens, using H_2 as electron donor (Borrel *et al*., 2012; Dridi *et al*., 2012; Paul *et al*., 2012; Borrel *et al*., 2013). This suggests that this metabolism is also common for the strains available in the millipede guts.

Interestingly, sequences from the order *Methanosarcinales* cluster with the *Methanomicrococcus blatticola* (Fig. 4; Sustr *et al*., 2014), an isolate from the cockroach *Periplaneta americana* (Sprenger *et al*., 2000). *M. blatticola* is also known to be an obligate methyl-reducing methanogen, using H_2 as electron donor (Sprenger *et al*., 2000). This reveals that the methyl-reducing pathway is commonly used in millipedes. However, clones from the millipede gut most probably form a new species within the

genus *Methanomicrococcus* (sequence similarity to *M. blatticola* 95-96%), so further investigation is required to determine if these strains use the same metabolism.

Clones affiliated with the order *Methanomicrobiales* (Fig. 5) fall within the genus *Methanocorpusculum* and cluster with sequences from the cockroach gut. DGGE patterns of Sustr and colleagues also show an affiliation of the sequences from the millipede guts with the order *Methanomicrobiales,* but they could not assign them to a specific genus (Sustr *et al*., 2014). Strains of the genus *Methanocorpusculum* are also known for their hydrogenotrophic lifestyle (Zellner *et al*., 1987), supporting the importance of H_2 formation in the millipedes. Clones from higher termites are not closely related to the millipede sequences and are affiliated with the genus *Methanospirillum*. This is interesting because phylogenetic analyses of the other orders always show a close relationship between sequences from termites and millipedes. Future analysis of millipedes may reveal sequences that are also related to *Methanospirillum*.

Conclusion. In the millipede gut *Enterobacteriaceae, Desulfovibrionaceae, Lachnospiraceae* and *Ruminococcaceae* are highly abundant bacteria, which form H_2 as a major fermentation product. The stimulation of CH_4 production by H_2 in *A. monilicornis* underline the importance of hydrogen-dependent methanogenesis in millipedes, supported by the fact that all of the amplified sequences are affiliated with the hydrogentrophic or methyl-reducing methanogens of the genera *Methanobrevibacter*, *Methanomicrococcus, Methanocorpusculum* and the intestinal cluster of the order *Methanomassiliicoccales*. Aceticlastic methanogenesis could not be verified in any of these millipedes. It is suspected that the short retention times in intestinal tracts do not favor the colonization of slow-growing aceticlastic methanogens (Liu and Whitman, 2008). Furthermore, our analysis confirms the fact that mostly tropical arthropod species emit CH_4 and supports the theory that methanogenic archaea are exclusively located in the hindguts of arthropods, as indicated by the analysis of CH_4 production rates of the isolated gut sections. Sequences from the millipede gut were closely affiliated with sequences from other CH_4 emitting arthropods, like termites, cockroaches, and scarab beetle larvae, suggesting a similar methanogenic community in all of these animals.

Most of the data were collected by Lena Mikulski as part of a supervised bachelor's thesis. Additional experiments will be performed, including O_2, redox potential, and pH microsensor measurements from the different gut compartments as well as stimulation experiments of the gut sections with H_2, formate and methanol. These experiments will address questions about the internal H_2 transfer in the millipede gut, the redox potential of the hindgut and substrates that stimulate methanogenesis.

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Chapter 5

Manuscript in preparation

Not coevolution but host specificity and habitat selection drive archaeal community structure in arthropod guts

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Running Title: *Archaeal community structure in arthropod guts*

Contributions:

C.D. designed and conducted the high-throughput sequencing analysis; created a reference database including all previously published data; conducted the final phylogenetic analysis, all bioinformatics, and statistical analyses; and wrote the manuscript.

J.O.N. created the majority of the clone libraries, and contributed to the phylogenetic analysis and the evaluation of the results.

K.L. created clone libraries and contributed to the phylogenetic analysis.

L.M. created clone libraries.

K.M. prepared libraries for the high-throughput sequencing analysis.

T.K. improved the primer set for the high-throughput sequencing analysis.

H.I.B. collected termites and created clone libraries.

D.K.N. collected termites and prepared DNA.

D.S.D. collected termites.

A.B. conceived the study, supervised the experiments, and discussed the results.

5.1 Abstract

The arthropod gut is streamlined to anaerobically mineralize mostly plant-derived organic matter. In only five major arthropod groups (millipedes, scarab beetle larvae, cockroaches, and lower and higher termites) are considerable amounts of methane formed by methanogenic archaea at the end of this process. Bacterial communities in the guts of termites and cockroaches mirror major events in the evolutionary history of their host. Whether this is also true for archaeal communities or whether diet is the key factor is unknown. Here, we used both clone libraries and high-throughput sequencing to document that the archaeal community structure in arthropod guts and the phylogeny of archaeal lineages is dependent on the host group and to a lesser extent on diet. With the exception of lower termites, all major arthropod groups contained at least one group each of hydrogenotrophic and methylotrophic methanogens regardless of the host diet. Hydrogenotrophic methanogenesis is almost exclusively carried out by members of the genus *Methanobrevibacter*, whereas methyl reduction is accomplished by different genera of the orders *Methanomassiliicoccales* and *Methanosarcinales*. The occurrence of specific genera of these obligately hydrogen-dependent methylotrophs differs among the hosts, which indicated that host habitat selection is the major driving force for arthropod archaea. Analysis of the phylogeny of the most abundant archaeal lineages in the arthropod host gut revealed host-group-specific clusters of archaeal lineages. Since cocladogenesis was absent in the resulting phylogenetic trees, coevolution could be excluded as the driving force. This underlines that the mechanisms for selection of archaeal lineages must be host-habitat-specific, as, e.g., the highly alkaline gut compartment of most higher termites, which selects for alkali-tolerant strains. In contrast to other studies, we did not find a uniform archaeal community in the guts of flagellate-containing lower termites; the archaeal community was similar to that of other host groups. Therefore, in lower termites, other mechanisms must select for archaeal lineages, e.g., microhabitats provided by flagellates. This hypothesis was supported by phylogenetic analysis of the corresponding representative operational taxonomic units, which often have long branches — an indication of a different rate of evolution that is frequently observed in endosymbionts.

5.2 Introduction

The gut of terrestrial arthropods is a diverse and important ecosystem, where mostly plant-derived organic matter is transformed and mineralized. This breakdown is mostly facilitated in concert with their complex gut microbiota in a series of fermentative processes (for a detailed description, see Brune, 2014 and references therein). The terminal processes include acetogenesis and methanogenesis from a variety of substrates. Although the generally accepted scheme of anaerobic breakdown suggests a steady ratio of acetate formed to methane produced, the dominance of one of these two processes in termites varies (Pester and Brune, 2007). Methane emission is restricted to five groups of terrestrial arthropods: millipedes, scarab beetle larvae, cockroaches, lower termites, and higher termites (Hackstein and Stumm, 1994; Hackstein *et al*., 2006). These groups differ greatly in their methane emission rates (summarized in Brune, 2010), but the driving forces behind this phenomenon are mostly unidentified.

Methane is formed exclusively by methanogenic archaea, which in termites may account for up to 3% of the total microbial community (Brauman *et al*., 2001). Archaeal lineages detected in arthropod guts can be classified to either the Miscellaneous Crenarchaeotic Group (MCG), the Soil Crenarcheotic Group (SCG) of the archaeal phylum *Thaumarchaeota,* or one of the four methanogenic orders *Methanobacteriales*, *Methanomassiliicoccales*, *Methanomicrobiales,* and *Methanosarcinales*. The distribution of these lineages differs in the different hosts. For millipedes, no clone library data have been obtained, but recently a DGGE analysis and sequencing revealed the presence of *Methanosarcinales*, *Methanobacteriales, Methanomicrobiales,* and some unclassified archaea (Sustr *et al*., 2014). The archaeal community of the scarab beetle *Pachnoda ephippiata* larva is dominated by members of the order *Methanobacteriales* (Egert *et al*., 2003). In the cockroach gut, members of the *Methanosarcinales* are the most abundant archaea (Hara *et al*., 2002). In lower termites, almost exclusively members of the *Methanobacteriales* were identified (Ohkuma and Kudo, 1998; Shinzato *et al*., 1999; Tokura *et al*., 2000; Shinzato *et al*., 2001). In higher termites, in contrast, the most abundant methanogenic groups differ among the host subfamilies: *Methanobacteriales*

(*Termitinae*), *Methanosarcinales* (*Macrotermitinae*), or *Methanomicrobiales* (*Nasutitermitinae*). Members of the non-methanogenic phylum of *Thaumarchaeota* were detected only in the hindguts of higher termites and the midgut of *Pachnoda ephippiata* (Egert *et al*., 2003). However, these results cannot be generalized as most of the earlier studies suffer from low sequencing depths (in some cases, five clones per sample), which does not highly support confidence. The still hidden diversity could be in the worst case as high as 20% of the archaeal community.

Earlier studies have also addressed the distribution of archaea along the different gut compartments of arthropods (Friedrich *et al*., 2001; Egert *et al*., 2003), possibly caused by the availability of different substrates and by differences in microenvironments (Schmitt-Wagner and Brune, 1999). Within the gut compartments, archaea (especially methanogens) can occur on the gut wall (or cuticular hair), associated with protists (Leadbetter *et al*., 1998), or in the lumen (Hackstein *et al*., 2006); in lower termites and cockroaches, they can also occur as symbionts of protists (Odelson and Breznak, 1985; Gijzen *et al*., 1991). Especially protists could influence the archaeal community structure in lower termites since the presence of endosymbiotic methanogens favors the protist host and is therefore beneficial for the termite (Odelson and Breznak, 1985; Messer and Lee, 1989). The success of this lifestyle in lower termites is also supported by the high numbers of methanogens associated with protists (10–50 per protist) and the high number of protists per gut (sometimes > 100,000) (Tokura *et al*., 2000).

Recently, it has been shown that bacterial communities mirror major events in the evolutionary history of termites and cockroaches (Dietrich *et al*., 2014). Whether also the archaeal community is determined by the host evolutionary history is unknown, but has been proposed (Hackstein *et al*., 2006). However, the resemblance of microbial communities in related arthropods does not necessarily result from cospeciation between arthropod hosts and their microbial lineages. Instead, the selection of certain archaeal lineages could be influenced by the specific gut habitat within the host (Rawls, 2006).

To identify the mechanisms that drive the methanogenic archaeal community structure in terrestrial arthropods and archaeal phylogeny, we used a hybrid approach consisting

of high-throughput sequencing and clone libraries to profile the communities across a wide range of all major groups of methane-emitting hosts.

5.3 Material and Methods

Insect samples. Termites were from laboratory colonies or field collections or purchased from commercial breeders (Tables 1 and 2). Insect hindguts were dissected immediately upon arrival or collection (Schauer *et al*., 2012; Köhler *et al*., 2012). Samples were identified by sequencing the cytochrome oxidase subunit II gene (COII) (Pester and Brune, 2006). COII genes not represented in public databases were submitted to NCBI GenBank.

DNA extraction. Hindguts were dissected with sterile forceps. Owing to the large differences in size of the different insect hosts, the number of animals of each host group used differed; for millipedes, scarab beetles and cockroaches, one animal was used, and for termites, 3–10 animals were used. Guts were homogenized and DNA was extracted using a bead-beating protocol with subsequent phenol–chloroform purification (Paul *et al*., 2012).

PCR amplification and cloning. Archaeal 16S rRNA genes were amplified according to Paul *et al*. (2012) using the primer set Ar109f′(5′-AMDGCTCAGTAACACGT-3′) of Imachi *et al*. (2006) and Ar912r (5′-CTCCCCCGCCAATTCCTTTA-3′) of Lueders and Friedrich (2000) or the primer set Ar109f and 1490R with the modification of Hatamoto *et al.* (2007) (5′-GGHTACCTTGTTACGACTT-3′). Briefly, each PCR mixture (50 µl) contained reaction buffer, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase (all Invitrogen, Carlsbad, CA), 50 µM deoxynucleoside triphosphates , 0.3 µM each primer, 0.8 mg ml^{-1} bovine serum albumin, and 20 ng DNA. PCR was carried out with an initial denaturation step (94 °C for 3 min), followed by 30 cycles of denaturation (94 °C for 20 s), annealing (52 °C for 30 s), extension (72 °C for 45 s), and a final extension step (72 °C for 7 min).

Sequence data from published studies. For the reanalysis of published clone library sequences (Table 1) in the community structure analysis, the respective data were downloaded from NCBI GenBank. In some cases, only a representative phylotypes were available; therefore, quantitative information on these phylotype sequences was

taken from the respective publications. Clone libraries were recreated by creating count tables, which used the *deunique.seqs* command in the *mothur* software suite version 1.33.3 (Schloss *et al*., 2009). The resulting data were treated the same way as the clone library data obtained in this study. Other available clone libraries that did not contain information about arthropod gut community structure (such as clone libraries from picked flagellates) were not used to visualize archaeal order-level differences in the arthropod hosts, but were included in the construction of the phylogenetic trees.

Phylogenetic analysis of sequence data. Raw sequences derived from clone libraries were analyzed and edited using *Seqman* (DNAStar) software. After importing the sequence into the current ARB-SILVA database (version 119, used throughout; Pruesse *et al*., 2007; http://www.arb-silva.de) using the *ARB* software package tool (Ludwig *et al*., 2004), sequences were aligned against the current SILVA alignment (Pruesse *et al*., 2007). If necessary, sequences were corrected manually. Location of sequences in the main SILVA tree was checked using the *ARB* parsimony tool. Afterwards, sequences belonging to different archaeal order or class levels were exported with the respective and adequate outgroup sequences. To conservatively exclude highly variable columns in the alignment, sequences were first clustered at 97% using the usearch software version 7.0.1090 (Edgar, 2010). The resulting representative sequences were used to construct a 30% mask for the alignment, which was applied on all sequences of interest. Sequences with no ambiguous positions were used because of phylogenetic resolution, filtered by the respective mask, and analyzed phylogenetically using the 16-state GTR-Γ model with 1,000 bootstraps in *RAxML* v8.1.3 (Stamatakis, 2014). Sequences that did not fit the quality criteria were inserted after treeing using the *ARB* parsimony tool by applying the same filter used to create the phylogenetic tree. Trees were rooted using type strains of the other methanogenic orders; for trees of archaea related to *Thaumarchaeota,* type strains from all methanogenic orders were employed.

Primer design for high-throughput sequencing. For high-throughput profiling of the archaeal community in the major arthropod groups, a primer set was needed that fit the requirements of the Illumina Miseq platform, i.e., that can deliver up to 300 nt paired-end reads. We aimed at an overlap of 100–150 nt for the paired-end reads. For this purpose,

we slightly modified the primer pair A533b/A934b described by Grosskopf *et al*. (1998) to maximize the number of sequences bound in SILVA database and to better bind the termite-specific sequences from clone libraries of this study and other published sequences from arthropod guts. The new forward primer A533f_mod (5′-TTACCGCGGCGGCTGVCA-3′) was modified at position 16, where an ambiguity character replaces the former G. The reverse primer was changed by introducing the ambiguity character Y at positions 5 and 7, resulting in the reverse primer A934b_mod (5′-GTGCYCYCCCGCCAATTCCT-3′). The resulting primer pair targets the V4–V5 region of the archaeal 16S rRNA gene. The performance of the primer was tested against SILVA database using TestPrime version 1.0 (Klindworth, 2013; http://www.arb-silva.de/search/testprime/). We followed the strategy of Daigle et al. (2011), which allowed us to multiplex. Briefly, primers A533f_mod and A934b_mod were flanked by universal M13 primers, which allows a very specific addition of multiplex identifiers (MIDs). The final primers consists of A533_M13f_mod (5′-cgccagggttttcccagtcacgacTTACCGCGGCGGCTGVCA-3′) and A934_M13b_mod (5′-tcacacaggaaacagctatgacGTGCYCYCCCGCCAATTCCT-3′).

High-throughput sequencing. The V4–V5 region of the archaeal 16S rRNA gene was amplified using the flanked primer set A533_M13f_mod and A934_M13b_mod. For this step, 20 ng DNA was prepared as recommended by the Herculase II Fusion DNA Polymerase Kit (Agilent Technologies, USA) and amplified with an initial denaturation step (94 °C for 3 min), followed by 28 cycles of denaturation (94 °C for 20 s), annealing (58 °C for 20 s), and extension (72 °C for 50 s). The quality of the final products was by gel electrophoresis. To allow multiplexing in the sequencing run, we used the decamers as MIDs, as recommended by Roche (2009), flanked by the universal M13 primer, and again followed the protocol of Daigle *et al*. (2011). Final amplicons were mixed in equimolar amounts and commercially sequenced (Illumina Miseq; GATC Biotech, Konstanz, Germany). The resulting reads were processed according to the *UPARSE* pipeline (Edgar, 2013) by applying very stringent quality criteria (reads > 400 nt, no ambiguous bases, and maximum expected error rate 0.5). Subsequently, reads were clustered at different OTU dissimilarity levels (1%, 3%, and 5%) to obtain a classification-independent estimate of diversity.

Classification. Sequence reads were classified with the Naive Bayesian Classifier implemented in *mothur*, using a bootstrap value of 60% as cutoff. Since the classification success with public reference databases for arthropod clusters was limited owing to a lack of both annotation and sequences, we slightly modified the SILVA database by adding relevant published studies and sequences obtained in this study. The taxonomy of relevant lineages was refined by adding or renaming groups that have been identified either in published phylogenies or by groups found in this study. The resulting reference database is available upon request.

Statistics and visualization of the data. For all statistical analyses, *R* version 3.0.1 (*R*, 2013) was used. Some graphics were produced using the ggplot2 package (Wickham, 2009). Trees were exported with the node and bootstrap information in Newick format and plotted with all meta data using the *APE* package (Paradis, 2004) with some customized functions for plotting clusters and annotations. The ecological analysis was analyzed with the vegan package (Oksanen et al., 2013) in conjunction with the Soergel distance function written in pure *R*. For cluster analysis of genus-level groups, abundance data was normalized approximately to the smallest sample size (1,000 seqs), and the Soergel distance was calculated sample wise. We chose the Soergel distance as a β-diversity measure since it performed well in a recent comparison study (Parks, 2013). For the logarithmic version of the Soergel distance (which was only used for the Supplementary Information), we expressed the data in per mill to circumvent negative values after the log transformation since many β-diversity measures rely on minimum, maximum, and sum terms, which would result in non-interpretable distances. When the data was finally logarithmized, we followed the recommended procedure of Costea et al. (2014). Briefly, a pseudo-count just a bit smaller than the smallest value of the dataset was added to circumvent log(0). To visualize the resulting distance matrices, we carried out a neighbor-joining analysis using the bionj implementation in ape since the classical hierarchical clustering is not well suited for biological (Rajaram, 2010) and especially compositional data sets (Friedman, 2012).

Phylogenetic analysis of high-throughput sequencing derived sequences. Sequences were clustered sample wise into operational taxonomic units (OTUs) at the

1% level using the Uparse strategy (Edgar, 2013) and classified into genus-level bins. All sequences in the same genus-level bin were aligned, and filtered to remove potentially poorly aligned columns (20% gap criterion). Sequences were subjected to a maximum-likelihood analysis using *RAxML* (Stamatakis, 2014) and rooted with a sequence from the methanogenic order *Methanopyrales*. Data were visualized using the *R* package *APE* (Paradis, 2004).

Correlation of archaeal with bacterial genus-level groups. In order to find possible dependencies of the archaeal genus-level groups on certain bacterial genus-level groups, a correlation analysis based on the SparCC algorithm was carried out since classical correlation analyses are not designed or even valid for compositional data (Friedman and Alm, 2012). For this purpose, we used the data set of this study and the data sets of the bacterial community structure (mostly taken from Dietrich *et al*., 2014). Both the archaeal and the bacterial data sets were classified into genus-level bins and exported in two ratios of 50:50 and 3:97 (archaea:bacteria) in the input format of sparCC. The choice of the latter ratio is natural as it has been reported to occur in termite guts (Brauman *et al*., 2001). The different ratios were used to test whether the ratio has an effect on the result, but no obvious differences were found in the genera combinations finally picked (minimum occurrence of a minimum of one 0.4 per genus). The resulting SparCC-r values were visualized using the *R* software package.

5.4 Results

Distribution of major archaeal groups in the clone libraries. We used a total of 31 clone libraries of archaeal 16S rRNA genes for the phylogenetic analysis of the archaeal communities of arthropods (Table 1). Seventeen libraries stemming from published studies and having a size ranging between 5 and 341 clones were downloaded from NCBI GenBank. These clone libraries included 1 scarab beetle larva, 2 cockroaches, and 13 termites. If necessary, the community structure of the phylotypes was recreated using tables in the respective publications.

The remaining 14 clone libraries consisting of 11 to 221 clones were created from a variety of different host groups, including 4 different millipede genera from 3 different

families, 1 new cockroach species, and clone libraries of higher termites belonging to 4 subfamilies of higher termites. One of these subfamilies (*Apicotermitinae*) has never been investigated before. In addition to the subfamilies that have already been investigated in the literature, we added information about new dietary groups such as grass- and wood-feeding *Termitinae* and *Nasutitermitinae* (Table 1).

Table 1. Characteristics of the 16S rRNA gene clone libraries of the archaeal hindgut microbiota of each host species. The same identifiers are used to identify the samples in all tables and figures of clone libraries. For the origin of samples that were not part of a previous publication, see the legend of Table 2.

Host species	Compart-ment[a]	Identi-fier	Clones	Diet group	Origin/ Reference[b]
Millipedes					
Pachybolidae					
Aphistogonoiulus corallipes	Wg	Aphco	33	Litter	B1
Rhinocricidae					
Anadenobolus monilicornis	Hg	Anamo	29	Litter	B1
Spirostreptidae					
Microtrullius uncinatus	Hg	Micun	22	Litter	B1
Harpagophoridae					
Harpagophorida sp.	Wg	Harsp	30	Litter	B1
Scarab beetle larvae					
Scarabaeidae					
Pachnoda ephippiata	M,Hg	Pacep	68[c]	Humus	(Egert *et al*., 2003)
Cockroaches					
Blaberidae					
Panesthia angustipennis	Hg	Panan	27	Wood	(Hara *et al*., 2002)
Salganea esakii	Hg	Sales	11	Wood	B3
Salganea taiwanensis	Hg	Salta	69	Wood	(Hara *et al*., 2002)
Lower termites					
Hodotermitidae					
Hodotermopsis sjoestedti	Wg	Hodsj1	12	Wood	(Tokura *et al*., 2000)
Hodotermopsis sjoestedti	Wg	Hodsj2	5	Wood	(Shinzato *et al*., 2001)
Kalotermitidae					
Cryptotermes domesticus	Lumen	Crydo	37[c]	Wood	(Ohkuma and Kudo, 1998)
Neotermes koshunensis	Wg	Neoko	5	Wood	(Shinzato *et al*., 2001)
Rhinotermitidae					
Coptotermes formosanus	Wg	Copfo	5	Wood	(Shinzato *et al*., 2001)
Reticulitermes kanmonensis	Wg	Retka	5	Wood	(Shinzato *et al*., 2001)
Reticulitermes speratus	Wg	Retspe1	60[c]	Wood	(Shinzato *et al*., 1999)

Reticulitermes speratus	Wg	Retspe2	24	Wood	(Tokura *et al*., 2000)
Higher termites (Termitidae)					
Macrotermitinae					
Macrotermes sp.	Hg	Macsp	39	Wood/Fungus	L1
Macrotermes subhyalinus	Hg	Macsu	39	Wood/Fungus	F5
Odontotermes formosanus	Wg	Odofo	20	Wood/Fungus	(Ohkuma *et al*., 1999)
Odontotermes sp.	Hg	Odosp	48	Wood/Fungus	F4
Apicotermitinae					
Alyscotermes trestus	Hg	Alytr	41	Humus	F5
Termitinae					
Amitermes sp.	C—P5	Amisp	164[c]	Interface	F8
Cubitermes fungifaber	Hg	Cubfu	50	Humus	(Donovan *et al*., 2004)
Cubitermes orthognathus	P1—P5	Cubor	110[a]	Humus	(Friedrich *et al*., 2001)
Cubitermes ugandensis	C—P5	Cubug	190[c]	Humus	F6
Microcerotermes sp.	Wg	Micsp	41	Wood	F5
Ophiotermes sp.	C—P5	Ophsp	221[c]	Humus	F7
Pericapritermes nitobei	Wg	Perni	18	Humus	(Ohkuma *et al*., 1999)
Nasutitermitinae					
Nasutitermes takasagoensis	Wg	Nasta1	12	Wood	(Ohkuma *et al*., 1999)
Nasutitermes takasagoensis	Wg	Nasta2	341[c]	Wood	(Miyata *et al*., 2007)
Trinervitermes sp.	Hg	Trisp	39	Grass	F5

[a] Gut compartment: Wg, Whole gut; Hg, Hindgut; M, Midgut; C–P5, all adjacent compartments from crop to P5 were separately analyzed; P1–P5, all adjacent compartments from P1 to P5 were separately analyzed.

[b] Origins of samples: B, commercial breeders (B1: b.t.b.e. Insektenzucht, Schnürpflingen, Germany); B3, Jörg Bernhardt, Helbigsdorf, Germany [http://www.schaben-spinnen.de]);F, field collections (F4, near Kajiado, Kenya; F5, near Nairobi, Kenya [by J. O. Nonoh]; F6, Lhiranda Hill, Kakamega, Kenya [by J. O. Nonoh];F8, near Eldoret, Kenya [by D.K. Ngugi]).L, laboratory colonies (L1, R. Plarre, Federal Institute for Materials Research and Testing, Berlin, Germany).

[c] Clones were distributed over either different compartments or samples of the same species. *Pachnoda ephippiata* (Pacep)*:* M: 24, Hg: 44; *Cryptotermes domesticus* (Crydo), 23 of 37 clones were classified as archaeal; *Reticulitermes speratus* (Retspe1), sample RS1–RS6, each with 10 clones (RS1 was chosen); *Amitermes* sp. (Amisp), C: 2, M: 26, P1: 26, P3: 41, P4: 41, P5: 28; *Cubitermes orthognathus* (Cubor)*,* P1: 27, P3: 26, P4: 26, P5: 31; *Cubitermes ugandensis*, (Cubug), C: 20, M: 15, P1: 35, P3: 48, P4: 42, P5: 30; *Ophiotermes* sp. (Ophsp), C: 26, M: 29, P1: 39, P3: 45, P4: 39, P5: 43; *Nasutitermes takasagoensis* (Nasta2), 341 clones in total from six libraries (The control group, wood-fed, had a total of 71 clones).

After classification into four methanogenic orders and the phylum *Thaumarchaeota,* differences between the compositions of the archaeal communities were obvious already at the order level (Fig. 1). *Methanobacteriales* was the predominant order, followed by *Methanosarcinales, Methanomicrobiales,* and *Methanomassiliicoccales*. Millipedes harbored either a combination of *Methanobacteriales* together with *Methanomicrobiales* and *Methanosarcinales* in high abundance (Aphco and Micun) or *Methanosarcinales* together with *Methanomassiliicoccales* and *Methanomicrobiales* (Anamo), or a relatively even community of all four methanogenic orders in the dataset (Harsp).

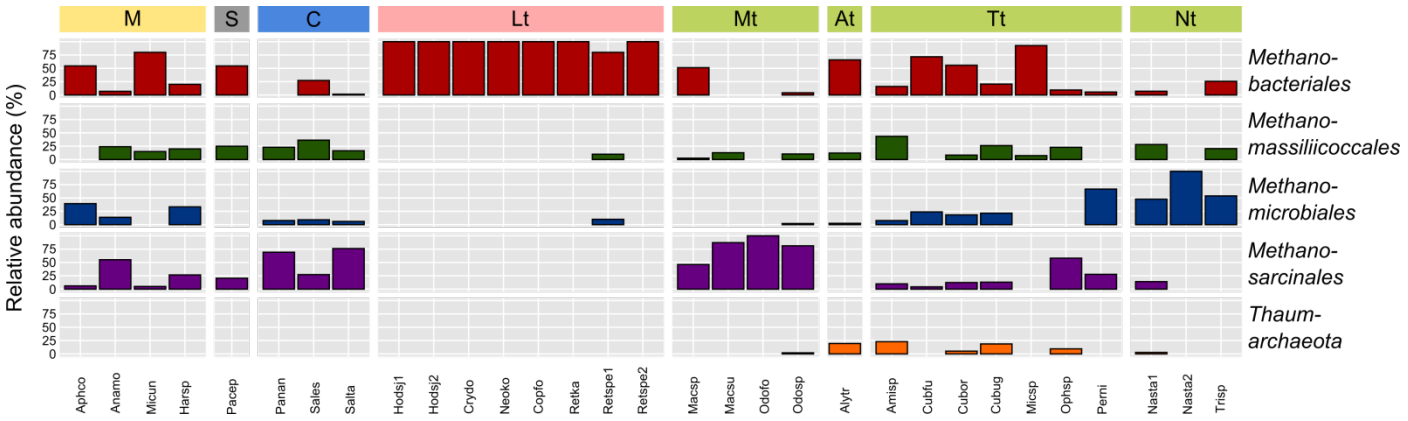

Figure 1. Structure of the archaeal communities in millipedes, scarab beetle larvae, cockroaches, and termites based on phylogenetic analysis of the 16S rRNA gene. The bar plots represent the order-level classification of the clone library sequences of this study and those obtained from various publications (see Table 1). The data is split vertically according to host group (M, Millipedes; S, Scarab beetles; C, Cockroaches; Lt: Lower termites; Mt, *Macrotermitinae*; At, *Apicotermitinae*; Tt, *Termitinae*; Nt, *Nasutitermitinae*) and horizontally according to the phylogenetic result at the order level (except *Thaumarchaeota*). Sample names are abbreviated as in Table 1.

Similar to the gut community of the millipedes, the scarab beetle larvae hindgut (Pacep) was dominated by *Methanobacteriales,* and *Methanomassiliicoccales* and *Methanosarcinales* were almost equivalently abundant. When Egert *et al*. (2003) performed this analysis; they found only two methanogenic orders (*Methanobacteriales* and *Methanosarcinales)* and members that were then assigned to the order of *Thermoplasmatales.* It is now known that these sequences belong to the recently

discovered methanogenic order *Methanomassiliicoccales* (Paul *et al*., 2012; Iino *et al*., 2013). Clones from wood-feeding cockroaches (Panan, Sales, Salta) were classified to all four methanogenic orders. In the data sets of *Panesthia angustipennis* (Panan) and *Salganea taiwanensis* (Salta) published by Hara *et al*. (2002), members of *Methanosarcinales* and *Methanomassiliioccales* (in their article called cluster XSAT3A and XSAT3B, respectively, of *Thermoplasmatales*) dominate. Our clone library of *Salganea esakii* (Sales) differed from the other two clone libraries. Whether this difference is due to the use of different primers or whether this reflects true biological variability cannot be discerned.

In the clone libraries of lower termites (all obtained from literature), mostly the order *Methanobacteriales* was detected. However, the clone library of *Reticulitermes speratus* (Retspe1) from Shinzato *et al*. (1999) also shows the presence of members of the orders *Methanomicrobiales* and *Methanomassiliicoccales*. Notably, in that study, the archaeal communities of six different colonies from different regions in Japan (data not shown) varied. The authors concluded that the orders *Methanomassiliicoccales* and *Methanomicrobiales* form only minor and variable fractions of the community in *R. speratus*. Earlier studies of lower termites used a different primer set that has been recently identified as having a mismatch against *Methanomassiliicoccales*-related sequences (Paul *et al*., 2012). This indicates that lower termites might not consist exclusively of a *Methanobacteriales*-dominated archaeal community, as the respective studies concluded.

The archaeal communities in higher termites showed similarities at the subfamily level. For example, the *Macrotermitinae* revealed a high abundance of *Methanosarcinales,* followed by *Methanomassiliicoccales*. In the *Macrotermes* sp. (Macsp), we also found a high abundance of *Methanobacteriales*, which indicated that variation within the termite genus *Macrotermes* can since the other *Macrotermes subhyalinus* (Macsu) has a different composition of methanogens. The archaeal communities of *Macrotermitinae* were relatively similar to those of wood-feeding cockroaches. Also, the subfamilies *Apicotermitinae* and *Termitinae* had a similar composition of archaeal order-level taxa. The analysis of several clone libraries indicated that members of the

Methanobacteriales are the most abundant group of methanogens in these termites, but also other orders are represented. A clear change in community structure is evident in the higher termite subfamily *Nasutitermitinae*, in which the archaeal communities were dominated by the order *Methanomicrobiales*.

Sequences classified as the phylum *Thaumarchaeota* were only detected in higher termites, making up 2–20% of the sequences in the respective clone libraries. Compared to the abundance of the methanogenic orders, members of *Thaumarchaeota* were never the predominant archaeal group.

Phylogenetic positions of arthropod archaeal sequences. Sequences derived from the clone libraries were classified at the order level and phylogenetically analyzed, yielding calculated phylogenetic trees of the methanogenic orders found in arthropods (Figs. 2–4). The phylum *Thaumarchaeota* was analyzed only superficially as these sequences in the SILVA database were not of high quality. Therefore, these sequences were analyzed only at the class level. An in-depth phylogenetic analysis is planned for an upcoming publication.

In general, sequences belonging to the order *Methanobacteriales* are clearly placed within the genus *Methanobrevibacter*. The closest cultured representatives include *Methanobrevibacter arboriphilus, Methanobrevibacter cuticularis, Methanobrevibacter filiformis,* and *Methanobrevibacter smithii.* All sequences from this study form either monophyletic clusters that consist of only sequences from specific host groups (e.g., higher termites) or from picked flagellates, or form intermixed clusters with sequences originating from many arthropod hosts. This indicates a specificity of the lineages in question to the arthropod gut systems in general, but cospeciation was not detected. Some clones from the study of Deevong *et al*. (2004) were found to be in radiated in the genus *Methanobacterium*, as was reported in that study.

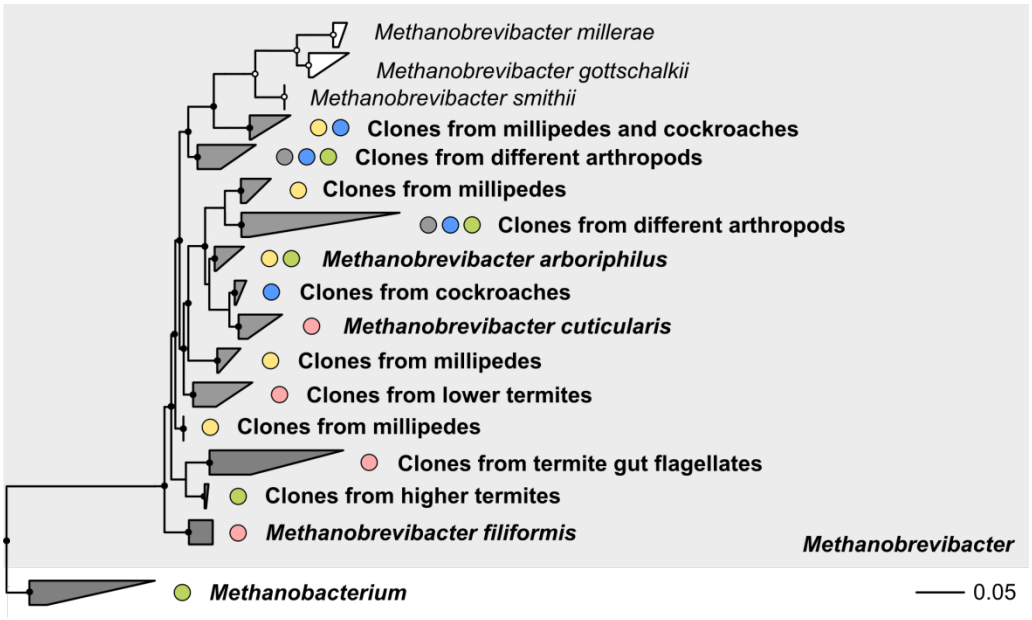

Figure 2. Phylogenetic tree based on 16S rRNA genes of *Methanobacteriales*-related sequences from clone libraries of this study and previously published sequences. Colored circles indicate the origin of sequences in the clusters; the same color code as in Fig. 1 is used. Filled and unfilled dots indicate bootstrap support of the nodes (< 70%, no dot; ≥ 70%, ○; ≥ 90%, ●).

Sequences that clustered within the order *Methanomicrobiales* are less diverse than the *Methanobacteriales*-related sequences. To date, only two clusters have been identified as containing arthropod sequences (Fig. 3). One cluster is within the genus *Methanocorpusculum*. The closest cultured representative of the arthropod sequences belonging to this genus is *Methanocorpusculum parvum,* as has also been documented by Shinzato *et al*. (1999). This type strain uses either hydrogen and carbon dioxide or 2-propanol and carbon dioxide for methanogenesis (Zellner *et al*., 1987). The second cluster – into which most of the *Methanomicrobiales*-related sequences of arthropod guts fell – was a monophyletic cluster consisting only of sequences from higher termites. This cluster is basal to the sequences of the genus *Methanospirillum*, which indicated that sequences of this cluster form a new genus-level group within the *Methanospirillaceae*. This is also underlined by the high degree of dissimilarity of 9–12% to *Methanospirillum stamsii,* compared to the distances within this cluster (up to 5%). Therefore, we tentatively name the cluster '*Methanospirillaceae* arthropod cluster'.

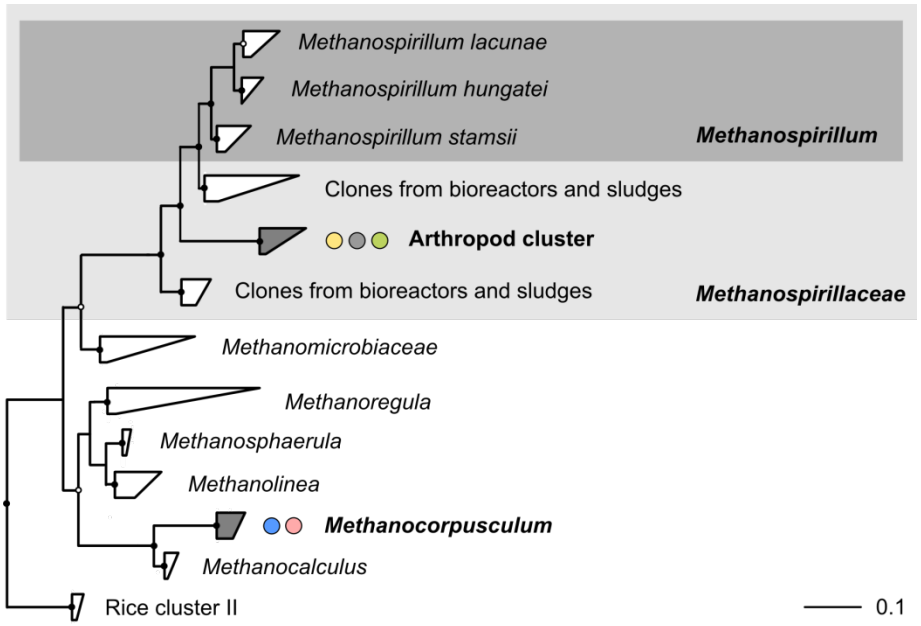

Figure 3. Phylogenetic tree based on 16S rRNA genes of *Methanomicrobiales*-related sequences from clone libraries of this study and previously published sequences. Colored circles indicate the origin of sequences in the clusters; the same color code as in Fig. 1 is used. Filled and unfilled dots indicate bootstrap support of the nodes (< 70%, no dot; ≥ 70%, ○; ≥ 90%, ●).

Within the *Methanosarcinales,* all sequences from clone libraries were phylogenetically located in the radiation of *Methanomicrococcus* (Fig. 4). *Methanomicrococcus blatticola* is the only cultured representative of this phylogenetic group and is able to reduce methanol and different methylamines with hydrogen as external electron donor (Sprenger *et al*., 2000). Arthropod-gut-derived sequences were located in three clusters. One group lay within a 3% radius around *M. blatticola* (mostly cockroach-gut-derived sequences). Another cluster consisted only of sequences originating from higher termite gut samples (1–2% difference to *M. blatticola*). The third group was apical to the other two groups and consisted only of sequences from millipede guts (2–4% dissimilarity to the groups of *M. blatticola* and the higher termite cluster). Since all arthropod-gut-derived sequences were highly similar to that of the type strain *M. blatticola*, we grouped these clusters within the genus-level group of *Methanomicrococcus*.

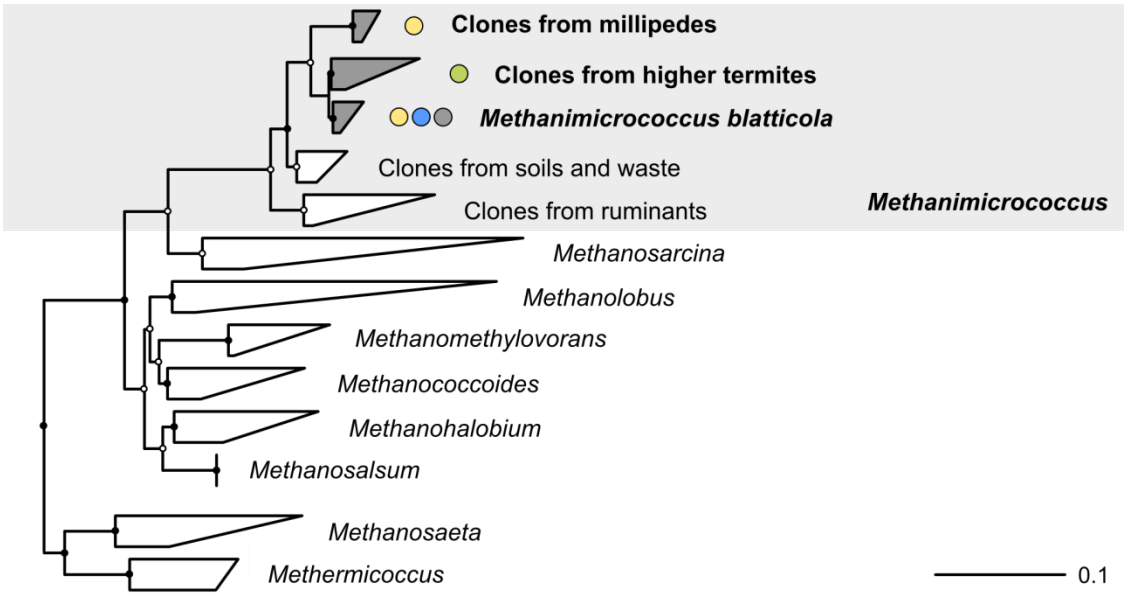

Figure 4. Phylogenetic tree based on 16S rRNA genes of *Methanosarcinales*-related sequences from clone libraries of this study and previously published sequences. Colored circles indicate the origin of sequences in the clusters; the same color code as in Fig. 1 is used. Filled and unfilled dots indicate bootstrap support of the nodes (< 70%, no dot; ≥ 70%, ○; ≥ 90%, ●).

Recently, a monophyletic group within the class *Thermoplasmata* was found to represent methanogenic archaea (Dridi *et al*., 2012; Paul *et al*., 2012). Past studies of arthropod guts always identified a moderate proportion of sequences highly similar to the *Thermoplasmatales*. These studies included scarab beetle larvae (Egert *et al*., 2003), cockroaches (Hara *et al*., 2002), lower termites (Shinzato *et al*., 1999), and different higher termites (Ohkuma *et al*., 1999; Friedrich *et al*., 2001; Donovan *et al*., 2004; Miyata *et al*., 2007) and document that the novel order *(Methanomassiliicoccales)* is a widespread group of methanogens within arthropod guts. This is also reflected in the phylogenetic placement of the sequences from arthropod-gut-derived clones (Fig. 5). We found a total of ten minor clusters. The most apical group contained sequences highly similar to the enrichment culture "*Candidatus* Methanoplasma termitum" (clustered at a radius of 3%), consisting of sequences from millipedes, higher termites, and wood-feeding cockroaches.

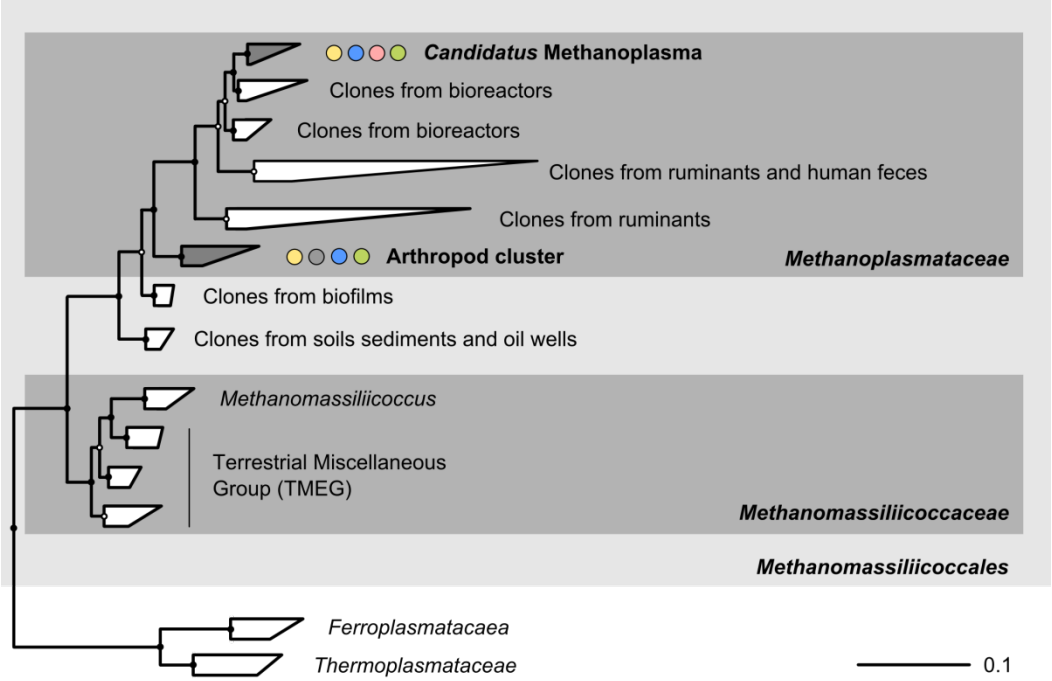

Figure 5. Phylogenetic tree based on 16S rRNA genes of *Methanomassiliicoccales*-related sequences from clone libraries of this study and previously published sequences. Colored circles indicate the origin of sequences in the clusters; the same color code as in Fig. 1 is used. Filled and unfilled dots indicate bootstrap support of the nodes (< 70%, no dot; ≥ 70%,○; ≥ 90%, ●).

The latter sequences were formerly described as the XSAT3A cluster (Hara *et al*., 2002). Since these sequences showed a difference of maximally 5% to "*Candidatus* Methanoplasma termitum", we considered the whole cluster to be a genus-level group and name it '*Candidatus* Methanoplasma'. Basal to this group, a cluster containing sequences from ruminants, bioreactors, and human feces was located, including 16S rRNA sequences of the recently enriched methanogens "*Candidatus* Methanomethylophilus alvus" (Borrel *et al*., 2012) and "*Candidatus* Methanogranum caenicola" (Iino *et al*., 2013). The second major group of arthropod sequences was basal to the first group and contained also clones from different arthropod hosts. Each host group was represented by at least one cluster. The clones from wood-feeding cockroaches were formerly classified as the XSAT3B cluster (Hara *et al*. 2002). Since the sequences in this cluster differed by 1% to 6%, we defined this group as a new genus-level group 'arthropod cluster' within the *Methanoplasmataceae* (see Fig. 5). The least common node of all enrichment cultures and the isolate *Methanomassiliicoccus*

(*Mmc*.) *luminyensis* (Dridi *et al*., 2012) were used to conservatively define the order *Methanomassiliicoccales*. Recently, Iino *et al*. (2013) proposed the family *Methanomassiliicoccaceae* based on an analysis of the gene encoding the alpha-subunit of methyl-coenzyme M reductase (*mcrA*) and the 16S rRNA gene. In both analyses, the authors find their enrichment culture *Ca.* Methanogranum caenicola clearly separated from the first isolate, *Mmc. luminyensis* (Dridi *et al*., 2012). We decided to place the cluster in which *Mmc. luminyensis* is located at the family level *Methanomassiliicoccaceae.* Subsequently, the least common node of the *Methanoplasmataceae* arthropod cluster and of the enrichment cultures *Ca.* Methanogranum caenicola*, Ca.* Methanoplasma termitum, and *Ca.* Methano-methylophilus alvus was used to define the *Methanoplasmataceae* at the family level.

Sequences that were classified to the phylum *Thaumarchaeota* are not described or discussed in detail as these will be part of another study.

Table 2. Characteristics of the high-throughput sequencing libraries used in this study. The same identifiers are used to identify the samples in all tables and figures of the high-throughput sequencing data.

Insect species	No.	Diet group	Origin[a]	Sequences	No. of 5% OTUs	No. of genus-level groups	No. of 3% OTUs	Diver-sity	Acces-sion No.
Millipedes									
Spirostreptidae									
Anadenobolus monilicornis	1	Litter	B1	14,804	9	6	15	1.1	
Harpagophoridae									
Harpagophorida sp.	2	Litter	B1	13,886	9	6	15	1.1	
Scarab beetle larvae									
Cetoniidae									
Dicronorhina derbyana	3	Humus	B2	3,390	4	2	4	1.0	
Genyodonta lequexi	4	Humus	B2	5,881	3	3	3	0.6	
Scarabaeidae									
Pachnoda aemula	5	Humus	B2	2,689	4	3	5	0.1	
Pachnoda ephippiata falkei	6	Humus	B2	3,632	10	6	14	1.2	
Gnorimus tibialis	7	Humus	B2	5,221	3	2	4	0.8	
Xylotrupes gideon	8	Humus	B2	2,212	3	3	5	1.4	
Cockroaches									
Polyphagidae									
Ergaula capucina	9	Litter	B3	4,030	12	8	22	1.9	
Blaberidae									
Elliptorhina chopardi	10	Litter	B3	2,168	9	7	13	2.0	
Panchlora sp.	11	Litter	B3	4,101	7	5	10	0.3	
Opisthoplatia orientalis	12	Litter	B3	2,940	10	6	16	1.7	
Nauphoeta cinerea	13	Litter	B3	10,653	13	8	23	1.6	

Gromphadorhina portentosa	14	Litter	B3	2,196	8	6	15	0.9
Diploptera punctata	15	Litter	B3	1,760	8	5	10	1.6
Panesthia angustipennis	16	Wood	B3	8,010	11	5	13	0.9
Salganea esakii	17	Wood	B3	4,515	12	7	20	2.0
Eublaberus posticus	18	Litter	B3	2,560	9	7	14	1.9
Schultesia lampyrodiformis	19	Litter	B3	13,700	11	5	17	1.5
Henschoutedenia flexivitta	20	Litter	B3	2,750	9	7	15	1.8
Blattidae								
Blatta orientalis	21	Litter	B3	3,330	8	6	16	1.4
Eurycotis floridiana	22	Litter	B3	5,025	12	3	19	1.8
Shelfordella lateralis	23	Litter	B3	16,745	10	6	17	0.9
Cryptocercidae								
Cryptocercus punctulatus	24	Wood	F1	14,294	10	5	13	0.3
Lower termites								
Mastotermitidae								
Mastotermes darwiniensis	25	Wood	L1	3,129	9	5	16	1.1
Hodotermitidae								
Hodotermopsis sjoestedti	26	Wood	L1	15,463	10	5	17	0.8
Hodotermes mossambicus	27	Grass	F2	5,844	10	6	16	0.7
Termopsidae								
Zootermopsis nevadensis	28	Wood	L2	2,269	5	4	8	0.9
Kalotermitidae								
Neotermes jouteli	29	Wood	F3	1,5181	10	7	16	0.8
Rhinotermitidae								
Reticulitermes santonensis	30	Wood	L2	2,067	9	8	14	1.4
Coptotermes niger	31	Wood	L1	1,575	8	5	11	0.5
Higher termites (*Termitidae*)								
Macrotermitinae								
Odontotermes sp.	32	Litter/Fungus	F4	1,533	8	7	14	1.4
Macrotermes sp.	33	Litter/Fungus	L1	3,231	7	7	16	0.9
Apicotermitinae								

Alyscotermes trestus	34	Humus	F5	2,188	11	9	17	1.7
Syntermitinae								
Cornitermes sp.	35	Humus	L3	2,415	7	6	8	1.7
Termitinae								
Cubitermes sp.	36	Humus	F6	3,932	5	5	8	1.0
Cubitermes sp.	37	Humus	F7	2,799	7	5	6	1.3
Cubitermes sp.	38	Humus	F8	5,181	7	5	7	1.1
Ophiotermes sp.	39	Humus	F9	3,402	12	8	18	2.2
Neocapritermes taracua	40	Humus	L3	4,801	10	6	11	1.5
Proboscitermes sp.	41	Humus	L3	3,745	7	6	10	1.3
Amitermes meridionalis	42	Grass	F10	1,345	12	7	16	1.7
Microcerotermes parvus (TD193)	43	Wood	L3	2,995	8	6	11	1.7
Nasutitermitinae								
Trinervitermes sp.	44	Grass	F5	1,571	9	7	15	1.5
Atlantitermes sp. (TD202)	45	Humus	L3	4,383	2	2	2	0.7
Unclassified Nasutitermintinae (TD194)	46	Humus	L3	4,654	6	5	6	1.0
Nasutitermes corniger	47	Wood	L4	1,457	9	7	12	1.8
Nasutitermes takasagoensis	48	Wood	F11	1,026	10	8	12	2.2

[a]Origin of samples: B, commercial breeders (B1: b.t.b.e. Insektenzucht, Schnürpflingen, Germany; B2: Wirbellosen Welt, Rödinghausen, Germany; B3, Jörg Bernhardt, Helbigsdorf, Germany [http://www.schaben-spinnen.de]); F, field collections (F1, Heywood County, NC, USA by C. Nalepa; F2, near Pretoria, South Africa by J. Rohland;F3, Fort Lauderdale, FL, USA by R. H. Scheffrahn; F4, near Kajiado, Kenya; F5, near Nairobi, Kenya by J. O. Nonoh; F6, Lhiranda Hill, Kakamega, Kenya by J. O. Nonoh; F7, South Africa, by M. Poulsen; F8, near Eldoret, Kenya by D.K. Ngugi; F9, Kalunja Glade, Kakamega, Kenya by D. K. Ngugi; F10, near Darwin, Australia by A. Brune; F11, near Nishihara, Japan by G. Tokuda); L, laboratory colonies (L1, R. Plarre, Federal Institute for Materials Research and Testing, Berlin, Germany; L2, MPI Marburg; L3, D. Sillam-Dussez, Bondy, France; L4, R. H. Scheffrahn, University of Florida, Fort Lauderdale, FL, USA).

Table 3. Coverage of the primer set A533f_mod A934b_mod of major archaeal groups in the SILVA database. Data differentiates between the coverage obtained with no mismatch or one mismatch allowed. Domains and phylum-level groups are set in bold whereas order level are set in regular font shape.

Taxonomic level	Coverage (%)	
	No mismatch	One mismatch
Archaea	81.7	90.8
Ancient Archaeal Group (AAG)	0.0	0.0
Crenarchaeota	86.2	95.3
Euryarchaeota	81.0	92.3
Methanobacteriales	90.9	96.8
Methanococcales	1.4	86.1
Methanocellales	83.3	86.7
Methanomicrobiales	89.5	94.1
Methanosarcinales	86.5	92.7
Methanopyrales	100.0	100.0
Methanomassiliicoccales	95.2	98.6
Korarchaeota	3.9	90.2
Marine Hydrothermal Vent Group 1 (MHVG-1)	7.7	7.7
Marine Hydrothermal Vent Group 2 (MHVG-2)	0.0	0.0
Nanoarchaeota	2.9	5.7
Thaumarchaeota	83.9	88.9
Bacteria	0.0	0.0
Eukaryota	0.0	0.3

Primer design. Based on the existing primer set A533b/A934b from Grosskopf *et al*. (1998), we designed new primers for the analysis of the archaeal community structure. We slightly modified the primers by maximizing the number of sequences targeted, with emphasis on arthropod gut archaeal groups and methanogens. In general, the primer set had a high specificity towards the domain Archaea (Table 3). In both scenarios, with and without a mismatch, no sequences originating from the bacterial domain were bound *in silico*. With one mismatch, 0.3% of the eukaryotic sequences were bound, but we excluded those sequences belonging to phylum *Arthropoda*. All archaeal phylum-level groups are well covered by this primer set, except the Ancient Archaeal Group

(AAG), the Marine Hydrothermal Vent Groups I and II (MHVG-1 and MHVG-2), and the phylum-level group *Nanoarchaeota*. Since these phylum-level groups are not reported to occur in arthropod gut systems, we considered our primer set appropriate for the profiling of the archaeal communities in arthropod guts.

Reference database. The current SILVA database contains 184 archaeal genus-level groups. We gave a name to the order *Methanomassiliicoccales,* which was not described in the database. Our phylogenetic analysis led to the addition of the following new genus-level groups to the database: arthropod cluster (order *Methanomassiliicoccales* and family *Methanoplasmataceae*) and arthropod cluster (order *Methanomicrobiales* and family *Methanospirillaceae*). The performance of this database is evaluated in the next subsection.

Profiling of the archaeal communities in arthropods. Since a comparison of the different clone libraries is challenging due to the use of different primer sets and sampling efforts (sometimes only 5 clones per clone library), we carried out a large-scale sequencing experiment with representatives of all major arthropod groups known to emit methane (Hackstein and Stumm, 1994). We collected 48 samples and sequenced DNA using the newly designed primer pair A533f_mod and A934b_mod. We obtained 1,026–16,745 sequences per sample (Table 2). Sequences were first clustered into OTUs at the 3% level, resulting in minimally 2 and up to 23 archaeal OTUs per sample. Subsequently, OTUs were classified into genus-level bins with the reference database created in this study. We were able to classify between 92.4% (*Ergaula capucina*, No. 9) and 100% (almost all samples) of all sequences at the genus level (Table 4). This indicated that almost all genus-level groups in the data set could be identified by the classification approach.

Table 4. Classification success of the high-throughput sequencing data.

Sample	No.	Classification success (%)				
		Phylum	Class	Order	Family	Genus
Millipedes						
Spirostreptidae						
Anadenobolus monilicornis	1	100.0	100.0	100.0	100.0	100.0
Harpagophoridae						
Harpagophorida sp.	2	100.0	100.0	100.0	100.0	100.0
Scarab beetle larvae						
Cetoniidae						
Dicronorhina derbyana	3	100.0	100.0	100.0	100.0	100.0
Genyodonta lequexi	4	100.0	100.0	100.0	100.0	100.0
Scarabaeidae						
Pachnoda aemula	5	100.0	100.0	100.0	100.0	100.0
Pachnoda ephippiata falkei	6	100.0	100.0	100.0	100.0	99.3
Gnorimus tibialis	7	100.0	100.0	100.0	100.0	100.0
Xylotrupes gideon	8	100.0	100.0	100.0	100.0	100.0
Cockroaches						
Polyphagidae						
Ergaula capucina	9	100.0	100.0	100.0	100.0	92.2
Blaberidae						
Elliptorhina chopardi	10	100.0	100.0	100.0	100.0	100.0
Panchlora sp.	11	100.0	100.0	100.0	100.0	100.0
Opisthoplatia orientalis	12	100.0	100.0	100.0	100.0	100.0
Nauphoeta cinera	13	100.0	100.0	100.0	100.0	100.0
Gromphadorhina portentosa	14	100.0	100.0	100.0	100.0	100.0
Diploptera punctata	15	100.0	100.0	100.0	100.0	100.0
Panesthia angustipennis	16	100.0	100.0	100.0	100.0	99.9
Salganea esakii	17	100.0	100.0	100.0	100.0	99.9
Eublaberus posticus	18	100.0	100.0	100.0	100.0	100.0
Schultesia lampyridiformis	19	100.0	100.0	100.0	100.0	100.0
Henschoutedenia flexivitta	20	100.0	100.0	100.0	100.0	100.0
Blattidae						
Blatta orientalis	21	100.0	100.0	100.0	100.0	100.0
Eurycotis floridana	22	100.0	100.0	100.0	100.0	100.0
Shelfordella lateralis	23	100.0	100.0	100.0	100.0	100.0
Cryptocercidae						
Cryptocercus punctulatus	24	100.0	100.0	100.0	100.0	99.9

Lower termites						
Mastotermitidae						
Mastotermes darwiniensis	25	99.9	99.9	99.9	99.9	95.4
Hodotermitidae						
Hodotermopsis sjoestedti	26	100.0	100.0	100.0	100.0	93.6
Hodotermes mossambicus	27	100.0	100.0	100.0	100.0	100.0
Termopsidae						
Zootermopsis nevadensis	28	100.0	100.0	100.0	100.0	97.0
Kalotermitidae						
Neotermes jouteli	29	100.0	100.0	100.0	100.0	93.6
Rhinotermitidae						
Reticulitermes santonensis	30	100.0	100.0	100.0	100.0	100.0
Coptotermes niger	31	100.0	100.0	100.0	100.0	100.0
Higher termites (*Termitidae*)						
Macrotermitinae						
Odontotermes sp.	32	100.0	100.0	100.0	100.0	100.0
Macrotermes sp.	33	100.0	100.0	100.0	100.0	99.9
Apicotermitinae						
Alyscotermes trestus	34	100.0	100.0	100.0	100.0	100.0
Syntermitinae						
Cornitermes sp.	35	100.0	100.0	100.0	100.0	92.6
Termitinae						
Cubitermes sp.	36	100.0	100.0	100.0	100.0	100.0
Cubitermes sp.	37	100.0	100.0	100.0	100.0	100.0
Cubitermes sp.	38	100.0	100.0	100.0	100.0	100.0
Ophiotermes sp.	39	100.0	100.0	100.0	100.0	100.0
Neocapritermes taracua	40	100.0	100.0	100.0	100.0	99.4
Proboscitermes sp.	41	100.0	100.0	100.0	100.0	100.0
Amitermes meridionalis	42	100.0	100.0	100.0	100.0	100.0
Microcerotermes parvus	43	100.0	100.0	100.0	100.0	100.0
Nasutitermitinae						
Trinervitermes sp.	44	100.0	100.0	100.0	100.0	100.0
Atlantitermes sp.	45	100.0	100.0	100.0	100.0	100.0
Unidentified *Nasutitermitinae*	46	100.0	100.0	100.0	100.0	100.0
Nasutitermes corniger	47	100.0	100.0	100.0	100.0	94.2
Nasutitermes takasagoensis	48	100.0	100.0	100.0	100.0	92.4

Eleven different genera were found in the total dataset. Each sample had a minimum of two and a maximum of nine different genus levels (Table 2). These numbers were in agreement with the number of OTUs at the 5% level, which showed that the genus-level groups of the reference database reflect the natural diversity well. The distribution of the archaeal genus-level groups indicated that most of the sequences were classified as methanogenic genera. The distribution of the genus-level groups in all samples revealed that the predominant lineages in this dataset are *Methanobrevibacter, Methanomicrococcus*, *Ca*. Methanoplasma, *Methanoplasmataceae* arthropod cluster, and the Miscellaneous Crenarchaeotic Group of the *Thaumarchaeota*. The archaeal community structure was consistent within each major arthropod group (Fig. 6). Millipedes harbored mainly *Methanomicrococcus,* followed by *Methanobrevibacter*. The other genus-level groups were present only in low abundance. These sequencing results are in agreement with the results of the millipede clone libraries.

The archaeal communities of scarab beetle larvae hindguts revealed a high abundance of both *Methanobrevibacter* and the *Methanoplasmataceae* arthropod cluster or only *Methanobrevibacter*. This is not in agreement with the results of Egert *et al*. (2003) (Fig. 1), who found additionally members of the order *Methanosarcinales* in high abundance in the hindgut of *Pachnoda ephippiata*. Since the order *Methanosarcinales* was one of the dominating orders in the total data set, although not in any scarab beetle larvae in this sequencing study (No. 3–8), and since the archaeal communities of other soil-feeding larvae sampled in this study also showed variation, we concluded that the archaeal community of scarab beetle larvae is variable.

The archaeal community of most cockroaches was dominated by *Methanomicrococcus* and *Methanobrevibacter*, like the millipede samples. Major exceptions were the leaf-feeding cockroach *Ergaula capucina* (No. 9), the wood-feeding cockroaches *Panesthia angustipennis* and *Salganea esakii* (No. 16 and 17), and the generalist blattid cockroach *Blatta orientalis* (No. 21), which either had a high abundance of the archaeal genus *Methanocorpusculum* (No. 9 and 21) or a high abundance of the *Methanoplasmataceae* arthropod cluster (No. 16 and 17). These results are in good agreement with the only other data available on archaeal community structure of cockroaches, namely *Salganea*

taiwanensis, and *P. angustipennis* (Hara *et al*., 2002), and with the clone library data of our study.

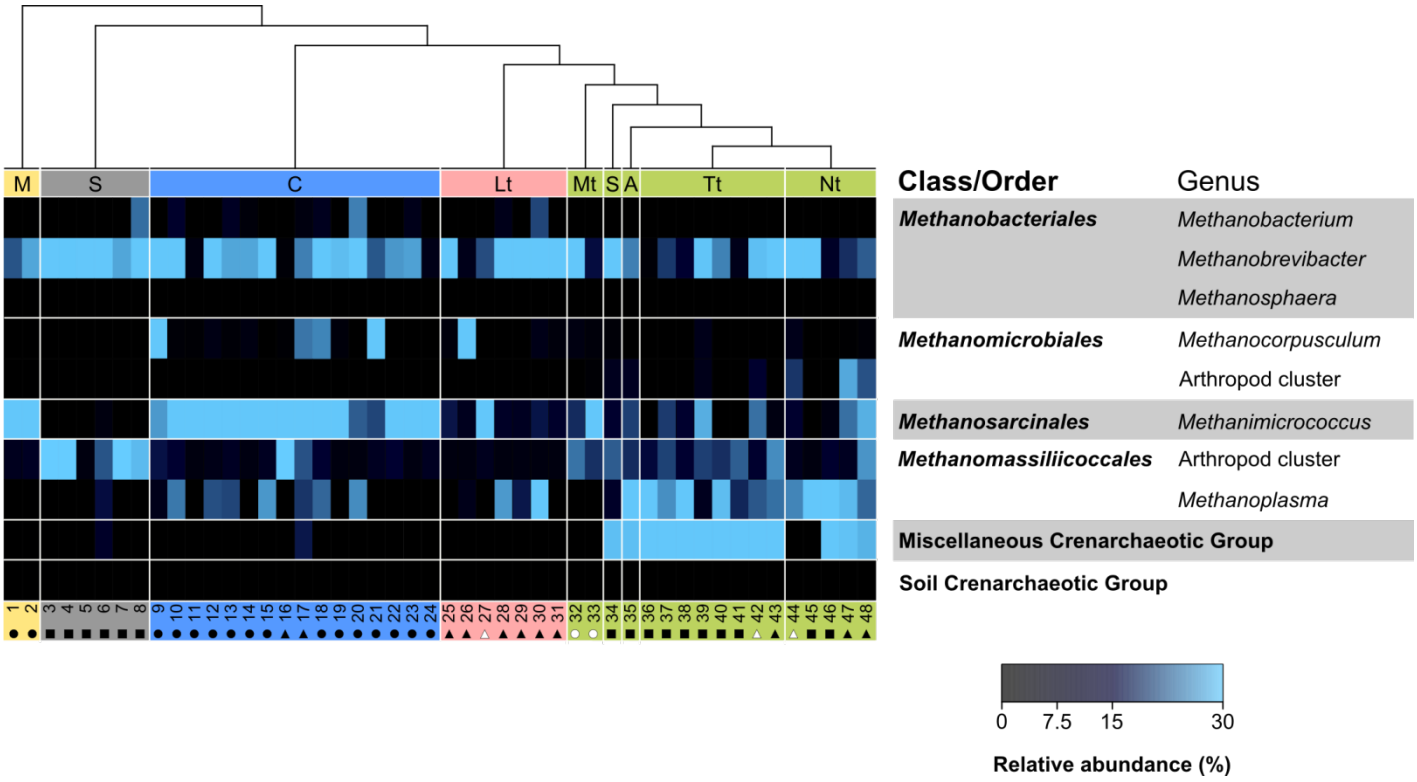

Figure 6. Distribution of archaeal genus-level groups in the arthropod gut samples from this study. The two panels show the same dataset but are differently horizontally arranged. The left panel shows the dataset ordered according to the taxonomy of the hosts (M, millipedes; S, scarab beetle larvae; C, cockroaches; Lt, lower termites; Mt, *Macrotermitinae*; A, *Apicotermitinae*; S (in green), *Syntermitinae*; Tt, *Termitinae*; Nt, *Nasutitermitinae*). The numbers below each panel are the sample identifiers used in Table 2. The symbols below the sample identifiers indicate the dietary specialization of the arthropod host (●, litter; ■, humus; ▲, wood; △, grass; ○, fungus/wood).

The archaeal communities in most of the lower termites consisted of *Methanobrevibacter,* which is in agreement with results in the literature (Ohkuma and Kudo, 1998; Shinzato *et al*., 1999; Tokura *et al*., 2000; Shinzato *et al*., 2001). The only exceptions were *H. sjoestedti* (No. 26), *H. mossambicus* (No. 27), and *R. santonensis* (No. 30). *H. sjoestedti* had a high abundance of *Methanocorpusculum*, *H. mossambicus* had a high abundance of *Methanomicrococcus,* and *R. santonensis* had a mixture of

both *Methanobrevibacter* and *Candidatus* Methanoplasma in high abundance. The results of *H. sjoestedti* contradict results of Tokura *et al*. (2000) and Shinzato *et al*. (2001); however, these studies used different primers and were at a different resolution. The archaeal community of *R. santonensis* had not yet been studied, but in studies of other species of the same genus (Shinzato *et al*., 1999; Tokura *et al*., 2000; Shinzato *et al*., 2001), *Methanobrevibacter* was the predominant order. However, Shinzato *et al*. (1999) revealed substantial differences in the community structure of different *R. speratus* samples depending on the sampling location and the termite colonies; members of the *Methanomassiliicoccales* were sometimes found. This suggests that low resolution of the analysis and the choice of primer sets in the past led to an underestimation of certain members of the archaeal communities.

The most typical characteristic of higher termite archaeal communities was the presence of group MCG of the phylum *Thaumarchaeota.* Except for *Macrotermitinae,* all higher termite subfamilies had a high abundance of this class, followed by *Ca.* Methanoplasma and *Methanobrevibacter*. Also other methanogenic genus-level groups were detected in small amounts. The *Macrotermitinae* had either a high abundance of *Methanobrevibacter* (*Odontotermes* sp.) or *Methanomicrococcus* (*Macrotermes* sp.). The community structure of the two *Nasutitermes* species (No. 47 and 48) are in good agreement with the results of Miyata *et al*. (2007).

Cluster analysis and correlation of archaeal with bacterial genus-level groups. When the archaeal communities of the arthropod gut samples were subjected to neighbor-joining cluster analysis based on the Soergel distance, specific archaeal community clusters became apparent (Fig. 7). Four major clusters were found. In order to discuss the results, we partitioned the data set into four clusters (red dashed line in the dendrogram in Fig. 7), which also illustrates the clear separation of the data into four clusters.

One major cluster was formed by the archaeal communities of higher termites, which documents the distinctness of their archaeal communities. Another cluster was formed by most cockroaches, which indicated a high similarity in their composition. However, within the cockroach cluster, also non-cockroach samples often clustered, including the

lower termites *H. sjoestetdi* and *Hodotermes mossambicus*, the higher termite *Macrotermes* sp., and the two millipedes. This indicated that millipedes have a cockroach-like archaeal community. Also fungus-cultivating termites had an atypical archaeal community structure in contrast to other higher termites, as has already been documented for the bacterial community (Dietrich *et al*., 2014). The fourth cluster was formed by all scarab beetles. The only non-scarab beetle larvae archaeal communities were some lower termites and a fungus-cultivating termite. The archaeal community structures of the lower termites were not similar to each other since they did not show a specific clustering. When the lower termite samples were removed from the analysis (Fig. S1b), each archaeal community mostly reflected the membership of their host in a major host group.

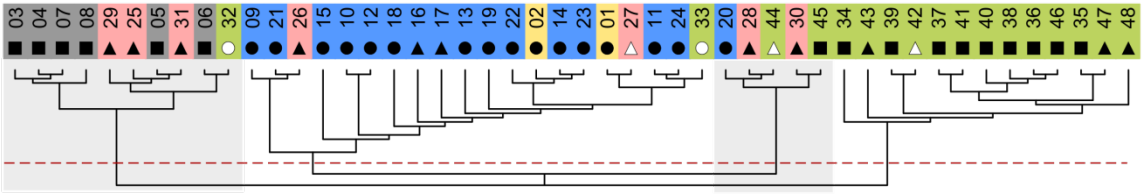

Fig. 7 Community structure based host specificity. Unrooted BioNJ tree displayed as cladogram of the pairwise Soergel distances of the archaeal community structure in arthropod guts. The dashed line aids in distinguishing the data set into four clusters (see also Fig. S1). The color code of hosts is the same as in Fig. 1; numbering is the same as in Table 2; and diet symbols are the same as in Fig. 6.

Host specificity of archaeal lineages. Since we found similarities in the archaeal community structure of arthropods belonging to the same major host group, we suspected also an abundance-based specificity of single archaeal genus-level groups for the major host groups. Therefore, we used the median abundance of each genus-level per host group and compared them with each other. The 5 most abundant genus-level groups belonged the three different orders *Methanobacteriales* (*Mb*), *Methanomassiliicoccales* and *Methanosarcinales* and the class MCG (Figure 8). The genus-level groups were *Methanobrevibacter, Methanomassiliicoccales* Arthropod

cluster, *Candidatus* Methanoplasma, *Methanomicrococcus,* and the Miscellaneous Crenarchaeotic Group (MCG) Athropod cluster. Each of the five groups occurs mostly only in certain host groups in high abundance. *Methanobrevibacter* was highly abundant in all host groups. In contrast, *Methanomicrococcus* occurred in high abundance only in cockroaches and millipedes, underlining the similarity of these host groups; The *Methanomassiliicoccales* Arthropod cluster clearly was mostly highly abundant in the guts of scarab beetle larvae, and both *Candidatus* Methanoplasma and the MCG Arthropod cluster occurred in high abundance in higher termites.

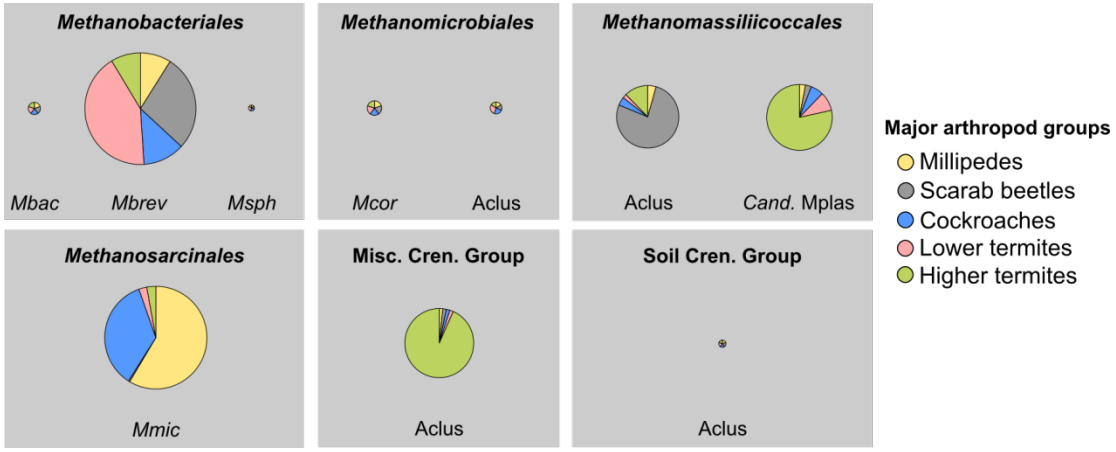

Figure 8. Abundance-based host specificity of archaeal lineages illustrated as pie charts of different genus-level groups that occur in the high-throughput sequencing data set. The area of the charts is scaled by the abundance in the total normalized dataset. The fractions of the different pie charts illustrate the median abundance in the different major arthropod groups. Genus-level group abbreviations: *Mbac*, *Methanobacterium*; *Mbrev*, *Methanobrevibacter*; *Msph*; *Methanosphaera*; Aclus, Arthropod cluster; *Cand* Mplas, *Candidatus* Methanoplasma*; Mcor, Methanocorpusculum*; *Mmic*, *Methanomicroccous*.

Phylogenetic analysis of the short reads. To elucidate not only whether archaeal lineages are preferentially abundant in certain host groups but also whether distinct phylotypes belonging to the different genus level groups are host specific, short reads were clustered sample-wise in 1%-level OTUs and were also subjected genus-level-wise to maximum-likelihood analyses. The resulting trees show representatives of OTUs that clustered at the 1%-level in the host sample (Fig. 9; see Fig. S2a–h for fully annotated trees). The genus *Methanobacterium* was represented by only a small of number of sequences and was basically a trifurcation, which does not allow any

conclusions about host-specific clusters and/or cocladogenesis. The genus *Methanobrevibacter* was represented by enough OTU sequences to conclude that clusters exclusively consisted of sequences belonging to either the host group cockroaches or to lower termites. Lower termite sequences showed long branches compared to the sequences that originated from sample of other host groups might be caused by a different rate of evolution (Fig. S2b), which would indicated that these sequences might stem from flagellate symbionts, since the endosymbiotic lifestyle is often associated with increased mutation rates. However, in the lower termite cluster and the cockroach cluster of *Methanobrevibacter*, no evidence of cocladogenesis could be found. *Methanobrevibacter*-related OTUs from the other host groups formed mainly clusters with OTU sequences from many host groups, which indicated that these *Methanobrevibacter* sequences are not shaped by host-specific mechanisms.

We observed similar phenomena for the order *Methanomassiliicoccales*. In the *Methanomassiliicoccales* arthropod cluster, a monophyletic group of sequences that originated only from higher termites samples occurred. The remaining two major clusters were consisting by either sequences from scarab beetle larvae or from the wood-feeding cockroach *Panesthia angustipennis* (No. 16). This indicates that scarab beetles and higher termites have host-specific lineages within the *Methanomassiliicoccales* Arthropod cluster with the exception of one OTU of *Panesthia angustipennis*. However, the more abundant and perhaps more important OTU from this host is located in another cluster. Notably, the OTU of *Macrotermes* sp. (No. 33) is located in the proximity of those of *Panesthia angustipennis* and *Pachnoda ephippiata falkei*. Cocladogenesis could not be observed also in this genus-level group.

In the *Ca.* Methanoplasma genus-level group (Fig. S2d), a higher termite cluster and a cockroach cluster were clearly evident. Neither of these showed a cocladogenesis signal. Interestingly, *Reticulitermes santonensis* (No. 30) is represented by two OTUs in this tree, the first as a very basal lineage and the second in a deep-branching bifurcation with an OTU from *Nasutitermes corniger*.

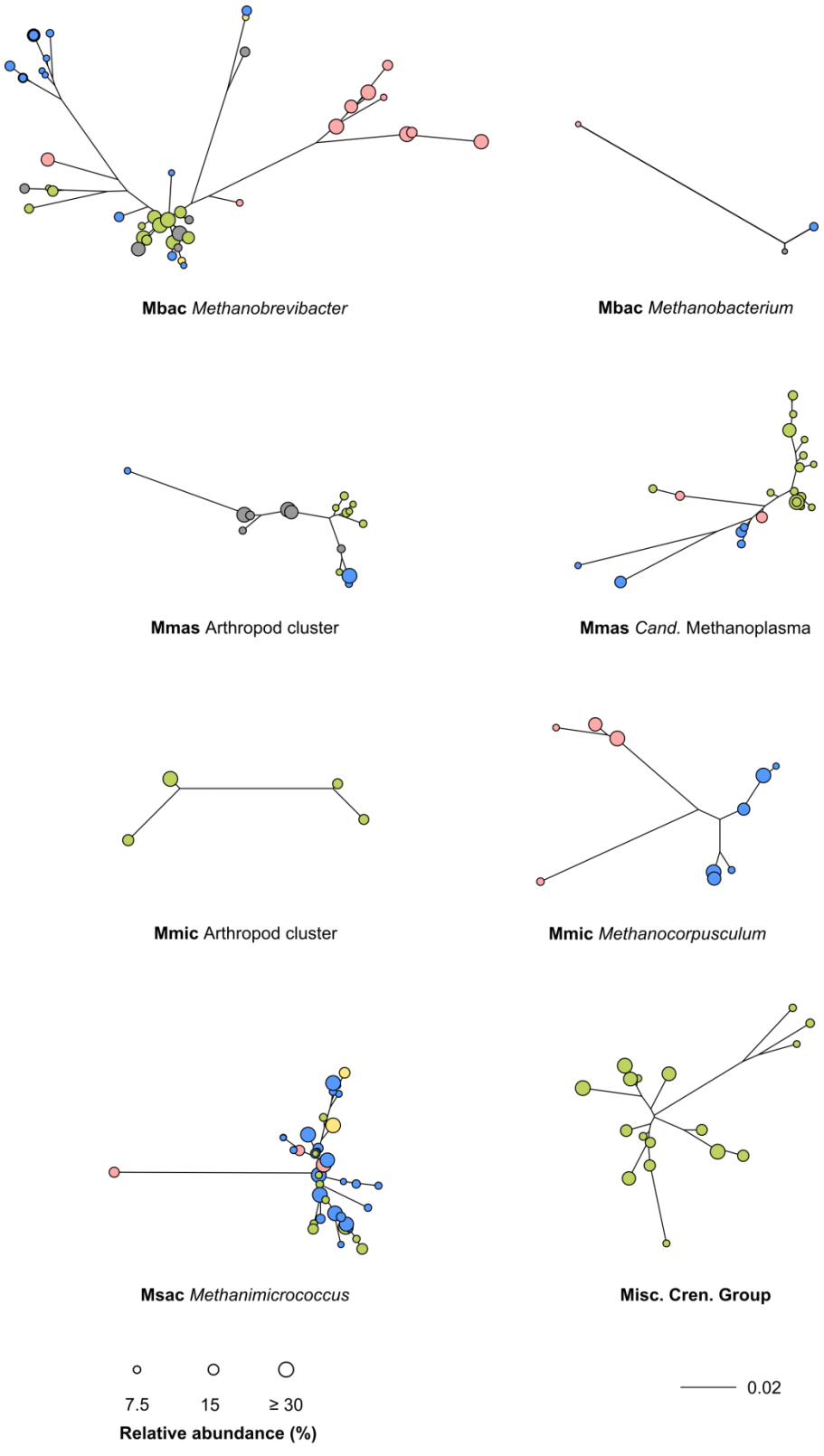
Mbac *Methanobrevibacter*
Mbac *Methanobacterium*
Mmas Arthropod cluster
Mmas *Cand.* Methanoplasma
Mmic Arthropod cluster
Mmic *Methanocorpusculum*
Msac *Methanimicrococcus*
Misc. Cren. Group
7.5
15
≥ 30
Relative abundance (%)
0.02

Figure 9. Phylogenetic host specificity of arthropod archaeal lineages. Shown are phylogenetic trees of the OTUs at the 1%-level for the most abundant archaeal genus-level groups in arthropod guts (Sequences that show a minimum relative abundance of 1% are taken). The area of the circles indicates the relative abundance of the OTU in the host sample. Color coding of the host-group membership is the same as in Fig. 1. Archaeal group name abbreviations: Mbac, *Methanobacteriales*; Mmas, *Methanomassilliicoccales*; Mmic, *Methanomicrobiales*; Msac, *Methanosarcinales*; Misc. Cren. Group., Miscellaneous Crenarcheotic Group. Note that the *Methanomicrobiales* arthropod cluster tree branch lengths were magnified by a factor of 10

The *Methanospirillaceae* arthropod cluster of the order *Methanomicrobiales* contained only sequences from higher termites; hence, the genus-level group itself seems to be a host-specific cluster (Fig. S2f). This is also supported by the clone library analysis in this study. The genus *Methanocorpusculum* contains a monophyletic group that only consists of cockroach gut derived OTUs. Sequences originating from *Hodotermopsis sjoestedi* (No. 26) showed that within the community of *Methanocopusculum,* more than one abundant OTU is present. Therefore, it is not surprising that these sequences cluster together.

The order *Methanosarcinales* is only represented by a single genus, *Methanomicrococcus*. No large cluster was detected that only contained sequences originating from guts of a particular host group (Fig. S2g). Surprisingly, OTU sequences from millipedes and cockroaches often represented a monophyletic group, which was reflected by the community structure analysis. In addition, OTU sequences from cockroaches and higher termites often formed a cluster, which documented also similarities between OTU sequences from these two host groups.

OTU representatives from the *Thaumarchaeota* group Miscellaneous Crenarchaeotic Group contained exclusively sequences from abundant OTUs from higher termites, with the exception of one cockroach-derived OTU sequence. Based on the distances in the tree, we conclude that sequences fall within one major genus-level group. This is supported by the sequences that showed a maximal dissimilarity of 6% from each other.

Possible interactions with bacteria. Another driver of the archaeal community structure could be the dependence on the availability of substrates. The most important

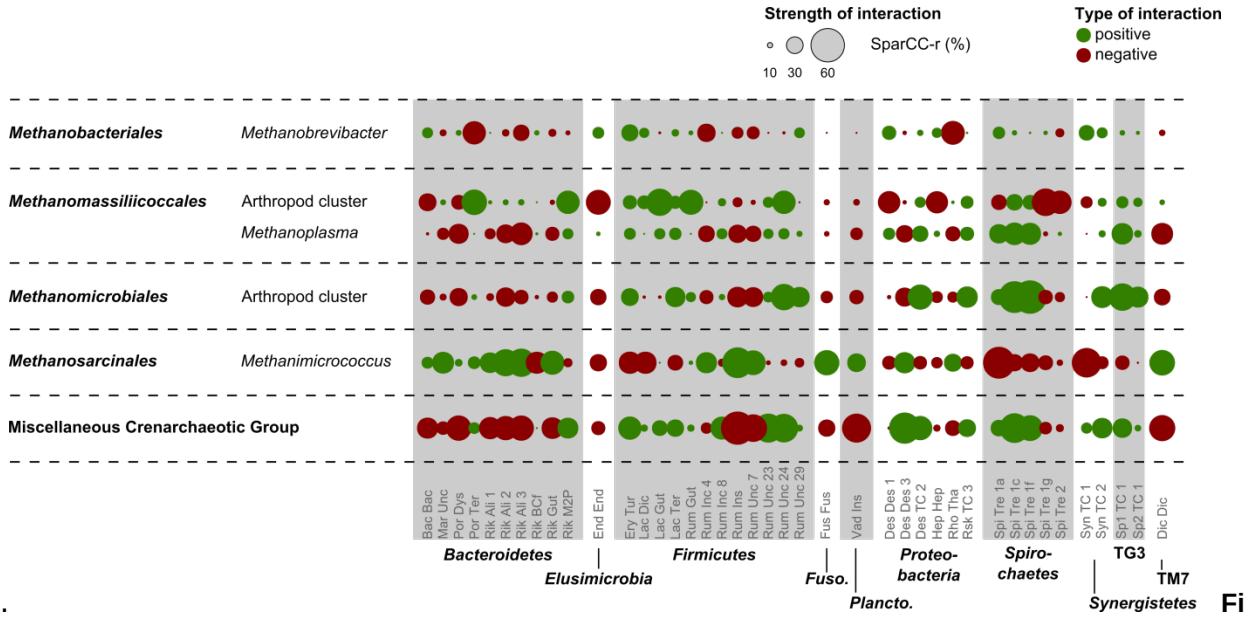

Figure 10. Potential dependencies between the archaeal and bacterial members of the gut microbiota of arthropods shown as a correlogram of the SparCC correlation results. Data were filtered by applying a minimum threshold of |r|= 0.4 for each archaeal and bacterial genus-level group. Colors of the points indicate directionality of the possible interaction: red, negative; green, positive. Point size indicates the strength of interaction as measured by SparCC-r. Bacterial genus-level groups are abbreviated according to the first three letters of the family and the genus-level group abbreviation (Table S1).

methanogenic substrates are molecular hydrogen, methanol (or methyl derivatives), and acetate. Although the acetate is found in high concentrations in arthropods (Egert *et al*., 2003; Pester and Brune, 2007; Köhler *et al*., 2012; Schauer *et al*., 2012), aceticlastic methanogenesis has never been detected. Our study confirmed that since no genus-level groups were detected potentially carry out this pathway. It is believed that aceticlastic methanogenesis might not occur in termites owing to the low growth rates of the responsible organisms, which would not allow the organisms to cope with the short retention time of the whole digestion process (Brune, 2010). However, methanogens depending on only hydrogen and/or hydrogen together with methanol as substrates have been reported in arthropods and were identified in all major host groups sampled in this study. These substrates are released during the serial breakdown of biomass, which is carried out almost exclusively by the bacterial microbiota. Therefore, we asked whether archaeal genus-level groups show a dependency on certain bacterial lineages.

For this purpose, we used previously published sequencing results (Dietrich *et al*., 2014) and correlated both fractions using the SparCC algorithm (Fig. 10). We found six archaeal genus-level groups with high correlations with bacterial microbiota. We identified 41 bacterial genus-level groups belonging to ten different phyla that showed a high correlation to at least one archaeal genus-level group. First, we carried out a classical correlation analysis using the Spearman correlation coefficient. This analysis was not successful which underlines that this analysis is not useful or valid for compositional such as relative abundances (Friedman and Alm, 2012). This underlines the need for the *SparCC*-algorithm-based correlation that can deal with compositional data.

Based on the *SparCC*-algorithm we found a high number of potential correlations between members of the bacterial and the archaeal microbiota. The genus *Methanobrevibacter* had fewer potential dependencies on bacterial groups than the other archaeal genus-level groups. The largest correlation was negative, which means that these bacterial genus-level groups might only occur when the relative abundance of *Methanobrevibacter* is low. The bacterial genus-level that are correlated with *Methanobrevibacter* originated from the phyla *Bacteroidetes*, *Firmicutes,* and *Proteobacteria*.

We found that the two genus-level groups of the methanogenic order *Methanomassiliicoccales*, Arthropod cluster and *Ca*. Methanoplasma, showed each ar highly abundant, but in different host groups. This difference between both genus-level groups also visible for the bacterial genus-level lineages, which showed a high correlation with those genus-level groups.

Whereas the arthropod cluster mostly correlated with genus-level groups of the phyla *Bacteroidetes* and *Firmicutes*, *Ca.* Methanoplasma had the strongest correlations with *Treponema* clusters 1a, 1c, and 1f and the termite cluster of subphylum 2 of the *Candidate Division* TG3. *Treponema* cluster 1a is the only *Treponema* cluster that contains isolated representatives: *Treponema primitia* and *Treponema azotonutricium* (Graber *et al*., 2004). Both isolates are reported to be homoacetogenic and to metabolize to a limited extent also oligosaccharides (Graber and Breznak, 2004).

However, as neither of these isolates is known to produce methanol or hydrogen, the causal links between these groups is unclear. *Ca.* Methanoplasma reduces methyl groups with external hydrogen, but it is not clear whether organisms of the arthropod cluster have this same metabolism.

In the methanogenic order *Methanomicrobiales,* only the *Methanospirillaceae* arthropod cluster showed noteworthy correlations with the bacterial microbiota. The *Methanospirillaceae* arthropod cluster strongly correlated with *Treponema* clusters 1a, 1c, and 1f and the *Ruminococcaceae* clusters uncultured 24 and 29. Little is known about the metabolic potential of the *Methanospirillaceae* arthropod cluster. The distantly related species *Methanospirillum hungatei* carries out methanogenesis from formate or hydrogen and carbon dioxide as substrates (Ferry, 1974). We wonder whether the *Methanospirillaceae* arthropod cluster might also be formicotrophic or hydrogenotrophic.

Interestingly, the genus-level group of *Methanomicrococcus* (*Methanosarcinales*) highly correlated with genus-level groups of the phyla *Bacteroidetes*, *Firmicutes,* and the *Candidate division* TM7. The *Bacteroidetes* genus-level groups *Alistipes* clusters 1, 2, and 3 and the *Candidate division* TM7 are able or have the potential to metabolize oligosaccharides (Mishra, 2012; Albertsen, 2013). However, strains of *Alistipes* are often associated with carnivores, and this implies also proteolytic abilities. In a small genome survey of the genus *Alistipes*, we found genes for pectin esterases in many genomes; therefore, these organisms might able to break down pectin and release methanol.

Members of the Miscellaneous Crenarchaeotic Group (MCG) showed the highest number of correlations, both negative and positive, within the data set. Positive correlations were found with the putative proteolytic and homoacetogenic genus-level groups from the phyla *Spirochaetes* and *Firmicutes*. Knowledge about the metabolism of this MCG is minimal. A recent metagenomic study implicated this group in the breakdown of aromatic compounds (Meng, 2014). If the members of the MCG are involved in the breakdown of aromatic compounds in higher termites, they would be placed at the top of the food chain in contrast to methanogens that usually occur at the end of the food chain.

5.5 Discussion

Although the abundance of the archaeal community in arthropods is usually below 3% of the total microbial community (Brauman *et al*., 2001), the functions carried out by this group are very unique. However, these functions do not seem to be essential for the arthropod host (Messer and Lee, 1989). Since the archaeal community does not seem to be essential, we concluded that the adaption of the archaeal community is not as strong as the adaption of the bacterial microbiota. In this study, we used a curated reference database to classify reads derived from high-throughput sequencing to profile the archaeal community of representatives of all known major methane-emitting terrestrial arthropod groups. The results indicated that the archaeal communities of these insects have a high specificity for their hosts through host group membership on all possible levels.

Host-specific and diet-specific community structure. The archaeal community structure followed to a large extent the major host groups, although that of millipedes was always similar to that of cockroaches, and those of lower termites had high similarities to those of other groups. The similarity of the archaeal community structure of millipedes to that of cockroaches (Fig. 7 and Fig. S1) might be explained by the similar, litter-feeding diet of the two groups. Interestingly, when low-abundant genus-level groups were emphasized in the analysis (Fig. S1c–d), the archaeal community structure of the humus-feeding scarab beetle larvae was more similar to that of higher termites, which also consisted of many soil-feeding taxa. We concluded that community structure is shaped by host-group membership and to a lesser extent by diet. This also becomes apparent when the two wood-feeding cockroaches were studied. Both wood feeders clustered with other cockroaches but were always (with and without emphasis on rare taxa) next to each other, indicating their similarity.

Lower termites in general did not show a consistent archaeal community structure. This clearly contradicts earlier studies that almost exclusively found only *Methanobrevibacter* sp. (Ohkuma and Kudo, 1998; Shinzato *et al*., 1999; Tokura *et al*., 2000; Shinzato *et al*., 2001). However, several of these studies consist only of a few clone libraries, and the utilized primer sets employed in some of these studies have a mismatch towards at

least one archaeal group (Paul *et al*., 2012). Our results suggest that the lower termite archaeal communities are in general not very similar and consistent. We believe that the main reason for the large heterogeneity of community structure in lower termites is the presence of flagellates. These symbiotic protists can be an important microhabitat for methanogens. However, the number of flagellate cells harboring methanogenic endosymbionts varies substantially. In *Reticulitermes speratus,* only 4% of the flagellate cells contain methanogens, whereas in *Hodotermopsis sjoestedti,* this association is found in 42% of the flagellate cells (Tokura *et al*., 2000). Furthermore, we showed that the lower termite *Reticulitermes santonensis* has a high abundance of strains belonging to the genus *Ca.* Methanoplasma (*Methanomassiliicoccales*). Interestingly, Shinzato *et al*. (1999) also found this group to be at least a variable part of the archaeal community in *R. speratus*. It has been already shown that *R. santonensis* has a different archaeal community than *R. speratus*. In *R. speratus,* a small number of flagellates harbor methanogens (Tokura *et al*., 2000), whereas in *R. santonensis,* no archaeal lineages associated with flagellates have been identified (Leadbetter and Breznak, 1996; Pester and Brune, 2006). Furthermore, our analysis revealed other genera present in high abundance in the lower termites sampled, such as *Methanocorpusculum* and *Methanomicrococcus*. These results challenge the concept of a purely hydrogenotrophic methanogenesis in lower termites, and this finding should be studied at the metabolic level in future studies.

Another characteristic of the archaeal communities in arthropod guts is the presence of at least two genus-level groups that carry out hydrogenotrophic and methyl-reducing methanogenesis. Surprisingly, the latter seems to be carried out by different genus-level groups that differ depending on the membership of the host to a particular major host group. In scarab beetle larvae, the *Methanoplasmataceae* arthropod cluster seems to carry out this pathway, whereas in millipedes and cockroaches, *Methanomicrococcus* was highly abundant. In higher termites, in contrast, the methyl reduction is most likely performed by the members of the genus-level group *Ca.* Methanoplasma. Hydrogenotrophic methanogenesis seem to be almost exclusively linked to *Methanobrevibacter,* which occurred in all arthropod groups.

Lineage specificity to major arthropod host groups. The most predominant archaeal groups were in high abundance only in certain host groups, which indicated a clear specificity of these groups to specific hosts. An exception was *Methanobrevibacter*. Although this genus was very abundant in the total data set, it was present in all major arthropod groups in high abundance. In contrast, the genus *Methanomicrococcus* clearly showed a preference for millipedes and cockroaches, which is also one of the reasons why millipedes and cockroaches formed a cluster in the cluster analysis (Fig. 7). Members of this genus utilize both methanol and hydrogen for methanogenesis (Sprenger *et al*., 2000). Since these host groups mainly feed on litter that might contain sources of pectin (e.g., leaves), the required methanol most likely stems from pectin esterase activities in both host groups. Interestingly *Methanomicrococcus* also had a high correlation with members of the bacterial genus-level groups *Alistipes* clusters 1, 2, and 3. In public genome databases, genes for pectin methyl esterases are commonly found in these organisms. Both *Alistipes* and *Methanomicrococcus*, might therefore be linked via methanol.

Ca. Methanoplasma and the *Methanoplasmataceae* arthropod cluster require the same substrate combination as *Methanomicrococcus*, hydrogen and methanol, which seems to be a typical feature of the whole order (Dridi *et al*., 2012; Paul *et al*., 2012; Iino *et al*., 2013). Interestingly, the arthropod cluster was mainly specific to scarab beetles, whereas *Candidatus* Methanoplasma was more specific to higher termites. Both groups also differed in their correlation pattern, which indicated that these two lineages might have different niches or even different metabolic requirements. *Ca.* Methanoplasma is mostly correlated with different genus-level groups of *Treponema,* including *Treponema* cluster 1a, which contains isolates that are hydrogenotrophic acetogens (Leadbetter *et al*., 1999). This is interesting since both groups use the same substrate, hydrogen. Notably, *Ca.* Methanoplasma correlates with *Treponema* cluster Ic, which is part of the fiber fraction in *Nasutitermes corniger* (Mikaelyan *et al*., 2014). However, it has not been shown whether *Treponema* cluster Ic is be able to produce hydrogen.

Compared to genus-level groups of methanogenic orders, virtually nothing is known about the Miscellaneous Crenarchaeotic Group. A recent publication revealed that this

group is involved in the degradation of aromatic compounds (Meng, 2014). In the hindguts of arthropods, this group only occurs in high abundance in the non-fungus-cultivating higher termites, which indicated a high specificity of this class/genus-level group for higher termites. This group showed the most and strongest correlations with bacterial genus-level groups. A positive correlation was found for some of the *Ruminococcaceae* genus-level groups, which are known for their proteolytic abilities. Soil-feeding termites and their microbiota are reported to most likely feed on polypeptide residues conserved in humus and their subsequent intermediates (Ji *et al*., 2000). How these energy-rich nitrogenous compounds are broken down is still unknown and would require also the polyphenol lattice of soil organic matter to be degraded. Members of the MCG might act in concert with main organisms or use the released phenol monomers that might be highly solubilized in the alkaline gut compartments of higher termites. However, since the MCG is a very diverse group, it is also questionable whether this group generally shares a common metabolism.

Host-specific phylogenetic clusters of major archaeal lineages. Our results showed that the archaeal community structure mostly mirrors major host groups and is dependent to a lesser extent on diet. This is apparent in the strong similarity of the archaeal community of millipedes and cockroaches, and indicated by an emphasis on lower abundant genus-level groups leads to a higher similarity between humus-feeding scarab beetle larvae and humus-feeding higher termites (Fig. S1). These findings lead us to question whether coevolution between archaeal lineages and their hosts could be a feasible scenario, as it has been proposed earlier for bacterial termite lineages (Hongoh *et al*., 2005). When we constructed phylogenetic trees of the genus-level groups with representative OTUs from the high-throughput sequencing reads, we recognized that many trees clearly contained clusters of OTUs from the same host group. This indicated that archaeal lineages found in arthropod hosts form unique host-specific clusters. A prominent example was the lower termite cluster of *Methanobrevibacter* (Fig. 9 and Fig S2b). With a closer look, no cocladogenesis with the respective hosts was visible, which indicated that coevolution does not seem to be a driving force, at least for *Methanobrevibacter.* However, members of *Methanobrevibacter* have been reported to be endosymbionts of termite gut flagellates

(Tokura *et al*., 2000), which might cause a coevolution between *Methanobrevibacter* and the flagellate host. Therefore, such a tripartite symbiosis could mask a coevolution signal between the *Methanobrevibacter* lineages and the arthropod host. Yet, also the other lineages do not show cocladogenesis, and hence coevolution between the archaeal lineages and their hosts can generally be excluded as a driving force of the phylogeny of arthropod archaeal lineages, or, alternatively, these associations have not yet had enough time to show a coevolution signal.

Determinants of arthropod gut archaeal communities. The peculiarity of host-specific clusters of archaeal lineages without obvious coevolution is puzzling. On one hand, there are large host-specific clusters in the phylogenetic trees, but on the other hand, cocladogenesis is missing. This raises the question which mechanisms shape the arthropod archaeal communities. Also selection by host habitat has been previously considered to be a determinant of the community structure (Hongoh *et al*., 2005). Although Dietrich *et al*. (2014) showed that major evolutionary events are mirrored by the bacterial community structure of cockroaches and termites, the causes of these patterns remain unknown. We found a large number of correlations between archaeal and bacterial genus-level groups. Some of these correlations pointed out possible metabolic links, such as the correlation between *Methanomicrococcus* and *Alistipes* clusters 1, 2, and 3. Close relatives of this bacterial genus carry genes for pectin methyl esterases in their genome and produce methanol. This indicates that the availability of certain substrates plays a large role. At least for higher termites, it has been shown that the addition of different substrates alters the methane emission rate of gut compartments and whole guts (Schmitt-Wagner and Brune, 1999). This indicates the presence of a strong dependency of the methanogenic community on substrates and shows that these substrates are even limited for methanogens. Furthermore, the different microenvironmental conditions found in the compartments of higher termites (Brune, 2014 and references therein) might select strains that are adopted to and specialized on these conditions. A very important condition would definitely be the alkalinity found in the P1 compartment of higher termites, especially in the soil-feeding *Cubitermes* species (Brune and Kühl, 1996).

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5.8 Supplementary Information

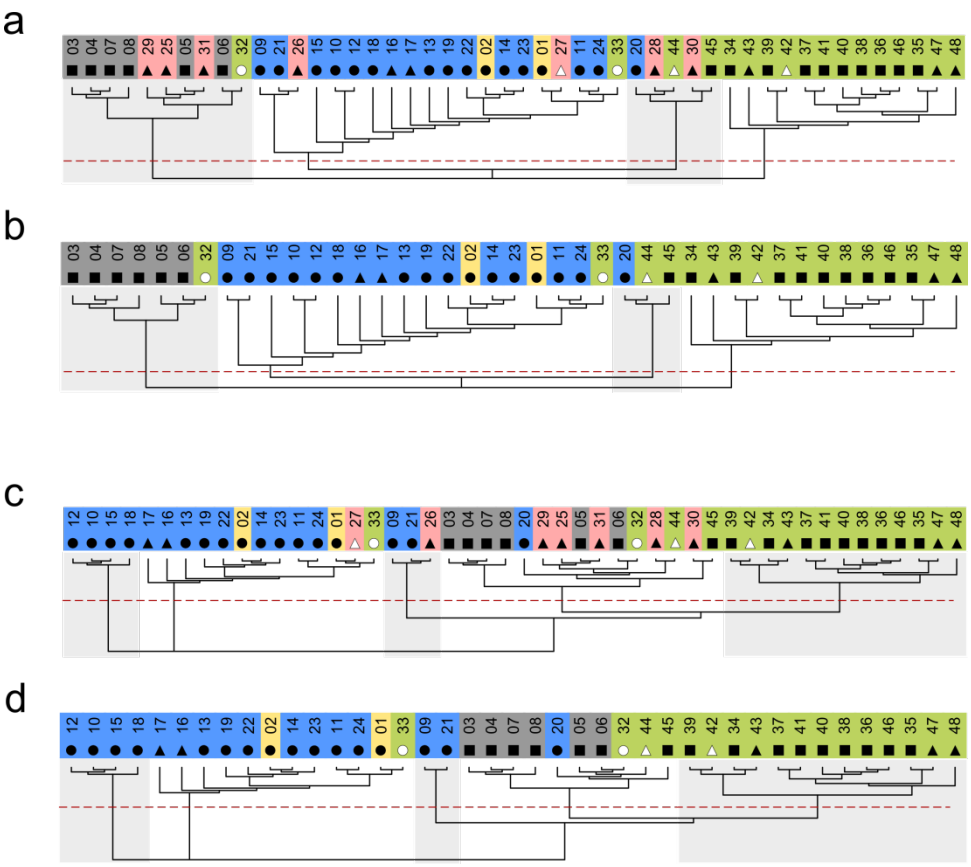


Figure. S1. Host- and diet-specific clustering of the archaeal community of arthropods. Unrooted BioNJ trees of the community structure displayed as cladograms based on the pairwise Soergel distances of (a) non-transformed data, (b) non-transformed dataset without lower termite samples, (c) logarithmized data set to emphasize lower abundant taxa and (d) logarithmized data set and lower termites removed. The dashed line aids in distinguishing the clusters in the data set. Color coding is the same as in Fig. 1. Numbering is the same as in Table 2. The symbols below the numbering indicate dietary specialization of most of the arthropod hosts and are the same as in Fig. 6.

Figure S2. Phylogenetic trees of 1%-level OTUs from high-throughput sequencing of arthropod archaeal lineages. Use the pdf bookmarks to navigate. A description of the archaeal genus-level groups is found also in the bookmarks. The numerical code of the different samples is as in Table 2. Please download Figure S2 from http://www.termites.de/brune/publ/suppl/Lang_Diss_Chap5_Fig_S2.html.

Table S1 Bacterial genus-level groups showing the highest correlation with the archaeal arthropod gut genus-level groups.

Abbreviation	Family	Genus
Bacteroidetes		
Bac Bac	*Bacteroidaceae*	*Bacteroides*
Mar Unc	*Marinilabiaceae*	Uncultured 1
Por Dys	*Porphyromonadaceae*	*Dysgonomonas*
Por Ter	*Porphyromonadaceae*	Termite cockroach cluster 3
Rik Ali 1	*Rikenellaceae*	*Alistipes* 1
Rik Ali 2	*Rikenellaceae*	*Alistipes* 2
Rik Ali 3	*Rikenellaceae*	*Alistipes* 3
Rik BCf	*Rikenellaceae*	BCf9-17 termite group
Rik Gut	*Rikenellaceae*	Gut Cluster C
Rik M2P	*Rikenellaceae*	M2PB4-61 termite group
Elusimicrobia		
End End	*Endomicrobiaceae*	*Endomicrobium*
Firmicutes		
Ery Tur	*Erysipelotrichaceae*	*Turicibacter*
Lac Dic	*Lachnospiraceae*	Dictyoptera cluster
Lac Gut	*Lachnospiraceae*	Gut cluster 1
Lac Ter	*Lachnospiraceae*	Termite cluster
Rum Gut	*Ruminococcaceae*	Gut cluster 2
Rum Inc 4	*Ruminococcaceae*	Incertae Sedis 4
Rum Inc 8	*Ruminococcaceae*	Incertae Sedis 8
Rum Ins	*Ruminococcaceae*	Insect guts cluster
Rum Unc 7	*Ruminococcaceae*	Uncultured 7
Rum Unc 23	*Ruminococcaceae*	Uncultured 23
Rum Unc 24	*Ruminococcaceae*	Uncultured 24
Rum Unc 29	*Ruminococcaceae*	Uncultured 29
Fusobacteria		
Fus Fus	*Fusobacteriaceae*	*Fusobacterium* 1
Planctomycetes		
Vad Ins	vadinHA49	Insect gut cluster
Proteobacteria		
Des Des 1	*Desulfovibrionaceae*	*Desulfovibrio 1*
Des Des 3	*Desulfovibrionaceae*	*Desulfovibrio 3*
Des TC 2	*Desulfovibrionaceae*	*Termite cluster 2*
Hep Hep	*Candidatus Hepatincola*	*Candidatus Hepatincola*
Rho Tha	*Rhodospirillales*	*Thalassospira*
Rsk TC 3	*Rs-K70 termite group*	*Termite cluster 3*
Spirochaetes		
Spi Tre 1a	*Treponema* 1	*Treponema* 1a
Spi Tre 1c	*Treponema* 1	*Treponema* 1c
Spi Tre 1f	*Treponema* 1	*Treponema* 1f
Spi Tre 1g	*Treponema* 1	*Treponema* 1g
Spi Tre 2	*Treponema* 2	*Treponema* 2
Synergistetes		
Syn TC1	*Synergistaceae*	Termite cluster 1
Syn TC2	*Synergistaceae*	Termite cluster 3

TM7		
Dic Dic	Dictyoptera cluster	Dictyoptera cluster
TG3		
Sp1 TC 1	SP1 – Termite cockroach cluster	Termite Cluster 1
Sp2 TC 1	SP2 – Termite cockroach cluster	Termite Cluster 1

Chapter 6

General Discussion

Kristina Lang

The results of this work finally prove the hypothesis that the deep-branching lineage affiliated with *Thermoplasmatales* represents the seventh order of methanogens. The methanogenic nature of this lineage was confirmed by a comprehensive phylogenetic analysis and the enrichment of two methanogenic strains from the gut of the higher termite *Cubitermes ugandensis* (*Candidatus* Methanoplasma termitum strain MpT1) and the millipede *Anadenobolus monilicornis* (Strain MpM2).

The analysis of the genome of *Candidatus* Methanoplasma termitum strain MpT1, a member of this novel order, from the gut of *C. ugandensis*, and the comparison of its genome to three recently amplified genomes from the human gut (Borrel *et al*., 2012; Gorlas *et al*., 2012; Borrel *et al*., 2013a) revealed a novel mode of energy conservation in methyl reduction methanogens that lack cytochromes. Furthermore, an ultrastructure analysis of *Candidatus* Methanoplasma termitum and strain MpM2 revealed that both strains possess an unusual two membrane system and seem to lack a cell wall.

Methanogenic archaea are widely distributed in CH_4-emitting arthropods and are well characterized in termites, cockroaches and scarab beetle larvae (Shinzato *et al*., 1999; Friedrich *et al*. 2001; Hara *et al*., 2002; Egert *et al*., 2003; Miyata *et al*., 2007). However, not much is known about the methanogenic community in millipedes. This work documents a diverse methanogenic community in millipedes and shows its similarity to termites, cockroaches and scarab beetle larvae.

In cooperation with Carsten Dietrich and James Nonoh, mechanisms that select archaeal lineages and shape the community structure in CH_4-emitting arthropods were investigated, indicating that the archaeal community structure and phylogeny is shaped more by the major host groups than by coevolution and diet.

Individual results documented in this work have been discussed in detail in the respective chapters. The following discussion provides a general overview about the distribution of obligately hydrogen-dependent methylotrophs, such as *Methanomassiliicoccales*, in different environments, the evolution of this pathway within the methanogens, and why archaea without cell walls might be more common than originally thought.

6.1 The methyl-reducing pathway

As described in detail in Chapter 2, the *Methanomassiliicoccales* represent the seventh order of methanogens. The novel order was provisionally named as "*Methanoplasmatales*" (Chapter 2). However, the bacteriological code (Lapage *et al*., 1992) dictates that the taxonomic name of an order, no matter how unwieldy, must be derived from the genus name of the first isolate, *Methanomassiliicoccus luminyensis* (Dridi *et al*., 2012). Therefore, we adopted the name *Methanomassiliicoccales* for the seventh order of methanogens. Different culture-based studies of *Methano-massiliicoccales* have documented their strict dependence on methanol or other methylamines with H_2 as external electron donor (Chapter 1; Borrel *et al*., 2012; Gorlas *et al*., 2012; Borrel *et al*., 2013; Iino *et al*., 2013).

Obligately hydrogen-dependent methylotrophs have so far been exclusively isolated or enriched from intestinal tracts, like *Methanosphaera stadtmanae* (Miller and Meyer, 1985) from the human gut, *Methanomicrococcus blatticola* from the cockroach gut (Sprenger *et al*., 2000) and *Methanomassiliicoccales*. These include *M. luminyensis* (Dridi *et al*., 2012), *Ca.* Methanomassiliicoccus intestinalis (Borrel *et al*., 2013a), *Ca.* Methanomethylophilus alvus (Borrel *et al*., 2012) all enriched from human feces, *Ca.* Mp. termitum (Chapter 2) from the gut of a higher termite, and MpM2 from the millipede gut (Chapter 2). Furthermore, sequences from the *Methanomassiliicoccales* are also present in the intestinal tracts of other mammals and arthropods (for more details see Chapter 2, 4 and 5). *Methanosarcina barkeri*, which was isolated from freshwater, marine mud, lagoons but also from sewage digesters and rumen (Keltjens and Vogels, 1993), is able to grow on H_2 and CO_2, acetate, and methanol (Balch *et al*., 1979), but can also use methanol + H_2 (Müller *et al*., 1986). The isolation of this organism from sewage digesters and the intestinal tract from cattle reveals also a correlation between methyl-reducing methanogenesis and intestinal tracts.

The colonization of methanogens in the intestinal tracts seems to be favored by a fiber rich diet, which is in accordance with the apparent absence of methanogenic archaea from carnivorous animals (Hackstein and van Aalen, 1996). Pectin and xylan are major components of this fiber rich diet; pectin is present in many fruits, fruit juice, vegetables

and it is part of the plant cell wall, andxylan is a hemicellulose and present in all rooted plants. The chemical structure of pectin reveals that its carboxyl groups of the polygalacturonic acid chains are partly or fully esterified with methanol (Deuel and Stutz, 1958). Xylan also contains substantial amounts of O-methylated glucuronic acid residues (Rosell, 1974). Through the fermentative breakdown of pectin and xylan in the intestinal tracts substantial amounts methanol could be released. This supported by the fact that the concentration of methanol in the human gut increased by one order of magnitude after the consumption of fruit (Lindinger *et al*., 1997). The release of methanol also favors the colonization of methyl-reducing methanogens in the termite gut, as shown for the higher termite *Nasutitermes takasagoensis*, where the feeding on xylan increased the relative abundance of the *Methanomassiliicoccales* (Miyata *et al*., 2007).

Our comprehensive analysis of the archaeal community structure in arthropods guts (Chapter 5) revealed that obligately hydrogen-dependent methylotrophs, like *Methanomassiliicoccales* or *Methanomicrococcus*, are highly abundant in these environments. The complete absence of *M. stadtmanae* and *Ms. barkeri* from arthropod guts and the fact that only one group of methyl-reducing methanogens is present in these guts, either *Methanomicrococcus* or *Methanomassiliicoccales*, suggests that there is a high competition for this niche. As outlined in Chapter 5, the presence of different methanogenic groups is more dependent on the host than on diet. This is in agreement with presence of the same methanogenic substrates, like methanol and H_2, in all intestines and does not explain why for example one group of obligately hydrogen-dependent methylotrophs is preferred instead of another. While the concentrations of these substrates contribute to the shape of community structure, they cannot be the sole driving factors. Therefore it can be speculated that conditions within the guts shape the community structure.

Oxygen could be one possible driver that can influence the community structure within the guts. Interestingly, *Ms. barkeri* and *M. stadtmanae* are only present in the gut of mammals, which possess a larger intestinal tract than arthropods. So there is a larger anoxic interior than in the arthropod gut, suggesting that *Ms. barkeri* and *M. stadtmanae* could be less adapted to oxygen than *Methanomassiliicoccales* and *M. blatticola*, which

are present in smaller arthropod gut. However, methanogens can possess different enzymes involved in oxygen detoxification (Brioukhanov *et al*., 2000; Seedorf *et al*., 2004). At least one of these enzymes, involved in this process, is present in *M. stadtmanae* (Fricke *et al*., 2006), in *M. barkeri* (Brioukhanov *et al*., 2006) and also in the *Methanomassiliicoccales* except for Ca. Mp. termitum (source: database of the Integrated Microbial Genome webpage; http://img.jgi.doe.gov). This indicates that both, *M. stadtmanae* and *Ms. barkeri* are able to deal with oxygen. The complete absence of these genes from *Ca.* Mp. termitum underlines that oxygen does not have a main influence on the community structure, suggesting that other environmental drivers within the gut favor the colonization of different methyl-reducing methanogens. Potential factors that may shape the community include redox potential and pH. In soil-feeding higher termites, for example, methanogens have to deal with highly alkaline conditions in parts of the hindgut (Brune and Kühl, 1996). Methanogens are mainly located in P3 and P4 gut sections (unpublished data of quantitative PCR analyses) of these termites, which have a pH of 6–9. However, to reach these sections, they first have to pass the highly alkaline P1 segment (pH >12) (Brune and Kühl, 1996). In contrast to this, in the human intestine, methanogens have to deal with the acidic conditions in the stomach before they attain the gut. Another important factor can be the influence of the H_2 partial pressure as discussed in detail in Chapter 3. Nevertheless, the *Methanomassiliicoccales* are the only group of obligately hydrogen-dependent methylotrophs present in diverse intestinal tracts, indicating that these organisms can better deal with different environmental conditions. This may explains why they have also been amplified from diverse other environments, like landfill leachate (Luton *et al*., 2002), sediments (Castro *et al*., 2004), rice paddy soil (Grosskopf *et al*., 1998) and eutrophic lakes (Earl *et al*., 2003) (for more details see Chapter 2). The recent enrichment of *Candidatus* Methanogranum caenicola from anaerobic sludge (Iino *et al*., 2013) indicates that this type of methanogenesis is not restricted to organisms from intestinal tracts, and is instead common to the complete order. The occurrence of the methyl-reducing pathway in environments other than the intestine is not surprising because the hydrogen-dependent reduction of methanol to CH_4 is more favorable than the disproportionation of methanol to CH_4 under standard conditions (Thauer *et al*., 1977). So far little is known about the environmental conditions, like H_2 partial pressure, in these environments. To

understand the environmental drivers that favor the colonization of a specific group of obligately hydrogen-dependent methylotrophs, these environments should be better investigated. Although the *Methanomassiliicoccales* are present in diverse environments, they seem to be completely absent from marine environment (Lloyd *et al*., 2013; Ragon *et al*., 2013), suggesting that they maybe cannot deal with high salt concentrations.

Further strains of *Methanosarcina,* as well as strains of *Methanolobus*, *Methanococcoides*, *Methanohalophilus* and *Methanosalsum* are all known to disproportionate methanol (Kendall and Boone, 2006), but it has not yet been tested whether these groups are also able to form CH_4 from methanol with external H_2. To fully understand the appearance of the obligately hydrogen-dependent methylotrophs in the environment and to identify the environmental drivers favoring the colonization of specific methyl-reducing methanogens, it would be interesting to know whether these methanogens are also able to perform the methyl-reducing pathway. Interestingly, these strains have been enriched from diverse environments (Kendall and Boone, 2006). If they are able to use the methyl-reducing pathway, this would support the statement that organisms, reducing methanol with external H_2, are not restricted to intestinal tracts.

Except of the *Methanomassiliicoccales*, all other obligately hydrogen-dependent methylotrophs, like *M. stadtmanae* and *M. blatticola,* seem to be restricted to intestinal tracts. The recent identification of this group and their energy metabolism explains now, why obligately hydrogen-dependent methylotrophs have been so far exclusively isolated from this environment. Before the identification of *Methanomassiliicoccales*, it was not possible to assign their 16S rRNA genes to their metabolic function. Therefore, methyl-reducing methanogens have escaped attention in other environments. In addition, there are no studies that tried to use H_2 + methanol as a substrate combination to isolate methanogenic archaea from environments other than intestinal tracts.

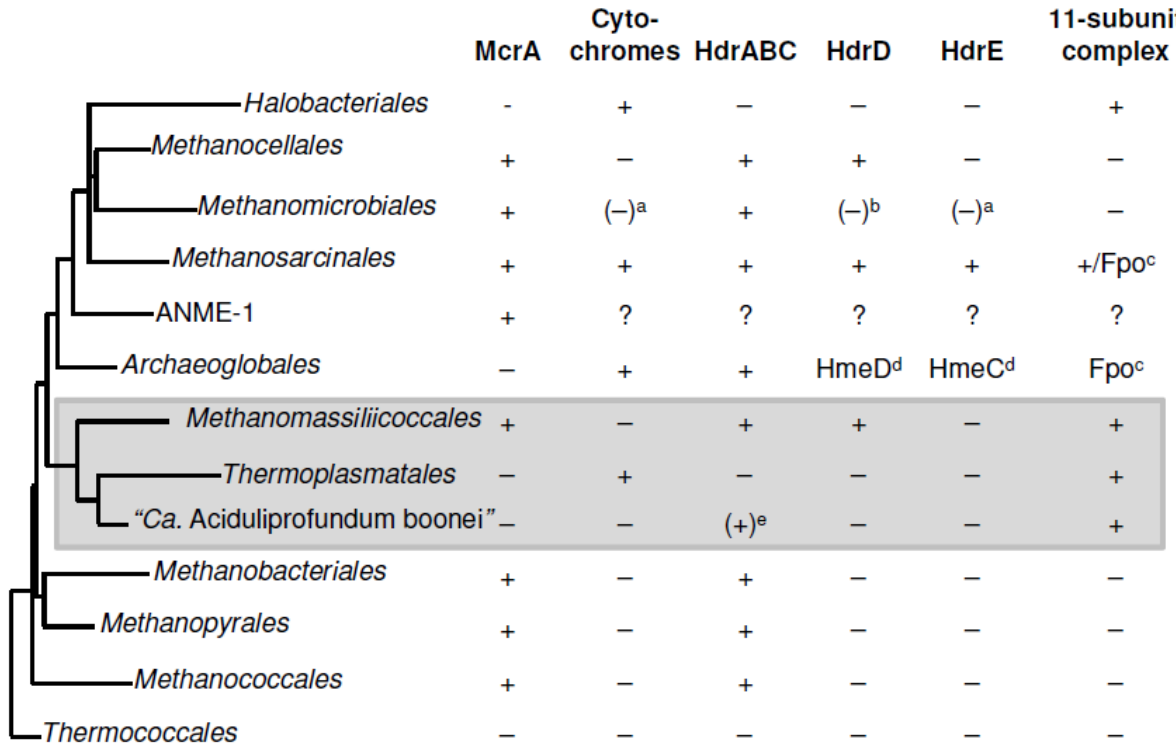
6.2 Evolution of genes involved in methanogenesis

As shown in Chapter 3, the *Methanomassiliicoccales* posses a novel mode of energy conservation different from those of other obligately hydrogen-dependent methylotrophs

(Thauer *et al*., 2008). This raises the question why there so many modes of energy conservation in methyl-reducing methanogens and how did they evolve.

A phylogenetic analysis of the evolution of methanogenesis and methanogens by Bapteste *et al*. (2005) suggests that the last common ancestor of the *Euryarchaeota* was methanogenic and that genes involved methanogenesis have subsequently been lost in the non-methanogenic lineages. Furthermore, they proposed that hydrogenotrophic methanogenesis evolved first and that other modes of energy conservation subsequently evolved from this pathway. This hypothesis is consistent with the finding that the *Methanomassiliicoccales,* like all other methanogens, possess the subunits A, B and C of the heterodisulfide reductase (Hdr) (Fig.1), an important enzyme in the energy metabolism of methanogens (Hedderich *et al*., 2005). These genes are also present in the genomes of *Candidatus* Aciduliprofundum boonei and in the genomes of *Archaeoglobales* (Fig. 1). However, the genes for *hdrA* and *hdrC* of Ca. A. boonei are unlikely to be function because of sequence modifications (Blastp analysis). These results support the theory that the genes were secondarily lost in non-methanogenic archaea. Although it is known that the presences of the cytochrome *b* dependent subunit HdrE in *Methanosarcinales* enabled these organisms to use substrates other than H_2 + CO_2 for growth (for more details see Thauer *et al*., 2008), this subunit is absent from the *Methanomassiliicoccales* (Chapter 3). However, HdrD, the soluble component of the heterodisulfide reductase in methanogens with cytochromes and catalyzes the reversible reduction of the heterodisulfide (Künkel *et al*., 1997), is present in all four genomes of the *Methanomassiliicoccales* (Chapter 3). Most of the apical *Euryarchaeota* (above the *Methanomassiliicoccales* in Fig. 1) also possess the D subunit or a homologous protein (*Archaeoglobales,* HmeD; Mander *et al*., 2002) except *Halobacteriales* and most of the *Methanomicrobiales*. However, in the genome of *Methanosphaerula palustris*, a member of *Methanomicrobiales*, the gene encoding for this subunit is also present (source: database of the Integrated Microbial Genome webpage; http://img.jgi.doe.gov/), indicating that the D subunit was first gained by the *Methanomassiliicoccales* and than acquired by the apical *Euryarchaeota*. Because the cytochrome dependent E subunit of the heterodisulfide reductase is missing in the *Methanomassiliicoccales*, it is likely that this gene was required later in evolution first by

the *Archaeoglobales* (Fig. 1), which possess an hdrE homologue (HmeC; Mander *et al*., 2004). Interestingly, the HmeCD complex of the *Archaeoglobales* works in a similar way to the HdrDE complex of *Methanosarcinales* (Mander *et al*., 2004). So far, the physiological electron acceptor has not been identified, but a disulfide is proposed (Mander *et al*., 2004), suggesting the same evolutionary origin in both organisms.



[a] *Methanocorpusculum parvum* possesses cyctochromes, but is missing the cytochrome-dependent subunit of the heterodisulfide reductase (HdrE) (Zellner *et al*., 1987). This subunit (HdrE) is present in the genome of *Methanosphaerula palustris* .

[b] The genome of *Methanosphaerula palustris* possesses the gene for *hdrD.*

[c] The 11-subunit complex is the ancestral form of F_{420} dehydrogenase (Fpo).

[d] The Hdr-like menaquinol-oxidizing enzyme (Hme) was identified in *Archaeglobus profundus* (Mander *et al.* 2002).

[e] *Ca.* A. boonei possesses the genes for subunits ABC of the heterodisulfide reductase, but the subunits AC seem not to be functional anymore

Figure 1. Evolutionary distribution of the alpha subunit of the methyl coenzyme M reductase, the rRNA operon, cytochromes, the subunits A,B,C,D and E of the heterodisulfide reductase and the 11-subunit complex in different genomes of the phylum *Euryarchaeota.* The grey box outlines the class of the *Thermoplasmata*. The phylogenetic tree is based on the phylogenomic analysis of Borrel *et al*., 2013b. The results were achieved through comprehensive literature search and through the genome annotation platform of the Joint Genome Institute.

As we proposed in Chapter 3, HdrD seems play an important role in the energy conservation of the *Methanomassiliicoccales* (Fig. 2) but with an interaction partner other than HdrE, because this gene was most likely required later in evolution. This interaction partner could be the Fpo-like complex or the 11-subunit complex (Moparthi and Hägerhäll, 2011, Chapter 3), which is also present in *Ca.* A. boonei, *Thermoplasmatales*, *Methanosaeta*, and the *Halobacteriales*, but is absent from all basal *Euryarchaeota (Methanbacteriales, Methanopyrales, Methanoccoccales* and *Thermococcales;* Fig. 1). It has been proposed that 11-subunit complexes are derived from [NiFe] hydrogenases that lost their [NiFe] cluster and gained new functions by the association with additional electron-transferring subunits, such as NuoEFG or FpoFO (Moparthi and Hägerhäll, 2011). One of these advancements is the $F_{420}H_2$ dehydrogenase (Fpo), which is present in *Methanosarcinales* and the *Archaeoglobales* (Fig. 1). The presence of the 11-subunit complex only in the apical *Euryarchaeota* suggests that this complex was gained later in evolution in addition to genes of the hydrogenotrophic pathway, possibly by horizontal gene transfer. The 11-subunit complex as well as preservation cytochromes could facilitate the development of different energy-conserving pathways in methanogens, the usage of new substrates, and consequently the exploitation of new environments.

Energy conservation does not only differ between methanogens without cytochromes, but also in the variety enzymes, which are involved in this process in the *Methanosarcinales* (for more details see Welte and Deppenmeier, 2014; Fig. 2). Recently, it has been proposed that *Methanosaeta* (genus of *Methanosarcinales)* uses also an Fpo-like complex, which gains its electrons from ferredoxin (Welte and Deppenmeier, 2011, Fig. 2). Although the subunit for $F_{420}H_2$ oxidation (FpoF) is still present in the genome, it does not interact with the Fpo-like complex (Welte and Deppenmeier, 2011). Because the large subunit of this complex is closely related to the large subunit of the Fpo-like complex of *Methanomassiliicoccus* (Chapter 3), this supports our theory that in the *Methanomassiliicoccales* this complex interacts with ferredoxin (Fig. 2). Although the *Thermoplasmatales* have been isolated a long time ago (Segerer *et al*., 1988), the problem of energy conservation, like in *Methanomassiliicoccales*, has not been solved yet. This suggests that the 11-subunit

complex could also play an important role in the *Thermoplasmatales*. Here, the complex most likely interacts with another electron acceptor, because HdrD or homologues gene is absent from all genomes of the *Thermoplasmatales* (Fig. 1).

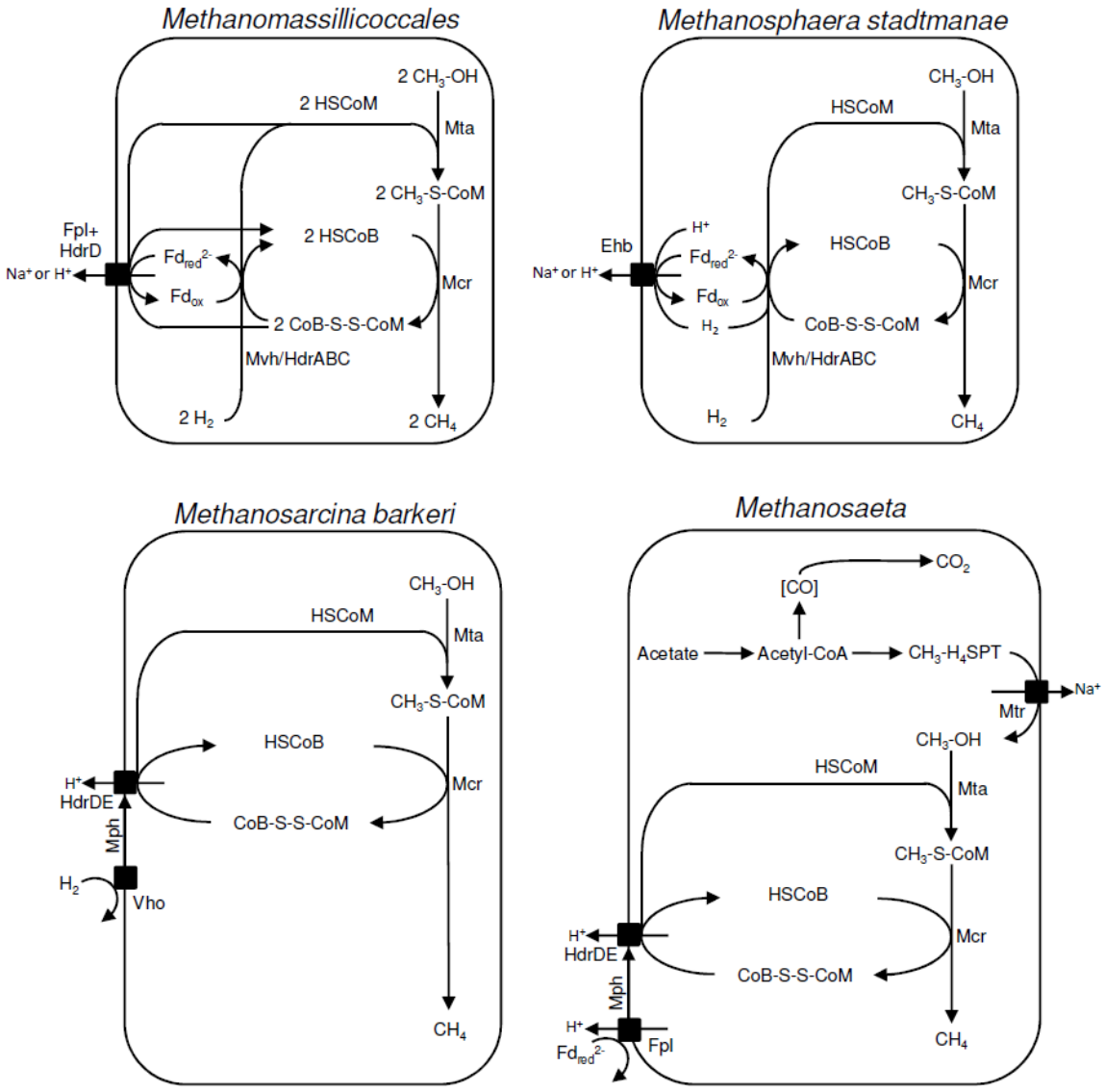


Figure 2. Different modes of energy conservation in the obligately hydrogen-dependent methylotrophs *Methanomassiliicoccales, Methanosphaera stadtmanae* (methanogens without cytochromes), *Methanosarcina barkeri* and the aceticlastic methanogens of *Methanosaeta* (latter two are methanogens with cytochromes). Abbreviations: Mta, methanol:CoM methyl-transferase; Mcr, methyl-CoM reductase; Mvh, non-F_{420}-reducing hydrogenase; Hdr, heterodisulfide reductase; Fpl, $F_{420}H_2$ dehydrogenase like complex; Ehb, energy-converting hydrogenase; Vho, methanophenazine-dependent hydrogenase; Mph, Methanophenazine; Mtr, methyl-H_4SPT:CoM methyltransferase; Fpl, $F_{420}H_2$ dehydrogenase-like complex.

In addition, the phylogenetic analysis of the large subunit of this complex in Chapter 3 shows that this subunit of the *Thermoplasmatales* is only distantly related to large subunit of the *Methanomassiliicoccales*. This further supported the theory that both organisms not necessarily use the same electron acceptor. As mentioned above, the 11-subunit complex or advancements of this complex are completely absent from all basal *Euryarchaeota*. In addition, none of the genomes contain genes that encode for HdrDE. However, these enzymes play important roles in the energy conservation of the *Methanomassiliicoccales* and *Methanosarcinales* and enable these organisms to use methanol and H_2 for methanogenesis. Interestingly, *M. stadtmanae* (*Methano-bacteriales*) also grows on methanol using H_2 as electron donor (Miller and Meyer, 1985), but belongs to the basal *Euryarchaeota* and is therefore missing a 11-subunit complex and HdrDE. It has been suggested that in this organism energy is conserved by a second hydrogenase, Ehb (Thauer *et al*., 2008; Fig. 2).

This enzyme has been proposed to have an anabolic function (Porat *et al*., 2006) in hydrogentrophic methanogens without cytochromes. Here, the reduction of the heterodisulfide is coupled via ferredoxin with Ehb, which produces H_2 (Thauer *et al*., 2008; Fig. 2). This leads to the conclusion that energy conservation in methyl reducing methanogens lacking cytochromes was developed independently from each other, first in the *M. stadtmanae* by the rearrangement of present enzymes, and secondly in the *Methanomassiliicoccales* by the gain of the Fpo-like complex and the D subunit of the heterodisulfide reductase (Chapter 3). The uptake of the cytochrome *b* dependent heterodisulfide reductase in addition to the *hdrD* gene and the gain of a novel hydrogenase (Vho) then allowed *Methanosarcina barkeri* to perform methyl-reducing methanogenesis using H_2 as electron donor (Fig. 2). Because the genome of *Methanomicrococcus blatticola* has so far not been sequenced it can only speculated how this organism conserves its energy. *M. blatticola* belongs to the order of the *Methanosarcinales*; therefore it can be suspected that energy conservation in this organism works in a similar way to *M. barkeri*.

Interestingly, other archaea and bacteria possess also an 11-subunit complex, but there is no electron acceptor interacting with the complex in common between all these organisms (Moparthi and Hägerhäll, 2011). Therefore, it is likely that the interaction

partner of the complex differs in the diverse microorganisms, like in the *Methanomassiliicoccales* (HdrD), in *Methanosarcinales* (FpoF), and in the NADH-oxidizing module of the complex 1 of the respiratory chain (NuoEFG). However, it seems that in all cases the complex has a role in energy conservation.

6.3 Cell wall less archaea

So far all isolated or enriched strains of *Methanomassiliicoccales* possess a roundish cell form (Chapter 1, Dridi *et al*., 2012; Iino *et al.*, 2013). For *M. luminyensis* it was carefully described that this organism possesses one electron dense layer and one thick transparent layer (Dridi *et al*., 2012; Fig 3A). Our transmission electron micrographs of *Ca.* Mp. Termitum (Fig. 3B) and strain MpM2 (Fig. 3C) in Chapter 3 shows that these layers are an unusual two membrane system and suggests the lack of a cell wall. It is likely that this unusual two membrane system is a common feature to all *Methanomassiliicoccales*. This seems to be similar to the two-membrane system of *Ignicoccus* species (Crenarchaeota; Rachel *et al.*, 2002; Fig. 3D). A similar kind of membrane system is also present in the ARMAN cells (archaeal Richmond Mine acidophilic nanoorganism), which have been identified in acid mine drainage (Comolli *et al*., 2009; Fig. 3E) and form a lineage without cultivated representatives that branches near the crenarchaeal/euryarchaeal divide (Baker *et al*., 2010). Like the *Methanomassiliicoccales, Ignicoccus* species (Rachel *et al.*, 2002) and the ARMAN cells (Comolli *et al*., 2009) do not possess the typically archaeal S-layer and a cell wall also seems to be absent. It appears that archaea with double membranes might be more common than originally thought. In 1981, Rose and Pirt isolated the methanogen *Methanoplasma elizabethii* from anaerobic sludge, which was assigned to the order *Mycoplasmatales* and described as a methanogen without a cell wall (Rose and Pirt, 1981). However, the name of this organism has never been validly published and no type strain was deposited in any culture collection (International Committee on Systematic Bacteriology, 1985). The lack of a cell wall suggests an affiliation to the *Methanomassiliicoccales*, but this can be excluded because it is growing on H_2 and CO_2 and the phase contrast micrographs of the culture reveals branching spindle-like cells.

The closest cultivated relatives of the *Methanomassiliicoccales*, the *Thermoplasmatales* (Fig. 1), also do not possess a cell wall (Huber and Stetter, 2006). However, this phenomenon is not common to the complete order. The genera *Thermoplasma* and *Ferroplasma* are missing a cell wall, whereas strains of the genus *Picrophilus* are cover by an S-layer protein. In contrast the *Methanomassiliicoccales*, *Ignicoccus* spp. and the ARMAN cells, these organisms possess only one membrane and lack a second one (Fig. 3F).

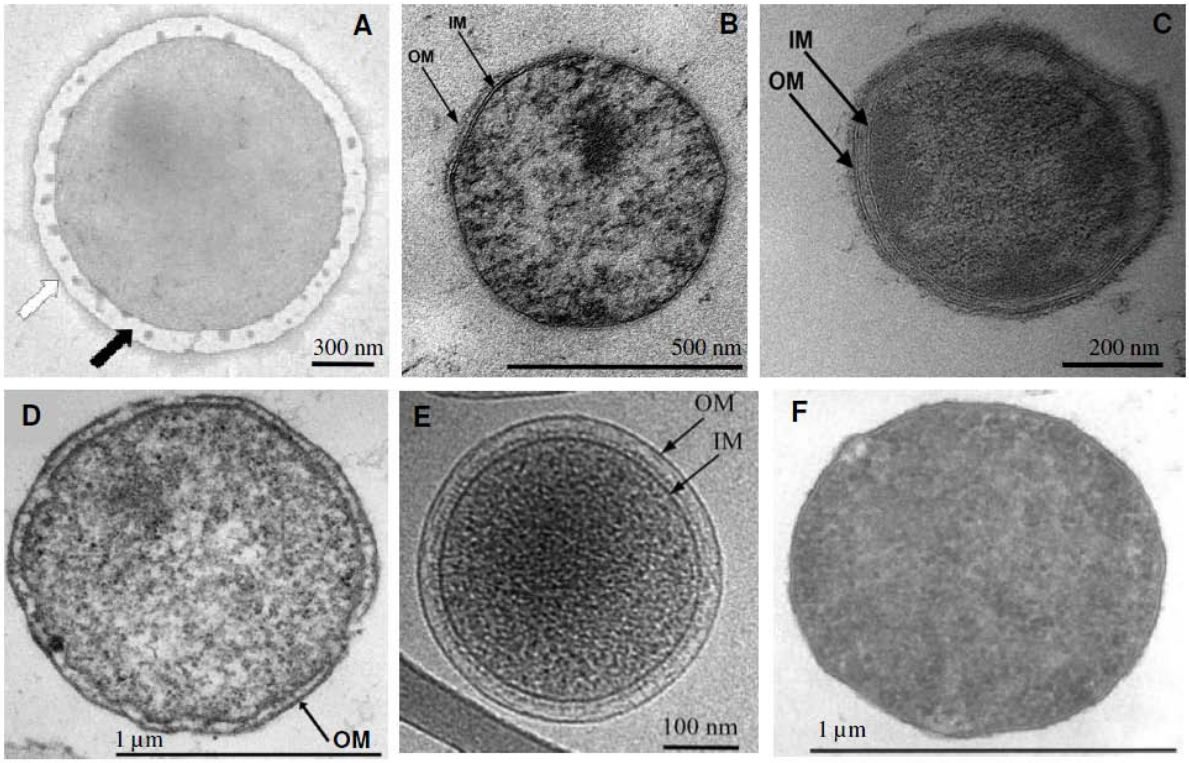


Figure 3. Transmission electron micrographs of different archaea missing a cell wall. A, *Candidatus* Methanoplasma termitum (chapter 3); B, Strain MpM2 (chapter 3); C, *Methanomassiliicoccus luminyensis* (Dridi *et al*., 2012) ; D, *Ignicoccus* sp. (Rachel *et al*., 2002); E, ARMAN cells (Comolli *et al*., 2009); F, *Thermoplasma acidophilum* (Huber and Stetter, 2006). Abbreviations: IM: cytoplasmic membrane, OM: outermost membrane (OM). Fig. 3A; black arrow: indicating the thick transparent layer; white arrow: showing the electron dense layer.

Interestingly, *Candidatus* Aciduliprofundum boonei from deep-sea hydrothermal vents harbors an S-layer (Reysenbach *et al*., 2006) like the strains from genus *Picrophilus*. This raises the question if previously all *Thermoplasmata* (Fig. 1) were missing a cell wall and gained the S-layer protein later or if all *Thermoplasmata* possessed a cell wall

and then lost it. The S-layer cell wall structure is widely distributed within all phyla of archaea, as well as in all major lineages of bacteria (Albers and Meyer, 2011). Therefore, the S-layer might be the earliest cell wall structure that was developed (Albers and Meyer, 2011), suggesting that the common ancestor of the *Thermoplasmata* harbored an S-layer cell wall, which was lost afterwards by several lineages of the *Thermoplasmata*. A second scenario could be the loss of the cell wall in the *Methanomassiliicoccales*, which seem to be separated first from the *Thermoplasmatales* and *Ca.* A. boonei (Fig. 1), the afterwards recovery of the S-layer in *Ca.* A. boonei and the additional loss of the second membrane by the *Thermoplasmatales*. Furthermore, strains of the genus *Picrophilus* (*Thermoplasmatales*) must have regained the S-layer again. However, this scenario is rather unlikely, because it is should be easier to lose a function than to recover it several times independently from each other. To fully answer this question, more cultured representatives of *Thermoplasmata* are needed. For example, it would be interesting to know if there is a cell wall present in the marine group II (Fig. 1, Chapter 3), which is the most basal group of the *Thermoplasmata*. It should be determined if there are more members of the *Thermoplasmatales* that possess a cell wall in addition to *Picrophilus.* Furhtermore it would be interesting to know if the strains belonging to the uncultivated 16S rRNA sequences from the deep-sea, located between the *Methanomassiliicoccales* and the *Thermoplasmatales* (Fig. 1, chapter 3) also lack a cell wall and if so, do they harbor one or two membranes.

Because archaea lacking cell walls have now been identified from different phylogenetically independent groups, this leads to the conclusion that they do not have a common origin. In addition, it raises the questions: What are the environmental drivers causing the loss of a cell wall and what are the advantages of the loss? Besides the absence of a cell well, none of these organisms share common general properties of microorganisms, such as metabolism, growth temperature, pH and isolation source. The *Ca.* Methanoplasma termitum, strain MpM2 and *M. luminyensis* are all obligate anaerobes, which perform methanogenesis and were isolated or enriched from intestinal tracts (Chapter 2+3; Dridi *et al*., 2012). They grow at neutral pH at mesophilic temperatures (Chapter 2, Dridi *et al*., 2012), whereas strains of the genus *Thermoplasma* are facultative anaerobes, performing sulfur respiration under anaerobic

conditions and grow at temperatures around 60°C. These organisms were isolated from acidic environments, and have an optimal pH between 1 and 2 (Huber and Stetter, 2006). Strains of the genus *Ferroplasma* also have an optimal pH ~1, but they are aerobes and grow best around at mesophilic conditions (Huber and Stetter, 2006). Less is known about ARMAN cells except that they are also acidophilic and occur in similar environments as *Thermoplasma* (Baker *et al*., 2006; Comolli *et al*., 2009), suggesting a similar temperature range. Strains of the genus *Ignicoccus* were isolated from hyperthermophilic environments because *Ignicoccus* spp. have a temperature range from 70 – 98°C. They are anaerobes that reduce sulfur and have an optimal pH at 6 (Huber *et al*., 2000; Paper *et al*., 2007). Interestingly, archaea without cell walls seem to be adapted to extreme environments like high temperatures and acidic pH, but also occur in neutral environments, giving no information about environmental drives that favor the lack of a cell wall. However, this does not explain, why the *Methanomassiliicoccales*, which grow at neutral pH and at mesophilic temperatures, lack their cell wall. Interestingly, *Ignicoccus hospitalis* is also known as the host of the *Nanoarchaeum equitans* (Paper *et al*., 2007) and it has been shown that the outer membranes of both organisms are in direct contact, maybe as prerequisite for metabolite transport (Junglas *et al*., 2008). Furthermore, investigations of biofilms of acidic mines show that ARMAN cells are penetrated by *Thermoplasmatales*, suggesting the transfer of nutrients from the *Thermoplasmatales* to the ARMAN cells, parasitism of the ARMAN cell (like between *I. hospitalis* and *N. equitans*), or an exchange of molecules between them (Barker *et al*., 2010). Taking these results together, it can be speculated that the lack of a cell wall favors the exchange of molecules and parasitism, which could be especially advantageous in extreme environments.

6.4 Concluding remarks and future perspective

The recent identification of the *Methanomassiliicoccales* as seventh order of methanogens (Chapter 2) revealed that the obligately hydrogen-dependent methylotrophs are not only restricted to intestinal tracts but also quite common in nature. In addition, these organisms are highly abundant in millipede guts (Chapter 4), but also seem to play an important role in the guts of other arthropods, as shown in Chapter 5. However, the intestinal tracts of arthropods are mostly dominated by one group of

methyl-reducing methanogens (Chapter 5), suggesting different environmental conditions within the gut that favor the colonization of these organisms.

The identification of a novel mode of energy conservation in the *Methanomassiliicoccales* (Chapter 3) different from those of other obligately hydrogen-dependent methylotrophs, like *M. stadtmanae* and *Ms. barkeri*, indicates that the pathways have evolved independently from each other, for example through the gain of a Fpo-like complex or the uptake of cytochromes. The isolation of *Ca.* Mp. termitum in pure culture would allow further experiments about the physiology of the *Methanomassiliicoccales* and the Fpo-like complex.

The *Methanomassiliicoccales* are the first documented methanogens with the unusual two-membrane system and the lack of a cell wall. The absence of a cell wall has been identified in several archaea and may favor the exchange of molecules and parasitism. Further investigations into the role *Ca.* Mp. termitum in the termite gut will give additional information about the interactions between this organism and other microbes.

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Erklärung der Eigenständigkeit

Ich versichere, dass ich meine Dissertation

„Diversity, ultrastructure, and comparative genomics of “*Methanoplasmatales*”, the seventh order of methanogens”

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat, falls nicht anders beschrieben, noch keinen sonstigen Prüfungszwecken gedient.

Marburg, Oktober 2014