

**“*Methanoplasmatales*,” *Thermoplasmatales*-Related Archaea in Termite Guts and Other Environments, Are the Seventh Order of Methanogens**

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**The *Euryarchaeota* comprise both methanogenic and nonmethanogenic 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 func- tional marker for methanogenesis, obtained from the same habitats. The identical topologies 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 cock- roaches, strongly suggested that they stem from the same microorganisms. This was further corroborated by two highly enriched cultures of closely related methanogens from the guts of a higher termite (*Cubitermes ugandensis*) and a millipede (*Anadenobo- lus* 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 data sets from the intestinal tracts of mammals (e.g., rumen cluster C) and other environments. Together with the recently de- scribed *Methanomassiliicoccus luminyensis* isolated from human feces, which falls into rice cluster III, the results of our study strongly support the idea that the entire clade of “uncultured *Thermoplasmatales*” in fact represents the seventh order of metha- nogenic archaea, for which the provisional name “*Methanoplasmatales*” is proposed.**

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ethanogenesis is an important process in the carbon cycle, with a significant impact on global warming. Methane is produced exclusively by methanogenic archaea—strictly anaero- bic 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

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sludge, and in the digestive tracts of animals and humans (33).

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) (3); and *Methanosarcinales* (class

III) (2), with the recently recognized sister group *Methanocellales* (50). It has been hypothesized that the genes for hydro- genotrophic methanogenesis were already present in a common ancestor and were vertically inherited in a broader monophyletic unit encompassing all methanogens (3). 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) (3). In addition, there are many deep-branching lineages of archaea that are exclusively represented by their 16S rRNA genes (19, 53,

60) and 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*. Origi- nally discovered in the marine environment (9, 17) and the deep subsurface (59), related clones were subsequently obtained from rice field soil (20), the water column and sediment of freshwater lakes (27, 44), and soil and leachate of landfills (24, 37). Other members of this clade were found in the guts of termites (16, 42, 54), wood-feeding cockroaches (22), and scarab beetle larvae (14). Also, studies of the mammalian digestive tract reported sequences of uncultured archaea distantly related to the *Thermoplasmatales*

in cattle (10, 26, 58, 62), sheep (63), and wallabies (15) and in the guts and subgingival pockets of humans (32, 39, 40, 51). 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 a-subunit of methyl coen- zyme M (methyl-CoM) reductase, has been established as a mo- lecular marker for methanogenic archaea (36). Studies of the di- versity 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 (37). Related sequences were soon discovered in a eutrophic lake (13) and in salt marsh sediments (7). Later studies of vertebrate guts also revealed the presence of novel *mcrA* genes in the cow rumen (10); feces of pigs, chickens, and horses (61); the guts of humans (39, 51); and the foregut of wallabies (15).

Kemnitz et al. (28) observed a correlation between the abun- dance of rice cluster III (RC-III) archaea and the rate of methano- genesis in enrichment cultures. Mihajlovski et al. (39) claimed that a new *mcrA* phylotype and a new 16S rRNA phylotype obtained from the same stool sample belonged to the same organism and subsequently postulated that they represent a putative new order of methanogens (40). Also, Evans et al. (15) had speculated that the unknown *mcrA* gene sequences in the foreguts of wallabies and

Received 11 July 2012 Accepted 11 September 2012 Published ahead of print 21 September 2012 Address correspondence to Andreas Brune, [brune@mpi-marburg.mpg.de.](mailto:brune@mpi-marburg.mpg.de) Copyright © 2012, American Society for Microbiology. All Rights Reserved. [doi:10.1128/AEM.02193-12](http://dx.doi.org/10.1128/AEM.02193-12)

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ruminants belong to a lineage of uncultivated archaea encoun- tered 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 (36, 37). This allows the correlation of unknown *mcrA* sequences with the corresponding 16S rRNA gene sequences, a strategy that has been successfully employed to predict the methanogenic na- ture of the uncultivated archaea in rice cluster I (36), which even- tually led to the enrichment and isolation of *Methanocella paludi- cola* (50).

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* (5). In ad- dition, we initiated enrichment cultures from the hindguts of ter- mites and millipedes to isolate a potentially methanogenic mem- ber of this novel lineage.

**MATERIALS 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 cock- roaches *Salganea esakii* and *Panesthia angustipennis* were collected in the vicinity of the Keta Shrine in Ishikawa Prefecture, Japan, by Kiyoto Maekawa, Toyama University. The millipede *Anadenobolus* sp. was ob- tained from a commercial breeder (b.t.b.e. Insektenzucht, Schnürpflin- gen, Germany). All animals were kept in plastic containers at room tem- perature in the dark.

**DNA extraction and purification.** The hindguts of 10 to 20 termites were dissected with sterile fine-tipped forceps, pooled in 2-ml tubes con- taining 750 µl sodium phosphate buffer (120 mM; pH 8.0), and homog- enized. Homogenates of individual cockroach hindguts were prepared in a similar manner. DNA was prepared using a bead-beating protocol com- bined with phenol-chloroform extraction. The homogenate was trans- ferred 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) was 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-chloro- form-isoamyl alcohol (24:24:1 by volume; pH 8.0). After a second centrifu- gation 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) solu- tion (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 vac- uum. DNA was dissolved in 50 µl elution buffer (MinElute PCR Purification Kit; Qiagen, Hilden, Germany), checked photometrically for purity (Nano- drop; PeqLab, Erlangen, Germany), quantified fluorimetrically (Qubit; Invit- rogen, Eugene, OR), and stored at —20°C.

**PCR amplification and cloning.** 16S rRNA genes were amplified us-

ing either the archaeon-specific primer pair Ar109f (5=-AMDGCTCAGT AACACGT-3=) (25) and Ar912r (5=-CTCCCCCGCCAATTCCTTTA-3=)

(35) or the archaeon-specific primer Ar109f and the prokaryote-specific primer 1490R with the modification of Hatamoto et al. (23) (5 -GGHTA CCTTGTTACGACTT-3 ), a combination that yields only archaeal 16S rRNA genes (43). Each PCR mixture (50 µl) contained reaction buffer, 2.5 mM MgCl2,1U *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 DNA extract. 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). For the amplification of the *mcrA* gene, the primer pair *mcrA*-f (5 -GGTGGT GTMGGATTCACACARTAYGCWACAGC-3 ) and *mcrA*-r (5 -TTCAT TGCRTAGTTWGGRTAGTT-3 ; 37) was used; the reaction mixture and the PCR protocol were the same as described above, except for the anneal- ing 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. (52).

**Sequence analysis.** The 16S rRNA gene sequences obtained in this study were imported into the current Silva database (version 106) (48; [http://www.arb-silva.de](http://www.arb-silva.de/)) using the ARB software package (34). Sequences from other studies that were not included in Silva were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>). The sequences were automat- ically aligned, and the alignments were refined manually. A 30% consen- sus 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 (56). Tree topology and node support (100 bootstraps) were tested using the maximum-parsimony method (DNAPARS) implemented in ARB. The *mcrA* gene sequences were im- ported 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 (21) implemented in ARB. Tree topology and node support (100 bootstraps) were tested using the maximum-parsimony method (PROTPARS) implemented in ARB.

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**Cultivation.** Enrichment cultures were set up in anoxic, bicarbonate- buffered AM5 medium under an atmosphere of N2-CO2 (80:20 [vol/vol]) (4), but dithiothreitol (DTT) was omitted. The basal medium was supple- mented with Casamino Acids (2 g/liter), coenzyme M (10 mg/liter), cys- teine (2 mM), and palladium on activated charcoal (10 ml/liter) and (op- tionally) with yeast extract (2 g/liter) or rumen fluid (10%). The medium (4.5 ml) was dispensed into 15-ml rubber-stoppered glass vials, and hy- drogen 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/liter). 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. The methane content in the headspace was measured every week. The culture headspace (0.2 ml) was sampled with a gas-tight sy- ringe, and the methane content was analyzed using a gas chromatograph with a flame ionization detector (38).

**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. (28) using the primers A364aF (5 -CGGGGYGCASCAGGCGCGAA-3 ) (6) and A934b (5 -GTGCTCC

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CCCGCCAATTCCT-3 ) (20). Bacterial 16S rRNA genes were quantified as described by Stubner (57) using the primer pair 519fc (5 -CAGCMGC CGCGGTAANWC-3 ) and 907r (5 -CCGTCAATTCMTTTRAGTT-3 )

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(31). In addition, the bacterial community structure of the sample was determined by 454 pyrotag sequencing as described elsewhere (30).

**Nucleotide sequence accession numbers.** The sequences obtained in this study were submitted to GenBank. The accession numbers are JX266062 to JX266091 for 16S rRNA genes and JX266092 to JX266145 for *mcrA* genes from hindgut homogenates. The accession numbers for the

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**TABLE 1** Clone frequencies in libraries of archaeal 16S rRNA genes and *mcrA* genes obtained from the hindguts of higher termites, documenting the cooccurrence of a novel lineage of *Thermoplasmatales-*related archaea and a cluster of novel *mcrA* genes

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 16S rRNA genes (%)*b* |  |  |  |  | *mcrA* genes (%)*c* |  | | | |
|  |  |  | Novel | No. of |  |  |  | Novel | No. of |
| Termite species*a* | *Methanomicrobiales* | *Methanobacteriales* | *Methanosarcinales* | lineage | clones | *Methanomicrobiales* | *Methanobacteriales* | *Methanosarcinales* | cluster | clones |
| *C. 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 |
| *M. michaelseni* | 0 | 52 | 45 | 2 | 44 | 0 | 74 | 16 | 11 | 19 |
| *A. trestus* | 3 | 84 | 0 | 14 | 37 | 4 | 64 | 0 | 32 | 25 |

*a* Representing all major lineages of higher termites (*Termitidae*): *C. ugandensis* and *Ophiotermes* sp. (*Termitinae*), *Trinervitermes* sp. (*Nasutitermitinae*), *M. michaelseni*

(*Macrotermitinae*), and *A. trestus* (*Apicotermitinae*).

*b* Obtained with primer pair Ar109f and Ar912r. *c* Obtained with primer pair *mcrA*-f and *mcrA*-r.

corresponding genes of strains MpT1 and MpM2 are JX266068, JX266097, JX648297, and JX648298.

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**RESULTS**

**Comparison of 16S rRNA and *mcrA* clone frequencies.** Analysis of the archaeal 16S rRNA gene sequences from the hindguts of several higher termites revealed a diverse community of methano- archaea, consisting of *Methanobacteriales*, *Methanosarcinales*, and *Methanomicrobiales*, although not all lineages were represented in each species (Table 1). In addition, each termite species yielded a substantial proportion of clones that clustered with a deep- branching lineage distantly related to *Thermoplasmatales* previ- ously obtained from termite guts and other intestinal environ- ments. 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, unpublished data).

Clone libraries of the *mcrA* genes amplified from the same samples not only yielded the *mcrA* genes expected of the methano- gens 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 *Thermoplas- matales*.

**Phylogenetic analysis of the 16S rRNA genes.** Since the 16S rRNA gene sequences obtained in the first data set 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 *C. ugandensis* and *Ophiotermes* sp. and for the cockroaches *S. esakii* and *P. angustipennis* to obtain longer se- quences (1,380 bp), together with those already present from pre- vious studies, for all lineages of *Thermoplasmatales*-related ar- chaea affiliated with termites and cockroaches. We included 16S rRNA gene sequences from all established lineages of *Euryar- chaeota* from the Silva database and GenBank, including all se- quences 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 para- phyletic 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

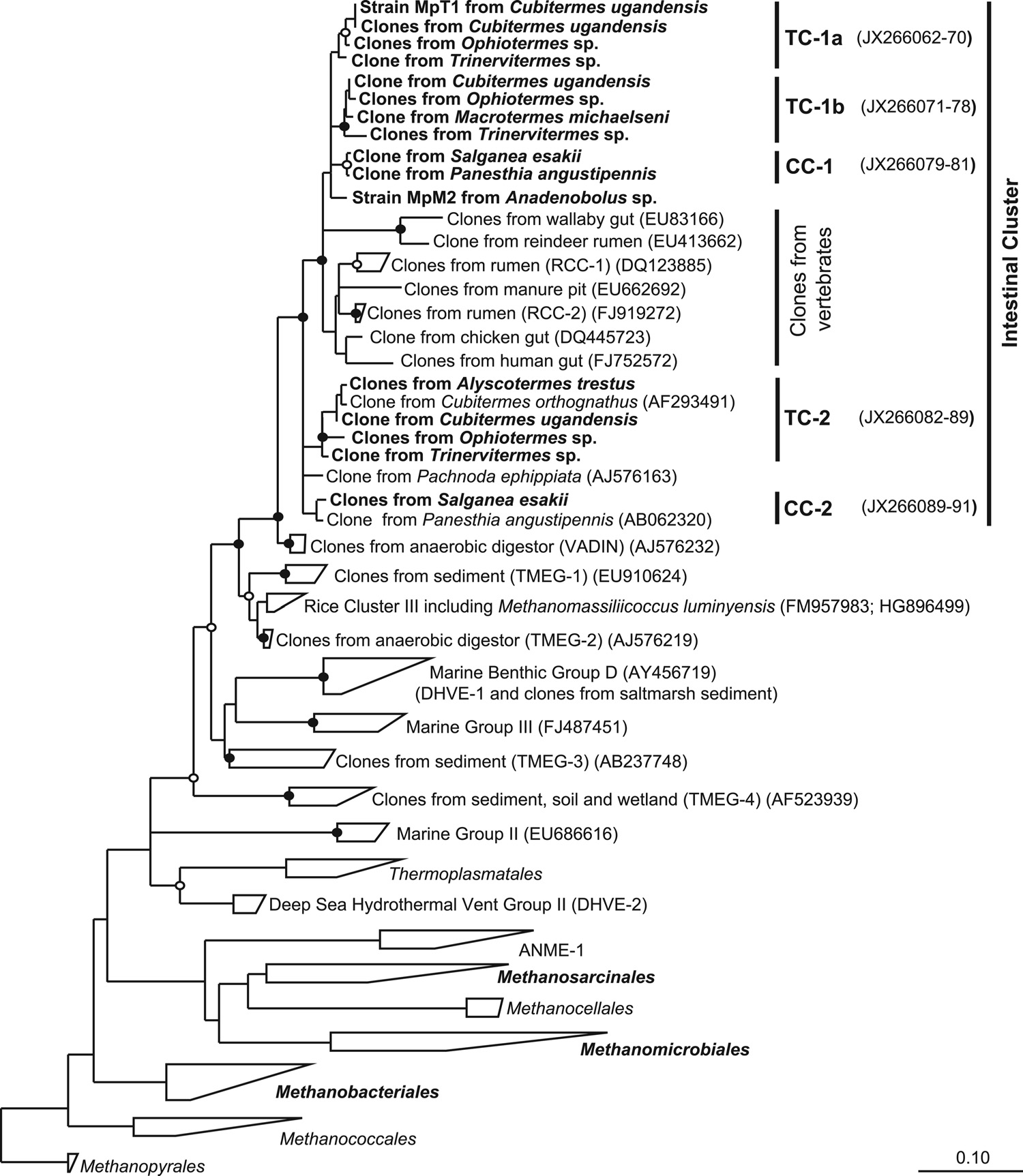
obtained exclusively from intestinal environments that was clearly separated from previously published clades containing sequences from diverse marine and freshwater habitats. Next, relatives of this clade were clones previously obtained from an anaerobic digestor (Vadin Group) (18). Within the intestinal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

**Phylogenetic analysis of the *mcrA* genes.** To test the phyloge- netic positions 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 methano- gens and including all *mcrA* genes of uncertain origin from envi- ronmental studies. Phylogenetic analysis confirmed the presence of *mcrA* genes in insect guts belonging to representatives of the orders *Methanosarcinales*, *Methanobacteriales*, and *Methanomi- crobiales*, 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 *S. esakii* and *P. 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 tracts 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 *Thermoplasma- tales*-related archaea, the novel *mcrA* genes from intestinal envi- ronments 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.

**Enrichment of novel methanogens from arthropod guts.** A hindgut homogenate of *C. 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 H2 and CO2. After a lag phase of several weeks, the culture containing methanol and yeast extract started to form CH4. No methane formation was observed under any other con- ditions, even after 6 months of incubation, and also not if rumen fluid was added to the cultures. Subsequent transfers of the culture on the same medium led to robust CH4 formation (up to 17-kPa

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**FIG 1** Phylogenetic tree showing the relationships among uncultured archaea related to *Thermoplasmatales* and to representatives of all other orders of methanogenic archaea and the ANME-1 group. Clusters of clones from termite (TC) and cockroach (CC) guts are indicated. The tree is based on a maximum- likelihood analysis of an alignment of archaeal 16S rRNA genes (1,250 bp) of archaea in public databases; sequences obtained in this study are marked in boldface. Sequences of *Trinervitermes* sp., *M. michaelseni*, and *A. trestus* were shorter and were added to the tree using the ARB parsimony tool. The bullets indicate bootstrap support (●, >95%; Œ, >70%). The scale bar indicates substitutions per site.

headspace partial pressure); rumen fluid was not required. Trans- fers of the enrichment culture to medium lacking methanol showed no methanogenesis; transfers to medium containing methanol in the absence of H2 produced much less methane than with H2. No methanogenesis occurred with acetate as the sole substrate (Fig. 3).

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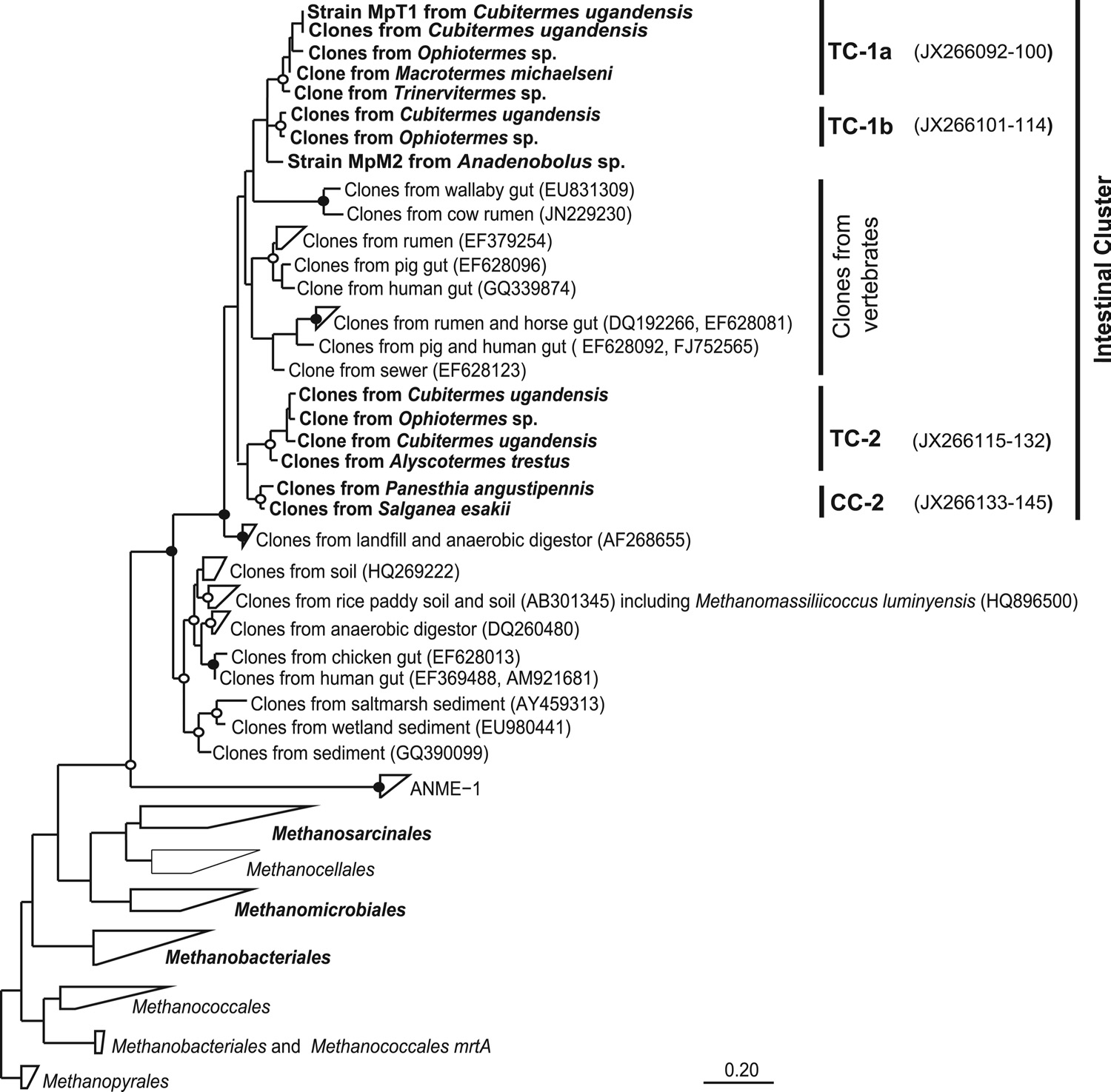
After the initial transfers, the culture already consisted mostly of small, roundish cells (ca. 0.6 to 1 µm in diameter) (Fig. 4A). DNA was extracted from several subcultures, and the archaeal 16S rRNA genes were amplified using specific primers (Ar109f and 1490R). Each PCR product could be sequenced 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 re- vealed that the archaeal 16S rRNA sequence (phylotype MpT1)

fell into the apical cluster of putative methanogens consisting ex- clusively of clones from higher termites (Fig. 1, TC-1a). 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 (Fig. 2, TC-1a).

When the abundance of archaeal 16S rRNA genes in the en- richment 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. Classifica- tion of the bacterial sequences revealed that the bacteria remain- ing in the enrichment culture represented several lineages of so-far uncultivated *Clostridiales* (Fig. 5). All attempts to isolate strain MpT1 in pure culture have so far been unsuccessful.

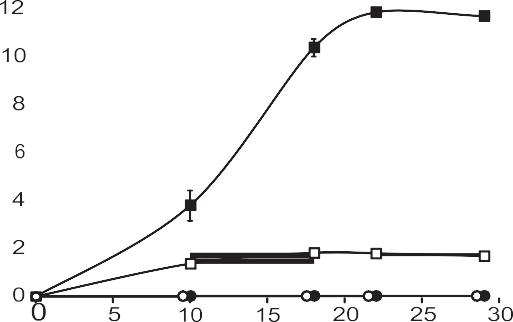
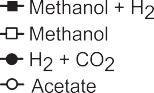
Meanwhile, we also obtained a second methanogenic enrich-

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**FIG 2** Phylogenetic tree showing the relationships among the novel *mcrA* genes and to representative *mcrA* genes of all other orders of methanogenic archaea and the ANME-1 group. Clusters of clones from termite (TC) and cockroach (CC) guts 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 boldface. The bullets indicate bootstrap support (●, >95%; Œ, >70%). The scale bar indicates substitutions per site.

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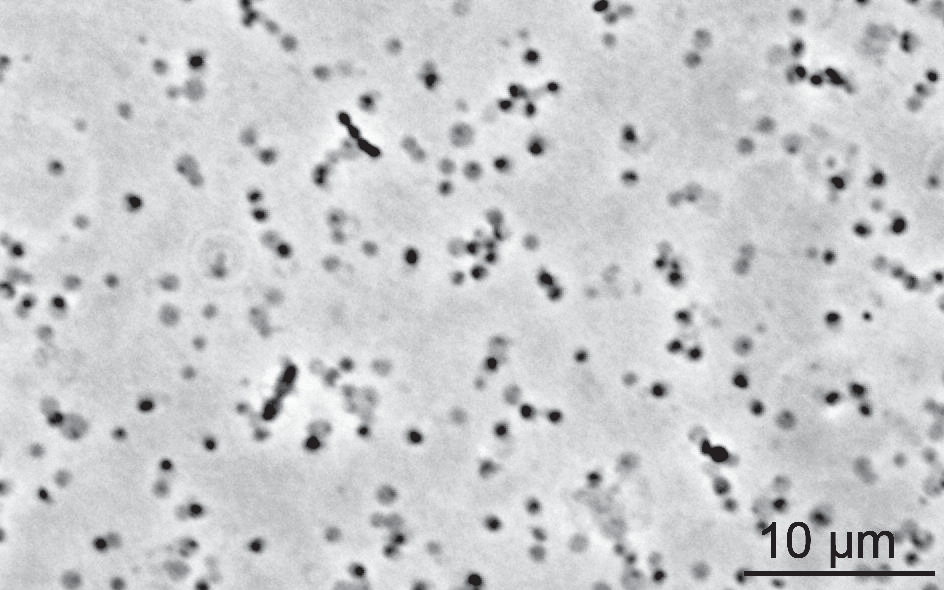
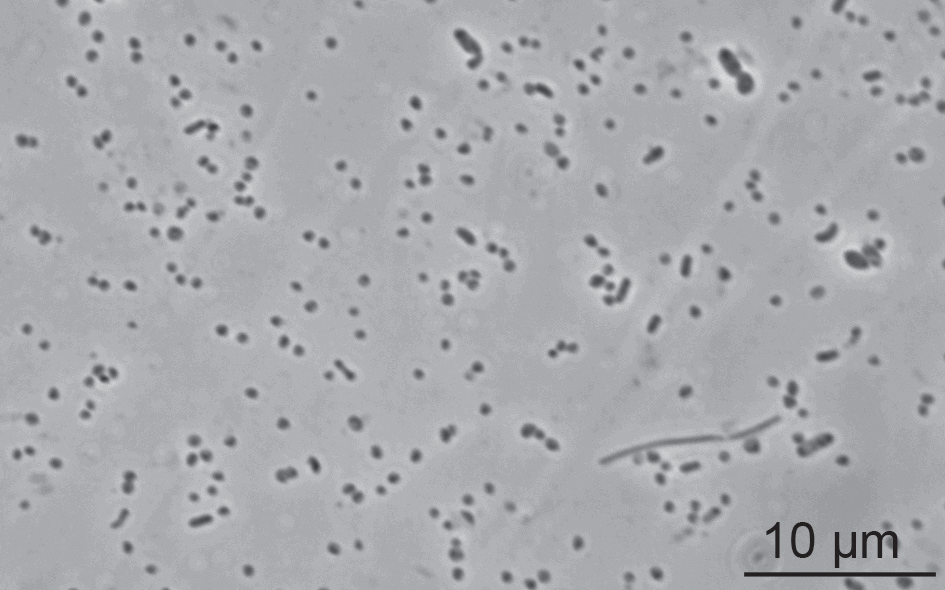
**FIG 3** Time course of methane partial pressure in the headspace of the en- richment culture MpT1 (N2-CO2; 80/20) inoculated from a methanol-starved preculture into basal medium supplemented with different substrates: H2 (50 kPa in headspace), methanol (50 mM), or acetate (30 mM). The values are means of two cultures; mean standard deviations are shown only if they are larger than the symbols.

ment 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 larger amounts of methane (45 kPa in the headspace) than strain MpT1. Again, the PCR products obtained with specific primers for ar- chaeal 16S rRNA genes and *mcrA* genes could be sequenced with- out cloning, which indicated that this enrichment culture was also dominated by a single strain of methanogens. Strain MpM2 had the same coccoid morphology as strain MpT1 but was slightly larger (Fig. 4B); both strains did not show the typical cofactor F420 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 radia- tion of sequences from termites and cockroaches (Fig. 1, TC-1 and CC-1). The *mcrA* gene of strain MpM2 clustered with the corre- sponding *mcrA* genes of the TC-1 subcluster (Fig. 2).

**DISCUSSION**

The results of this study are the final proof that the deep-branch- ing lineage of so far uncultured *Euryarchaeota* distantly related to the *Thermoplasmatales* represents the seventh order of methano- gens. This is supported by the congruence of the phylogenies of

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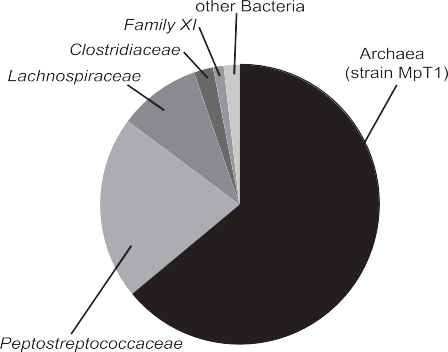
**FIG 4** Phase-contrast photomicrographs of the methanogenic enrichment cultures MpT1 (A) and MpM2 (B) after several transfers in basal medium supple- mented with H2 and methanol. Both cultures consisted mostly of small roundish cells (diameter, 0.6 to 1.0 µm).

16S rRNA and *mcrA* genes, which indicates that the corresponding gene sets obtained from termite and cockroach guts (this study) and from mammalian guts and several other environments (pre- vious 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 hind- guts of termites and millipedes and the isolate *Methanomassiliicoc-* *cus luminyensis* from human feces (12) (see below).

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**Novel archaea in the guts of termites and cockroaches.** Pre- vious studies of archaeal diversity in the hindguts of *C. orthog- nathus* (subfamily *Termitinae*) and *Nasutitermes takasagoensis* (subfamily *Nasutitermitinae*) had revealed the presence of four major lineages of *Euryarchaeota* in higher termites: *Methanosarci- nales*, *Methanomicrobiales*, *Methanobacteriales* (for references, see reference 5), and a deep-branching clade distantly related to *Ther- moplasmatales* (16, 42). Clones from the same lineages were also recovered from *C. ugandensis* and *Ophiotermes* sp., *Trinervitermes* sp., *M. michaelseni*, and *A. trestus* (this study), which indicated that representatives of the clade are consistently present in all sub- families of higher termites. In addition, clones of this lineage were also obtained from the wood-feeding cockroaches *S. esakii* and *P. angustipennis* (22; this study), which are distantly related to ter- mites.

Interestingly, the novel archaea from insect guts form two dis- tinct lineages, each comprising clones from higher termites and wood-feeding cockroaches that seem to be specific for their re- spective hosts. The general absence of the group from lower ter-



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

mites is in agreement with previous studies reporting that the insects are exclusively colonized by members of the genus *Me- thanobrevibacter* (45); the single clone of *Thermoplasmatales*-re- lated archaea obtained from *Reticulitermes speratus* (54) is affili- ated with cluster TC-1b (Fig. 1).

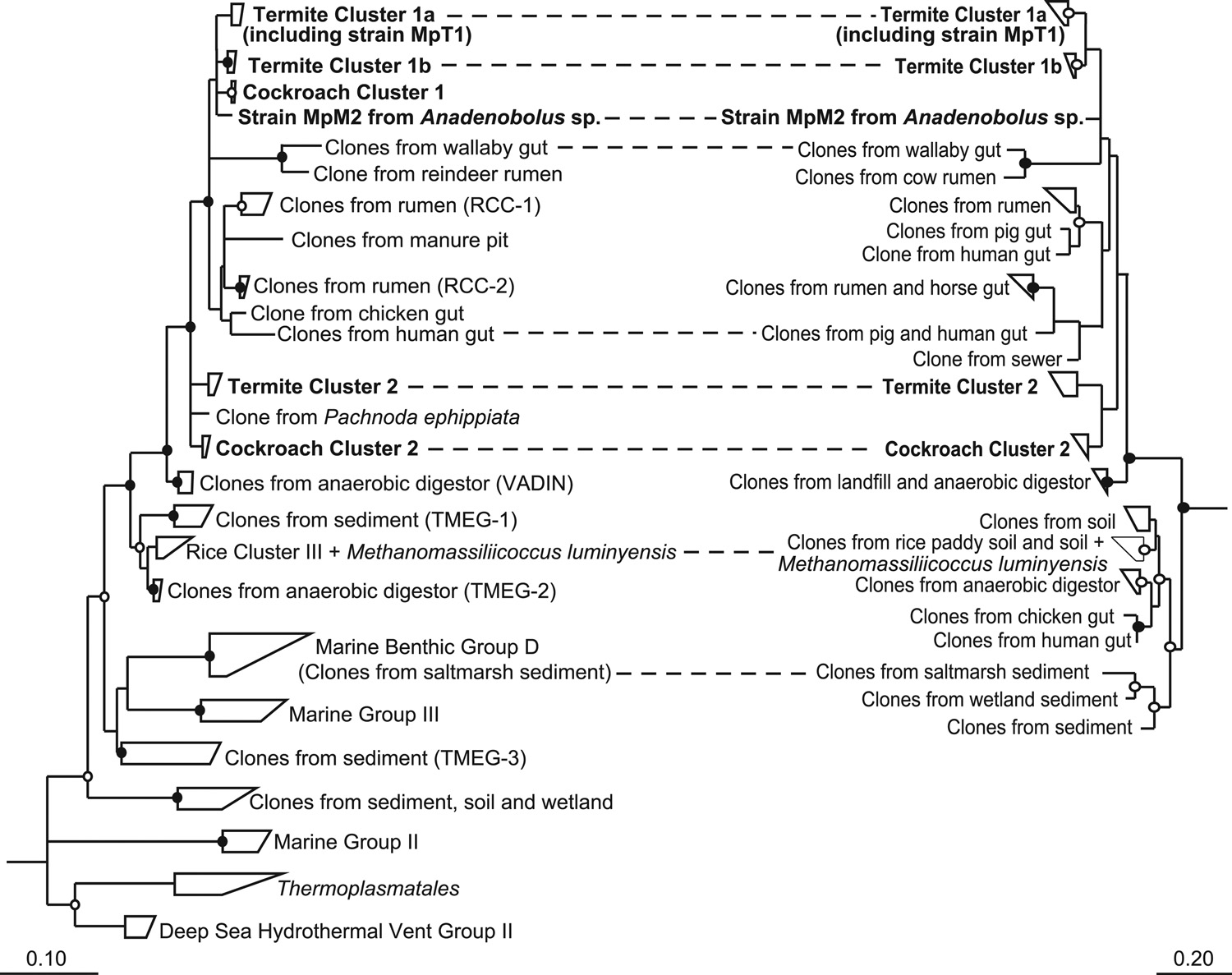
**Methanogenic nature of novel archaea.** The tree topologies of the 16S rRNA genes of novel archaea (Fig. 1) and the *mcrA* genes of unknown origin (Fig. 2) strongly resemble each other. A simpli- fied tanglegram of the two trees illustrates that the phylogenetic positions of the major clusters of 16S 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 (15), human gut (39, 40), and salt marsh sediment (7). In addition, other opposing clusters in the tree contain clones that originated from the same (e.g., the rumen) or related (i.e., from the guts and the manure of farm animals) habitats. Also the internal topologies of the respective groups are highly coincident (Fig. 1 and 2), which provides strong support for the idea 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 termite gut spe- cies (Table 1), although the results are probably affected by differ- ences in copy numbers of the 16S rRNA gene in *Methanosarcinales* and *Methanobacteriales* (1).

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 (Fig. 1 and 2, TC-1 and CC-1). They are part of the “intestinal cluster” of puta- tive methanogens that also comprises clones from the rumen (RCC) (58) and the human gut (40). More distant relatives are found in anaerobic digestors (VADIN), rice field soil (RC-III), sediments, and other terrestrial environments (TMEG-1 and -2). Since matching *mcrA* genes were obtained from most of these habitats, it is safe to assume that all these lineages are methano- genic.

The final piece of evidence for the methanogenic nature of the new lineage was provided by the study of Dridi et al. (12), which was published during the revision stage of the present study. They isolated and described a new genus and species of methanogens,

*M. luminyensis*, from human feces and reported that its 16S rRNA

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

gene sequence was most closely related to several clones of uncul- tured *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 phy- logenetic 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 (Fig. 1 and 2), which comprises most of the clones previously obtained from the diges- tive tracts of mammals. Instead, the isolate falls within the radia- tion of RC-III, where it clusters with clones from rice field soil (20,

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8) and a single clone previously obtained from the human gut (39).

The methanogenic character of euryarchaeota in RC-III had been suggested earlier by Kemnitz et al. (28), who observed that the abundance of RC-III clones in a methanogenic enrichment culture from rice field soil was reduced by the addition of bromo- ethanesulfonate (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 salt marsh samples (7). 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 the deeper-branching lineages are also methanogenic.

Interestingly, we observed a consistent and moderately sup- ported 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 (29). However, in view of the methanogenic properties of *M. luminyensis* (12) and our enrichment cultures and the cumulative evidence for the absence of methane oxidation in termite guts (47), 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, methano- genesis was strongly stimulated by the simultaneous supply of both H2 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 en- richment 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. luminy- ensis* (RC-III) and obligately H2-requiring methylotrophic me- thanogens from other lineages, like *Methanosphaera stadtmanae* (*Methanobacteriales*) and *Methanomicrococcus blatticola* (*Me- thanosarcinales*). Interestingly, such organisms have so far been isolated exclusively from the intestinal tracts of humans (12, 41) and cockroaches (55). It is likely that this mode of methanogenesis is an adaptation to the intestinal habitat.

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In a study of the archaeal diversity in the hindgut of the termite

*N. takasagoensis*, the relative abundance of uncultured *Thermo- plasmatales* (Fig. 1, clusters TC-1a and TC-2) increased when the animals were fed with xylan (42), a substrate that contains sub- stantial amounts of *O*-methylated glucuronic acid residues (49). 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 re- peatedly proposed that environmental clones distantly affiliated with the *Thermoplasmatales* represent a separate order of meth- anogens (15, 39), none of these studies provided enough evidence to substantiate this claim. Analysis of the entire set of sequences available to date clearly documents the diversity of the new lin- eage, 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* (12) and the enrichment cultures of strains MpT1 and MpM2 (this study). Based on this evidence, we propose the provisional name “*Methanoplasmatales*” for the en- tire deep-branching lineage of euryarchaeota outlined in Fig. 6. Although a first representative of the lineage has been isolated and described (12), 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 pres- ence 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 also use such combinations of methanogenic substrates for enrichments from other habitats. There are several other deep-branching lineages 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 (60). This is underlined by two stud- ies of archaeal diversity in termite guts that had failed to detect clones affiliated with “*Methanoplasmatales*.” In one case (46), this was most likely due to a mismatch in the reverse primer to the consensus sequence of “*Methanoplasmatales*,” whereas in the other case (11), the sequence of the forward primer differed slightly from that of the forward primer successfully used by Hara et al. (22).

It isstrikingthatthemajorityofthe *mcrA* genesofthe“*Methano- plasmatales*” have so far been retrieved only from intestinal sam- ples. The fact that there are only a few clones from other environ- ments 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 least to investigate their metabolic relationship to *Thermoplas- matales*, a clade of *Euryarchaeota* that may have experienced a secondary loss of the capacity for methanogenesis (3).

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