Comparative Genomic Analysis of Members of the Genera *Methanosphaera* and *Methanobrevibacter* Reveals Distinct Clades with Specific Potential Metabolic Functions

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*Methanobrevibacter* and *Methanosphaera* species represent some of the most prevalent methanogenic archaea in the gastrointestinal tract of animals and humans and play an important role in this environment. The aim of this study was to identify genomic features that are shared or specific for members of each genus with a special emphasis of the analysis on the assimilation of nitrogen and acetate and the utilization of methanol and ethanol for methanogenesis. Here, draft genome sequences of *Methanobrevibacter thaueri* strain DSM 11995^T^, *Methanobrevibacter woesei* strain DSM 11979^T^, and *Methanosphaera cuniculi* strain 4103^T^ are reported and compared to those of 16 other *Methanobrevibacter* and *Methanosphaera* genomes, including genomes of the 13 currently available types of strains of the two genera. The comparative genome analyses indicate that among other genes, the absence of molybdopterin cofactor biosynthesis is conserved in *Methanosphaera* species but reveals also that the three species share a core set of more than 300 genes that distinguishes the genus *Methanosphaera* from the genus *Methanobrevibacter*. Multilocus sequence analysis shows that the genus *Methanobrevibacter* can be subdivided into clades, potentially new genera, which may display characteristic specific metabolic features. These features include not only the potential ability of nitrogen fixation and acetate assimilation in a clade comprised of *Methanobrevibacter* species from the termite gut and *Methanobrevibacter arboriphilus* strains but also the potential capability to utilize ethanol and methanol in a clade comprising *Methanobrevibacter wolini* strain DSM 11976^T^, *Mbb.* sp. AbM4, and *Mbb.* boviskoreani strain DSM 25824^T^.

1. Introduction

The microbial ecology of the intestinal tract of humans and animals has been subject to extensive research in recent years, and it is becoming increasingly evident that commensal intestinal microbes have a strong impact on host physiology and well-being. One microbial group, the methanogenic archaea (methanogens), has been of particular interest in this regard. Methanogens have not only been implicated in greenhouse gas emission from livestock animals [1, 2] but several studies have also linked these microorganisms to specific bodyweight phenotypes in humans [3] and to differences in feed conversion efficiency in ruminants [4, 5].

The majority of the rumen and intestinal methanogens appear to belong to two of the seven known orders, the Methanobacteriales [6] and the recently described Methanomassiliicoccales [7]. Two genera of the order Methanobacteriales, *Methanobrevibacter* and *Methanosphaera*, comprise a large proportion of intestinal and rumen methanogenic archaea [8, 9]. Species of the genus *Methanobrevibacter* have been isolated from not only a wide range of different environments, including invertebrate and vertebrate guts, but also non-host-associated environments. The majority of the members of the genus *Methanobrevibacter* grow primarily hydrogenotrophically [10], using CO₂ and H₂ as substrates. A few strains and/or species have been shown by
cultivation-independent methods to be the predominant in specific intestinal environments, such as Mbb. smithii from the human gut [8] or Mbb. ruminantium, and Mbb. gottschalkii from the rumen of sheep and cows [11]. The former three species have gained considerable attention in the analysis of the human and rumen microbiome, but there are also Mbb. isolates that are less well investigated by cultivation-independent studies, for example, Mbb. thaueri strain DSM 11995T and Mbb. woesei strain DSM 11979T. Mbb. thaueri strain DSM 11995T was isolated from cow feces while Mbb. woesei strain DSM 11976T had been isolated from cow feces [12]. The 16S rRNA gene of either of the two species shares the highest sequence identity to Mbb. smithii strain DSM 861T, Mbb. gottschalkii strain DSM 11977T, and Mbb. millerae strain DSM 16643T [12–15]. Both species have been detected in a few cultivation-independent studies on fecal and rumen samples (for Mbb. thaueri, see [16, 17] and for Mbb. woesei, see [18–20]), but besides their original description, there is very little information on the physiology and ecology of these two species.

The genus Methanosphaera is less well characterized than the genus Methanobrevibacter, and only few species have been isolated or detected by cultivation-independent methods. Two species, Msp. stadtmamae DSM 3091T and Msp. cuniculi DSM 4103T, are currently the only formally described Methanosphaera-type species [21, 22]. The first genome-sequenced Methanosphaera species, Msp. stadtmamae DSM3091T, was isolated from human feces and has been shown to be restricted to growth on methanol and hydrogen under in vitro conditions [22]. This substrate restriction can to some extent be considered to be a typical trait of the genus Methanosphaera, but its genetic basis remained poorly understood until the genome of Msp. stadtmamae strain DSM 3091T was analyzed [23]. Comparative genome analysis revealed that most genes for biosynthesis of the molybdopterin cofactor (Moco), the cofactor of formylmethanofuran dehydrogenase (fmd), are absent from the genome of this methanogen. This explained why Msp. stadtmamae strain DSM3091T is not able to grow by disproportionation of methanol nor of CO2 and hydrogen as substrates. Moreover did the analysis reveal that the genome encodes four homologues of each subunit of the methanol:coenzyme-M methyltransferase (mtaABC) [23]. The MtaABC proteins form the key enzyme complex for the utilization of methanol and had before primarily been detected in species of the order Methanosarcinales.

The second genome of a genus Methanosphaera representative, Methanosphaera sp. WGK6, has only recently become available. This methanogen’s genome sequence is to large extent nearly identical to that of Msp. stadtmamae DSM 3091T, but some of the few genomic differences result in phenotypic differences between the two species. This regards mainly two genes, putative alcohol (walC) and aldehyde (walD) dehydrogenases [24]. These two genes were apparently acquired via horizontal gene transfer and seem to enable Msp. sp. WGK6 to utilize ethanol as substrate for methanogenesis (in addition to its ability to grow on methanol and hydrogen) [24]. Homologs of walC and walD genes have also been detected in genomes of other methanogens but have not been investigated systematically.

Here, draft genomes of Mbb. thaueri strain DSM 11995T, Mbb. woesei strain DSM 11979T, and Msp. cuniculi strain DSM 4103T (a draft genome of M. cuniculi DSM 4103T of similar quality was also recently published by Gilmore et al. [25]) are presented and compared to those of all other currently available Methanobrevibacter- and Methanobrevibacter-type strain genomes. The comparative analyses aim at investigating and identifying some of the distinctive features of the Methanobrevibacter and Methanosphaera genomes that allow differentiating the two closely related genera. The major emphasis was set on key genes that may have large impact on the overall physiology of the analyzed species, such as those involved in substrate utilization and nitrogen and acetate assimilation.

2. Materials and Methods

2.1. Cultivation of Microorganism and DNA Extraction. Mbb. thaueri DSM 11995T, Mbb. woesei DSM 11979T, and Msp. cuniculi DSM 4103T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Genomic DNA was ordered by the DSMZ (Braunschweig) or was isolated using the MasterPure complete DNA purification kit (Epicentre, Madison, WI, USA).

2.2. Genome Sequencing. Extracted DNA was used to generate Illumina-shotgun libraries (Nextera_XT) according to the manufacturer’s protocol (Illumina, San Diego, CA, USA). Sequencing was conducted using a MiSeq and MiSeq Reagent kit v3 (2×300 bp paired end) as recommended by the manufacturer (Illumina). Trimmomatic 0.32 [26] was used to filter low-quality reads and for clipping of adapter contaminations. The assembly was performed with the SPAdes genome assembler software 3.11 [27]. Coverages were determined using QualiMap version 2.1 [27–29], and automatic annotation was performed using the software tool PROKKA [30], and data analysis was partly performed using the IMG/ER system (Integrated Microbial Genomes & Microbiomes) [31]. The quality and the completeness of the draft genomes have been validated with CheckM [32].

2.3. Multilocus Sequence Analysis. For multilocus sequence analysis (MLSA), total protein sequences from 19 genomes were extracted from the corresponding GenBank files using cds_extractor.pl v0.6 [33] and used for downstream analysis with an in-house pipeline at the Goettingen Genomics Laboratory [34]. In detail, proteinortho version 5 (default specification: blast = blastp v2.2.24, E value = 1e−10, alg._conn. = 0.1, coverage = 0.5, percent_identity = 50, adaptive_similarity = 0.95, inc_pairs = 1, inc_singles = 1, selfblast = 1, and unambiguous = 0) [35] was used to generate clusters of orthologous groups, inparalogues were removed, and MUSCLE [36] used to align the remaining sequences and poorly aligned positions were automatically filtered from the alignments using Gblocks [37]. A maximum-likelihood tree from 574 orthologues was inferred with 500 bootstraps with RAxML [38]. A phylogenetic tree was inferred with neighbour joining and 500 bootstraps. PO_2_MLSA.py is available
3.2. Comparative Analysis of Methanobrevibacter Genomes.

Methanobrevibacter species were compared using the parameters "-m GTRGAMMA -# 500 -f a -x 2 -p 2" were used.

2.4. Nucleotide Sequence Accession Number. The annotated genomes of *Mbb. thaueri* DSM 11995 and *Mbb. woesei* DSM 11979 have been deposited at DDBJ/EMBL/GenBank under the accession MZGS00000000 and MZGU00000000, respectively. The versions described in this paper are versions MZGS01000000 and MZGU01000000, respectively. The annotated genome of *Msp. cuniculi* DSM 4103 has been deposited at DDBJ/EMBL/GenBank under the accession LWMS00000000. The version described in this paper is version LWMS01000000.

3. Results

3.1. General Genome Features. The *Methanosphaera cuniculi* strain DSM 4103 draft genome has been assembled into 48 contigs, with a N50 of 111,976 bp. The GC content of the draft genome is 28%, which is similar to the G+C content of the other two published *Methanosphaera* genome sequences. The genomes of *Mbb. thaueri* strain DSM 11995 and *Mbb. woesei* strain DSM 11979 were assembled into 39 contigs and 10 contigs with N50 of 178,217 bp and 240,239 bp, respectively. The genomes were not detected from the assembled contigs in either of the two draft genomes. General features of the *Mbb. thaueri* strain DSM 11995, *Mbb. woesei* strain DSM 11979, and *Methanosphaera cuniculi* strain DSM 4103 genomes and comparison with other *Methanobrevibacter* and *Methanosphaera* strains are shown in Table 1, and results of CheckM analysis (including all genomes used in the analysis) are shown in Table S1. Circular representation of the three genomes is shown in Supporting Figures S1 and S2.

3.2. Comparative Analysis of Methanobrevibacter Genomes.

Sixteen different *Methanobrevibacter* and three *Methanosphaera* genomes were included for the comparative analyses. The *Methanobrevibacter* strains had been isolated from a range of different intestinal environments, for example, bovine and ovine rumen [12, 41], human gut [42], and termite hindguts [43, 44], but include also the genome of the non-host-associated *Mbb. arboriphilus* strain DSM 1125 [45, 46]. Results of the single- and multilocus analyses are shown in Figure 1. Both analyses revealed clear separation between the two genera.

The MLSA also supports distinct clades within the genus *Methanobrevibacter* (Figure 1). There is limited association of the observed clades with a specific host origin, for example, some species from the rumen form distinct clades while others cluster with *Methanobrevibacter* species isolated from other hosts. A BLAST-based analysis of each clade identifies genes specific for clades and genes that are shared between them (Figure 2). Among the clade-specific genes of clade 1 are catalase, nitrogenase, and also a tRNA-specific endonuclease (VapC), but most other clade-specific genes of clade 1 and the other clades are without functional annotation and await further characterization.

It was also investigated whether certain metabolic traits could be associated with specific clades as pointed out in Figure 1. This concerns mainly the metabolism of alcohols and acetate assimilation. Carbon monoxide/acyetyl-CoA-synthetase complex (CODH-ACS) is—in addition to other enzymes—important for acetate assimilation, and its presence is one prerequisite for autotrophic growth. CODH-ACS is found in non-host-associated and autotrophic methanogens, for example, *Methanothermobacter* species [47–49], and is detected in four out of five clade 1 species, but gene homologues of the enzyme appear to be absent from the genomes of methanogens in clades 2–4.

The potential utilization of alcohols, specifically methanol and ethanol, is also less clearly distributed among the 19 genomes (and the four different clades) and there are currently no known *Methanobrevibacter* species that are restricted to growth on H₂ and methanol/ethanol [10]. Genes for methanol utilization of the *Methanosphaera/Thermaanaerobacter* type (mtaABC) are distributed broadly within the genus *Methanobrevibacter* as outlined in Figure 1 and have also already been reported for some strains [46, 50]. Physiological characterizations of the strains that harbor mtaABC genes (Mbb. arboriphilus strains DSM 1125 and ANOR1, Mbb. smithii strain DSM 861, Mbb. wolinii strain DSM 11976) is still outstanding to confirm that these species are capable of growth on methanol/methanol-hydrogen.

The utilization of ethanol by *Methanobrevibacter* species has only recently gained additional attention and it has been shown that *Mbb. sp. AbM4* is capable of growth in the absence of hydrogen but in presence of methanol/ethanol [51, 52]. Details regarding the exact metabolism remains speculative, but the walC and walD genes recently identified in *Msp. WKG6* [24] are also present in *Mbb. sp. AbM4* and in the closely related *Mbb. boviskoreani* strain DSM 25824, *Mbb. olleyae* strain DSM 16632, and *Mbb. wolinii* strain DSM11976 conferring this metabolic trait potentially to the entire clade. *Mbb. olleyae* strain DSM 16632 is currently the only *Mbb. species outside this clade that is harboring the walCD genes.

Only few additional genome sequences of *Methanobrevibacter* isolates other than for *Mbb. smithii* are currently available, but the clade-distinguishing features appear to be conserved in these isolates as well (see Table S2 for general genome features and Table S3 for shared genes).

3.3. Shared and Distinctive Genome Features in the Genomes of Methanosphaera Species. The currently known
| Genome                  | Size (bp)     | GC content (%) | Coding percentage (%) | CDS (pseudo) | Genes (pseudo) | rRNA | tRNA | Accession       | Contigs/scaffolds | CRISPR region | Original strain ID |
|-------------------------|---------------|----------------|------------------------|--------------|----------------|------|------|----------------|------------------|---------------|--------------------|
| Methanobrevibacter filiformis DSM 11501<sup>T</sup> | 2,606,143     | 26.99          | 69.94                  | 1933         | 1965           | 3    | 29   | LWMT00000000    | 237              | 6              | RFM-3               |
| Methanobrevibacter curvatus DSM 11111<sup>T</sup> | 2,414,608     | 25.72          | 70.65                  | 1969         | 2004           | 4    | 31   | LWMV00000000    | 187              | 5              | RFM-2               |
| Methanobrevibacter cuticularis DSM 11139<sup>T</sup> | 2,608,702     | 26.79          | 68.12                  | 2061         | 2094           | 3    | 30   | LWMW00000000    | 120              | 6              | RFM-1               |
| Methanobrevibacter arborphilus DSM 1125<sup>T</sup> | 2,445,031     | 25.44          | 74.96                  | 1963         | 2005           | 5    | 37   | JXMW00000000    | 40               | 9              | DH1                |
| Methanobrevibacter arborphilus ANOR1 | 2,221,072     | 25.53          | 74.02                  | 1993         | 2038           | 7    | 35   | CBVX00000000    | 5                | 3              | ANOR1              |
| Methanobrevibacter ruminantium DSM 1093<sup>T</sup> | 2,937,203     | 32.64          | 78.12                  | 2217         | 2283 (5)       | 8    | 53   | CP001719        | 1                | 4              | M1                 |
| Methanobrevibacter oleae DSM 16632<sup>T</sup> | 2,122,444     | 26.87          | 76.85                  | 1813         | 1854           | 4    | 33   | FOTL00000000    | 49               | 4              | KM1H5-1P           |
| Methanobrevibacter millerae DSM 16643<sup>T</sup> | 2,725,667     | 36.54          | 89.32                  | 2383         | 2467           | 4    | 77   | CP011266        | 1                | 1              | ZA-10              |
| Methanobrevibacter thaueri DSM 11995<sup>T</sup> | 2,243,115     | 36.87          | 87.52                  | 2138         | 2171           | 2    | 31   | MZGS00000000    | 39               | 4              | CW                 |
| Methanobrevibacter gottschalkii DSM 11977<sup>T</sup> | 1,879,371     | 30.02          | 88.01                  | 1845         | 1889           | 7    | 34   | FOAK00000000    | 19               | NA             | HO                 |
| Methanobrevibacter oralis DSM 7256<sup>T</sup> | 2,110,861     | 27.73          | 84.49                  | 2036         | 1994           | 9    | 32   | LWMU00000000    | 99               | 2              | ZR                 |
| Methanobrevibacter smithii DSM 861<sup>T</sup> | 1,853,160     | 31.03          | 90.31                  | 1795         | 1841           | 8    | 36   | CP000678        | 1                | 1              | PS                 |
| Methanobrevibacter woesei DSM 11979<sup>T</sup> | 1,543,150     | 29.90          | 89.61                  | 1581         | 1614           | 2    | 31   | MZGU00000000    | 10               | 2              | GS                 |
| Methanobrevibacter wolini DSM 11976<sup>T</sup> | 2,041,814     | 24.21          | 75.94                  | 1700         | 1747           | 8    | 36   | JHWX00000000    | 32               | 2              | SH                 |
| Methanobrevibacter boviskoreani DSM 25824<sup>T</sup> | 2,045,801     | 28.98          | 78.00                  | 1756         | 1799           | 4    | 36   | BAGX00000000    | 54               | 2              | JH1                |
| Methanobrevibacter sp. AbM4 | 1,998,189     | 29.04          | 76.7                   | 1671         | 1716           | 49   | 36   | CP004050        | 1                | 6              | N.A.               |
| Methanosphaera sp. WGK6 | 1,729,155     | 27.70          | 78.05                  | 1456         | 1616 (114)     | 3    | 42   | JRWK00000000    | 37               | 1              | N.A.               |
| Methanosphaera stadtmannae DSM 3091<sup>T</sup> | 1,767,403     | 27.63          | 84.10                  | 1534         | 1592 (2)       | 12   | 42   | CP00102        | 1                | 4              | MCB-3              |
| Methanosphaera cuniculi DSM 4103<sup>T</sup> | 1,881,497     | 28.08          | 81.36                  | 1585         | 1629           | 5    | 39   | LWMS00000000    | 48               | 0              | 1R-7               |
and only bootstrap values ≥ 70% are shown. The 16S rRNA tree was rooted with five Methanobacterium sequences. The scale bar indicates 0.10 inferred nucleotide substitutions per position. Red-colored dots indicate the presence of mtaABC genes in the species/clade, green-colored dots indicate the presence of walB and walC gene homologues (potential utilization of ethanol), blue-colored dots indicate the presence of nitrogenase genes, and black-colored dots indicate the presence of carbon monoxide/acetyl CO-DH genes (see also Table S3 for details).

**Figure 1:** Single- and multilocus sequence analysis of Methanobrevibacter and Methanosphaera species. A maximum likelihood tree (left) of 16 Methanobrevibacter and three Methanosphaera genomes was inferred with 500 bootstraps with RAxML and visualized with Dendroscope. Phylogeny of Methanobrevibacter and Methanosphaera based on the 16S rRNA gene is shown on the right. The tree was resampled 500 times, and only bootstrap values ≥ 70% are shown. The 16S rRNA tree was rooted with five Methanobacterium sequences. The scale bar indicates 0.10 inferred nucleotide substitutions per position. Red-colored dots indicate the presence of mtaABC genes in the species/clade, green-colored dots indicate the presence of walB and walC gene homologues (potential utilization of ethanol), blue-colored dots indicate the presence of nitrogenase genes, and black-colored dots indicate the presence of carbon monoxide/acetyl CO-DH genes (see also Table S3 for details).

**Figure 2:** Pan/core genome analysis of four different Methanobrevibacter clades. Venn diagram showing the numbers of orthologous genes (OGs) in the core, dispensable, and specific genome of compared strains. Ortholog detection was done with the Proteinortho software (blastp) with a similarity cut-off of 50% and an E value of 1e-10. The total numbers of genes and paralogs are depicted under the corresponding species name. Open-reading frames that were classified as pseudogenes were not included in this analysis. See also Table S3 for details on shared genes.

**Methanosphaera** species are characterized by their limited ability to use substrates for methanogenesis, for example, *Msp. stadtmanae* and *Msp. cuniculi* are able to utilize only methanol and hydrogen for growth, *Msp. sp. WKG6* can also utilize ethanol, but none of the species is capable of utilizing hydrogen and CO₂ or formate like organisms from closely related genera. The analysis of the *Msp. stadtmanae* genome helped explaining some of the distinctive physiological features of this methanogen, but comparative genome analysis with other closely related *Methanosphaera* and *Methanobrevibacter* species was not possible at that time due to the lack of sequenced genomes. After more than a decade, several *Methanosphaera* and *Methanobrevibacter* genomes have become available [24, 41, 46, 50, 53–58] and comparative analyses allow determining whether certain traits are species specific or shared by members of either or both genera. Comparative genome analysis reveals that some of the observations made for the *Msp. stadtmanae* genome appear to be consistent for all three available *Methanosphaera* genomes. Overall, more than 1000 genes are shared among all three *Methanosphaera* species (Figure 3), but the comparison with *Methanobrevibacter* genomes shows that several key genes are missing. Notably, this concerns the lack of genes for molybdopterin cofactor biosynthesis, energy-converting hydrogenase A (ehaA-Q), formate dehydrogenase (fdh), and methyl-coenzyme M reductase isoenzyme 1 mcrABCG (only genes encoding isoenzyme II are present (mtrABDG)). Despite the presence of formylmethanofuran dehydrogenase genes, it is not possible for *Methanosphaera* species to produce a functional enzyme due to the absence of the molybdopterin cofactor. The absence of functional formylmethanofuran dehydrogenase leads to the inability of these methanogens to grow hydrogenotrophically or to disproportionate methanol. The absence of Moco biosynthesis also seems to be specific to *Methanosphaera* species as all of the analyzed *Methanobrevibacter* genomes are capable of synthesizing this cofactor.

One of the noteworthy differences between the known *Methanosphaera* species is the capability of *Msp. WKG6* to
cies in this clade; however, there are only few studies that
of the Mbb. wolinii tested) [59]. It is noteworthy that all three sequenced strains
(growth on methanol but not methanol and hydrogen was
was not detected despite the presence of mtaABC genes
functions, for example, growth of Mbb. smithii
strains to utilize alcohols may not have been
brevibacter strains, as shown for
other genes required for hydrogenotrophic or methylo-
etrophy with other microorganisms (and potentially the host)
provide favorable growth conditions as well as ammon-
nium and acetate for the methanogen. It is also noteworthy
Mbb. curvatus in the “autotrophic clade” has nitrogenase
genes, but apparently, no CODH complex genes and may therefore require externally supplied acetate. This
could represent an intermediate stage between the potentially
autotrophic strains and the other clades and/or may be an
adaptation to a specific niche.

In addition to differences between species, there is cur-
rently only little information on strain diversity within a spe-
cies but studies indicate that there may be considerable
differences as shown for Mbb. smithii and Mbb. arboriphilus
[46, 61]. However, undertaking pan-genome approaches,
such as the one for Mbb. smithii by Hansen et al. [61],
requires substantial cultivation efforts as there are only few
strains available for each of the described methanogen spe-
cies. As development of sequencing technologies continues
to advance, it may also become feasible to obtain high-
quality closed genomes from low amounts of starting DNA
or through metagenomic approaches. Having such genomes
will allow greater certainty in determining the presence and
absence of specific genome features and will also enable
detection of small genomic differences between strains that
gate unnoticed in draft genomes.

Lastly, the results of our analyses corroborate the hypoth-
esis that the absence of molybdopterin cofactor biosynthesis is a
characteristic trait shared by members of the genus Metha-
nosphaera, while all sequenced Methanobrevibacter genomes
seem to encode genes for Moco biosynthesis. However, it
also needs to be considered that Methanosphaera species
encode the genes for formylmethanofuran dehydrogenase and
other genes required for hydrogenotrophic or methyl-
trrophic methanogenesis [62, 63]. The presence of these
genes may indicate that Methanosphaera species may still
be able to utilize the aforementioned methanogenic path-
ways, if molybdopterin cofactor is present and taken up by
the methanogen (as suggested by Fricke et al. 2006). Meta-
transcriptional profiling could be used to determine, if fmd
and other genes for hydrogenotrophic growth are expressed
at all in Methanosphaera or specifically in the absence and
presence of Moco or Moco-synthesizing microorganisms. It
needs to be emphasized that the analyses of this study are
based on the currently available three genomes of the three
Methanosphaera species that have been isolated from three

4. Discussion

The presented comparative genome analysis reveals insights
into the genome content of different Methanobrevibacter
species and how it compares to that of closely related Metha-
nosphaera species. The analysis suggests that phenotypic var-
iation among Methanobrevibacter strains may be larger than
previously assumed. The comparative analysis indicates the
presence of genes for methanol (in four Mbb. genomes) and
ethanol utilization (in four Mbb. genomes) in the sixteen ana-
lyzed Methanobrevibacter genomes, with the Mbb. wolинii
genome harboring both wLCD and mtaABC gene homol-
ogues. More physiological analysis will be required to deter-
mine if the detected genes are conferring the ability to utilize
ethanol/methanol to all these strains. The ability of Methano-
brevibacter strains to utilize alcohols may not have been
investigated in an in-depth manner previously as this trait
is more typically associated with other orders of methano-
gens, but some studies suggest that the genes may in some
cases also not be functional or may have unknown different
functions, for example, growth of Mbb. smithii on methanol
was not detected despite the presence of mtaABC genes
(growth on methanol but not methanol and hydrogen was
tested) [59]. It is noteworthy that all three sequenced strains
of the Mbb. wolinii clade harbor the genes for ethanol utiliza-
tion, which could point to a specific ecological role of the spe-
cies in this clade; however, there are only few studies that

have detected significant numbers of either of these three
species in a natural environment [5, 60] making it currently
difficult to determine potential cooccurrences or specific syn-
 trophic interactions with other microorganisms.

The presence of genes that could contribute to autotro-
phic growth, for example, nitrogenase and CODH-ACS in
clade 1 containing Mbb. species from termites and in Mbb.
arboriphilus strain, has been reported previously [46], and
these genes appear to be absent from species clades 2 to 4.
It can only be speculated why Mbb. species of clade 1 did
not undergo the same loss of these key genes like their coun-
terparts in the clades that have primarily been isolated from
the vertebrate intestinal tract. However, auxotrophy of some
Mbb. species may be the result of a close symbiotic inter-
action with other microorganisms (and potentially the host)
that provide favorable growth conditions as well as ammo-
nium and acetate for the methanogen. It is also noteworthy
that Mbb. curvatus in the “autotrophic clade” has nitrogenase
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the methanogen (as suggested by Fricke et al. 2006). Meta-
transcriptional profiling could be used to determine, if fmd
and other genes for hydrogenotrophic growth are expressed
at all in Methanosphaera or specifically in the absence and
presence of Moco or Moco-synthesizing microorganisms. It
needs to be emphasized that the analyses of this study are
based on the currently available three genomes of the three
Methanosphaera species that have been isolated from three

![Figure 3: Pan/core genome analysis of three different Methanosphaera species. Venn diagram showing the numbers of orthologous genes (OGs) in the core, dispensable, and specific genome of compared strains. Ortholog detection was done with the Proteinortho software (blastp) with a similarity cut-off of 50% and an E value of 1e – 10. The total numbers of genes and paralogs are depicted under the corresponding species name. Open-reading frames that were classified as pseudo genes were not included in this analysis. See also Table S3 for details on shared genes.](image-url)
different host species [23–25]. It provides strong evidence that the physiology will be similar for other Msp. species in other environments, but it can also not be ruled out that other Msp. species exist that harbor genes for Moco biosynthesis and that grow hydrogenotrophically or methylotrophically. The ability of Msp. sp. WGK6 to utilize ethanol does indicate that more phenotypic variation exists among Methanosphaera species and that further isolation and characterization of new Msp. species is necessary.

5. Conclusion

The presented study includes genomes of all currently available types of strain (and other selected isolate) of Methanobrevibacter and Methanosphaera genomes and allows comprehensive insights into the genera Methanobrevibacter and Methanosphaera. The analyses reveal that distinct clades within the genus Methanobrevibacter exist and that the lack of molybdopterin cofactor biosynthesis may be a specific trait for the genus Methanosphaera. Additional isolates and further physiological and genomic analyses will be required to determine if division of the genus Methanobrevibacter in more than one genus could be justified. The primary use of the type of strains (and other isolates) for the analysis in this study does warrant access of the scientific community to most of the analyzed Methanobrevibacter and Methanosphaera isolates and will facilitate testing of the predicted physiological phenotypes.

Data Availability

The data used to the support the findings of this study are available in the supplemental information or -as in the case of the genomes- are downloadable using the accession numbers provided in Section 2.4.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary 1. Figure S1: circular display of the Mbb. thaueri strain DSM 11995T and Mbb. woesei strain DSM 11979T genomes and comparison with other Methanobrevibacter genomes. The Mbb. thaueri strain DSM 11995T and the Mbb. woesei strain DSM 11979T genomes are shown in panels A and B, respectively. The genes encoded by the leading and the lagging strand of the respective species are shown in circles 1 and 2, and rRNA (pink) and tRNA (green) encoding genes are shown in circle 3. The presence of orthologous genes (red: high similarity; orange: medium similarity; yellow: low similarity; see color code below) is indicated for the other Methanobrevibacter genomes (circles 4 to 19) in comparison to that of the respective Methanobrevibacter genome. The two innermost plots represent the GC content and the GC skew (circle 20 and 21). Color code according to E values of the blastp analysis performed using Proteortho4.26. Gray: 1e−20 to 1; light yellow: 1e−21 to 1e−50; gold: 1e−51 to 1e−90; light orange: 1e−91 to 1e−100; orange: 1e−101 to 1e−120; red: >1e−120.

Supplementary 2. Figure S2: circular display of the Msp. cuniculi strain DSM 4103T genome and comparison with the genomes of Msp. stadtmanae strain DSM 3091T and Msp. sp. WGK6. The genes encoded by the leading and the lagging strand of Msp. cuniculi strain DSM 4103T are shown in circles 1 and 2, and rRNA (pink) and tRNA (green) encoding genes are shown in circle 3. The presence of orthologous genes (red: high similarity; orange: medium similarity; yellow: low similarity; see color code below) is indicated for the genomes of Msp. stadtmanae strain DSM 3091T and Msp. sp. WGK6 (circles 4 and 5 in comparison to the Msp. cuniculi strain DSM 4103T genome). The two innermost plots represent the GC content and the GC skew (circles 6 and 7). Color code according to E values of the blastp analysis performed using Proteortho4.26. Gray: 1e−20 to 1; light yellow: 1e−21 to 1e−50; gold: 1e−51 to 1e−90; light orange: 1e−91 to 1e−100; orange: 1e−101 to 1e−120; red: >1e−120.

Supplementary 3. Table S1: CheckM analysis to determine the completeness of genomes. Table S1A shows results from analysis of genomes from the main manuscript while Table S1B lists the results for genomes mainly mentioned in the supporting information. Table S2: general features of additional Methanobrevibacter genomes. General features of genomes mainly mentioned in the supporting information. Table S3: table of shared and unshared Methanobrevibacter and Methanosphaera genes. Highlighted in colors corresponding to those of Figure 1 are genes for CODH-ACS complex, nitrogenase, methanol, and ethanol utilization. Annotations were taken from the Mbb. arboriphilus DSM1125T genome.

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