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Abstract

Methane (CH_4), a very potent greenhouse gas, is mainly produced biotically by methanogenic archaea as an end-product of their anaerobic respiration. Wetlands and ruminants are the major natural and anthropogenic CH_4 sources, respectively. Recently a novel, previously unrecognised, 7th order of methanogenic archaea, within the class of *Thermoplasmata*, was proposed - the *Methanoplasmatales* (Mp). Until now, only one Mp isolate could be obtained (from human feces), producing CH_4 from methanol (MetOH) and hydrogen (H_2). Furthermore, a metatranscriptomic study provided compelling evidence that rumen Mp reduce methylamines to CH_4. The main objectives of this work were (I) the investigation of the environmental distribution of Mp and (II) their cultivation from bovine rumen as basis for physiological and genomic characterisations.

Different wetland types (acidic/neutral; temperate/arctic) and feces of various animal species were screened for *Methanoplasmatales* with PCR by targeting two established phylogenetic marker genes: 16S rRNA and *mcrA* (encoding Methyl:Coenzmye M reductase, the key enzyme of methanogenesis). More than 42 PCR products were cloned, >240 clones sequenced, and phylogenetic trees were constructed. Mp 16S rRNA genes were detected in all temperate and arctic wetlands as well as in most investigated animal feces (elephant, cow, rat and tortoises). In all animals and in several wetlands *mcrA* genes affiliated with Mp were also detected. Phylogenetic analyses of both genes showed that the obtained sequences formed two distinct clusters; one dominated by animal sequences (animal cluster) and the other by wetland sequences (wetland cluster). The occurrence of two distinct Mp sequence clusters point to Mp ecotypes with special adaptations to their environment (endosymbiotic vs. free-living). However, further studies on abundances and activity of Mp in wetlands are necessary to assess their role in CH_4 formation in these environments.

For rumen enrichment cultures Mp medium was inoculated with bovine rumen fluid and incubated either with trimethylamine (TMA) or MetOH. Mp abundances and enrichments of subcultures were monitored via qPCR. Physiological tests, different procedures to increase Mp enrichments and metagenomic studies were performed on different stages of enrichment cultures. Initially, the microbiota of bovine rumen fluid consisted of less than 1 % *Methanoplasmatales*, nevertheless comprising about 40 % of all rumen methanogens. Sequencing showed that all Mp belonged to the animal cluster. The Mp could be enriched both with TMA (up to 34 % of prokaryotic community) and with MetOH (up to 52 %). Under both conditions more than 99 % of the methanogens consisted of Mp. Considerable Mp growth was only observed if cultures were supplemented with sterile filtered rumen fluid and H_2. Whenever bacteria were inhibited, no considerable Mp growth was observed. Metagenomic analyses showed that the Mp and the bacterial community composition

changed during the enrichment trials; after 13 transfers the Mp community was dominated by wetland cluster Mp (69 %). Two partial rumen Mp genomes (about 80 % completeness) could be obtained from a metagenome; one related to *Candidatus* Methanomethylophilus alvus (animal cluster Mp) the other to *Methanomassiliicoccus luminyensis* (wetland cluster Mp). Genes for methanogenesis from methylated compounds (MetOH, methylamines and dimethyl sulfide) were found in the genomes of *M. luminyensis* and *Ca.* M. alvus as well as in the two partial rumen Mp genomes. However, both partial rumen Mp genomes lack genes necessary for the reduction of TMA to CH_4. The loss of TMA utilization during the enrichment trials was also indicated by the loss of *mttB* (trimethylamine:corrinoid methyltransferase) genes (negative PCR amplification) and TMA measurements. Acetate was identified as possible carbon source for Mp due to the presence of acetyl-coenzym A synthetase and pyruvate:ferredoxin oxidoreductase genes in the partial genomes and the genomes of *M. luminyensis* and *Ca.* M. alvus. It is very likely that endosymbiotic Mp, like rumen Mp, live in close metabolic interactions (e.g. syntrophy) with other intestinal microorganisms, as indicated by the enrichment trials, which revealed a link between rumen Mp and rumen bacterial growth and a dependency of Mp growth on supplementation with sterile rumen fluid possibly containing specific metabolites essential for Mp. Rumen Mp utilizing methylamines seem to occupy a unique metabolic niche in the bovine rumen. The only other methanogenic group known to possess this metabolic capacity (*Methanosarcinales*) is usually not found in ruminants.

According to these results, *Methanoplasmatales* are widely distributed in wetlands and animal intestinal tracts and are one major methanogenic group in the bovine rumen, suggesting that Mp play an important role in global CH_4 formation. This makes them a potentially important target for CH_4 mitigation strategies, especially in ruminants. Rumen Mp appear to be H_2-dependent methylotrophic methanogens with a wide range of methylated compounds as electron acceptors and acetate as C source. To gain more and better knowledge on the eco-physiology and interactions of rumen *Methanoplasmatales* further studies and especially cultivation-independent approaches are necessary, as cultivation biases emerged during the enrichment attempts.

1. Introduction

1.1. Methane and its Role as Greenhouse Gas

Humans have influenced and altered their environment since they appeared and developed on planet Earth. We only start to realize how tremendous the scale and consequences of these changes are. Among others atmospheric concentrations of gases such as carbon dioxide, nitrous oxide and methane have considerably increased during the industrial era (*1*). According to ice core records the pre-industrial atmospheric methane concentrations varied between 350 ppb (glacial periods) and 750 ppb (interglacial periods) over the last 650 ky (*2*), with a global pre-industrial value of 715 ± 4 ppb from 1700 to 1800 (*3*). In comparison present-day atmospheric methane concentrations are more than twice as high, the Intergovernmental Panel on Climate Change (IPCC) reported global concentrations of 1803 ± 4 ppb (in 2011) in their Fifth Assessment Report, 2013 (*4*).

Methane - CH_4 - is a hydrocarbon with tetrahedral shape; it is a colourless and odourless atmospheric trace gas and yet very considerable. Methane is a greenhouse gas; it can absorb thermal infrared radiation emitted from Earth's surface and re-emit this radiation, which increases the heating of the surface. Atmospheric methane contributes to about 20 % of the warming since pre-industrial times (*5*). Accordingly, methane is an especially potent greenhouse gas. It has a 12-year atmospheric lifetime and a global warming potential 26 times higher than carbon dioxide (*6*, *7*, *8*). Hence, methane mitigation strategies are very important and of great interest (*9*, *10*). To accomplish viable mitigation strategies it is necessary to understand where, by whom and how methane is produced and emitted to the atmosphere.

1.2. Methane Sources, Methanogens and Methanogenesis

Besides non-biogenic methane sources (e.g. fossil fuel mining and burning, biomass burning and geological sources), mainly biogenic methane sources contribute to atmospheric CH_4 concentrations. More than 70 % of atmospheric methane emissions originate from biogenic sources, in particular from wetlands, livestock, rice agriculture, landfills, forests, oceans and termites, with wetlands being the major natural source (*1*). It is estimated that >50 % of global CH_4 emissions are derived from anthropogenic activities, with enteric fermentation by ruminant animals accounting for ~37 % of global human-caused CH_4 emissions (*11*). Therefore, ruminants are the major anthropogenic source of atmospheric methane.

Methanogenesis, the biological formation of significant amounts of methane, is restricted to archaea, which produce CH_4 as an end-product of their anaerobic respiration. All known methanogenic archaea belong to the phylum of Euryarchaeota, until recently forming six distinct orders, namely *Methanomicrobiales*, *Methanobacteriales*, *Methanosarcinales*, *Methanococcales*, *Methanocellales* and *Methanopyrales* (fig. 1). Despite their ecological and phylogenetic diversity, there are basically three different ways of methanogenesis: hydrogenotrophic (CO_2 reduction pathway), methylotrophic and acetoclastic methanogenesis. The majority of methanogenic archaea are hydrogenotrophic; they produce CH_4 by reducing CO_2 preferentially with H_2 (or alternatively formate or less frequently secondary alcohols) as electron donor. Some methanogens are methylotroph; they reduce methylated compounds (e.g. methanol, methylamines and methylated sulfides) to CH_4, whereas additional methyl-groups are oxidized to CO_2 via reverse hydrogenotrophic methanogenesis to generate the required electrons. Methylotrophy is restricted to the order of *Methanosarcinales* except for some *Methanobacteriales* (*Methanosphaera* species). Acetoclastic methanogenesis, the third type of methanogenesis, is less common (restricted to two *Methanosarcinales* genera: *Methanosarcina* and *Methanosaeta*); acetate is split, the methyl-group is reduced to CH_4 and the carboxyl-group is oxidized to CO_2 to produce the reducing equivalents. For more details on methanogenic pathways see the review by Liu & Whitman (*12*). In all three methanogenic pathways the last step of methane formation is carried out by Methyl:Coenzyme M reductase (Mcr), the key enzyme of methanogenesis. Therefore, the gene encoding for the α-subunit of Mcr, *mcrA*, is used as functional and phylogenetic marker for methanogens (*13*, *14*).

1.3. *Methanoplasmatales* - a Novel Methanogenic Order

Very recently a seventh order of methanogenic Euryarchaeota, within the class of *Thermoplasmata*, was proposed (*14*). For years uncultured *Thermoplasmata* 16S rRNA gene sequences were obtained from various habitats and it was suggested these sequences belong to methanogens. First they were discovered in marine environments (*15*, *16*), later obtained from an anaerobic digestor (*17*) and from rice fields (*18*). Uncultured *Thermoplasmata* also occurred in the intestinal tracts of various animals, mainly in ruminants, for example in cow, sheep, reindeer and yak, but also in non-ruminants like wallaby, termite and cockroach (*14*, *19 - 23*). The recently described species *Methanomassiliicoccus luminyensis* isolated from human feces provided the ultimate proof for methanogens within the *Thermoplasmata* (*24*). Also recently, Paul et al. (*14*) succeeded in enriching methanogenic *Thermoplasmata* from arthropod guts and suggested the novel order of methanogens to be named *Methanoplasmatales*. The phylogenetic relationship between previously known methanogens and *Methanoplasmatales* is shown in figure 1.

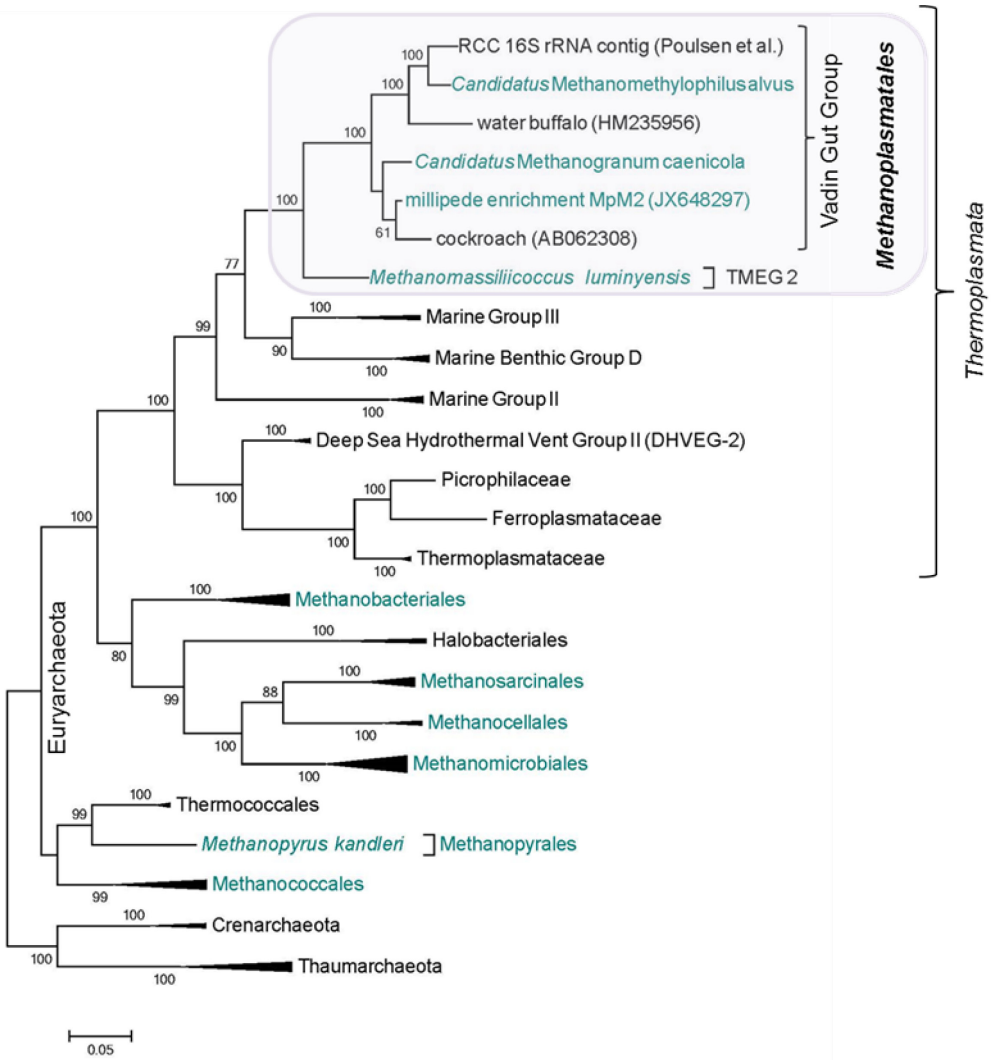


Figure 1. 16S rRNA gene tree showing the phylogenetic relationship of the six established methanogenic lineages within the Euryarchaeota and the previously discovered *Thermoplasmata* related methanogens - the *Methanoplasmatales*. Tree is based on nearly full-length 16S rRNA gene sequences (>1300 nt); see section 2.1.4. Methanogens are highlighted in blue.

Besides the currently sole isolate of *Methanoplasmatales*, *Methanomassiliicoccus luminyensis*, few genomes (e.g. of *Candidatus* Methanomethylophilus alvus and *Candidatus* Methanogranum caenicola) of enriched cultures and enrichments from termite (MpT1) and millipede gut (MpM2) were published (*14*, *25*, *26*). *Ca*. M. alvus, *Ca*. M. caenicola and millipede enrichment culture MpM2 cluster within a group of *Thermoplasmata* known as Vadin Gut Group, whereas *M*. *luminyensis* clusters within the Terrestrial Miscellaneous Group 2 (TEMG 2). This suggests that at least these two groups represent the novel methanogenic order within the class of *Thermoplasmata* - the *Methanoplasmatales* (in the following abbreviated Mp). Further uncultured *Thermoplasmata* 16S rRNA sequences are widespread and diverse, forming different clusters, e.g. Marine Group II and III, Deep Sea Hydrothermal Vent Group 2 (DHVEG-2), Marine Bentic Group D and DHVEG-1 and Terrestrial Miscellaneous Groups (1 - 11). At the moment, it is not known if and which of these groups contain also methanogenic members.

Methanoplasmatales are yet poorly characterised, especially because there is currently just one sole isolate of this novel methanogenic group available, *M. luminyensis*. *M. luminyensis* grows on methanol and H_2 (*24*) and similar finding were made for the other enrichment cultures (*14*, *25*, *26*). Poulsen et al. (*27*) provided compelling evidence that rumen *Methanoplasmatales* utilize methylamines (mono-, di-, and trimethylamine) for methanogenesis. Incubating rumen fluid with trimethylamine (TMA) led to a 3-fold increase of *mttB* genes and *Methanoplasmatales* 16S rRNA genes, while 16S rRNA genes of other methanogens showed no TMA-dependent increase. The *mttB* gene encodes for the Trimethylamine:corrinoid methyltransferase, an essential protein in TMA-utilizing methylotrophic methanogens, and, therefore, a possible functional marker (*27*).

Methylotrophy of *Methanoplasmatales* was very recently also suggested by genome analyses (*28*); *M. luminyensis* and *Ca.* M. alvus contain genes for the reduction of methanol, methylamines and dimethyl sulfide to CH_4.

1.4. Role of *Methanoplasmatales* in Major CH_4 Producing Environments

Wetlands. Less than 9 % of the terrestrial earth surface is covered by wetlands. Estimates of total wetland area range between 5.3 million km^2 to 12.8 million km^2 depending on the definition of wetlands. Wetlands provide ecosystem services of global importance by supporting biodiversity, improving water quality, acting as flood control and they play an important role in the global carbon cycle, e.g. by acting as a carbon sink (*29*). However, wetlands are the major natural source of global atmospheric methane emissions (*1*). Phylogenetic diversity of wetland methanogens is very high; members of all well-established methanogenic lineages (except *Methanopyrales*) were found in different wetland types (see *30* - *36*). As already mentioned above *Thermoplasmata* related 16S rRNA gene sequences, possibly representing members of the novel methanogenic group of *Methanoplasmatales*, occur in a great variety of anaerobic habitats. Whether or not *Methanoplasmatales* are widely distributed in different wetland types (e.g. arctic/temperate/acidic/neutral wetlands like peat soils, bogs and fens) and play an important role in methane formation in these habitats has not been assessed yet.

Ruminants. Methane is derived from a complex anaerobic degradation pathway of plant biomass by the ruminal microbiota, with the terminal group being methanogenic archaea, producing CH_4 that is emitted by the cows mainly via the breath (*37*, *38*). Two prominent methanogenic orders in the rumen are the *Methanomicrobiales* and *Methanobacteriales*. All isolates obtained from bovine rumen to date fall into those two orders. *Methanomicrobiales* form CH_4 using hydrogen (H_2) and formate as electron donors and carbon dioxide (CO_2) as electron acceptor; *Methanobacteriales* predominately utilize H_2 to reduce CO_2 to CH_4 with the exception of *Methanosphaera* which uses H_2 to reduce methanol (*12*). Recently the rumen methanogenic community has been identified as more diverse than previously assumed. Uncultivated Euryarchaeota named 'Rumen Cluster C' (RCC; *39*) represent a third major group besides *Methanobacteriales* and *Methanomicrobiales* constituting up to 50 % (80 % in *27*) of the archaeal communities in the rumen of cattle (reviewed in *40*). A methylotrophic methanogenic lifestyle of RCC was shown by an *in vivo* metatranscriptomic study of scientists from the Department of Ecogenomics and Systems Biology, University of Vienna and the Department of Animal Science, University of Aarhus, Denmark (Poulsen et al.; *27*). With the isolation of *M. luminyensis*, the methanogenic nature of RCC was confirmed; they represent a cluster within the novel methanogenic order of *Methanoplasmatales*. Poulsen et al. (*27*) identified Mp as high potential targets for methane mitigation strategies and showed an inhibition of RCC Mp after dietary rapeseed oil supplementation. Methane mitigation strategies are critical to ensure environmentally and economically sustainable ruminant milk and meat production since practical and economic methods for decreasing ruminant enteric CH_4 emissions have yet to be developed. Until now, no representatives of the RCC *Methanoplasmatales* have been isolated from rumen and their metabolism, eco-physiology, and (metabolic) interactions with other ruminal microorganism are poorly understood.

1.5. Aims of the Study

The role of *Methanoplasmatales* in global atmospheric methane emissions is currently not known. Furthermore, their environmental distribution and their eco-physiology are incompletely characterised and understood. This study started soon after the novel order of *Methanoplasmatales* was proposed and the isolation of *M*. *luminyensis* was published, with the two major aims to **(I)** investigate the environmental distribution of *Methanoplasmatales* in wetlands and animal intestinal tracts and **(II)** cultivate representative(s) from bovine rumen fluid for further in-depth physiological characterisation.

(I) A screening for *Methanoplasmatales* in temperate and arctic peat soils (bogs and fens) and in several animal species was conducted, to assess their environmental distribution and infer about their possible contribution to methanogenesis in these habitats. For this purpose two established phylogenetic marker genes (16S rRNA and *mcrA)* were employed and the usability of the *mttB* gene as a functional marker of TMA-utilizing methylotrophic methanogens was explored.

(II) Based on the study of Poulsen et al. (*27*) the following hypothesis underlay my work on cultivating *Methanoplasmatales* from bovine rumen fluid: TMA serves as electron donor and electron acceptor for RCC *Methanoplasmatales*, or at least as electron acceptor whereby H_2 would be the electron donor. To test this hypothesis, enrichment trials on cow rumen fluid were conducted. TMA as substrate provides the possibility to select specifically for novel rumen Mp because only one other methanogenic order, the *Methanosarcinales*, are capable of CH_4 formation from methylamines and they are not known to be harboured by cattle (*12*). If the applied procedures were resulting in enrichments and/or isolates of *Methanoplasmatales,* a (meta-)genomic, morphological and physiological characterisation was planned.

2. Materials and Methods

2.1. Environmental Distribution of *Methanoplasmatales*

2.1.1. Animal & Wetland Samples

Animal fecal samples were received either from the Viennese Zoo (Schönbrunn) or the University of Vienna. The temperate soil sample originated from the Vienna Woods. Cow rumen fluid was obtained from fistulated cows housed at the Aarhus University, Denmark and the University of Veterinary Medicine Vienna (for details see section 2.2.1 and following). Arctic peat samples were derived from a study by Tveit et al. (*30*). All temperate wetland samples were kindly provided by Alexander Loy and Michael Pester (*41*). Table 2.1 summarizes all relevant information about the various samples and their origin.

Table 2.1. Characteristics of all samples (animals, temperate soil, arctic peat soils, temperate wetlands) screened during the investigations on the environmental distribution of *Methanoplasmatales*.

	sample	type	pH	origin
animals	kangaroo	feces	-	Viennese Zoo
	cricket	feces	-	University of Vienna
	elephant	feces	-	Viennese Zoo
	marmoset	feces	-	University of Vienna
	giant tortoise	feces	-	Viennese Zoo
	tortoise	feces [a]	-	Viennese Zoo
	rat	feces [a]	-	Viennese Zoo
	cow	cow rumen fluid [a]	-	University of Vienna
	AUT RF	cow rumen fluid	-	Univ. of VetMed Vienna
	DK RF [b]	cow rumen fluid	-	Aarhus University, Denmark
temperate soil	Vienna Woods	soil	-	Vienna Woods
arctic peat	Knudsenheia	peat	5.5 ± 0.2	Svalbard, Norway
	Solvatn	peat	5.2 ± 0.1	Svalbard, Norway
temperate wetlands	AUT peat 1	fen [a]	4.9 ± 0.2	Roßbrand
	AUT peat 2	fen [a]	4.9 ± 0.2	Roßbrand
	AUT peat 3	fen [a]	4.9 ± 0.8	Roßbrand
	AUT peat 4	bog [a]	5.3 ± 0.1	Große Heide
	AUT peat 5	bog [a]	5.3 ± 0.2	Große Heide
	AUT peat 6	bog [a]	4.7 ± 0.4	Schremser Hochmoor
	AUT peat 7	ferrous biofilm [a]	-	Roßbrand
	AUT peat 8	fen [a]	7.3 ± 0.1	Schallhof
	AUT peat 9	fen/wet meadow [a]	7.6 ± 0.0	Berndorf
	AUT peat 10	fen/wet meadow [a]	7.6 ± 0.0	Berndorf
	IT peat 1	bog [a]	4.0 ± 0.1	Krähmoos
	IT peat 2	fen [a]	4.3 ± 0.1	Rasner Möser
	IT peat 3	fen [a]	4.3 ± 0.1	Rasner Möser
	GER peat 1	fen [a]	acidic	Schlöppnerbrunnen

AUT, Austrian; DK, Danish; RF, rumen fluid; IT, Italian; GER, German; (-), pH not determined/known

[a] provided DNA extracts; feces: QIAamp DNA Stool Mini Kit (QIAGEN); wetlands: Power Soil DNA kit (MoBio Laboratories)

[b] rumen fluid incubated with trimethylamine (TMA); provided by Morten Poulsen; Aarhus University, Denmark

2.1.2. DNA Extraction & PCR Amplification

Arctic peat, soil and feces samples were weighed into Lysing Matrix Tubes E, MP Biomedicals (between 0.25 and 0.37 g). DNA was extracted using a phenol/ chloroform/isoamyl alcohol protocol. First, cells were chemically lysed by adding 500 µL CTAB/phosphate buffer (120 mM, pH 8) and suspending the samples. 500 µL phenol/chloroform/isoamyl alcohol (25:24:1) was added, followed by mechanical lyses via bead beating (30 seconds; 5 ms^{-1}; FastPrep®-24, MP Biomedicals). Samples were cooled on ice for 2 minutes and after centrifugation (10 minutes; full speed) the supernatant (aqueous phase, containing DNA) was extracted with equal volume of chloroform/isoamyl alcohol (24:1). After a second centrifugation step (5 minutes; full speed), DNA from supernatant was precipitated by mixing with PEG/NaCl solution, followed by two hours of incubation at room temperature. After 30 minutes centrifugation DNA was pelleted, supernatant was removed and DNA pellets were washed with ice-cold ethanol (70 %). Ethanol was removed by centrifugation and pellets were dried before re-suspended in 50 µL 1x TE buffer (pH 7.5). DNA extracts were checked by agarose gel electrophoresis, quantified photometrically (NanoDrop®, ND-1000, peqlab) and stored at -20 °C.

Methanoplasmatales (Mp) 16S rRNA, *mcrA* and *mttB* genes were PCR amplified using specific primers; additionally broader *mcrA* primers targeting all methanogens were used (tab. 2.2). PCR reactions were mixed as follows, 10 µL 5x Green GoTaq® Flexi Buffer (Promega), 4 µL MgCl (25 mM, Fermentas), 1 µL dNTPs (10 mM, Fermentas); 1 µL forward + 1 µL reverse primer (25 µM, Eurofins MWG Operon), 0.25 µL BSA (Thermo Scientific), 0.25 µL DreamTaq (5 $u\mu L^{-1}$, Thermo Scientific), 31.5 µL H_2O and 1 µL DNA extract. DNA extract from cow rumen fluid, previously confirmed to contain *Methanoplasmatales*, was used as positive control, MQ water as negative control, respectively. PCR was performed with a T-Professional Thermocycler (biometra), program details see table 2.3. Afterwards, PCR products were checked for correct fragment length via agarose gel electrophoresis and purified using QIAquick PCR Purification Kit (QIAGEN) following the manual. DNA was eluted in 30 µL Elution Buffer (EB) and stored at -20 °C.

Table 2.2. Primers, used in this study, to screen for *Methanoplasmatales* (Mp).

primer	sequence	length	target gene	reference
AS1 AS2	CAG CAG TCG CGA AAA CTT C AAC AAC TTC TCT CCG GCA	485 nt	16S rRNA Mp	(*42*)
Tp-mcrA-F Tp-mcrA-R	GAY RAC ATC CTB GAR GAY TA RTC GWA WCC RTA GAA TCC GAG	360 nt	*mcrA* Mp	in submission[a]
mlas-mod For mcrA-rev	GGY GGT GTM GGD TTC ACM CAR TA CGT TCA TBG CGT AGT TVG GRT AGT	470 nt	*mcrA* all methanogens	(*43*)
mtt for 1 mtt rev 2 mod	GYR CTT CMA CCA CAT CGA CC CHC CCA STC KCC GAK CAT G	800 nt	*mttB* Mp	(*27*)

[a] Petersen et al., Methane mitigation and methanogen community changes with acidification of pig slurry

Table 2.3. PCR programs[a] for the primers used to investigate the environmental distribution of *Methanoplasmatales*.

primers	denaturation	annealing	elongation	cycles
AS1/AS2	95°C; 30 sec	60°C; 45 sec	72°C; 45 sec	34
Tp-mcrA-F/Tp-mcrA-R	95°C; 30 sec	52°C; 60 sec	72°C; 45 sec	36
mlas-mod For/ mcrA-rev	95°C; 30 sec	60°C; 45 sec	72°C; 45 sec	34
mtt for 1/ mtt rev 2 mod	95°C; 30 sec	48°C; 60 sec	72°C; 60 sec	36
T7/SP6	95°C; 30 sec	55°C; 45 sec	72°C; 45 sec	32

[a] all programs started with lid pre-heating (95 °C; 5 min) and ended with a final elongation step (72 °C; 5 min)

2.1.3. Clone Library Construction & RFLP Analysis

To generate clone libraries, PCR products were cloned using pGEM®-T Vector System I (Promega) and chemically competent *E. coli* cells (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen). Via ampicillin resistance and blue-white screening clones containing both, vector and PCR product, were identified. Ligation reactions were prepared as follows, 5 µL 2x Rapid Ligation Buffer, 3 µL purified PCR product, 1 µL pGEM®-T Easy Vector, 1 µL T4 DNA Ligase. Ligations were incubated one hour at room temperature and overnight at 4 °C until transformation. 2 µL of ligation reaction were added to 25 µL of *E. coli* cells. After 10 minutes of incubation on ice cells were heat shocked (42 °C for 35 seconds) and immediately cooled again on ice (2 min). To recover the cells, 250 µL of SOC medium were added and cells were shaken (850 rpm; 30 minutes) at 37 °C. Subsequently the cells were plated on LB agar plates containing ampicillin (100 mgL^{-1}), IPTG (0.5 mM) and X-Gal (80 $mgmL^{-1}$) and incubated overnight at 37 °C. At least eight clones per sample were picked and colony PCR was performed to amplify the cloned inserts for further RFLP analysis and sequencing. PCR reactions were prepared as described above, using standard primers (T7 and SP6); BSA was substituted by water (PCR program see tab. 2.3). Colony PCR products were checked for correct fragment length via agarose gel electrophoresis. QIAquick PCR Purification Kit (QIAGEN) was used for purification, DNA was eluted in 50 µL EB. Cloned, purified 16S rRNA, *mcrA* and *mttB* gene fragments were quantified photometrically (NanoDrop®, ND-1000, peqlab) and stored at -20 °C until further analysis.

RFLP analysis was performed to identify different clones of each sample. Colony PCR products were digested with HpyCH4V (New England BioLabs); optimally between 500 and 600 ng DNA were restricted. 20 µL reactions were prepared containing 2 µL NEBuffer 4 (New England BioLabs), 0.2 µL restriction enzyme (5 $uµL^{-1}$), DNA and H_2O. The restriction was performed at 37 °C for 3 hours and stopped by increasing the temperature to 65 °C for 20 minutes. The entire reactions were loaded on an agarose gel (3 %) and the gel run for about 2½ hours at 100 V to separate the bands properly. To minimize costs and effort only clones with different restriction patterns were chosen for sequencing. However, if there were less than eight clones, RFLP was omitted and all isolates were sent for sequencing.

2.1.4. Sequencing, Sequence Analyses & Phylogenetic Tree Construction

Clones were sequenced by LGC genomics. The obtained sequences were screened for vector contaminations using vecScreen (NCBI). The contaminations were removed in BioEdit (*44*) manually. Sequences with unclear and poor chromatograms were excluded from further analyses. All remaining sequences were verified with BLAST (*45*), 16S rRNA and *mttB* sequences with blastn, *mcrA* sequences with blastx. Sequences were rejected if not matching to expected sequences. Further, all 16S rRNA sequences were taxonomically classified with CREST (*46*) using standard settings. Operational taxonomic units (OTUs) were defined with 98 % sequence identity. 16S rRNA sequences with ≥98 % sequence identity were clustered and a representative sequence of each cluster was identified by CD-HIT Suite, cd-hit-est (*47*). Except "sequence identity cut-off" of 98 % all other settings remained unchanged. McrA sequence identity matrix was compiled by BioEdit (calculated on amino acid level), sequences with >98 % identity were clustered.

Sequences were aligned with ClustalW, implemented in BioEdit, without changing the standard settings. Obtained 16S rRNA sequences were aligned with a set of reference sequences containing cultured and uncultured *Thermoplasmata* 16S rRNA sequences as well as with reference sequences from members of all methanogenic lineages and other Euryarchaeota. *McrA* sequences were translated into amino acid sequences (BioEdit) before they were aligned with a reference dataset, comprising MrcA protein sequences from known methanogens and of unknown origin. 16S rRNA and McrA reference sequences were obtained from the NCBI (*48*) and SILVA (*49*) database. 16S rRNA phylogenetic trees were built with MEGA5 (*50*) after manually refining and cutting the alignments in BioEdit. Maximum likelihood trees were constructed using standard settings and 100 bootstrap replications. First, an overview tree was built using almost full-length 16S rRNA gene sequences (>1300 nt; alignment: 1203 nt positions) to depict the positions of all established methanogenic lineages and *Thermoplasmata* subgroups within the Euryarchaeota (see figure 1.1). A phylogenetic tree containing cultured/uncultured *Thermoplasmata* sequences from the databases and obtained 16S rRNA gene sequences was built based on a shorter alignment (517 nt positions). McrA phylogenetic tree was constructed similarly; based on a McrA amino acid sequence alignment with 104 amino acid positions.

2.2. Isolation/Enrichment of *Methanoplasmatales* from Bovine Rumen

2.2.1. Rumen Fluid

Cow rumen fluid for initial enrichment cultures was obtained from a fistulated Braunvieh (German: “brown cattle”), called Ridi, housed at the University of Veterinary Medicine Vienna, Austria. Ridi was born in January 2004 and has been feeding on hay and dairy concentrate. After harvesting, cow rumen fluid was immediately centrifuged 5 minutes at 1000 rpm to remove bigger particles. Afterwards, serum bottles containing slightly modified *Methanobrevibacter* medium (complete medium; see tab. 2.5), like the medium used for isolation of *M. luminyensis* (*24*), were inoculated with the rumen fluid. Residual rumen fluid was centrifuged (to remove particles), sterile filtered through a 0.2 µm cellulose acetate membrane filter (VWR International) and used as a supplemental substrate during the cultivation experiments. Initial rumen fluid was provided by Kathrin Deckardt, further supplemental rumen fluid by Johannes Lorenz Khol, both researchers at the University of Veterinary Medicine Vienna, Austria.

2.2.2. Experimental Setup

General workflow of enrichment experiments is depicted in figure 2.1. Serum bottles containing 30 mL medium were supplemented with substrates and inoculated (inoculation volume: 10 - 20 %; inoculation start = t_0). Cultures were incubated under anaerobic conditions at 37 °C in the dark to simulate cow rumen environment. Right after the inoculation (t_0) and at the end of incubation (t_n) cultures were sampled and DNA was extracted. Cultures were screened for *Methanoplasmatales* (Mp), other methanogens and bacteria by quantitative PCR (qPCR). 16S rRNA gene copies mL^{-1} culture were determined at both time-points (t_0 & t_n). Positively enriched cultures and cultures thought to be leading to Mp enrichments were subcultured.

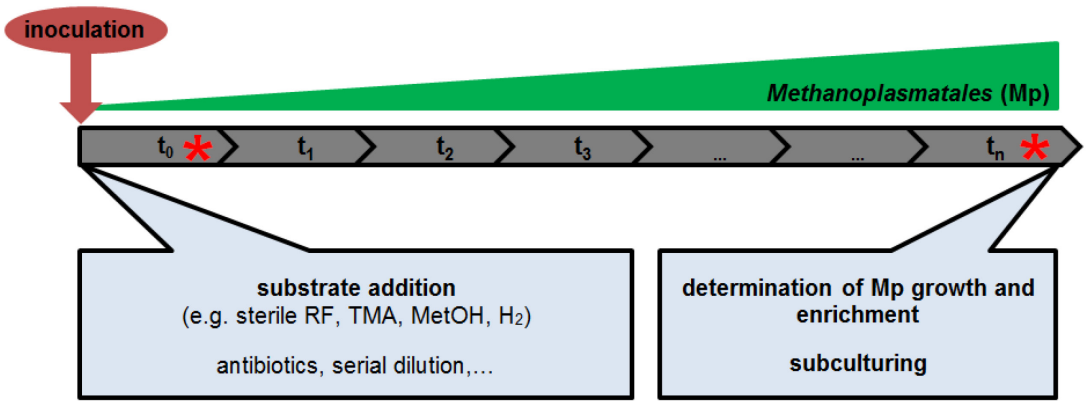


Figure 2.1. General workflow. At t_0 cultures were inoculated and substrates were supplemented. Red asterisks show sampling points. Quantitative PCR (qPCR) was performed at t_0 and t_n, to determine 16S rRNA gene copy numbers of *Methanoplasmatales*, other methanogens and bacteria. Several times substrate supplementations, enrichment procedures and qPCR were also performed at other time points during the incubation. RF, rumen fluid; TMA, trimethylamine; MetOH, methanol.

All working steps were performed in an anaerobic chamber (COY Laboratory Products; type COY A). Gas-, substrate- and antibiotic- additions as well as inoculation and sampling were done using sterile syringes.

2.2.3. Initial Cultures, Subculturing and Enrichment Conditions

Initially four serum bottles were inoculated with either 20 % or 100 % fresh cow rumen fluid and incubated with trimethylamine (TMA; 10 mM) under an atmosphere containing either N_2 gas or a mixture of N_2, H_2 and CO_2. Subculturing was performed many times (up to 35 transfers); at the beginning fresh medium was inoculated with 20 % of previous cultures, but after 16S rRNA gene copy numbers reached 10^7 mL^{-1}, inoculation volume was changed to 10 %. However, during the whole enrichment experiments inoculation volumes changed repeatedly. Generally cultures were incubated about one week, but incubation time varied from 4 days to a onetime maximum of 52 days. Different media, atmospheres, substrates as well as antibiotics and different enrichment procedures were applied to favour Mp growth and enrichments. Table 2.4 gives an overview of all varying parameters and enrichment trials, which were combined with each other in many different ways. Media, substrates and all applied procedures are described in the following section.

Table 2.4. Overview[a] of all varying parameters and enrichment trials during the rumen *Methanoplasmatales* cultivation attempts.

media[b]	atmospheres	substrates[c]	further enrichment trials
complete medium	N_2 (100 %)	sterile RF	serial dilutions
			filtration
minimal medium	N_2:H_2 (60:40)	TMA	addition of antibiotics
	H_2:CO_2 (80:20)		
extreme minimal medium	N_2:CO_2:H_2 (70:20:10)	MetOH	hop extract
			serial dilutions in solid medium

[a] for details see text; TMA, trimethylamine; MetOH, methanol; RF, rumen fluid

[b] for details see table 2.5

[c] all substrates were sterile filtered, deoxygenated and stored in sealed serum bottles at 4 °C; substrate concentrations (added per week): 7 - 10 % sterile rumen fluid; 10 - 60 mM TMA; 40 mM MetOH

2.2.3.1. Media

The initial medium and subsequently used modifications are described in table 2.5. Medium was prepared as in table 2.5; pH of the medium (excluding $NaHCO_3$, Na_2S .$9H_20$, sodium formate, vitamin solution) was adjusted to 7.5 with 10 M NaOH. After autoclaving, oxygen was removed from medium and headspace by flushing with N_2 gas with constant stirring. The medium was dispensed into serum bottles (30 mL each) and sealed with a thick rubber stopper in the anaerobic chamber. Serum bottles were autoclaved once more and sterile filtered supplements ($NaHCO_3$, Na_2S .$9H_20$, sodium formate, vitamin solution) were added.

Table 2.5. Composition[a] of anaerobic media used to enrich rumen *Methanoplasmatales*.

contents [L^{-1}]	complete medium[b]	minimal medium	extreme minimal medium
KH_2PO_4	0.5 g	0.5 g	0.5 g
$MgSO_4 .7H_2O$	0.4 g	0.4 g	-
NaCl	5 g	5 g	5 g
NH_4Cl	1 g	1 g	-
$CaCl_2 .2H_2O$	0.05 g	0.05 g	0.05 g
sodium acetate	1.6 g	1.6 g	-
cysteine-HCl	0.5 g	0.5 g	-
yeast extract	1 g	-	-
trypticase peptone	1 g	-	-
trace element solution[c]	1 mL	1 mL	1 mL
tungstate-selenite solution[d]	2 mL	2 mL	2 mL
$NaHCO_3$	4 g	4 g	4 g
$Na_2S .9H_20$	0.5 g	0.5 g	0.5 g
sodium formate	2 g	2 g	-
vitamin solution[e]	1 mL	1 mL	1 mL

[a] for preparation see text

[b] *Methanobrevibacter* medium, as used for *M. luminyensis* isolation (*24*), was slightly modified and termed complete medium; minimal and extreme minimal medium are further modifications of complete medium

[c] containing (L^{-1}) 8 mL HCl (12.5 M), 30 mg H_3BO_3, 100 mg $MnCl_2 .4H_2O$, 190 mg $CoCl_2 .4H_2O$, 24 mg $NiCl_2 .6H_2O$, 2 mg $CuCl_2 .2H_2O$, 144 mg $ZnSO_4 .7H_2O$, 36 mg $Na_2MoO_4 .2H_2O$, 987 mL dist. H_2O

[d] containing (L^{-1}) 0.4 g NaOH, 2 mg Na_2SeO_3 and 4 mg $Na_2WO_4 .5H_2O$

[e] containing (L^{-1}) 0.02 g biotin, 0.02 g folic acid, 0.1 g pyridoxine HCl, 0.05 g riboflavin, 0.05 g thiamine HCl, 0.05 g nicotinic acid, 0.05 g DL pantothenic acid, 0.05 g p aminobenzoic acid, 2 g choline chloride, 0.01 g vitamin B_{12}

2.2.3.2. Atmospheres & Substrates

Atmospheres. Used atmospheres were chosen based on recent literature on Mp. Most cultures were grown under an atmosphere containing 70 % N_2, 20 % CO_2 and 10 % H_2 or just N_2 gas, additionally atmospheres containing more than 10 % H_2 were tested (tab. 2.4).

Substrates. Serum bottles containing 30 mL medium were supplemented with substrates right before the inoculation (t_0). Sometimes additional substrates were added during incubation. Cultures were incubated using either one of the following substrates or a combination: sterile filtered rumen fluid (7 - 8 %); TMA (10 - 60 mM); methanol (40 mM). Additionally, H_2 and CO_2 were provided as substrates (see above).

2.2.4. Further Enrichment Trials

2.2.4.1. Serial Dilutions & Filtration

Serial Dilutions. Ten-fold serial dilutions (10 % → 1 % → 0.1 % → 0.01 % inoculum) were conducted several times.

Filtration. Once, inoculum was filtered using a 1.2 µm sterile-EO filter (Sartorius AG).

2.2.4.3. Antibiotics & Hop Extract

Antibiotics. Several subcultures grown on either complete or minimal medium were treated with antibiotics. Number of used antibiotics and concentrations varied from single ampicillin (50 mgL^{-1}) or kanamycin (25 mgL^{-1}) additions once a week, to a combination of ampicillin, kanamycin and streptomycin (100/50/100 mgL^{-1}) given four times a week. Further, different other combinations and concentrations of antibiotics were tested (tab. 2.6).

Hop Extract. Besides antibiotics, hop extract (plant extract from common hop; *Humulus lupulus*) was added to inhibit bacterial growth. Hop extract is known to have antimicrobial activity, among others against different *Clostridium* species (*51*). Hop extract and all antibiotics were sterile filtered, deoxygenated and stored in sealed serum bottles at 4 °C.

Table 2.6. Different antibiotic-treatments applied to inhibit rumen bacteria.

antibiotics	concentrations [mgL^{-1}]	addition ($week^{-1}$)
ampicillin	50	1 x
kanamycin	25	1 x
ampicillin + kanamycin	50/25	1 x
ampicillin + kanamycin + streptomycin	100/50/100	1/2/4 x
ampicillin + kanamycin + streptomycin + chloramphenicol	100/100/100/50	1 x

2.2.4.3. Serial Dilutions in Solid Medium

Serial dilutions in solid medium were one additional approach to cultivate rumen Mp. For this purpose 20 mL cultivation tubes containing 3 mL 4-%-agar were prepared. Agar was washed three times, autoclaved and deoxygenated before suspended into the tubes inside the anaerobic chamber. Afterwards, agar tubes were autoclaved once again and kept on 80 °C, to stay liquid. Concurrently, a ten-fold serial dilution was prepared in cultivation tubes, beginning with a pure liquid culture grown 8 days on minimal medium, 45 mM TMA, N_2:CO_2:H_2 (70:20:10) atmosphere and containing nearly 10^7 Mp 16S rRNA gene copies mL^{-1}. Dilutions were prepared from 10^{-1} - 10^{-6} containing about 10^6 - 10^1 Mp 16S rRNA gene copies mL^{-1}, dedicated minimal medium was supplemented with 15 mM TMA and 8 % sterile filtered rumen fluid. 7 mL of each dilution were added to 3 mL agar tubes and immediately mixed gently. Afterwards, tubes were either rolled on a cooled work space or just laid down at an angel until the mixture solidified to maximize the solid medium surface. After solidification, cultivation tubes were incubated several weeks at 37 °C.

2.2.5. TMA Measurements

Once, TMA concentrations of one diagnostic culture during 15 days of incubation and several TMA additions were measured using direct infusion Orbitrap MS (*27*). The measurements were performed by Lena Fragner from the Molecular Systems Biology Division, part of the Department of Ecogenomics and Systems Biology, University of Vienna.

2.2.6. Microscopy

Enrichment cultures were scanned under the microscope (Nikon Eclipse 50i); quantities and diversity of different morphologies were monitored. Besides, coenzyme F_{420} dependent autofluorescence was tested by exciting samples with ultraviolet light (as used to excite the DNA stain DAPI). At one point samples were stained with DAPI.

2.2.7. DNA Extraction

DNA was extracted from 1 mL culture samples, taken at the beginning and at the end of the incubations. The samples were centrifuged 8 minutes, supernatant (900 µL) was removed and stored at -20 °C for any further investigations. DNA of the pellet was extracted using QIAamp DNA Stool Mini Kit (QIAGEN), with chemical cell lysis and silica membrane based DNA purification. Protocol was slightly modified. Lysing Buffer was added to pelleted culture samples. Lysing temperature was increased to 95 °C, helping to lyse cells, e.g. gram-positive bacteria, which are rather difficult to lyse. Eventually DNA was eluted in 100 µL low-salt elution buffer. DNA extracts were stored at -20 °C.

2.2.8. Quantitative PCR – Growth and Enrichment Monitoring

Copy numbers of *Methanoplasmatales*, other methanogenic and bacterial 16S rRNA genes were determined by quantitative PCR (qPCR). Three specific primer pairs were used (tab. 2.7); qPCR reactions were mixed as follows, 10 µL QuantiFast SYBR Green PCR mix (QIAGEN), 1 µL forward and 1 µL reverse primer (0.25 µM; Fermentas), 7 µL H_2O and 1 µL of DNA extract. Standards were provided by Clarissa Schwab. Linearized plasmids containing PCR amplified Mp 16S rRNA genes and long fragments of PCR amplified bacterial and non-Mp 16S rRNA genes served to determine standard curves.

Table 2.7. Quantitative PCR primers targeting 16S rRNA genes of *Methanoplasmatales* (Mp), other methanogens (oM) and bacteria.

primer	sequence	target gene	reference
AS1 AS2	CAG CAG TCG CGA AAA CTT C AAC AAC TTC TCT CCG GCA	16S rRNA Mp	(*42*)
Meg915For 1100 AR	TGG GTC TCG CTC GTT G RTC GWA WCC RTA GAA TCC GAG	16S rRNA oM	(*52*)* (*53*)
Eu For EuREv	CGG YCC AGA CTC CTA CGG G TTA CCG CGG CTG CTG GCA C	16S rRNA bacteria	(*54*)

*modified by Clarissa Schwab

Quantitative PCR was performed using a Mastercycler ep realplex (Eppendorf). The same qPCR programme was used for all three reaction mixes, starting with 5 minutes initial denaturation (95 °C), followed by 40 cycles of denaturation (95 °C; 15 s), annealing (60 °C; 15 s) and elongation (72 °C; 15 s), ending with melting curve analysis (95 °C, 15 s; 60 °C, 15 s; 20 minutes melting curve and 95 °C, 15 s). Correct lengths of qPCR products were spot-checked by agarose gel electrophoresis. Efficiencies of all qPCR runs, analysed in the following, were higher than 95 %.

As qPCR was performed at incubation start (t_0) and after several days of incubation (t_n), growth of *Methanoplasmatales*, other methanogens and bacteria could be estimated (t_n / t_0 = x-fold increase). Moreover, Mp enrichments in percentage of methanogenic and prokaryotic community (methanogens + bacteria) were determined by following equations:

$$\%\ Mp\ of\ methanogens = \frac{t_n Mp}{t_n Mp + t_n \mathrm{oM}} * 100$$

$$\%\ Mp\ of\ prokaryotic\ community = \frac{t_n Mp}{t_n Mp + t_n \mathrm{oM} + t_n \mathrm{bac}} * 100$$

Mp, *Methanoplasmatales*; t_nMp / t_noM / $t_n$bac, Mp / other methanogenic / bacterial 16S rRNA gene copy at a certain point in time (t_n).

2.2.9. PCR Amplification, Cloning, Sequencing & Sequence Analyses

Mp 16S rRNA genes from initial cow rumen fluid were PCR amplified. Amplification and further steps (cloning, sequencing, sequence analyses) were performed as described in section 2.1.2 - 2.1.4. Additionally, the same procedures were applied to a Danish cow rumen fluid sample, pre-incubated with TMA. From both, Austrian (AUT) and Danish (DK) cow rumen fluid (RF), 26 clones were selected and sequenced.

Several enrichment cultures were screened for Mp *mttB* genes via PCR amplification (for PCR details see section 2.1.2).

2.2.10. IonTorrent Sequencing - Metagenomics

Two enrichment cultures, one from an earlier stage (7th transfer; RumEn_MG1) the other from a later stage (13th transfer; RumEn_MG2), were sequenced by Clarissa Schwab and Thomas Weinmaier using IonTorrent. Obtained 16S rRNA reads were taxonomically classified using CREST (*46*). Rumen *Methanoplasmatales* genomes were assembled and afterwards functionally annotated with RAST (*55*). Furthermore, the genomes of *M. luminyensis* and *Ca.* M. alvus were also annotated with RAST. Taxonomic classification, genome assembly and functional annotation were performed by Clarissa Schwab and Thomas Weinmaier. Afterwards, I compared bacterial and archaeal communities and used the annotated genomes to reconstruct the methanogenesis pathway of *Methanoplasmatales.*

3. Results

3.1. Environmental Distribution of *Methanoplasmatales*

Methanoplasmatales (Mp) 16S rRNA genes could be amplified, using primers targeting Mp/RCC (*42*), from seven out of ten animal samples, more precisely from feces of elephant, giant tortoise, tortoise, rat, and from rumen fluid of Danish and Austrian cows (see also 3.2.1); furthermore from all arctic and all temperate peat soils.

Taxonomic placements of all obtained 16S rRNA gene sequences by CREST were within the class of *Thermoplasmata*. Most of them were classified as members of VadinCA11 Gut Group or Terrestrial Miscellaneous Group (TMEG) 2 (tab. 3.1). The phylogenetic placement of obtained sequences was further investigated by constructing maximum likelihood trees (fig 3.1 and fig. 3.2).

Table 3.1. Taxonomic classification of 16S rRNA gene sequences obtained from animal and wetland samples by CREST.

***Thermoplasmata* subgroup**	**number of sequences**
VadinCA11 Gut Group	79
Terrestrial Miscellaneous Group 2 (TMEG 2)	55
Marine Benthic Group D and DHVEG-1	3
unknown *Thermoplasmatales* family	1

CD-HIT constructed 28 distinct OTUs with less than 98 % sequence identity. These OTUs contained either sequences from animals or wetlands or, more rare, from both; but no OTU contained sequences from different animal samples except for one rumen fluid OTU containing one rat clone (see tab. 3.2; AUT 02).

Mp specific 16S rRNA primers amplified only *Thermoplasmata* sequences, which formed two big, distinct clusters (fig. 3.1). One cluster was dominated by animal samples (animal cluster) and reference sequences belonging to the Vadin Gut Group, the other by wetland samples (wetland cluster) and TMEG 2 reference sequences. Additionally, few clones from two temperate wetland samples clustered with the Marine Bentic Group D and clones from AUT peat 9 with other Terrestrial Miscellaneous Groups (TMEGs). All animals were dominated by animal cluster RFLPs and all wetlands were dominated by wetland cluster RFLPs except AUT peat 7 with 60 % of RFLPs belonging to the animal cluster (fig. 3.3).

In all animal and both arctic peat samples *mcrA* genes could be amplified either with Mp specific or with general *mcrA* primers or with both. This extends to 10 out of 14 temperate wetland samples (fig. 3.3.). Both used *mcrA* primer pairs amplified *mcrA* sequences affiliated with *Methanoplasmatales* and with known methanogenic lineages (fig. 3.2).

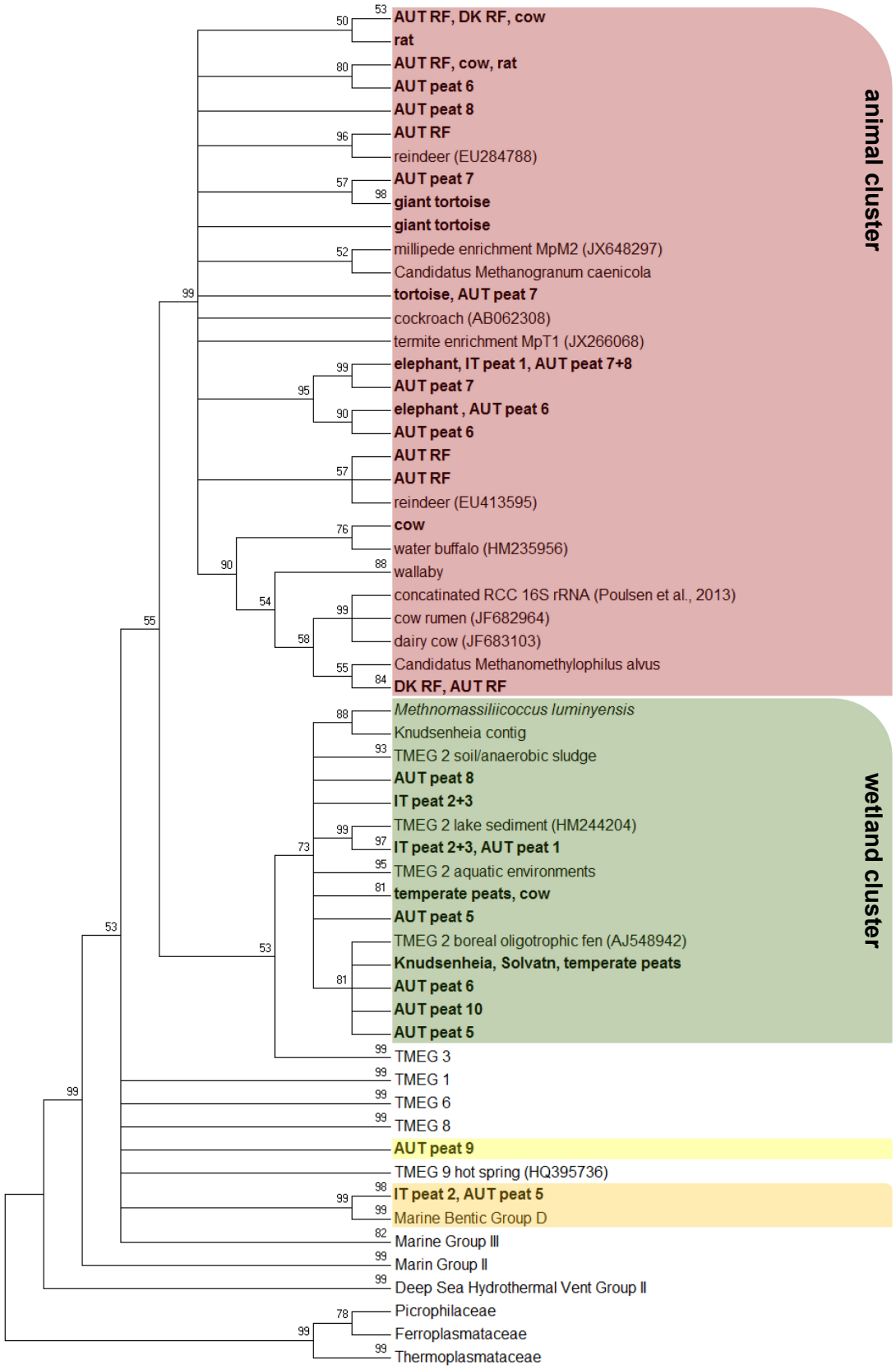


Figure 3.1. Maximum likelihood tree showing the phylogenetic relationship between obtained 16S rRNA gene sequences and cultured/uncultured *Thermoplasmata*. Tree is condensed with a 50 % cut-off value (numbers above the branches indicate bootstrap values) and based on an alignment of 517 nt positions.

Altogether, 95 *mcrA* clones were obtained. Of those clones the majority (63 clones) was associated with known methanogens. The obtained unknown *mcrA* sequences (32 clones) clustered together with the few known *Methanoplasmatales mcrA* sequences and were also forming an animal and a wetland specific sequence cluster.

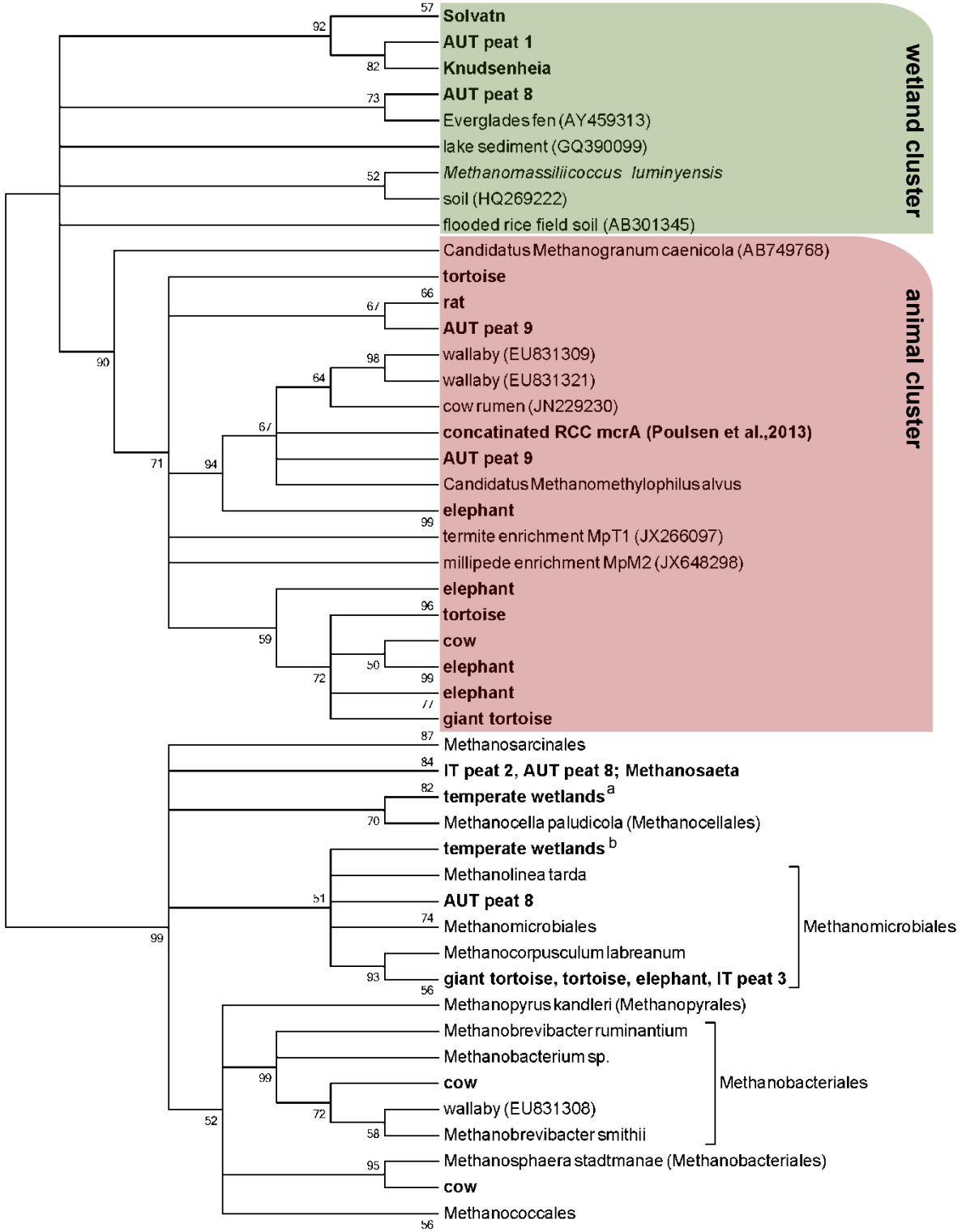


Figure 3.2. Maximum likelihood tree showing the phylogenetic relationship between obtained McrA protein sequences and McrA sequences of known and unknown origin from the databases. Tree is condensed with a 50 % cut-off value (numbers above the branches indicate bootstrap values) and based on an alignment of 104 amino acid positions. [a] including sequences from IT peat 2 and AUT peat 2 & [b] including sequences from AUT peat 3 and AUT peat 4 amplified with Mp specific *mcrA* primer.

Additionally, *mttB* amplicons of the expected size were detected in a few samples (elephant, giant tortoise, AUT peat 5/6/7, IT peat 3, GER peat 1). Sequence verification using BLAST showed that all of these amplicons were results of unspecific primer binding.

Figure 3.3 shows the location of all investigated temperate and arctic wetlands and gives an overview of what was amplified with the three successfully used primer pairs, with Mp specific 16S rRNA / *mcrA* primers and *mcrA* primers targeting all methanogens.

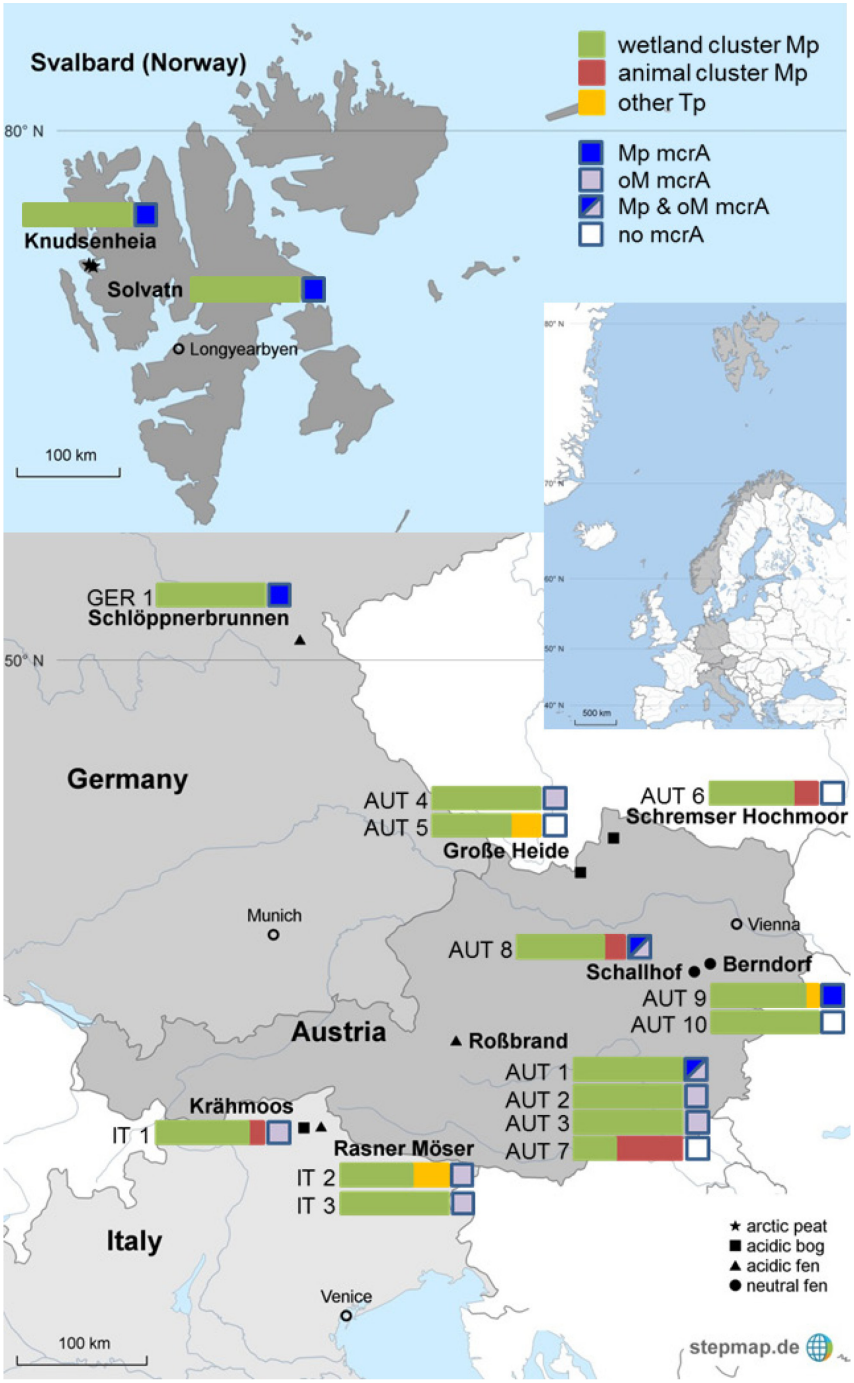


Figure 3.3. Localisation of arctic and temperate (acidic/neutral bogs/fens) wetland samples and PCR amplification of 16S rRNA and mcrA genes with Mp specific primes (16S rRNA, *mcrA*) and general *mcrA* primers targeting all methanogens. Proportion of 16S rRNA sequences belonging to animal or wetland cluster Mp or to other *Thermoplasmata* subgroups (other Tp) based on RFLP analysis is given (green/ red/yellow bar). Blue boxes indicate if and which *mcrA* sequences were amplified, either sequences associated with *Methanoplasmatales* (Mp) or other methanogens (oM) or with both.

3.2. Rumen *Methanoplasmatales* & *Methanoplasmatales* Enrichments

3.2.1. Cow Rumen Fluid - Community Composition

Diversity of *Methanoplasmatales*. In addition to the phylogenetic trees (section 3.1) this section provides an in-depth analysis of the obtained 16S rRNA sequences from bovine rumen fluid, to get insights into the nature and complexity of the rumen fluid used for the enrichment attempts. Analysing the *Methanoplasmatales* community composition of initial Austrian rumen fluid (AUT RF) and pre-incubated Danish cow rumen fluid (DK RF), revealed major differences (tab. 3.2). 26 AUT RF Mp 16S rRNA clones and 25 DK RF Mp 16S rRNA clones were sequenced, obtained sequence lengths varied between 479 and 483 nt. OTUs containing sequences with ≥ 98 % identity were constructed with CD-HIT (*47*). Nine AUT RF OTUs (AUT 01 - 09) were identified; AUT 01 and AUT 02 were predominant, containing ten and eight sequences, respectively. In contrast, only three DK RF OTUs (DK 01 - 03) were identified, and just one dominated - DK 02 - comprising 21 sequences (fig. 3.4).

Table 3.2. Austrian (AUT) and Danish (DK) rumen fluid OTUs (with <98 % sequence identity) and their best BLAST hits (uncultured and cultivated/enriched organisms).

sequences		best BLAST hit (uncultured)			nearest cultivated organism/enrichment[a]		
cluster	no.	clone / accession no.	identity	source	*Candidatus*	identity	source
AUT 01	10	clone QTPC108 / JF807170.1	99%	rumen	M. caenicola	93%	digested sludge
AUT 02[b]	8	clone ARE5A71TFB / JX907495.1	100%	bovine rumen	M. caenicola	94%	digested sludge
AUT 03	1	clone ARF5A82TFB / JX906851.1	100%	bovine rumen	M. caenicola	91%	digested sludge
AUT 04	1	clone CSIRO-Qld24 / AY995292.1	98%	sheep	M. caenicola	92%	digested sludge
AUT 05	2	clone ARE5A01TFB / JX907471.1	99%	bovine rumen	M. caenicola	94%	digested sludge
AUT 06	1	clone A4_1 / JN315193.1	99%	rumen	M. caenicola	92%	digested sludge
AUT 07	1	clone PE-CAN.08 / DQ123868.1	100%	cattle	M. caenicola	92%	digested sludge
AUT 08	1	clone C3ALE12 / JQ179701.1	100%	bovine rumen	M. caenicola	92%	digested sludge
AUT 09	1	clone CNSL54 / AB535363.1	99%	sheep	M. alvus	98%	human gut
DK 01	3	clone C1ALG07 / JQ179525.1	100%	bovine rumen	M. caenicola	92%	digested sludge
DK 02	21	clone CNSL54 / AB535363.1	99%	sheep	M. alvus	98%	human gut
DK 03	1	clone p14f-86sF / AY907247.1	98%	human gut	M. alvus	96%	human gut

[a] accession no: *Ca.* Methanogranum caenicola (AB749767.1); *Ca*. Methanomethylophilus alvus (CP004049.1)

[b] the only animal OTU containing one sequence from another animal species (one rat clone); see 3.1

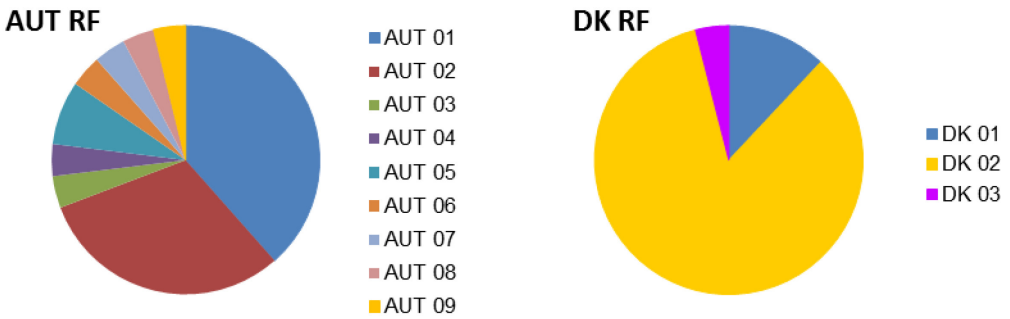


Figure 3.4. Austrian (AUT) and Danish (DK) rumen fluid (RF) OTUs (with <98 % sequence identity) and their relative abundances. For detailed cluster information see table 3.2.

All obtained 16S rRNA sequences from Austrian and Danish rumen fluid fell within the animal sequence cluster (see phylogenetic tree; fig. 3.1). The majority of AUT RF OTUs showed rather low sequence identities (91 - 94 %) to their nearest related organism within the NCBI database, *Candidatus* Methanogranum caenicola, an enrichment culture obtained from digested sludge. The majority of DK RF sequences showed 98 % sequence identity with the 16S rRNA gene of *Candidatus* Methanomethylophilus alvus, a human gut enrichment culture. Best uncultured BLAST hits for all sequences originate from similar environments - animal intestinal tracts.

Abundances of *Methanoplasmatales,* other Methanogens and Bacteria. Initial Austrian rumen fluid and pre-incubated Danish rumen fluid abundances of bacteria and methanogens are depicted in Figure 3.5. The prokaryotic community of initial AUT RF was dominated by bacteria, with over 10^9 bacterial 16S rRNA gene copies mL^{-1} rumen fluid. The community consisted of 99.5 % bacteria, 0.3 % other methanogens and just 0.2 % *Methanoplasmatales*. Methanogenic communities consisted of 42 % and 68 % Mp, with abundances of 8x 10^6 and 3x 10^7 16S rRNA copies mL^{-1} of AUT RF and DK RF, respectively.

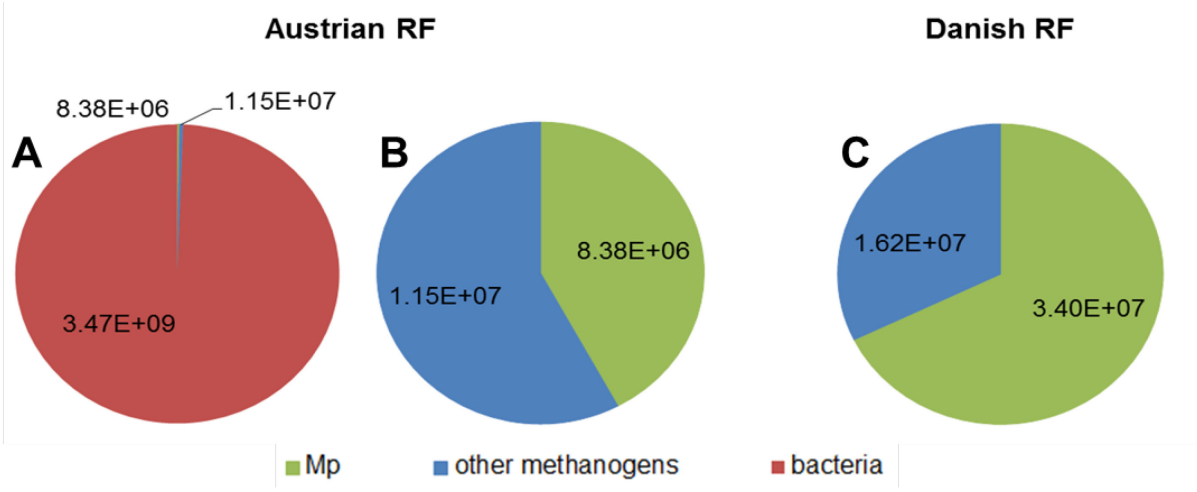


Figure 3.5. Community composition of initial Austrian rumen fluid and primary enriched Danish rumen fluid (pre-incubated with TMA). Distribution and 16S rRNA gene copies mL^{-1} are given (for details see text). 16S rRNA copies mL^{-1} were determined by qPCR. A) Prokaryotic community composition of Austrian rumen fluid; B) methanogenic community composition of Austrian rumen fluid; C) methanogenic community composition of Danish rumen fluid. Mp, *Methanoplasmatales*; RF, rumen fluid.

3.2.2. Establishing Enrichment Conditions and Screening

Modified *Methanobrevibacter* medium (complete medium; tab. 2.5) and TMA as substrate for methanogenesis were chosen to enrich *Methanoplasmatales*. It was assumed that TMA could serve as both - electron donor and electron acceptor. Different atmospheres were applied, containing either N_2 gas, or a mixture of N_2, CO_2 and H_2, to provide H_2 as electron donor, in case TMA just acts as electron acceptor. Quantitative PCR (qPCR) was chosen to screen enrichment cultures and turned out to be a proper tool to monitor Mp growth and enrichments (see 2.2.8).

Initially, complete medium was inoculated with 20 % Austrian cow rumen fluid and incubated one week with TMA under an atmosphere containing either N_2 or a mixture of N_2:CO_2:H_2 (70:20:10). These initial cultures were compared with same volumes of 100 % Austrian cow rumen fluid incubated under the same conditions. Complete medium inoculated with 20 % fresh rumen fluid and incubated under N_2:CO_2:H_2 atmosphere showed strongest increase of Mp 16S rRNA gene copies mL^{-1} after one week of incubation (16-fold). In contrast, incubation under N_2 atmosphere increased 6-fold. 16S rRNA gene copies mL^{-1} of pure rumen fluid cultures increased similarly; the applied atmosphere showed no influence (fig. 3.6). Apparently, adequate enrichment conditions were selected right from the beginning. *Methanoplasmatales* grew not only in undefined complex rumen fluid, but also in a more defined, artificial medium.

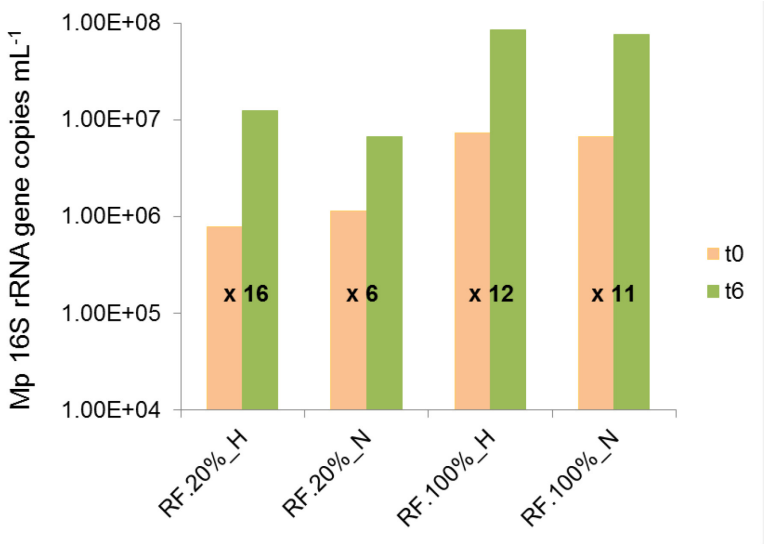


Figure 3.6. Increase of *Methanoplasmatales* (Mp) 16S rRNA gene copies mL^{-1} during one week of incubation (t0 - t6) with 10 mM TMA at 37 °C. RF.20%_H/_N, complete medium inoculated with 20 % Austrian rumen fluid (RF) under N_2:CO_2:H_2 (70:20:10) atmosphere (_H) or N_2 atmosphere (_N); RF.100%_H/_N, 100 % rumen fluid incubated under N_2:CO_2:H_2 or N_2 atmosphere. Within the bars x-fold increase is given (t_6 / t_0 = x n).

The subsequent cultures were grown on complete medium (tab. 2.5), incubated with 30 mM TMA under an atmosphere containing 70 % N_2, 20 % CO_2 and 10 % H_2. Subcultures, with 10 % inoculum, were taken after about one week of incubation. These conditions led to stable enriched cultures containing around 10 % *Methanoplasmatales* after the 4th transfer. The methanogenic communities of these enrichment cultures were >90 % enriched in Mp. But considerable growth and enrichments of Mp could only be obtained by adding 8 % sterile filtered cow rumen fluid as an additional substrate. Figure 3.7 depicts one representative of a stable enriched culture (A) compared to one culture treated the same way except addition of sterile filtered rumen fluid (B). The culture containing sterile filtered rumen fluid showed a 12-fold increase of Mp 16S rRNA gene copies mL^{-1}. In contrast, the culture without sterile filtered rumen fluid showed no considerable Mp growth (1.4-fold increase of 16S rRNA gene copies mL^{-1}).

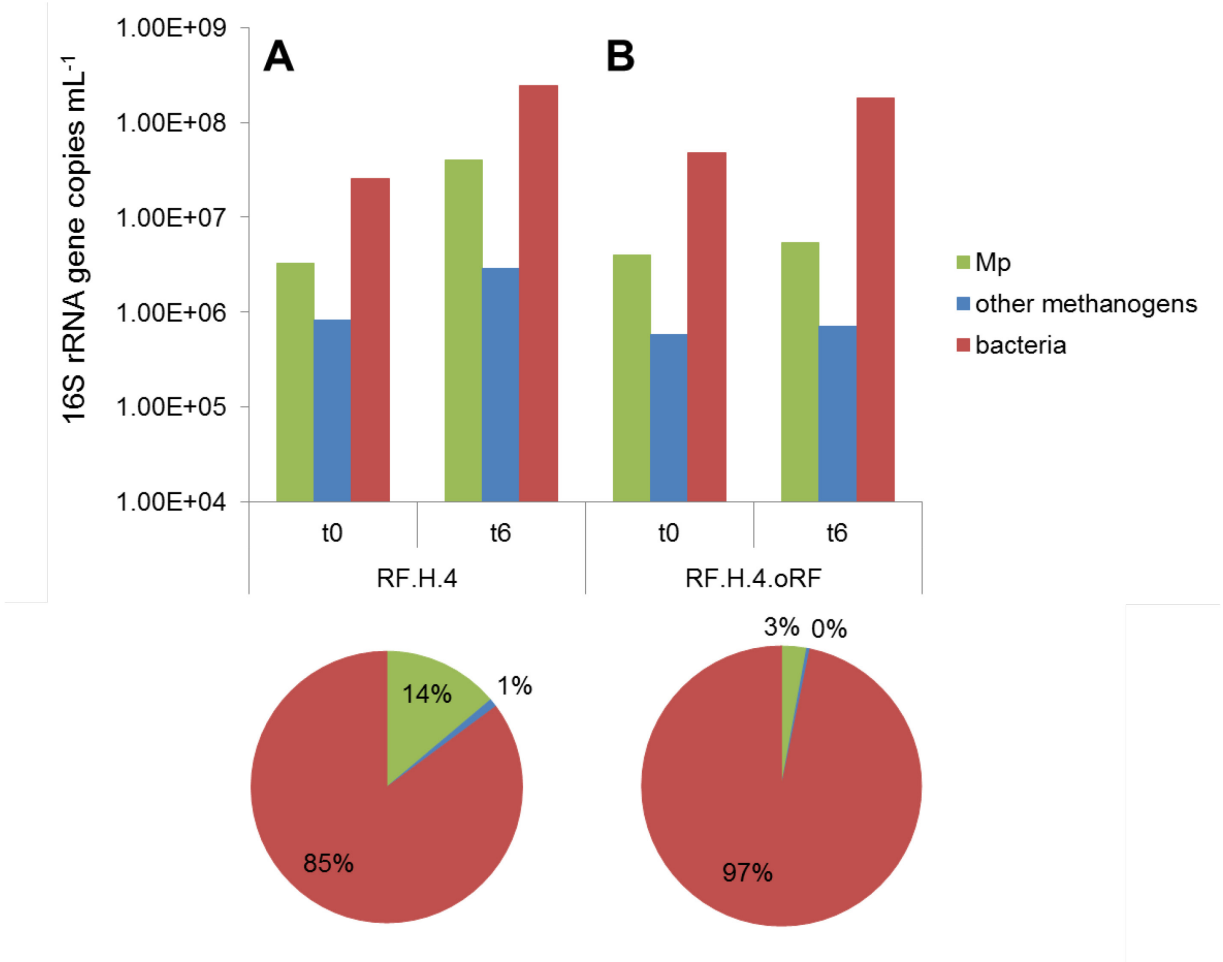


Figure 3.7. 16S rRNA gene copy increase of *Methanoplasmatales* (Mp), other methanogens and bacteria after seven days of incubation (t_0 - t_6) with 30 mM TMA under an atmosphere containing 70 % N_2, 20 % CO_2 and 10 % H_2. (A) Culture RF.H.4 contained 8 % sterile filtered cow rumen fluid; Mp 16S rRNA gene copies increased 12-fold. (B) Culture RF.H.4.oRF was grown without sterile filtered rumen fluid; Mp 16S rRNA gene copies did not increase (1.4-fold). Pie charts depict proportions of Mp, other methanogens and bacteria of cultures above (at t_6).

Even though Mp growth was already observed after 2 - 3 days, an incubation time of about one week seemed to be most suitable because considerable Mp growth (>5-fold increase of 16S rRNA gene copies mL^{-1}) could also be observed after 4 days of incubation. After 10 days of incubation cultures showed no more growth, neither growth of Mp nor of bacteria or other methanogens.

In these early enrichment cultures *Methanoplasmatales* were enriched from an initial 0.2 % to around 10 %, resulting in an enrichment factor of 50. However, enrichment conditions needed to be improved, to gain higher enriched cultures. Especially, bacterial growth inhibition was required because at this time bacteria and Mp increased at the same order of magnitude during incubation, and bacteria still dominated the cultures.

3.2.3. Medium & Atmosphere Adjustments

Medium. Complete medium was thought to be favouring heterotrophic bacteria because of its high amount of organic compounds (e.g. 1 gL^{-1} yeast extract and trypticase peptone). Therefore, it was reduced to minimal medium (tab. 2.5), to be more selective for *Methanoplasmatales*.

Indeed, enrichments >20 % up to 32 % were obtained immediately after changing from complete medium to minimal medium, within the 7th subculture generation (fig. 3.8). Reducing the complete medium resulted in an increase from 10 % Mp to 20 - 30 % *Methanoplasmatales* enrichment; giving an enrichment factor of 100 - 150, compared to initial cow rumen fluid.

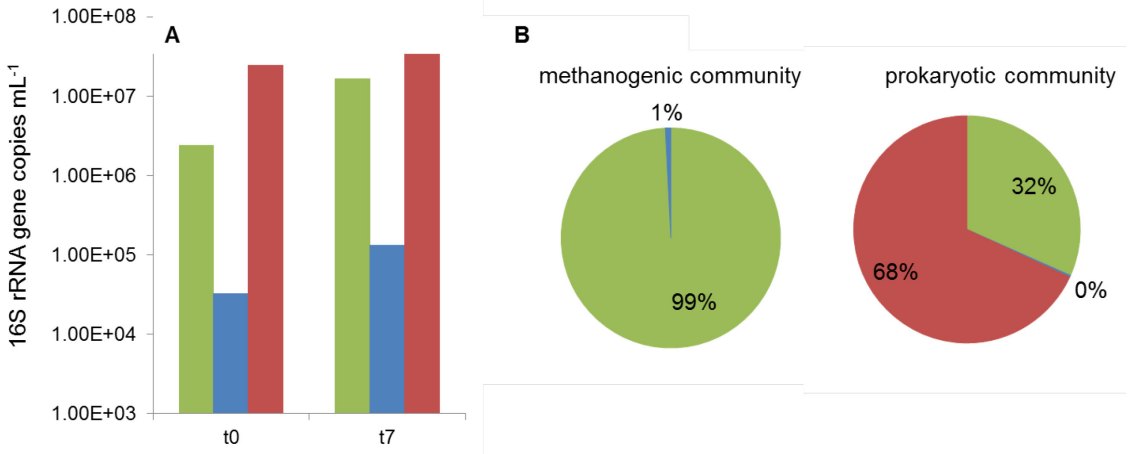


Figure 3.8. (A) Minimal medium was inoculated with 10 % complete medium culture resulting in depicted subculture RF.H.7.mm. Subculture RF.H.7.mm was grown under the same conditions as the previous culture: incubated about one week on 45 mM TMA under an atmosphere containing 70 % N_2, 20 % CO_2 and 10 % H_2. 16S rRNA gene copy increase of *Methanoplasmatales* (Mp), other methanogens and bacteria after eight days of incubation (t_0 - t_7) is shown. (B) Pie charts depict Mp proportion of methanogenic and the prokaryotic community.

Cultures grown on minimal medium showed higher mean and maximum Mp growth and enrichments and lower bacterial / other methanogen growth compared to cultures grown on complete medium (tab. 3.3). However, enrichments and increase of 16S rRNA gene copies mL^{-1} varied strongly. Subsequently, extreme minimal medium (tab. 2.5) was created by excluding other possible bacterial substrates. Cultures grown on extreme minimal medium showed little or no bacterial growth, but also no considerable Mp growth.

Table 3.3. Range (upper value) and mean (lower value) of *Methanoplasmatales* (Mp) enrichments and x-fold increase of 16S rRNA copies mL^{-1} during incubation on complete and minimal medium[a].

enrichment and increase		complete medium	minimal medium
Mp enrichment [%]	prokaryotes	0 - 15 3.5	0 - 52 14.0
	methanogens	56 - 100 90.2	12 - 100 94.3
x-fold increase of 16S rRNA copies mL^{-1} during incubation	Mp	0.5 - 12.1 6.2	0.0 - 36.0 5.9
	other methanogens	1.1 - 51.9 10.9	0.7 - 38.7 6.3
	bacteria	1.2 - 66.1 12.7	0.9 - 35.9 6.4

[a] serial dilutions, cultures treated with bacterial inhibitors as well as cultures incubated with divergent substrate concentrations were excluded. Complete medium (n = 21); minimal medium (n = 60)

Atmosphere. *M. luminyensis* and all published enrichments were grown under an atmosphere containing H_2. Considerable rumen *Methanoplasmatales* growth was observed if cultures were grown under an atmosphere containing either a mixture of N_2, CO_2 and H_2 (70:20:10) or just N_2 gas. However, higher Mp growth was observed if atmospheres contained 10 % H_2. Whenever H_2 concentrations in the headspace were increased to >10 %, growth of other methanogens was favoured (data not shown).

3.2.4. Further Enrichment Trials

Besides medium modifications and atmosphere adjustments, to gain a defined medium selecting for rumen *Methanoplasmatales*, other enrichment procedures were conducted.

3.2.4.1. Serial Dilutions & Filtration

Serial Dilutions. Several 10-fold serial dilutions were conducted because it was assumed that bacterial dilution and promotion of Mp growth, via TMA addition, could result in highly enriched cultures. Results of all serial dilutions were similar. Higher dilutions led to higher Mp growth, but much higher bacterial growth and even higher growth of other methanogens. Thus, relative Mp enrichments decreased with each dilution step (fig. 3.9).

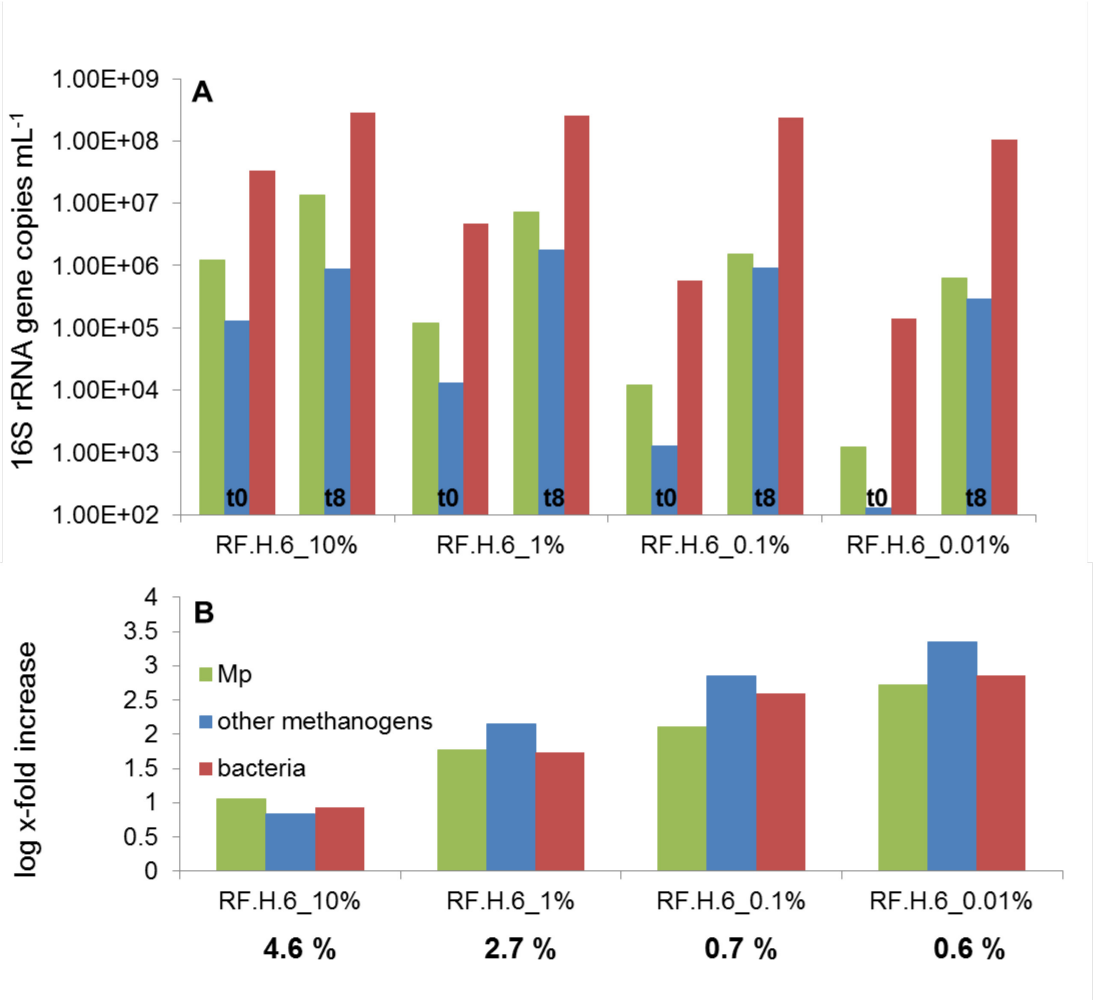


Figure 3.9. Representative 10-fold serial dilution trial (10 % → 1 % → 0.1 % → 0.01 % inoculum). (A) 16S rRNA gene copies mL^{-1} culture at incubation start (t_0) and after nine days of incubation (t_8) with 30 mM TMA, 8 % sterile filtered rumen fluid, under an atmosphere containing 70 % N_2, 20 % CO_2 and 10 % H_2. (B) Increase of 16S rRNA gene copies mL^{-1} after incubation (log x-fold increase). Percentages below show *Methanoplasmatales* (Mp) enrichments (of prokaryotic community).

Filtration. Rumen *Methanoplasmatales* were thought to be small cocci (see 3.2.7). Therefore, filtration was applied to separate big bacterial cells from small coccoid Mp cells. In fact, bacteria as well as Mp and other methanogens decreased after filtration (fig. 3.10 A). The filtered culture was incubated for one week. Similar effects as seen in serial dilutions occurred (B). Bacteria and especially other methanogens grew by far much better than *Methanoplasmatales*.

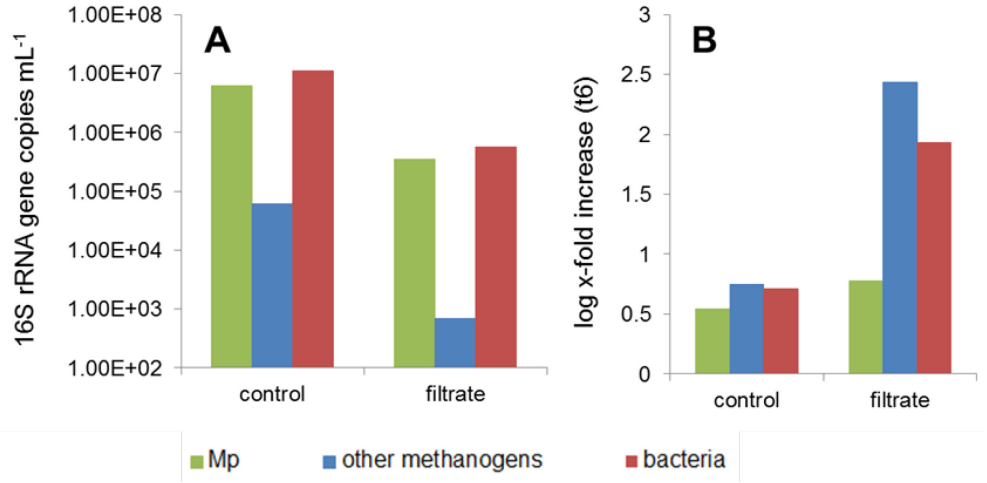


Figure 3.10. (A) 16S rRNA gene copies mL^{-1} culture at incubation start (t0). (B) Increase of 16S rRNA gene copies mL^{-1} after one week of incubation (log scale). Control and filtrate were incubated under the same conditions (minimal medium, 35 mM TMA, 8 % sterile filtered RF, N_2:CO_2:H_2).

3.2.4.2. Antibiotics and Hop Extract

Several attempts to inhibit bacterial growth via specific antibiotics were conducted (for details see 2.2.4.3). In many cases, no influence of antibiotic addition could be observed. Treated and untreated cultures grew to the same extent. Whenever antibiotic addition inhibited bacterial growth, also *Methanoplasmatales* growth was inhibited. Figure 3.11 shows one representative antibiotic-treated culture.

Addition of hop extract (an antimicrobial plant extract from common hop) was not inhibiting bacteria, and had no positive effect on *Methanoplasmatales* enrichments (data not shown).

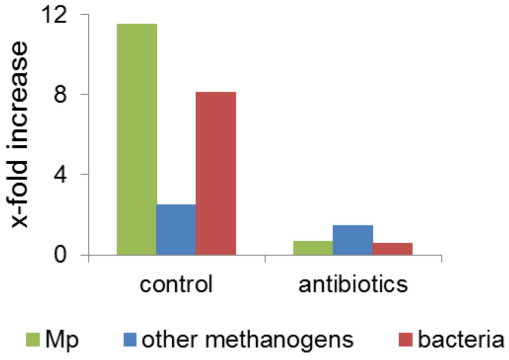


Figure 3.11. X-fold increase of 16S rRNA gene copies mL^{-1} after one week of incubation (minimal medium; 20 mM TMA; 8 % sterile filtered rumen fluid; N_2 atmosphere). Antibiotic-treated culture showed no considerable growth of *Methanoplasmatales* (Mp) and bacteria. Added antibiotics: 100 mgL^{-1} ampicillin, kanamycin and streptomycin; 50 mgL^{-1} chloramphenicol.

3.2.4.3. Serial Dilutions in Solid Medium

Serial dilutions (10^{-1} - 10^{-6}) of Mp enrichment cultures in solid medium were prepared to possibly obtain single colonies of *Methanoplasmatales* on and within the solid medium.

After four weeks of incubation at 37 °C inside four cultivation tubes (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) a sediment layer in the condensate could be observed (fig. 3.12). These sediments were investigated under the microscope (see 3.2.7). Remaining sediment was inoculated in serum bottles containing 30 mL minimal medium, 20 mM TMA, 10 % sterile RF, and an atmosphere of 70 % N_2, 20 % CO_2 and 10 % H_2. After one week of incubation qPCR analysis revealed >99 % bacteria within the cultures; *Methanoplasmatales* could not be enriched from cultivation tube sediments. Furthermore, few white colonies grew on solid medium, via microscopy they were identified as big rods, most likely bacteria.

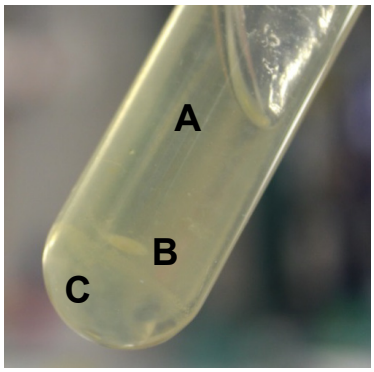


Figure 3.12. Cultivation tube (10^{-3} dilution) after 4 weeks of incubation. (A) liquid phase; condensate. (B) sediment. (C) solid medium. For details see text.

3.2.5. TMA Measurements

Trimethylamine concentrations, from one culture (13th round of subculturing) grown on minimal medium (containing 8 % sterile rumen fluid) under an atmosphere containing 70 % N_2, 20 % CO_2 and 10 % H_2, were measured over 11 days of incubation. At incubation start (t_0) and two times during incubation (t_3 and t_6) 15 mM TMA were added. Besides TMA measurements, 16S rRNA gene copies mL^{-1} of *Methanoplasmatales* and bacteria were determined several times (t0, t2, t3, t6, t11) to monitor growth (fig. 3.13).

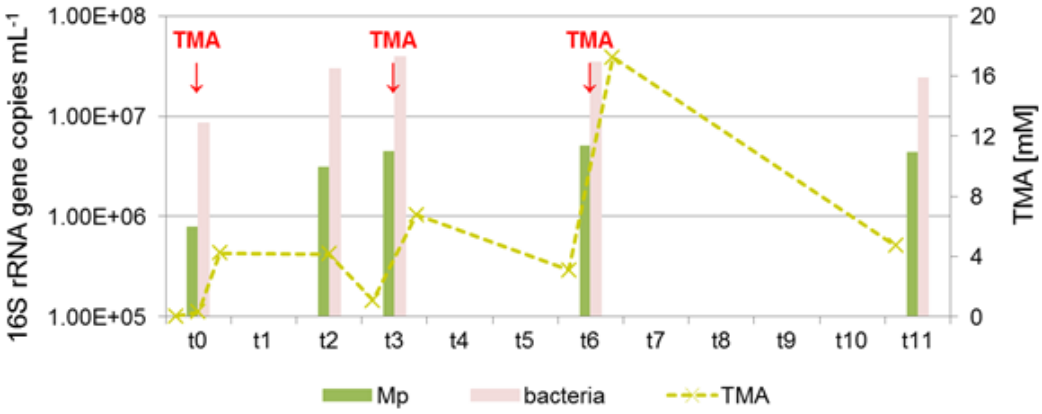


Figure 3.13. Culture grown 11 days on minimal medium (incl. 10 % sterile filtered rumen fluid) under an atmosphere containing a mixture of N_2:H_2:CO_2 (70:20:10). Three times 15 mM TMA were added, TMA concentrations were measured before inoculation, right after inoculation as well as before and after each TMA addition. *Methanoplasmatales* (Mp) and bacterial 16S rRNA gene copies mL^{-1} were determined by qPCR.

Mp 16S rRNA gene copies mL^{-1} increased 4-fold during the first two days of incubation; however, TMA concentrations did not change. On the third incubation day a TMA decrease was measured, and Mp grew little (1.4-fold increase of 16S rRNA gene copies mL^{-1}). After 2nd (t3) and 3rd (t6) TMA addition, TMA decreased the following days, but no considerable growth of Mp could be detected. At the same time TMA was measured, Mp enrichments were rather unstable, ranging from <5 % to around 30 % Mp (of prokaryotic community), even if cultures were treated the same way. PCR amplification of *mttB* genes from selected enrichment cultures indicated a loss of *mttB* genes after the 3rd transfer.

3.2.6. Enrichments on Methanol

Besides TMA methanol (MetOH) was supplemented as substrate for rumen Mp methanogenesis, as MetOH was confirmed as substrate of *M. luminyensis* and other Mp enrichment cultures (*14*, *24*, *25*). Several times subcultures were incubated with TMA or with MetOH in parallel. After some time a certain trend could be observed. Cultures incubated with MetOH grew better and reached higher Mp enrichments than cultures incubated with TMA (tab. 3.4). A onetime maximum enrichment of 52 % Mp could be obtained (fig. 3.14), with 5.7x 10^7 Mp 16S rRNA gene copies mL^{-1} culture.

Table 3.4. Obtained *Methanoplasmatales* enrichments, during parallel incubation with either trimethylamine (TMA; n = 12) or methanol (MetOH; n = 25).

substrate	*Methanoplasmatales* enrichments				
	>10 %	>20 %	>30 %	>40 %	>50 %
TMA	0	1	3	0	0
MetOH	6	1	3	4	1

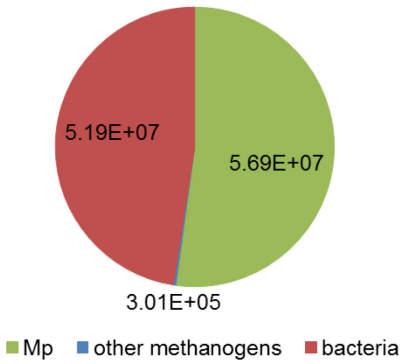


Figure 3.14. 16S rRNA gene copies mL^{-1} culture (RF.31.H.M) of *Methanoplasmatales* (Mp), other methanogens and bacteria. Mp constituted 52% of prokaryotic community. Culture was grown on minimal medium and incubated nine days with 40 mM MetOH under an atmosphere containing 70 % N_2, 20 % CO_2 and 10 % H_2.

3.2.7. Microscopy

Several cultures were analysed under the microscope. All of them showed high morphological diversity: rods and filaments of different sizes, spirochetes, cocci, diplococci and streptococci as well as aggregates of cocci. Because of coccoid shape of *M. luminyensis*, and termite/millipede enrichments, we expected rumen *Methanoplasmatales* also to be coccoid. Observed abundances of small cocci seemed to coincide with estimated percentage of Mp within the cultures via qPCR.

3.2.7.1. Microscopy of Cultivation Tube Sediments

Several sediment layers of cultivation tubes were investigated by microscopy. All of them looked similar; figure 3.15 shows some constantly recurring observations. Sediments consisted mainly of aggregates, potentially clusters of small cocci, and of big rods (A). In many cases some rods were attached to the aggregates. DAPI staining identified aggregates to be cell clusters, obviously clusters of cocci. Figure 3.15 C shows a very big cocci-cluster (phase contrast microscopy picture), if excited with ultraviolet light (D) the big cocci-cluster showed some kind of autofluorescence. This autofluorescence was observed from all investigated cocci-aggregates.

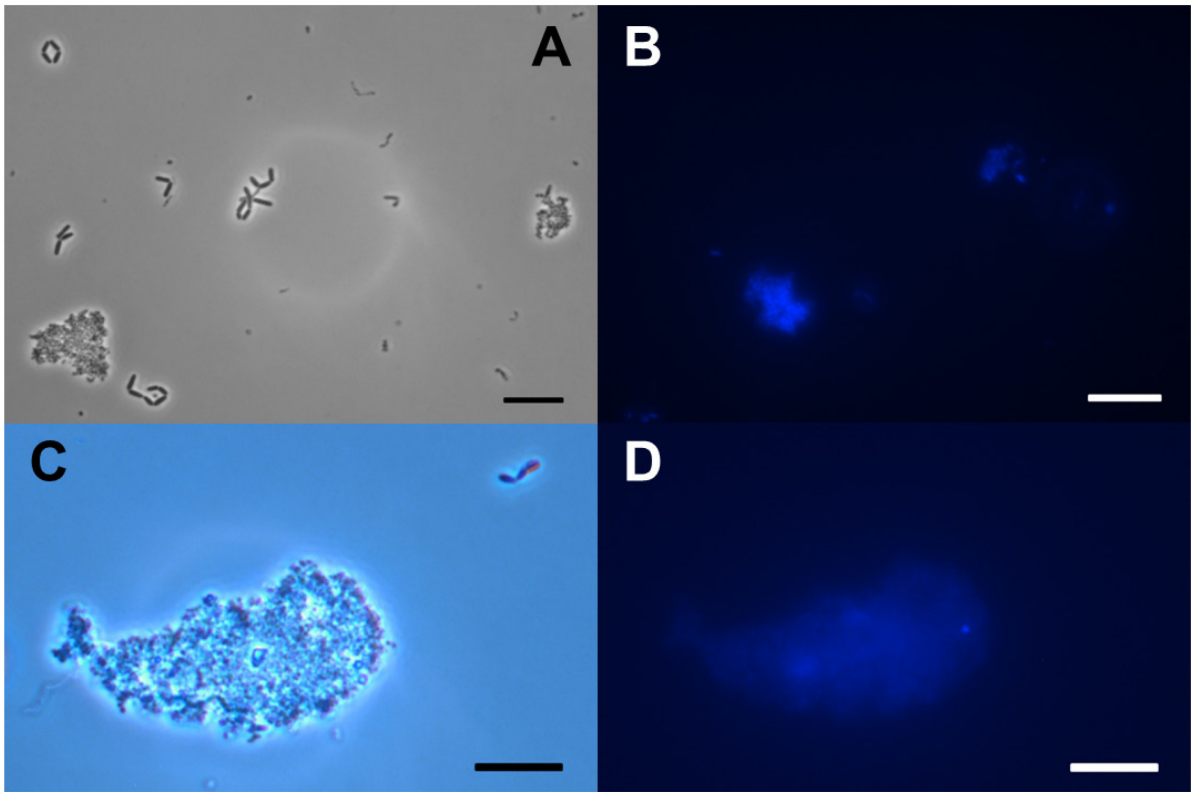


Figure 3.15. Microscopy of condensate sediments. A and C: phase contrast; B: DAPI stained sediment; D: autofluorescence of cell aggregate shown in C. Bars = 20 µm. For details see text.

3.2.8. IonTorrent Sequencing & Metagenomic Analyses

Table 3.5 shows the ouput of both sequencing runs on rumen *Methanoplasmatales* enrichment cultures, RumEn_MG1 and RumEn_MG2 (rumen enrichment metagenome 1/2). Both cultures were grown under similar conditions (minimal medium; 8 % sterile filtered rumen fluid; N_2:H_2:CO_2 (70:20:10); 60/30 mM TMA) and qPCR revealed Mp enrichments of around 20 %.

Table 3.5. General information on both rumen *Methanoplasmatales* enrichment metagenomes.

ID	subculture	no. of reads	average read length
RumEn_MG1	7th transfer	0.48 Mio	114 nt
RumEn_MG2	13th transfer	1.87 Mio	128 nt

3.2.8.1. Community Composition

Prokaryotic Community. CREST assigned 234 reads of RumEn_MG1 at prokaryotic domain level. 24 % of all 16S rRNA reads were assigned to Archaea; 76 % to Bacteria. The bacterial community consisted mainly of *Clostridiales* (33 %), *Desulfovibrionales* (15 %), *Lactobacillales* (9 %) and *Bacteroidales* (7 %); all archaeal 16S rRNA sequences were assigned to the order of *Thermoplasmatales*; see figure 3.16. In contrast, RumEn_ MG2 was much bigger; 1360 reads were assigned at prokaryotic domain level by CREST. 20 % of all 16S rRNA reads were assigned to Archaea; 80 % to Bacteria. The bacterial community consisted mainly of *Bacteroidales* (35 %), *Clostridiales* (24 %) and *Desulfovibrionales* (14 %); nearly all archaeal 16S rRNA sequences were assigned to the order of *Thermoplasmatales* (fig. 3.16.). There was a clear switch in bacterial community composition between the 7th transfer (RumEn_MG1) and the 13th transfer (RumEn_MG2); mainly by an increase of *Bacteroidales* and a decrease of *Lactobacillales* with time.

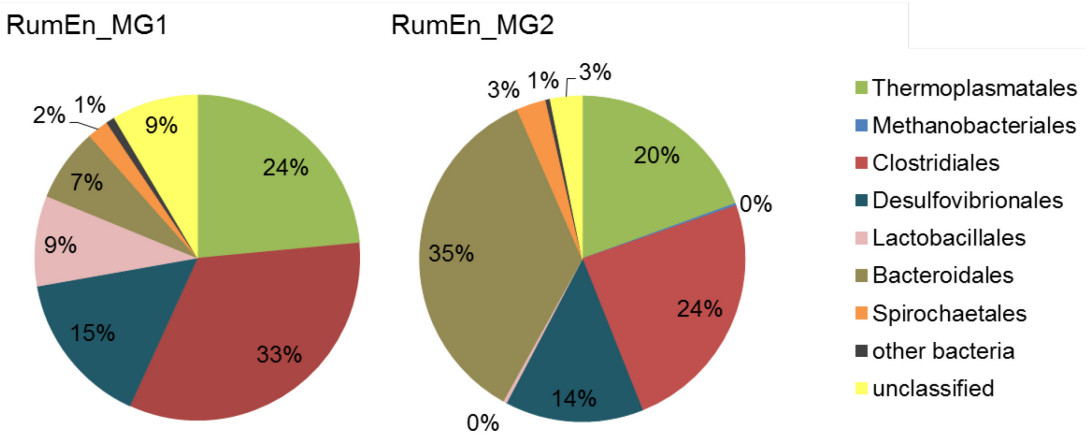


Figure 3.16. CREST assignment of 16S rRNA reads from both metagenomes, RumEn_MG1 (7th transfer) and RumEn_MG2 (13th transfer), on order level. For details see text.

Methanoplasmatales. There was not only a switch in bacterial community composition, but also a change in rumen Mp community. Figure 3.17 depicts the changes of Mp ecotypes during the experiments. Initially, all Mp detected in cow rumen fluid (see 3.2.1) belonged to the animal cluster (see 3.1 for more information about Mp clusters).

After repeated inoculation (7-times; RumEn_MG1) only 37 % of all Mp 16S rRNA sequences belonged to the animal cluster; after the 13th transfer (RumEn_MG2) even less were left (31 %). Now, the majority of Mp 16S rRNA sequences belonged to the wetland cluster.

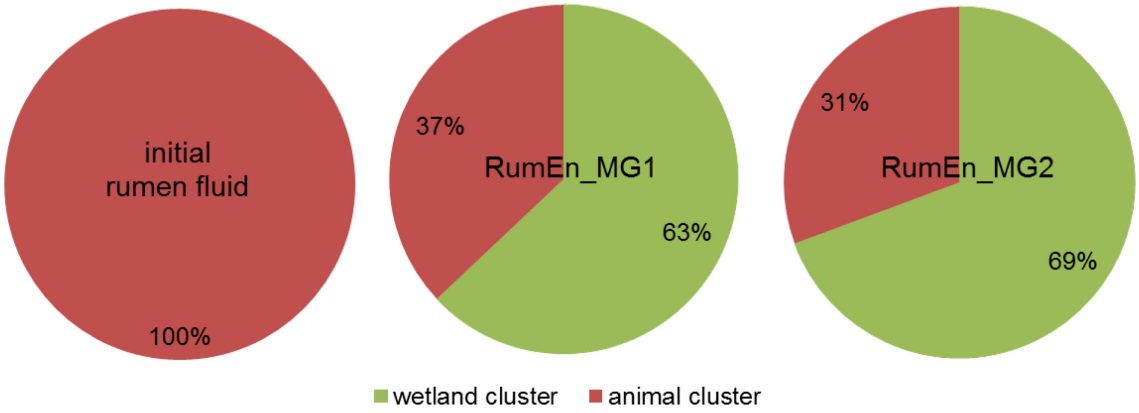


Figure 3.17. Change of *Methanoplasmatales* (Mp) ecotype proportion of enrichment cultures with time: Mp community of initial rumen fluid was obtained by sequencing of 26 clones containing rumen Mp 16S rRNA genes (see section 3.2.1). RumEn_MG1 and RumEn_MG2 = rumen enrichment metagenome 1 and 2 (IonTorrent Sequencing; 7th transfer: MG1; 13th transfer: MG2). For details see text.

3.2.8.2. Rumen Enrichment Mp Genomes - Functional Analyses

Based on different GC contents, Thomas Weinmaier was able to assemble two distinct partial rumen *Methanoplasmatales* genomes from the second metagenome RumEn_MG2. One partial genome, RumEN_Mp1, belongs to the wetland cluster (see 3.1) and is related to *M. luminyensis*, while the other partial genome, RumEN_Mp2, belongs to the animal cluster and is related to *Ca.* M. alvus. Table 3.6 gives an overview of both partial rumen Mp enrichment genomes and their nearest relatives *M. luminyensis* and *Ca.* M. alvus. All four genomes were annotated with RAST for further functional analyses.

Table 3.6. Genome features of both rumen *Methanoplasmatales* genomes (RumEN_Mp1, RumEN_Mp1), obtained from a cow rumen fluid Mp enrichment culture, and their nearest relatives from the public databases (*Methanomassiliicoccus luminyensis*, *Candidatus* Methanomethylophilus alvus).

genome	G+C content [%]	size [Mbp]	no. of contigs	status
RumEn_Mp1	62.46	2.21	182	partial (~80 %)
M. luminyensis	60.05	2.62	26	complete
RumEn_Mp2	54.75	1.28	18	partial (~80 %)
Ca. M. alvus	55.59	1.67	1	complete

An overview of genes involved in methanogenesis and carbon acquisition and their presence in the four annotated genomes is shown in table 3.7.
The resulting methanogenic pathway of Mp is discussed in section 4.4.

The genes involved in the last methanogenesis step (*mrcABCDG*), the formation of CH_4 from Methyl-coenzym M (CH_3-S-CoM), were found in all four analysed genomes. Most genes necessary for the reduction of methylamines (MMA, DMA, TMA), methanol and dimethyl sulfide to CH_4 were found in *M. luminyensis* and *Ca.* M. alvus. These genes were partially present in the RumEn_Mp1 genome, but could not be detected in RumEN_Mp2. In all genomes, genes involved in the regeneration of coenzyme M (HS-CoM) and coenzyme B (HS-CoB) are present (*hdrABCD*). Genes for methanogenesis from H_2/CO_2 as well as genes for the reverse H_2/CO_2 methanogenesis pathway (for production of reducing equivalents) are not present in any of the genomes (fig. 4.4; grey box). The gene encoding the alpha subunit of the energy-conserving hydrogenase (Ech) was detected in *M. luminyensis*. However, all other subunits (echBCDEF) were missing, as well as, the whole complex was missing in the two rumen Mp genomes and in *Ca.* M. alvus.
RumEN_Mp1 contained all genes encoding the two enzymes, acetyl-coenzym A synthetase and pyruvate:ferredoxin oxidoreductase, which are necessary for carbon acquisition from acetate; whereas RumEN_Mp2 and *Ca.* M. alvus lacked the acetyl-coenzym A synthetase gene.

Table 3.7. Genes involved in methanogenesis and carbon acquisition present in assembled rumen enrichment *Methanoplasmatales* genomes RumEn_Mp1 and RumEn_Mp2, *Methanomassiliicoccus luminyensis* (*M. lum.*) and *Candidatus* Methanomethylophilus alvus (*Ca.* M. a.).[a]

gene		RumEn_Mp1	*M. lum.*	RumEn_Mp2	*Ca.* M. a.
Methyl coenzyme M reductase alpha subunit	mcrA	+	+	+	+
Methyl coenzyme M reductase beta subunit	mcrB	+	+	+	+
Methyl coenzyme M reductase gamma subunit	mcrC	+	+	+	+
Methyl coenzyme M reductase operon protein C	mcrD	+	+	+	+
Methyl coenzyme M reductase operon protein D	mcrG	+	+	+	+
Monomethylamine:corrinoid methyltransferase	mtmB	+	+	-	+
Monomethylamine methyltransferase corrinoid protein	mtmC	-	-	-	-
Dimethylamine:corrinoid methyltransferase	mtbB	-	+	-	+
Dimethylamine methyltransferase corrinoid protein	mtbC	-	+	-	+
Trimethylamine:corrinoid methyltransferase	mttB	-	+	-	+
Trimethylamine methyltransferase corrinoid protein	mttC	-	+	-	-
Methanol:corrinoid methyltransferase	mtaB	+	+	-	+
Methanol methyltransferase corrinoid protein	mtaC	+	+	-	+
Methylthiol:coenzyme M methyltransferase corrinoid protein	mtsB	+	+	-	-
Methylcobalamin:coenzyme M methyltransferase, methylamine-specific	mtbA	+	-	-	-
Methylcobalamin:coenzyme M methyltransferase, methanol-specific	mtaA	+	-	-	-
Corrinoid protein:coenzyme M methyltransferase, dimethyl sulfide specific	mtsA	-	-	-	-
CoB--CoM heterodisulfide reductase subunit A	hdrA	+	+	+	-
CoB--CoM heterodisulfide reductase subunit C	hdrB	+	+	+	+
CoB--CoM heterodisulfide reductase subunit B	hdrC	+	+	+	+
CoB--CoM heterodisulfide reductase subunit D	hdrD	+	+	+	-
heterodisulfide reductase subunit E	hdrE	-	-	-	-
Energy-conserving hydrogenase (ferredoxin), subunit A	echA	-	+	-	-
Energy-conserving hydrogenase (ferredoxin), subunit B - F	echB-F	-	-	-	-
Acetyl-coenzym A synthetase		+	+	-	-
Pyruvate:ferredoxin oxidoreductase, subunit A	porA	+	+	+	+
Pyruvate:ferredoxin oxidoreductase, subunit B	porB	+	+	+	+
Pyruvate:ferredoxin oxidoreductase, subunit C	porC	+	+	+	+
Pyruvate:ferredoxin oxidoreductase, subunit D	porD	+	+	+	-
Pyruvate:ferredoxin oxidoreductase, subunit E	porE	+	-	-	-
Pyruvate:ferredoxin oxidoreductase, subunit F	porF	+	-	-	-

[a] for details see text

4. Discussion

4.1. Environmental Distribution of *Methanoplasmatales*

This study employed two established phylogenetic marker genes (16S rRNA and *mcrA)* and explored the usability of the *mttB* gene to investigate the distribution of *Methanoplasmatales* in wetlands and animal intestinal tracts.

CREST analysis showed that 95 % of the obtained 16S rRNA sequences belong either to Vadin Gut Group (79 sequences) or TEMG 2 (55 sequences), the two *Thermoplasmata* subgroups which were confirmed to comprise methanogens. Thus, the majority of the obtained 16S rRNA sequences belong to members of the novel 7th methanogenic order of *Methanoplasmatales*. With both *mcrA*-targeting primer pairs (Tp-mcrA-F/Tp-mcrA-R: Mp specific; mlas-mod For/mcrA-rev: all methanogens) *mcrA* genes affiliated with *Methanoplasmatales* could be amplified. However, the Mp specific *mcrA* primers not only amplified Mp *mcrA* genes (19 sequences) but also *mcrA* genes affiliated with other methanogenic lineages (*Methanomicrobiales*: 7 sequences; *Methanocellales*: 6 sequences). This unspecific primer binding was restricted to temperate wetland samples (see fig. 3.2). No Mp *mttB* genes could be amplified from the investigated samples, either because there were no Mp *mttB* genes present (or below the detection limit) or more likely because the respective primers were not suitable. Mp specific *mcrA* and *mttB* primer pairs need to be redesigned to ensure their specifity.

There are certainly different reasons why no *Methanoplasmatales* 16S rRNA and *mcrA* genes could be detected in Vienna Woods soil, kangaroo, cricket and marmoset feces. Vienna Woods soil was probably too aerobic to host methanogens. Further it is known that methanogens can be present in the foregut of kangaroos, but also be absent or below detection limit (*23*). The same seems to be true for the intestinal tracts of crickets and marmosets (*56*, *57*). However, the occurrence of PCR inhibitors within the DNA extracts could also be the reason for the lack of PCR products.

4.1.1. *Methanoplasmatales* in Wetlands

Wetlands like peat soils, bogs and fens are the major natural source of atmospheric methane emissions. Several studies could show the occurrence of *Methanosarcinales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales* and *Methanocellales* in temperate wetlands (*31* - *34*), *Methanosarcinales*, *Methanobacteriales*, *Methanomicrobiales* in arctic wetlands (*30*, *35*, *36*), respectively. We were now able to assign the previously unrecognised methanogenic lineage of *Methanoplasmatales* to various wetland types - ranging from arctic to temperate and acidic to neutral peat, fens, bogs, and wet meadows.

The lack of Mp specific *mcrA* genes in several wetlands (fig. 3.3) might be caused by too low Mp abundances and a dominance of other methanogens (i.e. *Methanomicrobiales* and *Methanocellales*) in combination with weak primer specifity; resulting in the amplification of *mcrA* genes affiliated with these dominant methanogenic lineages. Our results showed that Mp are widely distributed in different wetland types; however, information on the global role of wetland *Methanoplasmatales* in methane formation cannot yet be given. Further studies investigating the abundances and activity of *Methanoplasmatales* in wetlands are necessary to address this question.

4.1.2. *Methanoplasmatales* in Animals

Methanogens occur in the gastrointestinal tracts of a great variety of animals, especially in ruminants (e.g. cattle, sheep, buffalo, reindeer) but also in other non-ruminant mammals (e.g. wallabies, alpaca, camel, some rodents, orang-utan, human) and in *Sauropsida* (reptiles and birds; e.g. tortoise, boa, ostrich, chicken, duck), as reviewed (*40*, *58*). Furthermore, some insects are known to harbour methanogens in their hindgut, especially termites, but also cockroaches, scarab beetles and millipede (*14*, *59*). In their review on gut methanogens of herbivorous animals, 2013, St-Pierre and Wright (*40*) outline that gut methanogens are affiliated with certain predominant, phylogenetic groups; on the one hand with *Methanobacteriales* (especially with *Methanobrevibacter and Methanosphaera* species) on the other hand with a *Thermoplasmatales*-Affiliated Lineage (TALC) now known as *Methanoplasmatales*. Corresponding to that, we showed the occurrence of *Methanoplasmatales* in different vertebrates: in ruminants (Austrian and Danish cows) and non-ruminant mammals (elephant, rat) and in reptiles (giant tortoise, tortoise). Thus, for the first time, to our knowledge, *Methanoplasmatales* were assigned to elephants, rats and tortoises; whereas, the occurrence of *Methanoplasmatales* in ruminants was shown already in previous studies (e.g. *27*, *40*).

4.1.3. *Methanoplasmatales* Ecotypes

It is remarkable that the constructed 16S rRNA gene tree as well as the McrA protein tree showed two main clusters of Mp distinct by sequence origin (fig. 3.1 and 3.2). However, the separation of these two clusters was much sharper within the 16S rRNA gene tree. Apart from one cow clone all animal clones formed an animal specific cluster with bootstrap support of 99 % (90 % support in McrA tree), containing only Vadin Gut Group reference sequences. Few wetland clones fell within the animal cluster. The second main cluster was strongly dominated by wetland clones (wetland cluster) and contained only TMEG 2 reference sequences. RFLP analysis of all 16S rRNA clones across all investigated samples

showed that all animals were dominated by animal cluster RFLPs, all wetland by wetland cluster RFLPs; except AUT peat 7 with 60 % of RFLPs belonging to the animal cluster (fig. 3.3). This shift is very likely because AUT peat 7 differs a lot from all other temperate wetland samples. In contrast to the other fen soil samples collected from the same temperate fen, AUT peat 7 was a ferrous biofilm sample. The different chemical and physical properties might lead to a shift in methanogenic community. We also detected *Thermoplasmatales* related 16S rRNA gene sequences affiliated with other TMEGs and the Marine Benthic Group D. Yet, it is not known, if these groups comprise also methanogenic members. No Mp *mcrA* genes could be amplified from both peat soils (AUT peat 5, IT peat 2) containing 16S rRNA genes associated with Marine Benthic Group D, this could be seen as an evidence of a non-methanogenic nature of this group as suggested previously (*28*, *60*, *61*).
The existence of distinct animal and wetland clusters allows speculations about certain adaptations and specialisations of *Methanoplasmatales* according to their environment and suggests that there are at least two fundamentally different Mp ecotypes. The endosymbiotic Mp within animal intestinal tracts may live in closer contact and syntrophy with other microorganisms. This might also be true for *Methanoplasmatales* living in biofilms as represented by AUT peat 7. The same is indicated by the genome sizes of *Ca.* M. alvus (representative of Vadin Gut Group) and *M. luminyensis* (TMEG 2), with a size of 1.7 Mb (*25*) and 2.6 Mb (*24*), respectively. The genomes of (endo)symbionts are known to be much smaller than the genomes from their free-living relatives because (endo)symbionts can afford to reduce genes and pathways for products they obtain from their hosts/symbiosis partner (*62*). Although *M. luminyensis* was isolated from human feces, it might not yet be adapted to an endosymbiotic lifestyle.

Furthermore, all Mp OTUs comprised only sequences originating from the same animal (resulting in animal specific OTUs; cow-, elephant-, rat-, giant tortoise-, tortoise-OTU) except for one rat clone clustering with an Austrian rumen fluid OTU (AUT 02; see section 3.1 and tab. 3.2). This might suggest a host specifity of *Methanoplasmatales*, as it was also indicated by the study of Paul et al. (*14*) showing termite and cockroach specific Mp sequence clusters. However, in this study, far too few different animal species and clones were investigated to allow concluding that *Methanoplasmatales* are host specific; further investigations for a verification of this hypothesis are necessary.

4.2. Rumen *Methanoplasmatales*

4.2.1. Abundances of Rumen Mp and Global Role in CH_4 Formation

Methanogens are the major archaeal group in ruminants and there they are acting as the terminal group in anaerobic plant degradation (*39*). Despite their generally low abundances (0.3 - 3.3 % of total rumen prokaryotes; *39*) rumen methanogens are a very considerable group due to their ability to produce the greenhouse gas methane, which is released to the atmosphere mainly via the cow breath (*37*, *38*). In our study the novel methanogenic group of *Methanoplasmatales* contributed less than 1 % to the prokaryotic community of the investigated Austrian cow rumen fluid (fig. 3.5). Nevertheless, our results along with recent literature on *Methanoplasmatales* suggest that they play an important global role in methane formation, as they are widely distributed and a major archaeal / methanogenic group in ruminants (reviewed in *40*). It was reported that *Methanoplasmatales* constitute up to 80 % of the archaeal community in bovine rumen and in sheep, little less Mp were detected in goats (up to 60 %) and in reindeer (up to 50 %) (*21*, *27*, *40*, *63*, *64*); correspondingly we detected 42 % *Methanoplasmatales* in Austrian rumen fluid (fig. 3.5). Even more Mp were detected in Danish rumen fluid (68 %); possibly DK RF was primary enriched in Mp because it was pre-incubated with TMA. Ruminants are major atmospheric methane sources, especially ruminant livestock, which are accounting for ~37 % of global anthropogenic CH_4 emissions (*11*). Ruminant CH_4 emissions are predicted to increase as demands for milk and meat are expected to double by 2050 (*65*). Therefore, the occurrence of the novel, previously unrecognised group of *Methanoplasmatales*, their abundances and their dominance in many ruminants make them a very potent target for methane mitigation strategies as already suggested by Poulsen et al. (*27*) who could show an inhibition of RCC *Methanoplasmatales* after dietary fat supplementation (i.e. rapeseed oil, RSO). However, the mode of action of RSO is currently not understood and further investigations are needed.

4.2.2. Diversity of Rumen *Methanoplasmatales*

The diversity within *Methanoplasmatales* in Austrian cow rumen fluid is astonishingly high. Nine distinct OTUs with less than 98 % sequence identities were detected (see tab. 3.2 and fig. 3.4), showing mainly low sequence identity to their closest relative, *Ca.* M. caenicola. The reduced Mp diversity and the dominance of one cluster within the Danish cow rumen fluid suggest that the incubation with TMA selected for a certain type of Mp closely related to *Ca.* M. alvus. As expected, all 16S rRNA sequences from Austrian and Danish rumen belonged to the animal cluster, pointing out that animal cluster Mp are the predominant *Methanoplasmatales* in the rumen. However, this changed through the enrichment trials. After several subcultural transfers, wetland cluster Mp were the dominant *Methanoplasmatales* within the enrichment cultures (see fig. 3.17 and section 4.3.3).

4.3. Enrichment Trials & Inferences on Rumen *Methanoplasmatales*

Figure 4.1 shows key events/observations during the enrichment trials. In the following sections these events/observations are discussed in detail.

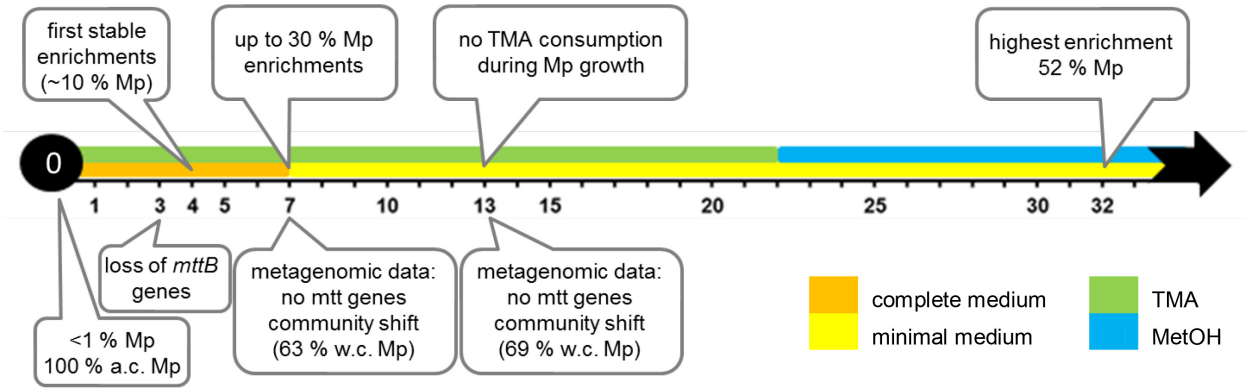


Figure 4.1. Key events/observation during rumen *Methanoplasmatales* (Mp) enrichment trials. Transfers of subcultures are given on the time scale. 0, start of enrichments, initial cultures; 1 - 33, transfers; a.c. animal cluster; w.c. wetland cluster. For details see section 4.3.1 - 4.3.4.

4.3.1. Medium Composition for Enrichments

To enrich *Methanoplasmatales* from bovine rumen fluid the medium used for *M. luminyensis* isolation (*24*) was slightly modified (complete medium; different vitamin and trace element solutions; tab. 2.5). Subsequently, organic components, possible substrates for heterotrophic bacteria, were reduced. The resulting minimal medium (tab. 2.5) seems to be a proper medium to enrich Mp from bovine rumen fluid. However, the medium is missing some essential components since considerable Mp growth could only be ensured by the addition of sterile filtered rumen fluid to the medium (see fig. 3.7). Supplementation of sterile rumen fluid is very common in cultivation of methanogens (and strictly anaerobic bacteria), even in cultivating non-ruminant methanogens (e.g. cockroach and millipede methanogens; *14*).

The further reduction of the minimal medium to extreme minimal medium could not increase Mp enrichments; in fact no considerable Mp growth was observed. One reason for that might be the lack of acetate (see tab. 2.5), which is, according to the genome analyses (see section 3.2.8.2 and 4.4), probably the carbon source of *Methanoplasmatales*.

4.3.2. Substrates of Rumen *Methanoplasmatales*

During the enrichment experiments the following possible substrates were investigated (see 2.2.3.2): TMA, H_2, CO_2, MetOH and sterile filtered rumen fluid.

Rumen Fluid. As already mentioned sterile filtered rumen fluid was necessary for considerable Mp growth. Rumen fluid is a very complex substrate, with varying, cow- and nutrition-specific, composition. Without any further fractionation of the supplemented rumen fluid it is not possible to identify the essential components for Mp and if they originate from the host or other intestinal microorganisms.

Hydrogen. Especially at the beginning it was hard to tell if there was any difference between cultures grown with or without H_2 (see fig. 3.6). With an increasing number of subcultures our results suggested that rumen Mp need H_2 for growth, consistently higher Mp growth and enrichments were obtained if H_2 was supplemented. Nevertheless, Mp grew also without H_2 addition. As bacteria were dominating the enrichment cultures it is very likely that the bacteria provided H_2 for Mp methanogenesis and, therefore, for Mp growth. These presumptions are consistent with the genome analyses of RumEN_Mp1, RumEN_Mp2, *M. luminyensis* and *Ca.* M. alvus (see sections 3.2.8.2, 4.4 and reference *28*) suggesting that Mp lack the reverse hydrogenotrophic methanogenic pathway; instead they are using H_2 from an external source as electron donor to reduce methylated compounds to CH_4. Furthermore, if more than 10 % H_2 (headspace concentration) was supplemented, much better growth of other methanogens than of Mp was observed. This might suggest that rumen *Methanoplasmatales* compete with other methanogens for H_2 and they are outcompeted at high H_2 concentrations.

Carbon Dioxide. The experimental setup was not designed to verify the influence of CO_2 on rumen Mp. CO_2 was always used in combination with H_2 and concentrations were not varied. The genome analyses (3.2.8.2, 4.4, *28*) suggest that *Methanoplasmatales* cannot use CO_2 for methanogenesis, since no genes for the reduction of CO_2 to CH_4 are present in any of the four analysed genomes. The RumEN_MG1 and *M. luminyensis* genomes contain many genes for CO_2-fixation via the Calvin-Benson cycle. However, the pathway is incomplete and it is more likely that Mp use acetate as carbon source. Although, CO_2 fixation might explain the relative enrichment of wetland cluster Mp during the incubation under a CO_2-containing atmosphere. The relevance of CO_2 for (rumen) *Methanoplasmatales* needs to be verified with further investigations.

TMA & MetOH. At the beginning of the enrichment attempts all cultures were incubated with TMA (based on the study of Poulsen et al.; *27*). The early enrichments (see fig. 3.7; 3.8) seemed to confirm the hypothesis that TMA serves as electron acceptor and H_2 as electron donor for rumen *Methanoplasmatales*. Enrichments between 10 - 30 % were obtained (see fig. 4.1). However, after several transfers, subcultures changed and react differently even under the same treatment; resulting in unpredictable Mp enrichments. Our observations increasingly made us wonder whether TMA is used as substrate or not. Eventually, we found increasing evidence for the falsification of our hypothesis. Firstly, the TMA measurements showed that no TMA was consumed during *Mp* growth (see fig. 3.13; t_0 - t_2; fig. 4.1). But, TMA concentration decreased after each later TMA addition (fig. 3.13; t_3 - t_6; t_6 - t_{11}). Even if Mp did not grow on TMA during exponential growth phase, TMA could have been used in the later stationary growth phase of Mp. But it is also possible that TMA was consumed or removed by other microbes of the enrichment cultures. However, determining the role of TMA based on TMA measurements of one single enrichment culture is impossible. Secondly, no *mttB* genes could be PCR amplified from selected subcultures after the 3rd transfer (see fig. 4.1. and section 3.2.5). Thirdly, no genes encoding for enzymes specific for reduction of TMA to CH_4 (*mttB* and *mttC*) were found in the metagenomes RumEn_MG1 and RumEn_MG2, obtained from the enrichments (7th and 13th transfer; see tab. 3.7 and fig. 4.1). All this suggests that TMA might have been utilized by rumen *Methanoplasmatales* in early enrichments, but could not be used anymore by Mp of later enrichments (see also 4.3.3). This might explain the unstable enrichments on TMA and the much better growth and higher enrichments of Mp (up to 52 % of prokaryotic community) after switching to the addition of MetOH as substrate for methanogenesis (fig. 4.1).

4.3.3. Eco-physiology and Interactions of Rumen *Methanoplasmatales*

The substrate switch (from TMA to MetOH) might correlate with the Mp community switch from animal cluster dominated cultures (initial cow rumen fluid and perhaps early enrichments) to wetland cluster dominated enrichments (fig. 4.1). The genome of *M. luminyensis*, the representative wetland cluster genome, is rather big, suggesting that wetland cluster Mp are metabolically more flexible and can react and adapt faster to changing environments, like the change from cow rumen to an artificial medium. This would explain the Mp community switch and the dominance of wetland cluster Mp, revealed by the enrichment metagenomes (fig 3.17). Another explanation is given by the medium itself. The used medium to enrich rumen Mp was originally designed for the isolation of *M. luminyensis*, a wetland cluster Mp, therefore, wetland cluster Mp instead of animal cluster Mp might have been favoured by the medium.

Whenever serial dilutions were performed (or filtration; see section 3.2.4.1) Mp growth was outcompeted by bacterial growth, and even more by the growth of other methanogens. It seems like rumen Mp have a disadvantage at high nutrient availability compared to other methanogens (e.g. high H_2 concentrations). This might suggest that in terms of methanogenesis *Methanoplasmatales* occupy spatial and temporal niches in the rumen with rather low nutrient/H_2 concentrations and/or that they grow slower than other methanogens.

Not only the Mp community changed during the enrichment trials, but also the bacterial community (see fig. 3.16) changed, conceivably several times. The rumen Mp seemed to be linked to rumen bacteria. Disturbing and decreasing bacteria (e.g. via antibiotics) also decreased Mp; no considerable Mp growth was observed whenever bacterial growth was inhibited. Bacterial community shifts could, therefore, be an additional explanation for the unstable and unpredictable rumen Mp enrichments. Anyway, it seems to be clear that rumen *Methanoplasmatales* live in close metabolic interactions with rumen bacteria (fig. 4.2). They might even live in syntrophy. In the rumen, bacteria (and protozoa) very likely supply Mp with H_2, TMA and MetOH. Rumen Mp might also depend on other bacterial products, indicated by the Mp dependency on sterile rumen fluid. Furthermore, the dependence might be vice versa. Rumen *Methanoplasmatales* might supply bacteria with ammonia (NH_3), as NH_3 is a by-product of methanogenesis from methylamines (see fig. 4.3) and keep the H_2 partial pressure low. Presumably, rumen *Methanoplasmatales* and bacteria live also in symbiosis with eukaryotic rumen microorganisms and are also depending on their host (fig. 4.2).

According to this complex rumen community, struggling with isolating a representative rumen Mp is not too surprising. Furthermore, the reason why no Mp colonies could be grown on solid medium (see section 3.2.4.3) might be a lack of supply with bacterial and host products essential for rumen *Methanoplasmatales*.

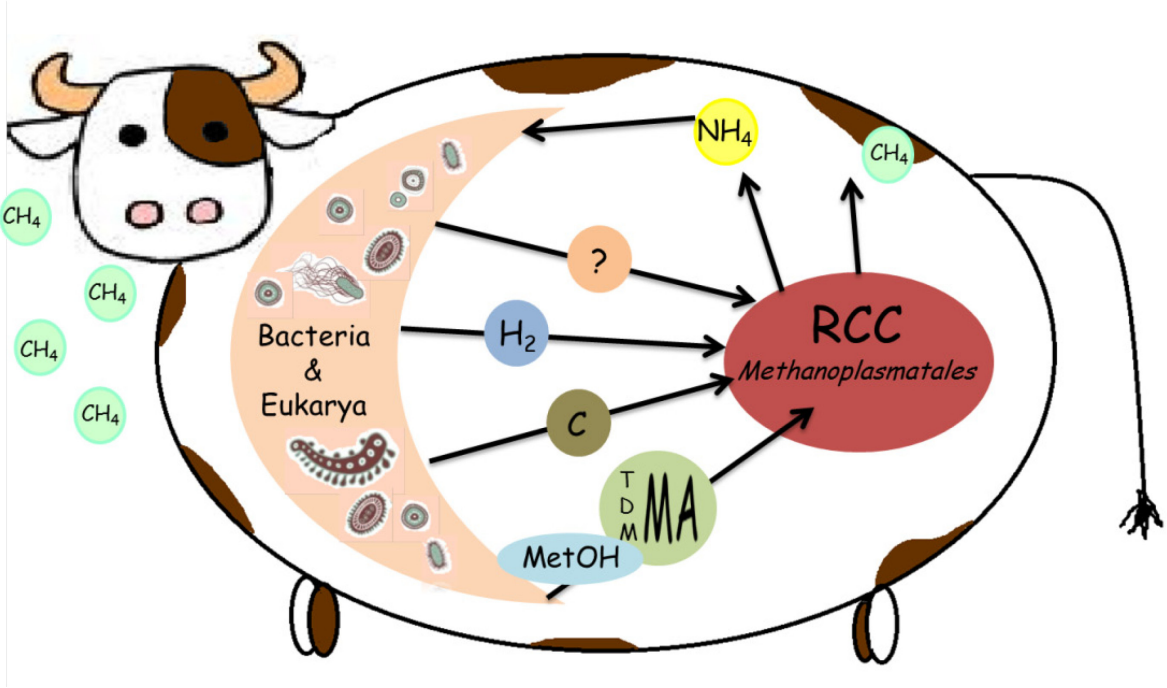


Figure 4.2. Schematic diagram of the putative interactions between rumen *Methanoplasmatales* (RCC) and other ruminal microorganisms (Bacteria and Eukarya). The complex ruminal microbial community provide substrates for methanogenesis (methanol, MetOH; mono-, di-, and trimethylamine, MMA, DMA, TMA; hydrogen, H_2) to the *Methanoplasmatales*; additionally they provide acetate as possible carbon(C)-source and perhaps other essential products (question mark). RCC *Methanoplasmatales* produce methane (CH_4) as an end-product of their anaerobic respiration, which is released to the atmosphere mainly via the breath of the cow. NH_4 is produced as a by-product of RCC methanogenesis from methylamines and might be supplied to the rumen microbiota. Besides interactions of RCC with other ruminal microorganisms interactions with the host are very likely.

4.3.4. Morphology of Rumen *Methanoplasmatales*

Enrichment cultures showed high morphological diversity. However, the numbers of observed small cocci seemed to coincide with numbers of *Methanoplasmatales* quantified with qPCR. According to that and the described morphologies of Mp (*14*, *24*) the observed small cocci are potentially rumen *Methanoplasmatales*. If so, rumen Mp seem to occur as single cells, but also in big cell aggregates (see fig. 3.15). These big cell aggregates were found in liquid enrichment cultures and especially in the sediment layers of the cultivation tubes (see fig. 3.12; fig. 3.15). Autofluorescence of the latter could be shown (fig. 3.15; D). However, it is not clear if this autofluorescence conforms to the F_{420}-dependent autofluorescence many methanogens show. Used wave length to excite the cells was below 420 nm, the absorption maximum of F_{420}. Therefore, other compounds could be the cause of the observed autofluorescence. No autofluorescence could be detected from single cocci, probably because autofluorescence was too faint or might be cell density dependent. Furthermore, no genes for F_{420} were found in the two investigated metagenomes of rumen fluid enrichments, pointing towards other compounds, as e.g. flavins, being responsible for the autofluorescence.

4.4. Methanogenic Pathways and C-source of *Methanoplasmatales*

The methanogenic pathway of Mp (fig. 4.4) was constructed based on the genome analyses of RumEN_Mp1, RumEN_Mp2, *M. luminyensis* and *Ca.* M. alvus. Furthermore, recent literature on methanogenesis and Mp was consulted (*12, 24, 25, 28, 66*).

As in all described methanogenic pathways the last step of methanogenesis, the formation of CH_4 from Methyl-coenzym M (CH_3-S-CoM), is performed by Mcr (Methyl:Coenzyme M reductase). The methyl-groups needed to generate CH_3-S-CoM seems to be derived from methylamines (mono-, di-, trimethylamine; MMA, DMA, TMA) and methanol (MetOH); furthermore, as depicted also by Borrel et al. (*28*) probably from dimethyl sulfide. However, RumEN_Mp1 lacked most of the genes necessary for the utilization of methylamines, RumEN_Mp2 contained no genes for the utilization of methylamines and methanol and both rumen Mp genomes lacked genes to produce CH_4 from dimethyl sulfide. Both obtained genomes were incomplete, what could be one explanation for the missing genes.

The predicted utilisation of various methylated compounds (MetOH, MMA, DMA, TMA, dimethyl sulfide) is very remarkable. Only one other methanogenic order, the *Methanosarcinales*, is capable of producing CH_4 from such a broad range of methylated substrates. Thus, it is very likely that rumen *Methanoplasmatales* reducing methylamines to CH_4 occupy a unique metabolic niche within the rumen, because *Methanosarcinales* are not known to be harboured by ruminants.

The enriched rumen *Methanoplasmatales* seemed to have lost their capability of reducing TMA to CH_4 during the enrichment trials. The same might be true for reduction of MMA and DMA to CH_4. In the late enrichments Mp grew only on MetOH. The genes (*mtbA* and *mtaA*) encoding the enzymes necessary for the transfer of the methyl-groups onto coenzyme M (HS-CoM) could not be identified definitely; only the genome of RumEN_Mp1 contained genes for a methylamine- and a methanol-specific methylcobalmin:coenzym M methyltransferase (tab. 3.7).
The lack of a dimethyl sulfide specific coenzyme M methyltransferase (*mtsA*) in all investigated genomes might be compensated by other methyltransferases (tab. 3.7), which might also transfer methyl-groups of dimethyl sulfide onto coenzyme M, allowing the utilisation of dimethyl sulfide for Mp methanogenesis.

As the reverse H_2/CO_2 methanogenesis pathway is not present in *Methanoplasmatales* it is very likely that the electrons needed for the reduction of CH_3-S-CoM to CH_4 are derived from additional H_2, which makes this methylotrophic methanogenic pathway H_2-dependent as described in (*66*). The hydrogenase necessary to split H_2 and generate electrons could not be identified. However, the gene encoding for the alpha subunit of one hydrogenase known and capable of performing this reaction, *echA*, was detected in the genome of *M. luminyensis*, but the other subunits were missing (*echB-F*).

It seems like acetate serves as carbon source for *Methanoplasmatales*, because RumEN_Mp1 and the *M. luminyensis* genome contained all genes necessary to assimilate acetate (tab. 3.7). In contrast, the gene encoding for acetyl-coenzyme A synthetase, which performs the formation of acetyl-coenzyme A from acetate, is missing in RumEN_Mp2 and the genome of *Ca*. M. alvus. However, acetate as carbon source was also suggested by the studies of Brune and colleagues (personal communications with Andreas Brune).
Rumen Mp could be grown on complete medium and minimal medium (medium composition see tab. 2.5; both contained acetate as a constant component) but could not be grown on extreme minimal medium, lacking acetate, which is another hint pointing at acetate as carbon source of *Methanoplasmatales*.

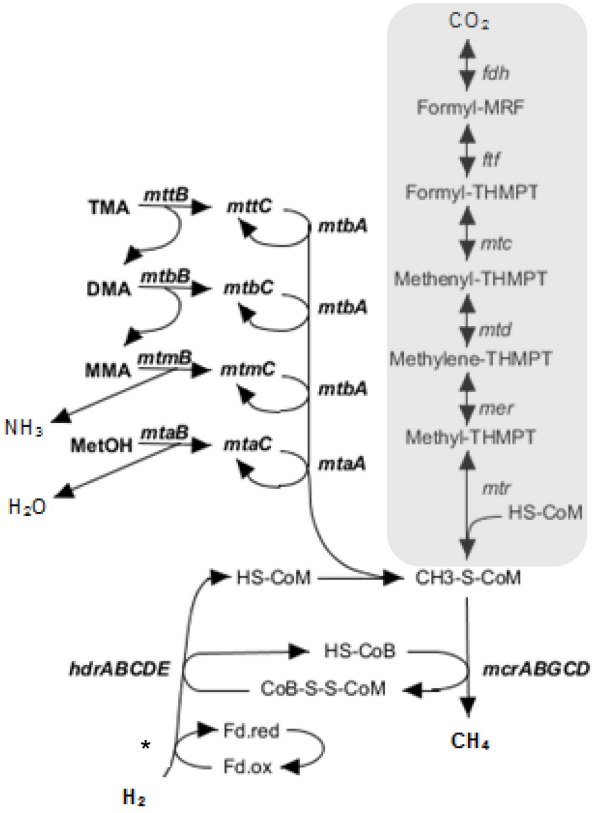


Figure 4.4. *Methanoplasmatales* (Mp) methanogenic pathway and involved genes. MRF, methanofuran; THMPT, tetrahydromethanopterin; HS-CoM, coenzyme M; HS-CoB, coenzyme B; CH3-S-CoM, Methyl-coenzym M; TMA, trimethylamine; DMA, dimethylamine; MMA, monomethylamine; MetOH, methanol; asterisk, hydrogenase (possibly Ech), grey box: pathway not present in Mp. For details see text (3.2.8.2; 4.4).

5. Conclusions

This study shows that *Methanoplasmatales* are widely distributed in wetlands, as well as in animal intestinal tracts. Furthermore, they represent one major methanogenic group within the bovine rumen. This suggests that they play an important role in global methane production and atmospheric methane emissions, as wetlands are the major natural and ruminants the major anthropogenic methane sources. This makes the previously overlooked *Methanoplasmatales* one considerable group of methanogens, especially in the context of global climate change. Due to global warming arctic permafrost soils are expected to thaw and turn partially into wetlands, thus potentially resulting in an increase of methane emissions from high latitudes. Additionally, CH_4 emissions from ruminants are predicted to increase as demands for milk and meat of a growing world population are expected to double by 2050 (*65*). To fully understand the global impact of *Methanoplasmatales* further investigations focusing on their environmental abundances and their physiological activities are necessary.

Counteracting increasing methane emissions from arctic wetlands is hardly feasible. However, rumen methanogens, and especially rumen *Methanoplasmatales* are promising targets for methane mitigation strategies. Proposed methane mitigation strategies include the removal of protozoa as carriers of methanogens (defaunation), the addition of plant fats and secondary metabolites as antimicrobials or for the limitation of methanogenesis substrates (reviewed in *9*, *65*, *67*). Poulsen et al. (*27*) showed an inhibitory effect of rapeseed oil on rumen *Methanoplasmatales*. Further investigations are necessary to clarify the mode of action of rapeseed oil supplementation and to develop efficient methane mitigation strategies ensuring ecologically and economically sustainable ruminant milk and meat production.
Furthermore it can be assumed that there are significant differences (e.g. physiological differences) between endosymbiotic (e.g. rumen Mp) and free-living *Methanoplasmatales*. It will be interesting to learn more about nature and extent of their adaptations to specific environments.

Methanoplasmatales seem to possess a truncated H_2-dependent methanogenesis pathway specialised on the utilisation of methylated compounds as electron acceptors and it is very likely that they are heterotrophs, with acetate being their carbon source. Within the rumen Mp seems to occupy a unique metabolic niche. Further efforts are necessary to eventually isolate one *Methanoplasmatales* representative from cow rumen. However, we already could see how the natural state (e.g. dominance of animal cluster Mp; usability of TMA) is disturbed by cultivation approaches, leading to cultivation biases. This suggests that further cultivation-independent studies are necessary for an in-depth eco-physiological characterisation of rumen *Methanoplasmatales* in their natural habitat.

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Furthermore, I owe thanks to everyone who provided samples and material for my work. I want to thank Michael Pester and Alexander Loy from the University of Vienna for proving all temperate wetland samples; Alexander Tveit from the University of Tromsø, Norway and Tim Urich for providing arctic wetland samples and 16S rRNA contigs; Morten Poulsen from the University of Aarhus, Denmark for providing the Danish cow rumen fluid; Kathrin Deckardt and Johannes Lorenz Khol from the University of Veterinary Medicine Vienna for providing the Austrian cow rumen fluid; Clarissa Schwab for providing the other animal samples; and the Division of Terrestrial Ecosystem Research, University of Vienna (especially Margarete Watzka) for supplying hydrogen. And, of course, I owe special thanks to Ridi the cow, who was donating the Austrian cow rumen fluid.

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8. Supplement

8.1. Zusammenfassung

Methan (CH_4) ist ein sehr starkes Treibhausgas und wird hauptsächlich von methanogenen Archaea produziert. Diese leben in einer Vielzahl von anaeroben Habitaten und bilden dort CH_4 als Endprodukt ihrer anaeroben Atmung. Wiederkäuer gelten als größte anthropogene Methanquelle (Viehzucht), Moor- und Sumpfgebiete als größte natürliche Quellen. Vor kurzem wurde eine bislang unbekannte Ordnung der methanogenen Archaea innerhalb der Klasse der *Thermoplasmata* entdeckt - die *Methanoplasmatales* (Mp). Bisher konnte nur ein einziger Vertreter der Mp isoliert werden (aus menschlichem Stuhl), welcher CH_4 aus Methanol und Wasserstoff (H_2) erzeugt. Des Weiteren weist eine Metatranskriptom-Studie auf in Wiederkäuern lebende Mp hin, welche auch Methylamine zu CH_4 reduzieren können. Hauptanliegen und -ziele dieser Arbeit waren (1) die Verbreitung der Mp zu untersuchen, sowie (2) ein Mitglied dieser unzureichend charakterisierten Gruppe aus dem Rinder-Pansen, für physiologische und genomische Analysen, zu isolieren.

Ein PCR-Screening nach 16S rRNA Genen (phylogenetische Marker) und *mcrA* Genen (phylogenetische und funktionelle Marker) von Mp wurde in verschiedenen Mooren (arktische und saure/neutrale warm-gemäßigte Standorte) und tierischen Stuhlproben durchgeführt. Mehr als 40 PCR-Produkte wurden kloniert, über 240 Klone sequenziert und anschließend phylogenetische Bäume berechnet. Mp 16S rRNA Gene wurden in allen arktischen und warm-gemäßigten Mooren sowie in den meisten der untersuchten Tierproben (Elefanten, Rinder, Ratten und Schildkröten) gefunden. In allen Tierproben, arktischen und in einigen der nemoralen Moorböden wurden zusätzlich Mp *mcrA* Gene detektiert. Die phylogenetischen Analysen beider Markergene zeigten übereinstimmend zwei voneinander klar getrennte Gruppen innerhalb der Mp - eine von Tier-Sequenzen („animal cluster Mp“), die andere von Moor-Sequenzen („wetland cluster Mp“) dominiert. Dies weist auf die Existenz von verschiedenen, speziell auf ihren Lebensraum angepassten Mp Ökotypen hin (freilebende und endosymbiontische Mp). Es sind jedoch weiter Studien nötig, um neben der weiten Verbreitung von Mp auch deren Abundanzen und Aktivität und somit deren globale Rolle in CH_4-Emissionen von Mooren und Sumpfgebieten beurteilen zu können.

Um *Methanoplasmatales* aus dem Rinder-Pansen anzureichern, wurde Mp Medium mit Pansen-Flüssigkeit inokuliert und entweder mit Trimetylamin (TMA) oder Methanol inkubiert. Mittels quantitativer PCR wurden Mp Abundanzen und Anreicherungen in den Subkulturen überwacht. Während unterschiedlicher Anreicherungsphasen wurden verschiedenste Methoden zur Steigerung der Mp Anreicherungen angewendet, physiologische Tests durchgeführt und metagenomische Untersuchungen vorgenommen. Die Mikrobiota der ursprünglichen Pansen-Flüssigkeit war bakteriell dominiert - die Mp machten weniger als 1 %

der Prokaryonten aus. Allerdings gehörten 40 % aller Methanogenen zur Ordnung der *Methanoplasmatales* (animal cluster). Mp konnten sowohl mit TMA (bis zu 34 % prokaryontischer 16S rRNA Gene) als auch mit Methanol (bis zu 52 %) angereichert werden. Unter beiden Bedingungen stieg der prozentuelle Anteil der Mp an den Methanogenen auf über 99 %. Nennenswertes Mp Wachstum konnte nur dann beobachtet werden, wenn den Anreicherungskulturen sterile Pansen-Flüssigkeit und H_2 beigegeben wurde. Wurden die Bakterien in den Anreicherungskulturen inhibiert, blieb auch das Wachstum der Mp aus. Die metagenomischen Analysen zeigten, dass sich im Laufe der Anreicherungsversuche die Zusammensetzung der bakteriellen und auch der methanogenen Gemeinschaften in den Subkulturen änderte. Die *Methanoplasmatales* beispielsweise wurden nach 13 Passagen von wetland cluster Mp dominiert (69 %). Aus den Metagenomen konnten zwei partielle Pansen-Mp Genome assembliert werden (ca. 80 % Vollständigkeit), wobei ein Genom große Ähnlichkeiten mit *Candidatus* Methanomethylophilus alvus (animal cluster Mp) aufwies, das andere mit *Methanomassiliicoccus luminyensis* (wetland cluster Mp). Sowohl in den Genomen von *Ca.* M. alvus und *M. luminyensis* als auch in den Pansen-Mp Genomen sind Gene für die CH_4 Bildung aus methylierten Verbindungen (Methanol, Methylamine, Dimethylsulfid) codiert. Jedoch fehlen in den partiellen Pansen-Mp Genomen die nötigen Gene, um aus TMA CH_4 erzeugen zu können. Die Pansen-Mp haben demnach die Fähigkeit zur TMA-Metabolisierung im Laufe der Anreicherungsversuche verloren, worauf auch TMA Messungen und spezifische PCR Amplifikationen hinwiesen. Das Vorhandensein der Gene für Acetyl-Coenzym A Synthetase und Pyruvate:ferredoxin Oxidoreductase lässt darauf schließen, dass Acetat den Mp als Kohlenstoffquelle dient. Endosymbiontische Mp, wie die Pansen-Mp, leben sehr wahrscheinlich in engen metabolischen Beziehungen mit anderen Mikroorganismen. Dies würde die während der Anreicherungsversuche deutlich werdende Abhängigkeit der Mp vom bakteriellen Wachstum sowie von der Zugabe der sterilen Pansen-Flüssigkeit, welche verschiedenste für Mp essentielle Verbindungen enthalten könnte, erklären. Die Fähigkeit der Pansen-Mp CH_4 aus Methylaminen herzustellen grenzt sie deutlich von anderen, im Pansen vorkommenden Methanogenen ab und erlaubt es ihnen somit eine spezifische ökologische Nische im Pansen zu besetzen.

Zusammenfassend konnte diese Arbeit zeigen, dass *Methanoplasmatales* in Mooren und tierischen Verdauungstrakten sehr weit verbreitet sind, und zumindest im Rinder-Pansen zu den abundantesten Methanogenen gehören. Demnach spielen sie weltweit eine wichtige Rolle als CH_4 Produzenten, was sie in Bezug auf Strategien zur Verringerung der CH_4 Emissionen zu einer besonders interessanten Organismengruppe macht. Weiter Studien, insbesondere kultivierungs-unabhängige Studien, sind nötig um mehr über die (Öko)-Physiologie und die Interaktionen der Pansen-Mp zu erfahren und dadurch brauchbare und umsetzbare Strategien zur Senkung der globalen CH_4 Emissionen entwickeln zu können.

8.2. Curriculum Vitae



Andrea Söllinger, BSc
born, 1988 in Upper Austria.

School Education:

1995 – 1999 Primary School, Gaspoltshofen, Upper Austria
1999 – 2003 Secondary Modern School, Gaspoltshofen, Upper Austria
2003 – 2007 Grammar School / High School, BORG Grieskirchen, Upper Austria (with special emphasis on Biology, Chemistry and Physics)
June 2007 A-levels (in German, English, Mathematics and Physics)

University Education:

Oct. 2007 – Jun. 2008 Study of Ecology and Biodiversity (Bachelor's program), at the University of Salzburg, Austria
Oct. 2008 – Jun. 2011 Bachelor's degree in Biology with focus on Ecology, at the University of Vienna, Austria; graduated in June, 2011.
Oct. 2011 – 2014 Study of Ecology with focus on Microbial, Molecular and Chemical Ecology (Master's program) at the University of Vienna, Austria
Jan. 2013 – 2014 Master's thesis at the Department of Ecogenomics & Systems Biology (former: Department of Genetics in Ecology), Faculty of Life Sciences, University of Vienna, Austria

Specific Education and Internships:

Jun. 2013 Bioinformatics course: Computational Methods in Microbial Ecology, Department of Microbiology and Ecosystem Science, University of Vienna. Subject (among others): Computational pipelines for amplicon sequencing.

Sep. 2012 - Jan. 2013 Internship at the Department of Genetics in Ecology, University of Vienna. Subject: Methanogenic *Thermoplasmata* in Wetlands and Animals – Environmental Distribution of a Novel Group of Methanogens.

Jul. 2012 Practical course: Phylogeny of Prokaryotes, Department of Microbial Ecology, University of Vienna. Subject (among others): Comparative sequence analysis and phylogenetic tree construction using ARB.

May 2012 Practical course: Biogeochemistry of Streams and Lakes, WasserCluster Lunz, Lunz am See, Austria. Subject: Metabolism and Nutrient-Dynamics in Streams.

Apr. - May 2012 Project Internship: Proteomics in Systembiology, Department of Molecular Systems Biology, University of Vienna. Subject: Proteome analysis of *Arabidopsis thaliana*.

Feb. 2012 Bioinformatics course: Metagenomics and Metatranscriptomics, CUBE – Division of Computational Systems Biology, University of Vienna.

Feb. 2012 Practical course at the Department of Genetics in Ecology, University of Vienna. Subject: Methods in ecogenetics – microbial communities and model organisms – molecular, genetic and postgenomic analyses.

Jan. 2012 Practical course: Stable Isotope Tracing, Department of Chemical Ecology & Ecosystem Research, University of Vienna. Subject: N-isotope fractionation during nitrification by ammonia oxidizing microorganisms.

Apr. 2011 Project Internship: Marine Biology, Marine Biology Station Piran, Slovenia. Subject: Carbon flux in coastal waters using the example of Piran.

Feb. 2011 Project Internship: Fluorescence *In Situ* Hybridisation (FISH), Department of Microbial Ecology, University of Vienna. Subjects: Identification of prokaryotes in environmental samples using FISH. Epifluorescence and confocal laser scanning microscopy.

Oral Presentations at workshops and conferences:

"Methanogenic *Thermoplasmata* in Wetlands and Animals". **Let's talk about symbiosis! - Workshop,** November 2013, Vienna Ecology Center, Austria.

"Methanogenic *Thermoplasmata* - Enrichment Cultures and Environmental Distribution of a Novel Group of Methanogens". **Science Day of the Vienna Ecology Center,** March 2013, Vienna Ecology Center, Austria.