Reclassification of *Methanogenium tationis* and *Methanogenium liminatans* as *Methanofollis tationis* gen. nov., comb. nov. and *Methanofollis liminatans* comb. nov. and description of a new strain of *Methanofollis liminatans*

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Sequencing of 16S rRNA genes and phylogenetic analysis of *Methanogenium tationis* DSM 2702^T (OCM 43^T) (T = type strain) and *Methanogenium liminatans* GKZPZ^T (= DSM 4140^T) as well as other members of the family *Methanomicrobiaceae* revealed that both species belong to a separate line of descent within this family. In addition, a new strain of *Methanogenium liminatans*, strain BM1 (= DSM 10196), was isolated from a butyrate-degrading, fluidized bed reactor and characterized. Cells of both species are mesophilic, highly irregular cocci that use H₂/CO₂ and formate for growth and methanogenesis. In addition, *Methanogenium liminatans* strains GKZPZ^T and BM1 used 2-propanol/CO₂, 2-butanol/CO₂ and cyclopentanol/CO₂. Both species contained diether and tetraether lipids. The polar lipids comprised amino-phosphopentaketol derivatives, which appear to be characteristic lipids within the family *Methanomicrobiaceae*. The pattern of glycolipids, phosphoglycolipids and amino-phosphoglycolipids was consistent with the assignment of these two species to a taxon within the family *Methanomicrobiaceae*, but also permitted them to be distinguished from other higher taxa within this family. The G+C contents of the DNA of *Methanogenium tationis* and *Methanogenium liminatans* were 54 and 60 mol% (T_m and HPLC), respectively. On the basis of the data presented, the transfer of *Methanogenium tationis* and *Methanogenium liminatans* to the genus *Methanofollis* gen. nov. as *Methanofollis tationis* comb. nov. and *Methanofollis liminatans* comb. nov., respectively, is proposed, with *Methanofollis tationis* as the type species.

Keywords: *Methanogenium tationis*, *Methanogenium liminatans*, *Methanofollis* gen. nov., lipids, phylogeny

INTRODUCTION

The DNA of irregularly coccoid methanogens has a wide range of G+C contents (38–61 mol%), which

indicates a wide evolutionary diversity among these methanogenic *Archaea*. However, only a limited number of phenotypic characteristics of irregularly coccoid methanogens are available for taxonomic purposes. In addition to morphology and substrate spectrum, characteristics such as polyamine content (Zellner *et al.*, 1989a; Blotevogel *et al.*, 1991; Boone *et al.*, 1993), cellular lipids (Grant *et al.*, 1985; Zellner *et al.*, 1989a, b, c; Blotevogel *et al.*, 1991; Koga *et al.*, 1993) and whole-cell protein patterns (Maestrojuán *et al.*,

Abbreviations: PAS, periodic acid–Schiff; UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL accession numbers for the 16S rDNA and rRNA sequences reported in this paper are Y16428, Y16429, AF095266–AF095272.

1990) have been used. The S-layer architecture and the apparent molecular mass of glycoprotein subunits have also been used (Zellner *et al.*, 1989a, c, 1990; Messner & Sleytr, 1992; Sleytr *et al.*, 1993). Due to the fact that few physiological tests can be used to differentiate taxa within the methanogenic *Archaea*, other phenotypic characters (e.g. cell wall structure, chemical composition, polyamines) and genotypic characters (e.g. 16S rDNA sequence) must be used to delineate the higher taxonomic ranks. Recent developments in the taxonomy of both *Archaea* and *Bacteria* indicate that this combination of phenotypic and genotypic methods is suitable for the delineation of taxa in a system which seeks to base these on the phyletic groupings detected by such data.

Analysis of the sequences of 16S rRNA or its genes revealed a high diversity of irregularly coccoid methanogenic strains, with some deeply rooted lines of descent in phylogenetic trees (Zellner *et al.*, 1989a; Rouvière *et al.*, 1992; Boone *et al.*, 1993). As a consequence, several coccoid methanogens were reclassified on the basis of DNA–DNA hybridization studies and/or 16S rDNA sequence analysis (Xun *et al.*, 1989; Zellner *et al.*, 1989c; Maestrojuán *et al.*, 1990; Boone *et al.*, 1993). This resulted in the description of the five current genera of hydrogenotrophic, irregularly coccoid methanogens of the order *Methanomicrobiales*: *Methanogenium*, *Methanoculleus*, *Methanolacinia*, *Methanoplanus* and *Methanocorpusculum*.

Recently, the reclassification of *Methanogenium tationis* (Zabel *et al.*, 1984) as *Methanofollis tationis* was suggested (Boone *et al.*, 1993) but not formally proposed. In this paper, the transfer of *Methanogenium tationis* and *Methanogenium liminatans* as *Methanofollis tationis* gen. nov., comb. nov. and *Methanofollis liminatans* comb. nov. is proposed on the basis of 16S rDNA sequencing and chemotaxonomic and physiological data. In addition, the isolation and characterization of a new *Methanofollis liminatans* strain, BM1, is reported.

METHODS

Reference strains. *Methanogenium tationis* strain (DSM 2702^T) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Methanogenium liminatans* strain GKZPZ^T (= DSM 4140^T) was isolated and (after deposition in the DSMZ) subcultured in this laboratory. The newly isolated strain BM1 has been deposited at the DSMZ as DSM 10196. The following strains were obtained from the Oregon Collection of Methanogens (Beaverton, OR, USA): *Methanoculleus olentangi* RC/ER^T (OCM 52^T), *Methanoculleus bourgensis* MS2^T (OCM 15^T), *Methanocorpusculum bavaricum* SZSXXZ^T (OCM 127^T), *Methanocorpusculum sinense* China Z^T (OCM 128^T) and *Methanocorpusculum labreanum* Z^T (OCM 1^T).

Media, media preparation and cultivation. A synthetic basal medium (WHP medium) containing tungstate, with acetate and L-cysteine (the latter as a component of the reductant) as the sole organic carbon sources, was used for enrichment

cultures, isolation procedures and cultivation of *Methanogenium liminatans* strains. WHP medium was based on the recipe described by Zellner & Jargon (1997) except that yeast extract, peptone and additional sodium sulfate were omitted. WHP medium contained 1 µM sodium tungstate and was used for the cultivation of strains DSM 2702^T, GKZPZ^T and BM1. Substrates (e.g. sodium formate, sodium acetate, 2-propanol, 2-butanol, cyclopentanol) were added from anaerobic, sterile stock solutions (20%, w/v or v/v) as indicated to give concentrations of 0.5% (w/v or v/v) in the serum bottles. Cells were grown under H₂/CO₂ (4:1, v/v, 300 kPa) in 120 ml serum bottles on a rotary shaker (150 r.p.m.). When other substrates were used instead of hydrogen, N₂/CO₂ (4:1, v/v, 300 kPa) served as the gas atmosphere. For the preparation of large masses of cells, cells were grown in H₂/CO₂ (4:1, v/v) at a flow rate of 50 l h⁻¹ in a 12 l fermenter (Biostat S; Braun).

Methanogenium tationis was grown as described previously (Zabel *et al.*, 1984). *Methanoculleus olentangi* and *Methanoculleus bourgensis* were grown as described by Corder *et al.* (1983) and Ollivier *et al.* (1986), respectively. *Methanocorpusculum labreanum* was grown as described by Zhao *et al.* (1989). *Methanocorpusculum sinense* and *Methanocorpusculum bavaricum* were grown as described by Zellner *et al.* (1989a).

Source and habitat of strain BM1. Strain BM1 (= DSM 10196) was isolated from a butyrate-degrading, fluidized bed reactor inoculated with a culture originally derived from the upflow anaerobic sludge blanket (UASB) reactor from the wastewater treatment plant at a sugar refinery in Brühl, Germany (Zellner *et al.*, 1991). The predominant methanogen in this bioreactor was an autofluorescent irregular coccus, similar in appearance to the new isolate (strain BM1) described here, and several other methanogens were identified immunologically (Zellner *et al.*, 1991, 1997).

Enrichment and isolation of strain BM1. WHP medium with H₂/CO₂ (4:1, v/v, 300 kPa) as substrate was inoculated with 1% (v/v) bioreactor liquid and incubated at 37 °C. The gas phase was regularly replaced and autofluorescent, irregularly coccoid cells increased in number simultaneously with the formation of methane. This enriched culture was serially diluted into WHP medium and the highest dilution showing growth of the fluorescent, irregularly coccoid cells was again serially diluted. Aliquots (0.1 ml) of the dilutions were then streaked on agar plates with WHP medium plus 2.5% (w/v) Oxoid agar (Unipath). All manipulations were performed in an anaerobic chamber (M. Braun) under a nitrogen atmosphere. The plates were transferred into a stainless steel anaerobic jar, pressurized to 300 kPa with H₂/CO₂ (4:1, v/v) and incubated at 37 °C. After 20 d incubation, the gas pressure had dropped to 150 kPa, indicating gas consumption. The anaerobic jar was transferred into the anaerobic chamber. Cells were picked with a syringe needle from a well-isolated colony, injected into a 120 ml serum bottle containing 20 ml WHP medium under H₂/CO₂ (4:1, v/v, 300 kPa) and incubated at 37 °C. This procedure resulted in the isolation of strain BM1.

Microscopy. Phase-contrast and epifluorescence microscopy were carried out by using a Zeiss Axiophot microscope as described previously (Zellner *et al.*, 1991). Motility was checked microscopically.

Analyses. Volatile fatty acids, alcohols, hydrogen and methane were analysed by GC (Zellner & Winter, 1987). The Colour Gram 2 staining set from bioMérieux was used for Gram staining according to the manufacturer's instructions.

Growth was measured as described previously (Zellner *et al.*, 1989a).

Extraction of polar lipids and presumptive lipoquinones. Freeze-dried cells (100 mg) were subjected to the two-stage extraction method described by Tindall (1990a, b). Respiratory lipoquinones were recovered in the first stage and polar lipids were recovered in the second stage. The presence or absence of respiratory lipoquinones was confirmed by TLC of the first stage extract on silica gel thin layers (Art. nr. 805 023, Macherey-Nagel) cut to 10 × 10 cm squares, with hexane:tert-butylmethylether (9:1, v/v) as the developing solvent.

Polar lipids were separated by two-dimensional TLC on silica gel thin layers (Macherey-Nagel, Art. nr. 818 135, which is a 10 × 10 cm format). The plates were developed in chloroform:methanol:water (65:25:4, by vol.) in the first direction, followed by chloroform:methanol:acetic acid:water (80:12:15:5, by vol.) in the second direction. Total lipids were visualized with 5% dodecamolybdatophosphoric acid and specific functional groups were detected with periodate-Schiff (α -glycols), ninhydrin (free amino groups), α -naphthol (sugars), Dragendorff (quaternary nitrogen) and anisaldehyde-H₂SO₄ (sugars and terpenoids).

Extraction of ether lipids and TLC analysis. Ether lipids were extracted from 100 mg freeze-dried cells by an acid hydrolysis method that did not hydrolyse diether lipids containing a hydroxyl group in the side chain. Diether lipids were subjected to double development in hexane:diethyl ether (4:1) to resolve phytanyl and sesterpanyl diether lipids (i.e. C₂₀:C₂₀, C₂₀:C₂₅, C₂₅:C₂₅). Tetraethers and diethers were separated in the solvent hexane:diethyl ether:acetic acid (25:25:1, by vol.).

Determination of G + C content of DNA. DNA from strain BM1 was isolated and purified and the mol% G + C content of the DNA was determined by HPLC according to Zellner *et al.* (1989a).

Sequence analysis of 16S rDNA and phylogenetic analysis. For *Methanogenium tationis* DSM 2702^T and *Methanogenium liminatans* strains GKZPZ^T and BM1, genomic DNA was extracted, the 16S rDNA was amplified by PCR and the sequences of the purified PCR products were analysed as described previously (Rainey *et al.*, 1996). The sequencing reaction products were electrophoresed with a model 373A automated DNA sequencer (Applied Biosystems). The almost complete 16S rRNA genes of *Methanogenium liminatans* GKZPZ^T (1396 nucleotides) and *Methanogenium liminatans* BM1 were determined. Approximately 1380 nucleotides of the *Methanogenium liminatans* GKZPZ^T 16S rRNA were also sequenced directly by the reverse transcriptase method (Rouvière *et al.*, 1992). This sequence was 99.7% identical to the sequence obtained for the 16S rRNA gene. The 16S rRNA gene sequence was used for the phylogenetic analysis. In addition, the 16S rRNA of *Methanogenium tationis* DSM 2702^T, *Methanoculleus olentangyi* RC/ER^T, *Methanoculleus bourgensis* MS2^T, *Methanocorpusculum bavaricum* SZSXXZ^T, *Methanocorpusculum sinense* China Z^T and *Methanocorpusculum labreanum* Z^T were sequenced directly by the reverse transcriptase method. About 1380 nucleotides were determined for each of these sequences.

The global phylogenetic positions of the strains were analysed by using the ARB database (Strunk & Ludwig, 1997). 16S rRNA and rDNA sequences were compared with the existing 16S rDNA database of members of the order

Methanomicrobiales (Rouvière *et al.*, 1992; Maidak *et al.*, 1997). A greater degree of resolution among closely related taxa was obtained by using the ae2 editor (Maidak *et al.*, 1997). A phylogenetic dendrogram was reconstructed using the treeing algorithm of De Soete (1983), based on similarity values which had been transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). The programs NEIGHBOR-JOINING and MAXIMUM-LIKELIHOOD contained in the PHYLIP package (Felsenstein, 1993) were used in the construction of phylogenetic dendrograms (Saitou & Nei, 1987). Bootstrap values were determined by using the programs NJFIND and NJBOOT of the PHYLIP package (Felsenstein, 1993).

Nucleotide sequence accession numbers. The sequences of 16S rDNA of *Methanogenium liminatans* strain DSM 4140^T and strain BM1 were deposited in the EMBL database under the accession numbers Y16428 and Y16429, respectively. The 16S rRNA sequences of *Methanogenium tationis* DSM 2702^T, *Methanoculleus bourgensis* MS2^T (= OCM 15^T, DSM 3045^T) and *Methanoculleus olentangyi* RC/ER^T (= OCM 52^T, DSM 2772^T), *Methanocorpusculum bavaricum* SZSXXZ^T, (= OCM 127^T, DSM 4179^T), *Methanocorpusculum sinense* China Z^T (= OCM 128^T, DSM 4274^T) and *Methanocorpusculum labreanum* Z^T (= OCM 1^T, DSM 4855^T) were deposited in GenBank under the accession numbers AF095266–AF095272, respectively. Other sequences utilized were obtained from EMBL (*Methanoculleus palmeoli*, Y16382) or the Ribosomal Database Project (Maidak *et al.*, 1997).

RESULTS AND DISCUSSION

Morphology of strains, S-layer architecture and composition

Cells of the newly isolated strain BM1 were highly irregular cocci. Some cells also had a ring-shaped appearance under phase-contrast microscopy. The cells were 1.25–2.0 µm in diameter, stained Gram-negative and were non-motile. The addition of 1% (w/v) SDS led to lysis of cells of *Methanogenium liminatans* GKZPZ^T and strain BM1, indicating a cell envelope with a proteinaceous cell wall. With the exception of *Methanogenium liminatans* strains, which appear as ring-shaped cells under phase-contrast microscopy (Zellner *et al.*, 1990), the species described to date of the genera *Methanogenium* and *Methanoculleus* are irregular cocci and are indistinguishable from each other (Boone *et al.*, 1993). However, some differences in cell envelope composition have been observed (Table 1). All irregularly coccoid methanogens within the order *Methanomicrobiales* possess hexagonal S-layer lattices consisting of glycoprotein subunits with *M_r* ranging from 90 000 to 155 000. However, only methanogens belonging to the family *Methanocorpusculaceae* have an S-layer glycoprotein with *M_r* ranging from 90 000 to 94 000. The *M_r* of S-layer glycoprotein subunits of genera of the family *Methanomicrobiaceae* range from 101 000 to 155 000.

During the course of compiling this paper, the incorrect orthography of *Methanoculleus thermophilicus* was noticed which, according to Rule 61 of the

Table 1. S-layers of irregularly coccoid methanogens of the families *Methanomicrobiaceae*, *Methanoplanaceae* and *Methanocorpusculaceae*

Strain	DSM no.	Lattice constant c/c (nm)*	Glycoprotein subunit M_r ($\times 10^{-3}$)	Reference
Genus <i>Methanofollis</i>				
<i>M. tationis</i> Chile 9 ^T	2702 ^T	ND	120	Zabel <i>et al.</i> (1984); Zellner <i>et al.</i> (1998)
<i>M. liminatans</i> GKZPZ ^T	4140 ^T	15.4	118	Zellner <i>et al.</i> (1990, 1998)
Genus <i>Methanoculleus</i>				
<i>M. marisnigri</i> JR1 ^T	1498 ^T	ND	138	Zellner <i>et al.</i> (1990)
<i>M. olentangyi</i> RC/ER ^T	2772 ^T	15.4	132	Zellner <i>et al.</i> (1990)
<i>M. bourgensis</i> MS2 ^T	3045 ^T	15.4	101	Zellner <i>et al.</i> (1990)
<i>M. thermophilus</i> CR-1 ^T	2373 ^T	ND	130	Zabel <i>et al.</i> (1985)
<i>M. thermophilus</i> UCLA	2624	ND	130	Zabel <i>et al.</i> (1985)
<i>M. thermophilus</i> Ratisbona	2640	ND	130	Zabel <i>et al.</i> (1985)
<i>M. palmolei</i> INSLUZ ^T	4273 ^T	15.2	120	Zellner <i>et al.</i> (1998)
Genus <i>Methanogenium</i>				
<i>M. cariaci</i> JR1 ^T	1497 ^T	14.0	117	Zellner <i>et al.</i> (1990)
<i>M. frittonii</i> FR-4 ^T	2832 ^T	15.4	106	Zellner <i>et al.</i> (1990)
Genus <i>Methanolacinia</i>				
<i>M. paynteri</i> G2000 ^T	2545 ^T	15.3	155†	Zellner <i>et al.</i> (1989c)
Genus <i>Methanoplanus</i>				
<i>M. limicola</i> M3 ^T	2279 ^T	14.0	143	Zellner <i>et al.</i> (1989a)
<i>M. endosymbiosus</i> MC1 ^T	3599 ^T	ND	110	Zellner <i>et al.</i> (1989a)
Genus <i>Methanocorpusculum</i>				
<i>M. parvum</i> XII ^T	3823 ^T	14.3	90	Zellner <i>et al.</i> (1989a)
<i>M. bavaricum</i> SZSXXZ ^T	4179 ^T	16.0	94	Zellner <i>et al.</i> (1989a)
<i>M. sinense</i> CHINAZ ^T	4274 ^T	15.8	92	Zellner <i>et al.</i> (1989a)
<i>M. aggregans</i> MS1 ^T	3027 ^T	15.4	92	P. Messner & G. Zellner, unpublished

ND, Not determined.

* Centre-to-centre spacing of hexagonally arranged protein subunits of S-layer.

† A second, weaker band with an M_r of 135 000 that reacted with PAS stain presumably represented a degradation product or a less glycosylated precursor.

Bacteriological Code (Lapage *et al.*, 1992), may be corrected to *Methanoculleus thermophilus* (corrig.).

Culture conditions and substrates for growth and methanogenesis

The optimal growth temperature of strain BM1 was about 37–40 °C. No growth was obtained below 20 °C or above 45 °C. The optimal pH was about 7. Growth and methane production were observed on H₂/CO₂, formate, 2-propanol/CO₂ and 2-butanol/CO₂. Growth and methane production by strain BM1 on potential substrates was as follows (maximal OD₅₇₈ obtained/total methane produced per 20 ml culture in a 120 ml serum bottle): H₂/CO₂ (0.86/3285 µmol), formate (0.14/172 µmol), 2-propanol/CO₂ (0.06/52 µmol), 2-butanol/CO₂ (0.04/44 µmol) and cyclopentanol/CO₂ (0.001/21 µmol). Thus, except for cyclopentanol/CO₂, which supported only methanogenesis, all these substrates supported both growth

and methanogenesis. The secondary and cyclic alcohols were oxidized to the respective ketones. Strain BM1 did not grow or produce methane in media with acetate, methanol, methylamines, ethanol or 1-propanol as substrate. In contrast, *Methanogenium tationis* DSM 2702^T did not grow or produce methane on 2-propanol/CO₂ or 2-butanol/CO₂ (Zellner & Winter, 1987) or cyclopentanol/CO₂. Acetate was required for growth of strain BM1 on formate. Thus, while strain BM1 resembled *Methanogenium liminatans* more closely in terms of substrate utilization, it could not be distinguished from other species of the genera *Methanoculleus* and *Methanogenium* on this basis (Table 2).

Cell physiology

The original descriptions of *Methanogenium tationis* and *Methanogenium liminatans* were supplemented by material on the utilization of secondary and cyclic

Table 2. Phenotypic features of irregularly coccoid strains of the families *Methanomicrobiaceae* and *Methanoplanaceae*

Strain	DSM no.	Cell size (µm)	Flagella	Substrates*				Min. t _D (h)	Growth temp. (°C)†	G + C content (mol%)‡	Reference
				H	F	2P	2B				
Genus <i>Methanofollis</i>											
<i>M. tationis</i> Chile 9 ^T	2702 ^T	1.5–3.0	+	+	+	–	–	12	37–40/25–45	54	Zabel <i>et al.</i> (1984)
<i>M. liminatans</i> GKZPZ ^T	4140 ^T	1.25–2.0	+	+	+	+	+	7.5	40/25–45	59.3	Zellner <i>et al.</i> (1990)
<i>M. liminatans</i> BM1	10196	1.25–2.0	ND	+	+	+	+	ND	40/20–45	60.0 ^b	This paper
Genus <i>Methanoculleus</i>											
<i>M. marisnigri</i> JR1 ^T	1498 ^T	1.3/1–2	+	+	+	+	+	10	20–25/10–45	61–62 ^a	Romesser <i>et al.</i> (1979)
<i>M. olentangyi</i> § RC/ER ^T	2772 ^T	1.0–1.5	–	+	+	(+)	(+)	10.9	37/30–45	54.4 ^a	Corder <i>et al.</i> (1983)
<i>M. bourgensis</i> § MS2 ^T	3045 ^T	1.0–2.0	+	+	+	+	+	18.0	37/25–55	59 ^a , 56–57	Ollivier <i>et al.</i> (1986)
<i>M. thermophilus</i> CR-1 ^T	2373 ^T	0.8–1.3	+	+	+	ND	ND	2.5	55/37–65	59.5 ^a , 57–59	Rivard & Smith (1982)
<i>M. thermophilus</i> UCLA	2624	0.7–1.8	+	+	+	–	–	1.8	55–60/<70	56, 59.7	Ferguson & Mah (1983)
<i>M. thermophilus</i> Ratisbona	2640	0.9–1.5	+	+	+	–	–	3.4	58/30–60	57	Zabel <i>et al.</i> (1985)
<i>M. thermophilus</i> TCI	3915	0.6–1.5	NR	+	+	+	+	NR	55/30–60	54.7	Widdel <i>et al.</i> (1988)
<i>M. oldenburgensis</i>	6216 ^T	1.0	–	+	+	–	–	3.8	45/20–50	48.6	Blotevogel <i>et al.</i> (1991)
<i>M. palmolei</i> INSLUZ ^T	4273 ^T	1.25–2.0	+	+	+	+	+	13.5	40/21–51	59.0	Zellner <i>et al.</i> (1998)
Genus <i>Methanogenium</i>											
<i>M. cariaci</i> JR1 ^T	1497 ^T	<2.6, 1–3	–	+	+	+	+	11.0	37–45/15–50	51.6 ^a	Romesser <i>et al.</i> (1979)
<i>M. organophilum</i> CV ^T	3596 ^T	0.5–1.5	–	+	+	+	–	ND	30–35/15–39	46.7	Widdel <i>et al.</i> (1988)
<i>M. frittonii</i> FR-4 ^T	2832 ^T	1–2.5	–	+	–	–	–	1–2	57/26–62	49.2	Harris <i>et al.</i> (1984)
<i>M. frigidum</i> Ace-2	[SMCC459W]	1.2–2.5	–	+	+	ND	ND	69.6	15/0–17	ND	Franzmann <i>et al.</i> (1997)
Genus <i>Methanolacinia</i>											
<i>M. paynteri</i> G-2000 ^T	2545 ^T	0.6 × 1.5–2.5	+	+	–	+	+	ND	40/20–45	45 ^a , 38	Zellner <i>et al.</i> (1989c)
Genus <i>Methanoplanus</i>											
<i>M. limicola</i> M3 ^T	2279 ^T	1–3 × 0.1–0.25	+	+	+	–	–	ND	40/17–41	47.5	Wildgruber <i>et al.</i> (1982)
<i>M. endosymbiosus</i> MC1 ^T	3599 ^T	1.6–3.4	+	+	+	ND	ND	7	32/16–36	38.7	van Bruggen <i>et al.</i> (1986)

NR, Not reported; ND, not determined.

* H, H₂/CO₂; F, formate; 2P, 2-propanol/CO₂; 2B, 2-butanol/CO₂. None of the strains utilized acetate, methanol or methylamines. *Methanogenium organophilum* utilized ethanol, 1-propanol and 1-butanol in addition. Data on alcohol utilization partly taken from Zellner & Winter (1987). In addition, both *Methanofollis liminatans* strains, GKZPZ and BM1, *Methanoculleus palmolei* and *Methanolacinia paynteri* utilized cyclopentanol/CO₂ with concomitant production of cyclopentanone and CH₄, while *Methanofollis tationis* could not utilize cyclopentanol/CO₂. (+), Weakly positive.

† Growth temperature is given as optimum temperature/temperature range.

‡ G + C content of DNA was determined by T_m unless otherwise indicated, in which case it was determined by buoyant density (a) or HPLC (b).

§ *Methanoculleus olentangyi* and *Methanoculleus bourgensis* are apparently subjective synonyms (Xun *et al.*, 1989; Boone *et al.*, 1993).

acids from Zellner & Winter (1987) and Bleicher *et al.* (1989). *Methanogenium liminatans* contains a secondary alcohol dehydrogenase activity that is devoid of zinc and is dependent on factor F₄₂₀ (Bleicher & Winter, 1991).

The pterin of *Methanogenium tationis* has a structural modification not found in other methanogens and is called tatiopterin. Tatiopterin lacks a 7-methyl substituent but has both a glutamyl and an aspartyl residue conjugated to the phosphoglutaryl moiety (Raemakers-Franken *et al.*, 1989) and can be distinguished from methanopterin and sarcinapterin (Keltjens *et al.*, 1983; DiMarco *et al.*, 1990).

Presumptive respiratory lipoquinones of *Methanogenium tationis* and *Methanogenium liminatans*

Examination of *Methanogenium tationis* DSM 2702^T and *Methanogenium liminatans* GKZPZ^T for the presence of respiratory lipoquinones indicated that none were present, a finding consistent with results obtained for all other members of the methanogenic *Archaea* examined to date (B. J. Tindall, unpublished data).

Lipids of *Methanogenium tationis* and *Methanogenium liminatans*

Both diether- and tetraether-linked glycerol lipids were present in *Methanogenium tationis* DSM 2702^T and *Methanogenium liminatans* GKZPZ^T (data not shown). The diether lipids were diphytanyl glycerol diethers, while the tetraether lipids showed no evidence of cyclization and only acyclic C₄₀:C₄₀ dibiphytanyl tetraethers were present. These results are consistent with the results from other members of the family *Methanomicrobiaceae* examined to date (Grant *et al.*, 1985; Zellner *et al.*, 1989c; Blotevogel *et al.*, 1991; B. J. Tindall & G. Zellner, unpublished data).

The major polar lipids present in *Methanogenium tationis* DSM 2702^T and *Methanogenium liminatans* GKZPZ^T were glycolipids, phospholipids and phosphoglycolipids (Fig. 1). The major phospholipids present were ether derivatives of phosphatidyl glycerol, phosphopentaketetrol amine and phosphopentaketetrol trimethylamine. The major glycolipid present was a diglycosyl ether lipid, the nature of the sugars and their mode of linkage not being determined. A single major phosphoglycolipid was also detected. The

Fig. 1. Thin-layer chromatograms of the polar lipids of (a) *Methanogenium tationis* DSM 2702^T and (b) *Methanogenium liminatans* GKZPZ^T. Spots are identified as: 1, phosphatidyl glycerol; 2, phosphatidyl pentanetetrol amine; 3, diglycosyl diether; 4, phosphatidyl pentanetetrol trimethylamine; 5, phosphoglycolipid; 6, phosphoglycolipid; 7, amino-phosphoglycolipid; 8, amino-phospholipid.

presence of aminopentanetetrol-containing phospholipids is characteristic of members of the family *Methanomicrobiaceae*. Moreover, a detailed analysis of the total polar lipid composition of the various taxa within this group indicates that there are clear differences between the various taxa currently described (Grant *et al.*, 1985; Zellner *et al.*, 1989a, c; Blotevogel *et al.*, 1991; B. J. Tindall & G. Zellner, unpublished data). Thus, the chemical composition of the cell may be a useful way of differentiating genera in this phyletic group.

Although it is not known whether members of the *Methanocorpusculaceae* also contain these unusual aminopentanetetrol-containing phospholipids, the polar lipid composition of members of this family is

Fig. 2. Dendrogram showing the phylogenetic positions of *Methanofollis tationis* DSM 2702^T, *Methanofollis liminatans* GKZPZ^T (= DSM 4140^T) and *Methanofollis liminatans* BM1 (= DSM 10196) within the family *Methanomicrobiaceae*. Numbers at branch points represent percentage bootstrap support. Bar, 10% sequence divergence.

sufficiently different to distinguish them from the members of the family *Methanomicrobiaceae* (Zellner *et al.*, 1989a). The fact that members of the family *Methanocorpusculaceae* have a distinct polar lipid pattern would appear to contradict the 16S rDNA sequence data, since this family appears to branch within the family *Methanomicrobiaceae*. However, the branching order of some groupings within the members of the order *Methanomicrobiales* is uncertain. Such uncertainties in branching order, particularly where the pendant edges of the dendrogram are long in relation to the length of internal branches, has been already documented.

The presence of identical polar lipids in both *Methanogenium tationis* and *Methanogenium liminatans* suggests that these features are not characteristic of the individual species but indicative of a higher taxonomic group. Although certain aspects of their polar lipid pattern are similar to other members of the family *Methanomicrobiaceae*, there are differences in the details of the polar lipid patterns which may be regarded as genus-specific markers.

Phylogenetic position

Sequencing of the 16S rRNA and rDNA was performed to determine the phylogenetic relationship of *Methanogenium tationis* strain DSM 2702^T and *Methanogenium liminatans* strains GKZPZ^T and BM1 to other members of the family *Methanomicrobiaceae*. Analysis of these sequences indicated that the three strains form a phylogenetically coherent group almost equidistant from the genus *Methanoculleus* and other *Methanogenium* species (Fig. 2). The G+C content of the DNA of strain BM1 was determined to be 60.0 mol %, i.e. nearly identical to that of *Methano-*

genium liminatans GKZPZ^T (Zellner *et al.*, 1990). Thus, the G + C content of DNA of this group ranges from 54 to 60 mol %.

On the basis of the similarity in G + C content of DNA, substrate utilization patterns and other phenotypic properties, strain BM1 is assigned to the same species as *Methanogenium liminatans* GKZPZ^T. On the basis of the 16S rDNA sequence and chemotaxonomic data, which indicate that *Methanogenium tationis* and *Methanogenium liminatans* represent a distinct phyletic group within the family *Methanomicrobiaceae*, it is proposed that they be placed in a new genus, for which the name *Methanofollis* gen. nov., is proposed. The strains are described as *Methanofollis tationis* DSM 2702^T gen. nov., comb. nov., *Methanofollis liminatans* GKZPZ^T comb. nov. and *Methanofollis liminatans* BM1.

The 16S rRNA sequences of *Methanocorpusculum bavaricum* and *Methanocorpusculum sinense* have a missing stretch of 40 bp. The remainders of their sequences are 100% and about 99.6% identical to *Methanocorpusculum parvum* and *Methanocorpusculum labreanum*, respectively, which are themselves 99.8% identical. Therefore, the four *Methanocorpusculum* species are highly related. About 1100 nucleotides were used for the alignment of *Methanocorpusculum bavaricum* and *Methanocorpusculum sinense* 16S rRNA sequences, while 1150 nucleotides were used for the *Methanocorpusculum parvum* and *Methanocorpusculum labreanum* sequences.

Description of *Methanofollis* gen. nov.

Methanofollis (Me.tha.no.fol'lis. M.L. n. *methanum* methane; M.L. n. *follis* a bag; *Methanofollis* a methane-producing bag).

Irregular cocci, 1.5–3.0 µm in diameter, with a proteinaceous, SDS-sensitive S-layer. Cells stain Gram-negative. Some strains are motile. Obligately anaerobic, no microaerophilic or aerobic growth. Mesophilic (range ≥ 15 to <45 °C). Substrates for growth and methane production are H₂/CO₂ or formate; some species may use 2-propanol/CO₂, 2-butanol/CO₂ and cyclopentanol/CO₂. Sulfate is not reduced. The ether lipids comprise diphytanyl diether and dibiphytanyl tetraethers. No evidence for significant quantities of other derivatives, including the presence of pentacyclic rings. The major polar lipids are phospholipids, glycolipids and phosphoglycolipids. Among these the predominant compounds are the ether derivatives of phosphatidyl glycerol, diglycosyl glycolipid, phosphopentametrol amine, phosphopentametrol trimethylamine and a phosphoglycolipid. Minor components include a glycolipid, a phospholipid, an amino-phospholipid, a phosphoglycolipid and an amino-phosphoglycolipid. This pattern differs in detail from other members of the family *Methanomicrobiaceae*. The G + C content of DNA of known strains ranges from 54 to 60 mol % (*T_m* and

HPLC). Habitats are anaerobic high-rate wastewater bioreactors or solfataric fields. The type species of the genus is *Methanofollis tationis* comb. nov.

Description of *Methanofollis tationis* comb. nov.

Irregularly coccoid cells, 1.5–3 µm in diameter, with a proteinaceous, SDS-sensitive S-layer. Cells stain Gram-negative. Cells are motile or non-motile. Obligately anaerobic, no microaerophilic or aerobic growth. Strains are mesophilic (range ≥ 15 to <45 °C). Substrates for growth and methane production are H₂/CO₂ and formate. Methanol, methylamines, ethanol, 2-propanol/CO₂, 2-butanol/CO₂ and cyclopentanol/CO₂ are not utilized. Cells contain tatioppterin, a modified pterin distinct from methanoppterin and sarcinapterin. Lipid composition as that described for the genus. The G + C content of DNA of the only strain known so far is 54 mol % (*T_m*). The type strain is DSM 2702^T and was isolated from a solfataric field on Mount Tatio in the Atacama desert in northern Chile.

Description of *Methanofollis liminatans* comb. nov.

Irregularly coccoid or ring-shaped cells, 1.25–2.0 µm in diameter, with a proteinaceous, SDS-sensitive S-layer. Cells stain Gram-negative. Some strains may be motile. Obligately anaerobic, no microaerophilic or aerobic growth. Cells are mesophilic (range ≥ 15 °C to <45 °C) with an optimum at 40 °C. Substrates for growth and methane production are H₂/CO₂, formate, 2-propanol/CO₂, 2-butanol/CO₂ and cyclopentanol/CO₂. Secondary and cyclic alcohols are oxidized to the respective ketones. Methanol, methylamines, ethanol and acetate are not utilized as catabolic substrates. Cells of GKZPZ^T contain a secondary alcohol dehydrogenase dependent on F₄₂₀ and devoid of Zn. Lipid composition as that described for the genus. The G + C content of DNA of the type strain is 60 mol % (*T_m*). The type strain is GKZPZ^T (= DSM 4140^T), isolated from an industrial wastewater bioreactor (Biohochreaktor, Hoechst, Kelsterbach, Frankfurt, Germany). Strain BM1 (= DSM 10196) is a reference strain, isolated from a methane-producing fluidized bed reactor operated with synthetic wastewater containing butyrate and present in a full-scale UASB reactor treating sugar refinery wastewater anaerobically in Brühl, Germany.

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