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Taxonomic Significance of the Distribution of Component Parts of Polar Ether Lipids in Methanogens

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Summary

The ether core lipids, phospholipid-polar head groups and glycolipid-sugar moieties (component parts of polar lipids) from 18 species of methanogens, belonging to 5 families, were analysed qualitatively at the total lipid level, without separation of lipids after extraction, and the results were cumulated to the data published previously from 13 other species. Archaeol, caldarchaeol, macrocyclic archaeol, and two kinds of hydroxyarchaeol were found as core lipids; *myo*-inositol, ethanolamine, serine, aminopentanetetrols, glycerol, and *N*-acetylglucosamine were identified as phospholipid-polar head groups; and glucose, galactose, and mannose as glycolipid-sugars in methanogens as a whole. The distribution of these component parts, regardless their arrangement in the lipid molecules, was characteristic of methanogen taxonomic groups at a family or genus level, and, therefore, coincided with the classification based on the 16S rRNA analysis. This shows that lipid component parts could become a new chemotaxonomic marker, which utilises more lipid-structure-oriented information than a thin-layer chromatographic pattern. A determinative key of methanogens, only based on lipid component analysis, is proposed.

Key words: Methanogens – Ether lipids – Chemotaxonomy – Ethanolamine – Inositol – Serine – Polar head groups – Phospholipids – Glycolipid-sugars – Aminopentanetetrol

Introduction

The lipids of methanogenic bacteria consist of di- and tetra-ethers of glycerol and isoprenoid alcohols. More than 30 complete structures of ether polar lipids from 7 species of methanogens have been determined to date. In these studies, a great variety of lipids have been shown, i. e., five kinds of core lipids (alkyl glycerol ethers), seven or more polar head groups and at least three sugar moieties have been identified. On the basis of the diversity of polar lipid structures of methanogens, it was considered that polar lipid composition may be employed for methanogen taxonomy. *Grant* et al. (1985) and *Koga* et al. (1987) have suggested that the thin-layer chromatographic (TLC) pattern of total polar lipids from a methanogen is characteristic to the family or genus level of the methanogen group. In spite of the simplicity of TLC procedure, TLC is essentially less informative regarding the chemical structures of lipids, and TLC patterns could be compared if they were carried out only in a standardised system (using standardised solvent systems and a certain batch of TLC plates). Although the best way to show the relationship between lipids is to compare their complete structures, the complete structural determination of a lipid is a laborious and time-consuming task that is not suitable for rapid taxonomic work. We intended to develop a method that is more informative in lipid structure than TLC and less time-consuming in analysis than complete structural determination, i. e., the analysis of the component parts of polar lipids in the total lipids. The combination of the occurrence of these component parts should give a significant information on a chemotaxonomic importance of polar lipids. We report here the analyses of polar head groups, monosaccharide units, and core portions from 18 species belonging to 5 families of methanogens as well as previously described complete or fragmentary information of lipid components from 13 species. The nomenclature for archaeal ether lipids proposed by *Nishihara* et al. (1987) is used throughout this paper.

Non-standard abbreviations: TLC = thin-layer chromatography; GLC = gas-liquid chromatography.

Materials and Methods

Strains and growth of methanogenic bacteria. Cultured cells of *Methanothermus fervidus* DSM 2088^T (^{T} = type strain), *Methanothrix (Methanosaeta) thermophila* P_T (DSM 6194^T) and "*Methanosarcina alcaliphila*" NY-728 and "*Methanosalinarium flagellum*" NY-218 (*Nakatsugawa*, 1991) were generous gifts from *H. König, Y. Kamagata,* and *N. Nakatsugawa*, respectively. Other methanogenic strains used in this study were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The growth media, methanogenic substrates, and growth conditions in this study are summarised in Table 1. Approximately 1 g of dry cells (containing 50 mg of total lipid) were obtained for the analyses of component parts of lipids described below. Medium M1 was described by *Balch* et al. (1979). Medium M2W was the same as medium 2 described by *Balch* et al. (1979) except that 2.64 mg/l $Na_2WO_4 \cdot 2H_2O$ was included. Medium 322D was a modification of the DSM medium number 322 from the DSM Catalogue of Strains (1989), from which rumen fluid was omitted and concentrations of vitamins except thiamine and biotin were reduced to the level of DSM medium number 141. Medium M728 for "*Methanosarcina alcaliphila*" and medium M218 for "*Methanosalinarium flagellum*" were described by *Nakatsugawa* (1991). Medium MM contained in 1 l of water NaCl 87 g, KCl 1.5 g, $MgCl_2 \cdot 6H_2O$ 6 g, $CaCl_2 \cdot 2H_2O$ 0.4 g, NH_4Cl 1 g, $K_2HPO_4 \cdot 3H_2O$ 0.4 g, $NaHCO_3$ 4 g, trace mineral salt solution (as in DSM 141 medium (the DSM Catalogue of Strains, 1989)) 10 ml, yeast extract 2 g, trypticase 2 g, coenzyme M 0.2 mg, $Na_2S \cdot 9H_2O$ 0.25 g, resazurin 1 mg, trimethylamine 2.5 g; the pH of the medium was adjusted to 7.0 before autoclaving (*H. Hippe,* pers. comm.). Cells were collected by centrifugation without washing due to the fragility of the cells of some strains. Harvested cells were kept at –20 °C.

Extraction of total lipid. Total lipid was extracted by the method of *Nishihara* and *Koga* (1987) using the trichloroacetic acid-acidified extraction solvent. Trichloroacetic acid in the crude lipid extract was removed by washing several times with water.

Analysis and identification of component parts. All analyses described here were made using total lipid without separation of individual lipids. Fig. 1 shows the items that were analysed, i. e., core lipids, hydrocarbon chains, phosphate-containing polar head groups and sugar moieties. The structures of the component

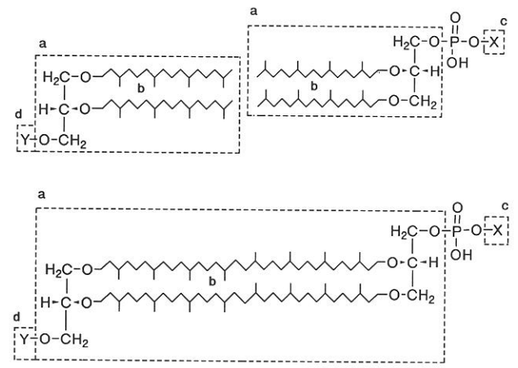

Fig. 1. Component parts of polar ether lipids of methanogens analysed in this study. a, core lipids; b, alkyl chains; c, phospholipid-polar head groups; d, glycolipid-sugars.

Table 1. Growth conditions of methanogens used in this study

Species	Strain	Medium[a]	Substrate[b]	Groth Temperature (°C)	Conditions[c]
Methanobacterium formicicum	DSM 1535^T	119	H_2+CO_2	37	A/shaking
Methanobacterium wolfeii	DSM 2970^T	M2W	H_2+CO_2	62	B/bubbling
Methanothermus fervidus	DSM 2088^T	M1	H_2+CO_2	84	C/bubbling
Methanosphaera stadtmaniae	DSM 3091^T	322D	Methanol + H_2	37	D/shaking
Methanococcus vannielii	DSM 1224^T	141	H_2+CO_2	37	B/bubbling
Methanococcus voltaei	DSM 1537^T	141	H_2+CO_2	37	E/bubbling
Methanococcus thermolithotrophicus	DSM 2095^T	141	H_2+CO_2	65	A/shaking
Methanococcus jannaschii	DSM 2661^T	282	H_2+CO_2	75	A/shaking
Methanolacinia paynteri	DSM 2545^T	274	H_2+CO_2	37	B/bubbling
Methanospirillum hungateii	DSM 1101	119	H_2+CO_2	37	E/bubbling
Methanogenium cariacii	DSM 1497^T	141	H_2+CO_2	23	A/static
Methanoculleus bourgense	DSM 3045^T	332	H_2+CO_2	37	A/shaking
Methanosarcina barkeri	DSM 800^T	120	Methanol	37	B/static
"*Methanosarcina alcaliphila*"	NY-728	M728	Methanol	37	F/static
Methanolobus tindarius	DSM 2278^T	233	Methanol	25	B/static
Methanohalophilus mahii	DSM 5219^T	MM	$(Me)_3N$	35	B/static
"*Methanosalinarium flagellum*"	NY-218	M218	$(Me)_3N$	37	F/static
Methanothrix thermophila	P_T^{T}	334	Acetate	55	A/static

[a] Medium numbers are those described in the DSM Catalogues of Strains 1989 except M1, M2W, 322D, M728, MM, and M128 (see Material and Methods).
[b] H_2+CO_2 = a gas mixture of H_2+CO_2 (4:1); $(Me)_3N$ = trimethylamine.
[c] Culture vessels and volumes of media: A, 1-litre serum bottle (100 ml medium for shaking culture and 500 ml medium for static culture); B, 10-litre carboy (5 l medium); C, 300-litre Fermenter; D, biphasic culture (*Miller* and *Wolin*, 1985); E, 15-litre fermenter (5 l medium); F, 2-litre bottle (1 l medium). In the case of the vessel A or D with H_2, cells were grown under a pressurised atmosphere.

parts of polar ether lipids detected in this work are shown in Fig. 2 and 3. Lipid component parts were identified by co-migration with standards and staining responses on a TLC plate or co-chromatography on gas-liquid chromatography (GLC), i. e., when a sample showed a retention time close to a standard, a 1 : 1 mixture of the sample and the standard was injected. If only a single sharp peak was detected, the identity of the both compounds was assumed to be confirmed.

TLC of total polar lipids. The total lipids were analysed by two-dimensional TLC on a Silica Gel 60 plate (Merck) as described by *Nishihara* and *Koga* (1987).

Core lipids. Alkyl glycerol ethers (core lipids) were prepared from total lipid by acetolysis and subsequent strong acid methanolysis (*Morii* et al., 1986). Core lipids were separated by one dimensional TLC as described by *Nishihara* and *Koga* (1991). A diagramatic representation of a TLC chromatogram of core lipids is shown in Fig. 4. Spots were detected by $NaIO_4$-Schiff reagent and acid charring. Due to the acid-lability of hydroxyarchaeols (*Sprott* et al., 1990; *Nishihara* and *Koga*, 1991), they were degraded into mainly monoalkylglycerol monoether and small amounts of by-products under the above condition (Fig. 4). Two isomers of hydroxyarchaeol were discriminated by the response of monoalkylglycerol to the $NaIO_4$-Schiff reagent. A positive response indicated the presence of 3-O-phytanyl *sn*-glycerol monoether derived from β-hydroxyarchaeol, while a negative response indicated that only the 2-O-phytanyl *sn*-glycerol monoether derived from α-hydroxyarchaeol was present. Core lipids were determined on the basis of TLC analysis and the results were confirmed by alkyl chain analysis as follows.

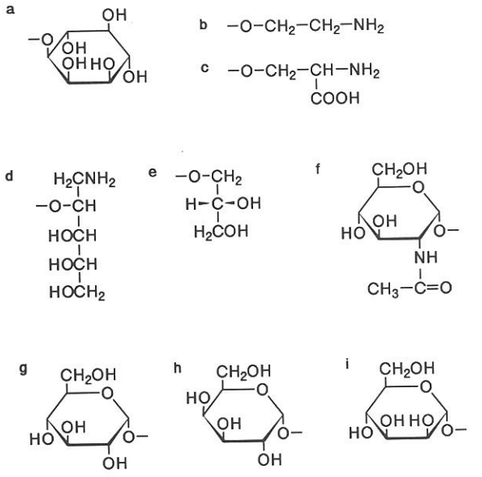

Fig. 3. Structures of polar head groups and sugar groups found in methanogens analysed. a, *myo*-inositol; b, ethanolamine; c, serine; d, aminopentanetetrol; e, glycerol; f, *N*-acetylglucosamine; g, glucose; h, galactose; i, mannose.

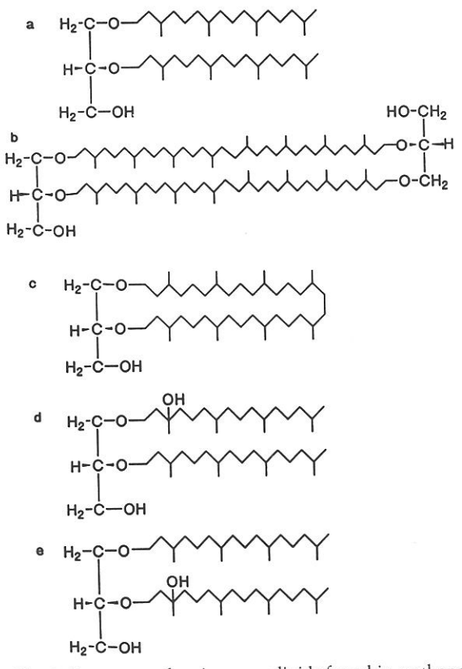

Fig. 2. Structures of various core lipids found in methanogens. a, archaeol; b, caldarchaeol; c, macrocyclic archaeol; d, α-hydroxyarchaeol; e, β-hydroxyarchaeol.

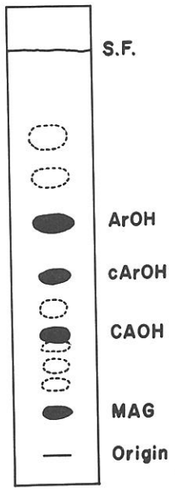

Fig. 4. Diagramatic representation of a thin-layer chromatogram of core lipids prepared by acetolysis and subsequent methanolysis. The closed spots and dotted spots are major and minor spots, respectively. This diagram represents locations of all the core lipids found in any methanogens on a TLC chromatogram, that is, no one species actually contains all these spots. ArOH, archaeol; cArOH, macrocyclic archaeol; CAOH, caldarchaeol; MAG, monoalkylglycerol; Orig, starting origin of the chromatography; S.F., solvent front.

Alkyl chains. Hydrocarbon chains were prepared by HI degradation followed by $LiAlH_4$ reduction, as previously described (*Nishihara* et al., 1989). The resulting hydrocarbons were analysed with a gas-liquid chromatograph (Shimadzu GC9A, Japan) with a column (200 cm) packed with OV-11 at a temperature increasing from 100 to 340 °C at a rate of 20 °C/min.

Glycerophosphate esters. The total lipids were dealkylated with BCl_3 (*Nishihara* and *Koga*, 1988) to prepare glycerophosphate esters, which were separated by electrophoresis on a cellulose TLC plate (Merck) at a potential of 37 V/cm at pH 3.6 for 60 min using a solvent, pyridine-acetic acid-water (1 : 10 : 89) in the first dimension (*Dittmer* and *Wells*, 1969), and then developed in the second dimension by chromatography with a solvent, phenol-water (100 : 38). Electrophoresis of polar groups was used before TLC to separate contaminating sugars from the glycerophosphate ester preparations, which interfere with the development of glycerophosphoinositol and glycerophosphoserine. Spots were detected by the $FeCl_3$-salicylsulfonic acid procedure (*Vorbeck* and *Marinetti*, 1965) for water-soluble phosphate esters and ninhydrin for amino groups. A diagramatic representation of the profile of two-dimensional electrophoresis-TLC of glycerophosphate esters is shown in Fig. 5.

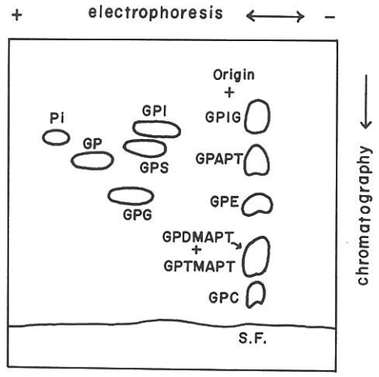

Fig. 5. Diagramatic representation of two-dimensional thin-layer electrophoresis and chromatography of dealkylated products of ether phospholipids on a cellulose plate. Electrophoresis was carried out in the first, horizontal direction (anode and cathode were right- and left-hand sides, respectively), and chromatography in the second, vertical direction (from top to bottom, ascending). GPIG, glycerophospho(glucosaminyl)inositol; GPI, glycerophosphoinositol; GPS, glycerophosphoserine; GP, glycerophosphate; GPAPT, glycerophospho(amino)pentanetetrol; GPG, glycerophosphoglycerol; GPE, glycerophosphoethanolamine; GPDMAPT, glycerophospho(dimethylamino)pentanetetrol; GPTMAPT, glycerophospho(trimethylamino)pentanetetrol; GPC, glycerophosphocholine; S.F., solvent front.

Inositol and monosaccharide units of glycolipids. Monosaccharide units including hexoses and *N*-acetylhexosamines from glycolipids or phosphoglycolipids in the total lipid were released by acid methanolysis to yield methylglycosides, which were reduced to alditol acetates after demethylation as described previously (*Nishihara* et al., 1987). Alditol acetates were analysed by GLC with a DB-1 capillary column (0.25 mm × 30 m, J & W Scientific, California). Column temperature was programmed from an initial temperature of 190 °C, held for 23 min, followed by a programmed increase of 8 °C/min to 230 °C, and finally held at that temperature for 6 min. Inositol phosphate, liberated from inositol containing phospholipids by acid methanolysis, was hydrolysed with 6 M aqueous HCl at 100 °C for 6 hr to remove the phosphate group. After acetylation, *myo*-inositol was identified by GLC under the same conditions as those for sugar analysis. Fig. 6 is a typical GLC chromatogram of sugars and inositol. Glucosamine not acetylated was liberated under the same condition as the hydrolysis of inositol phosphate (stronger conditions) but not under the condition of release of hexoses or *N*-acetylglucosamine.

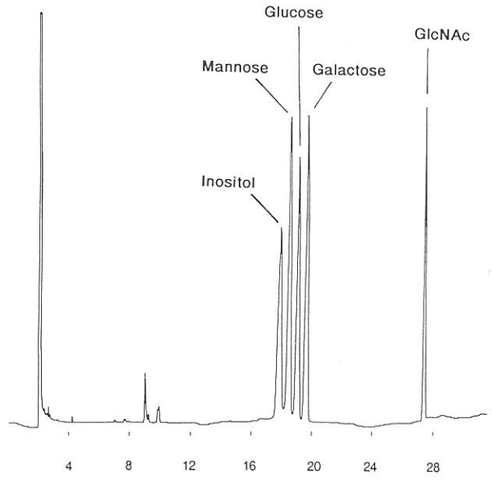

Fig. 6. A gas-liquid chromatogram of sugar-derived polyol acetates and *myo*-inositol acetate. The peaks are acetylated derivatives of mannitol derived from mannose, galactitol from galactose, glucitol from glucose, *N*-acetylaminoglucitol from *N*-acetylglucosamine (GlcNAc), and *myo*-inositol. This figure simply shows a chromatogram of the mixture of the above five compounds, which are never present in lipids from one species of methanogen, and alditol acetates and inositol acetate are separately analysed because of the differences in conditions of their preparation (see text).

Results

Table 2 summarises the results of the analyses for 31 species including 18 species analysed in this study, the simplified expression of the occurrence of component parts deduced from the complete structures of polar lipids of 3 species, and the results described in the previous papers of the tentative identification of polar lipids by TLC from 10 species (12 strains) that were not analysed in the present work. Lipid component parts analyses of 4 species (*Methanococcus voltaei*, *Methanococcus jannaschii*, *Methanospirillum hungatei*, and *Methanosarcina barkeri*) coincided with the complete structures which have been

Table 2. Distribution of component parts of polar lipids among methanogens. +, detected; −, not detected

Methanogenic strain[a]	Core lipid				Glycolipid-sugar			Phospholipid-polar head group					References
	ArOH	CAOH	hy-Ar	cArOH	Glc	Gal	Man	GlcNAc	Ino	$EtNH_2$	Ser	APT	Gro	
Methanobacteriaceae														
Mba. thermoautotrophicum DSM 1053^T	+	+	–	–	+	–	–	[b]	+	+	+	–	ND[b]	1
Mba. wolfeii DSM 2970^T	+	+	–	–	+	–	–	–	+	+	+	–	–	13
Mba. formicicum DSM 1535^T	+	+	–	–	+	–	–	–	+	+	+	–	ND	13
Mba. bryantii DSM 863^T	+	+							(+)[c]	(+)	(+)	(–)		2, 3
Mba. palustre DSM 3108^T	+	+							(+)	(+)				4
Mbr. arboriphilicus A2	+	+	–	–	+		–	–	+	–	+	–	–	5
Mbr. arboriphilicus DH1^T									(+)	(–)	(+)	(–)		3
Mbr. ruminantium DSM 1093^T	+	+							(+)	(+)	(+)	(–)		2, 3
Mbr. smithii DSM 861^T	+	+							(+)	(–)	(+)	(–)		2, 3
Methanothermaceae														
Mtm. fervidus DSM 2088^T	+	+	–	–	+	–	+	–	+	–	–	–	–	13
Undefined family														
Msph. stadtmaniae DSM 3091^T	+	+	+ α[d]	–	+	–	–	–	+	–	+	–	–	13
Mpy. kandleri DSM 6324^T	+	–												6
Methanococcaceae														
Mco. vannielii DSM 1224^T	+	–	+ β	–	+	–	–	ND	–	–	–	(+)	(+)	8
Mco. voltaei DSM 1537^T	+	–	+ β	–	+	–	–	–	–	–	+	+	+	8, 13
Mco. thermolithotrophicus DSM 2095^T	+	+	+ α	–	+	–	–	–	–	–	+	+	+	13
Mco. jannaschii DSM 2661^T	+	+	–	+	+	–	–	–	–	–	+	–	–	3, 8, 13
Mco. igneus Kol5^T	+	+		+										13
Methanomicrobiaceae														
Mmi. mobile DSM 1539^T	+	+	–	–	+	–	–	–	–	–	–	ND	ND	13
Mla. paynteri DSM 2545^T	+	+	–	–	+	–	–	–	–	–	–	ND	ND	7
Msp. bungatii DSM 1101	+	+	–	–	+	–	–	–	–	–	–			8
Mge. cariacii DSM 1497^T	+	+	–	–	+	–	–	–	–	–	–	(+)	(+)	8, 13
Mcu. bourgense DSM 3045^T	+	+	–	–	+	–	–	–	–	–	–	(+)	(+)	13
Mcu. oldenburgensis DSM 6216^T	+	+												9
Methanosarcinaceae														
Msa. barkeri DSM 800^T	+	–	+ β	–	+	–	–	–	+	+	+	–	+	13
Msa. mazeii S-6^T			+ β											10
"*Msa. alcaliphila*" NY-728	+	–	+ β	–	–	–	–	–	–	–	–	–	–	13
Mlo. tindarius DSM 2278^T	+	–	+ β	–	+	–	–	–	+	+	+	–	+	13
Mha. mahii DSM 5219^T	+	–	+ β	–	+	–	–	–	+	+	+	–	+	13
"*Msal. flagellum*" NY-218	+	–	+ β											2
Mcd. methylutens	+	–	+ α	–	+	–	–	–	–	–	–	–	–	11, 12
Mtx. concilii GP6														12
Methanothrix sp. MTAS	+	+	–	–	–	–	–	–	–	–	–	–	–	13
Mtx. thermophila P_t^T														

Abbreviations: ArOH, archaeol; CAOH, caldarchaeol; hy-Ar, hydroxyarchaeol; cArOH, macrocyclic archaeol; GlcNAc, N-acetylglucosamine; Ino, inositol; $EtNH_2$, ethanolamine; Ser, serine; APT, aminopentanetetrol irrespective to methylation; Gro, glycerol.
Abbreviations for genus names: *Mba.* = *Methanobacterium*; *Mbr.* = *Methanobrevibacter*; *Mtm.* = *Methanothermus*; *Msph.* = *Methanosphaera*; *Mpy.* = *Methanopyrus*; *Mco.* = *Methanococcus*; *Mmi.* = *Methanomicrobium*; *Mla.* = *Methanolacinia*; *Msp.* = *Methanospirillum*; *Mge.* = *Methanogenium*; *Mcu.* = *Methanoculleus*; *Msa.* = *Methanosarcina*; *Mlo.* = *Methanolobus*; *Mha.* = Methanohalophilus; *Msal.* = Methanosalinarium; *Mcd.* = Methanococcoides; *Mtx.* = Methanothrix.
[a] *Strain number Mcd. methylutens* was not specified by *Langworthy* (1985).
[b] Blank, not reported in the literatures cited. ND, not determined in this work.
[c] + or – in parentheses was based on the mobility and staining responses of TLC of total polar lipid.
[d] α, α-hydroxyarchaeol; β, β-hydroxyarchaeol.
References: 1, *Nishihara* et al. (1989); 2, *Langworthy* (1985); 3, *Koga* et al. (1987); 4, *Zellner* et al. (1989a); 5, *Morii* et al. (1986, 1988); 6, *Kurr* et al. (1991); 7, *Tricone* et al. (1992); 8, *Zellner* et al. (1989b); 9, *Blotevogel* et al. (1991); 10. *Sprott* et al., (1990); 11, *Ferrante* et al. (1988a, 1988b, 1989); 12, *Ohtsubo* et al. (1991); 13, this work.

determined by *Ferrante* et al. (1986, 1987, 1990), *Kushwaha* et al. (1981), and *Nishihara* and *Koga* (1991).

Methanobacteriaceae

Three species of the genus *Methanobacterium* (*M. thermoautotrophicum, M. wolfeii,* and *M. formicicum*) contained identical component parts of lipids. The TLC profiles of the other species (*M. bryantii* (*Koga* et al., 1987) and *M. palustre* (*Zellner* et al., 1989a)) of the genus were consistent with the results of above three species. Core lipids were archaeol and caldarchaeol, both of which were present in significant amounts, the latter being predominant. The only sugar moiety was glucose. The polar head groups were ethanolamine, serine and *myo*-inositol. Gentiobiosyl caldarchaetidylinositol (tetraether type of inositol-containing phosphoglycolipid) has been recognised as a signature lipid of this family (*Morii* et al., 1988). The present analysis of the component parts was consistent with the conclusion of *Morii* et al. (1988). Ethanolamine was absent in *Methanobrevibacter arboriphilicus* A2, while the other components were the same as those of *Methanobacterium*. TLC profiles have shown the absence of ethanolamine phospholipids in two other species of *Methanobrevibacter, M. smithii* and *M. ruminantium,* and another strains of *M. arboriphilicus* (strain DH1^T, DC, and AZ) (*Koga* et al., 1987). The presence or absence of ethanolamine is, therefore, a discriminating marker between members of the genera *Methanobacterium* and *Methanobrevibacter*.

Methanothermaceae

The analysis of one of two species of this family showed that it was significantly different in polar lipid composition from members of the family *Methanobacteriaceae* although the family *Methanothermaceae* belongs to *Methanobacteriales*. *Methanothermus fervidus* contained only inositol as a phospholipid-polar head group (Table 2), which resembled sulfur-dependent thermophilic *Archaea,* i. e., *Crenarchaeota*. The sugar moieties of the glycolipids of this species were glucose and *N*-acetylglucosamine.

Methanosphaera

In spite of the presence of pseudomurein in the cell walls, the family to which *Methanosphaera* belongs has not been defined. *Methanosphaera* differs from members of the above two families in utilisation of methanol + H_2 as methanogenic substrates and inability to grow on H_2 + CO_2 (*Miller* and *Wolin*, 1985). The component parts composition of *Methanosphaera stadtmaniae* was similar to that of *Methanobrevibacter* species, except that *M. stadtmaniae* contained a third core lipid α-hydroxyarchaeol (Table 2).

Methanococcaceae

The core lipid composition of this family was diverse (Table 2). The two mesophilic species, *Methanococcus vannielii* and *M. voltaei* contained β-hydroxyarchaeol as well as archaeol, while the two extremely thermophilic species (*M. jannaschii* and *M. igneus*) had the unique core lipid, macrocyclic archaeol but not hydroxyarchaeol. *M. jannaschii* contained archaeol, caldarchaeol, and macrocyclic archaeol, and the moderate thermophile, *M. thermolithotrophicus* contained hydroxyarchaeol instead of macrocyclic archaeol. Both had three kinds of core lipids. The main glycolipid-sugar was glucose like members of the family *Methanobacteriaceae*. *N*-Acetylglucosamine is linked to archaeol through a phosphodiester linkage in *M. voltaei* (*Ferrante* et al., 1986), and *N*-acetylglucosamine should, therefore, be listed as a phospholipid-polar head group, but since this was detected in the same analysis as the glycolipid-sugars by GLC, this was listed as a sugar group in Table 2. The most characteristic feature of the polar head groups in *Methanococcus* was the absence of *myo*-inositol. Ethanolamine was found in *M. jannaschii* so far analysed. As a whole, the component parts of lipids in this genus was more diverse than the other families of methanogens.

Methanomicrobiaceae

The core lipids of methanogens of this family were archaeol and caldarchaeol and no variation was found (Table 2). Two remarkable characteristics of polar lipid component parts were recognised. One of them was the presence of a galactose residue in glycolipids in addition to glucose. Phospholipid polar head groups were also unique: archaetidyl(dimethylamino)pentanetetrol and archaetidyl (trimethylamino)pentanetetrol were identified in *Methanospirillum hungateii* by *Ferrante* et al. (1987). Their derivative without methylation, archaetidyl(amino)-petanetetrol was also identified by us (unpublished data). This was the ninhydrin-positive phospholipid detected in *Methanospirillum hungateii, Methanogenium cariaci* and *Methanolacinia* (formerly *Methanomicrobium*) *paynteri* designated as "PX" in our previous paper (*Koga* et al., 1987). In this study, the presence or absence of aminopentanetetrol, regardless of methylation, was recorded. In this family, aminopentanetetrol was detected in all species so far analysed and reported by other authors (*Zellner* et al., 1989b; *Blotevogel* et al., 1991). Ethanolamine, serine and inositol found in other families were not present in members of the family *Methanomicrobiaceae*.

Methylotrophic Methanosarcinaceae

The core lipids of the methylotrophic methanogens (the genera *Methanosarcina, Methanolobus, Methanohalophilus,* and "*Methanosalinarium*") were archaeol and β-hydroxyarchaeol (Table 2). The phospholipid-polar head groups were similar to those of members of the family *Methanobacteriaceae*. A unique glucosaminylinositol group was identified in *Methanosarcina barkeri* (*Nishihara* et al., 1992). It was, however, not listed in Table 2 since it was detected only in the species and taxonomic significance is not clear. Serine was absent in the halophilic methylotrophic methanogens (*Methanohalophilus* and "*Methanosalinarium*"). The other significant difference between members of the order *Methanomicrobiales* and

members of the order *Methanobacteriales* in polar head groups was the presence of non-alkylated glycerol (Table 2). Glycerophosphoglycerol as a phospholipid backbone was found not only in five species of methylotrophic methanogens but also in four species of the family *Methanomicrobiaceae* so far analysed. The only sugar residue was glucose in the methylotrophic members of the family *Methanosarcinaceae*. No glycolipid was detected in a newly isolated alkaliphilic methanogen "*Methanosarcina alcaliphila*".

Methanothrix

There were three kinds of sugar (glucose, galactose and mannose) in glycolipids in the mesophile, aceticlastic *Methanothrix (Methanosaeta) concilii* (Table 2). Phospholipid-polar head groups were similar to those of *Methanosarcina* species (*Ferrante* et al., 1988a, 1989). The presence of serine was variable, being dependent on the strains of *Methanothrix* species studied (*Ohtsubo* et al., 1991). Core lipids also resembled *Methanosarcina* species but the hydroxyphytanyl chain was etherified at the *sn*-3 position of the glycerol backbone in *Methanothrix* (*Ferrante* et al., 1988b; α-hydroxyarchaeol). A newly described thermophilic species, *Methanothrix thermophila* strain P_T^T contained archaeol and caldarchaeol but no hydroxyarchaeol as core lipids. Galactose was the sole glycolipid-sugar in this species.

Discussion

The analyses described above on a variety of methanogens clearly demonstrated that composition of the component parts of polar lipids were generally specific at the family level, in some cases at genus level. In other words, the lipid composition of methanogens corresponds to the classification of methanogens based on phylogeny deduced from the 16S rRNA homology (*Balch* et al., 1979; *Woese* et al., 1991). The characteristic distribution of polar lipid component parts in methanogens are summarised in Table 3, in which a set of core lipids, phospholipid-hyphen polar head groups and glycolipid-sugars was correlated to a family-level taxonomy of methanogens.

By rearranging the results, a tentative determinative key may be constructed as shown in Fig. 7, based only on the lipid component analysis. First, the presence of inositol in the total lipid differentiates members of the order *Methanobacteriales* and the family *Methanosarcinaceae* from members of the order *Methanococcales* and the family *Methanomicrobiaceae*. Subsequently galactose, caldarchaeol, ethanolamine, serine, hydroxyarchaeol, and aminopentanetetrols could be markers that distinguish genera or families as shown in Fig. 7. Further differentiation of genera in the family *Methanomicrobiaceae* can not be made at present. In view of the fact that lipid structures of members of two other families, *Methanoplanaceae* (a TLC pattern of polar lipids from *Methanoplanus limicola* has been reported by *Grant* et al., 1985) and *Methanocorpusculaceae*, are not known at all, the possibility cannot be excluded that the determinative key shown in Fig. 7 should be revised after progress in the lipid analysis from more species of methanogens. Nevertheless, this analysis may, at present, give a rough sketch of methanogen lipid distribution as a whole.

Although members of the families *Methanomicrobiaceae* and *Methanosarcinaceae* have been grouped into the same order *Methanomicrobiales* (*Balch* et al., 1979), their lipid compositions were greatly different each other. Caldarchaeol, galactose, and aminopentanetetrol were present in members of the family *Methanomicrobiaceae* but not in members of the family *Methanosarcinaceae*, while hydroxyarchaeol, inositol, ethanolamine, and serine were found in members of the family *Methanosarcinaceae* but not in members of the family *Methanomicrobiaceae*. The common components for both families were only archaeol and glucose, which are ubiquitously distributed throughout methanogens. Methanogens of both families also differ in their substrate utilisation for methanogenesis and cell surface structures. Evolutionary distances among various methanogens based on 16S rRNA sequences also showed that the distance between the family *Methanomicrobiaceae* and the family *Methanosarcinaceae* may correspond to the difference between the orders *Methanobacteriales* and *Methanococcales* (*Oyaizu* et al., 1990; *Woese* et al., 1991). *Rouviere* et al. (1992) have shown that members of the order *Methanomicrobiales* are divided into two major clusters (the "methanosarcina" group and the

Table 3. Summary of the distribution of the lipid component parts among main groups of methanogens

Family	Core lipid			Glycolipid-sugar			Phospholipid-polar head group			
	CAOH	hy-Ar	cArOH	Glc	Gal	Man	Ino	$EtNH_2$	Ser	APT
Methanobacteriaceae	+	−	−	+	−	−	+	(+)	+	−
Methanothermaceae	+	−	−	+	−	−	+	−	−	−
Mesophilic *Methanococcaceae*	−	+	−	+	−	−	−	−	+	−
Thermophilic *Methanococcaceae*	+	(+)	(+)	+	−	−	−	(−)	+	−
Methanomicrobiaceae	+	−	−	+	+	−	−	−	−	+
Methylotrophic *Methanosarcinaceae*	−	+	−	(+)	−	−	+	+	(+)	−
Aceticlastic *Methanothrix*	(−)	+	−	(+)	+	(+)	+	+	(−)	−

Abbreviations are the same as in Table 2.
Symbols in parentheses indicate that there are some exceptions.

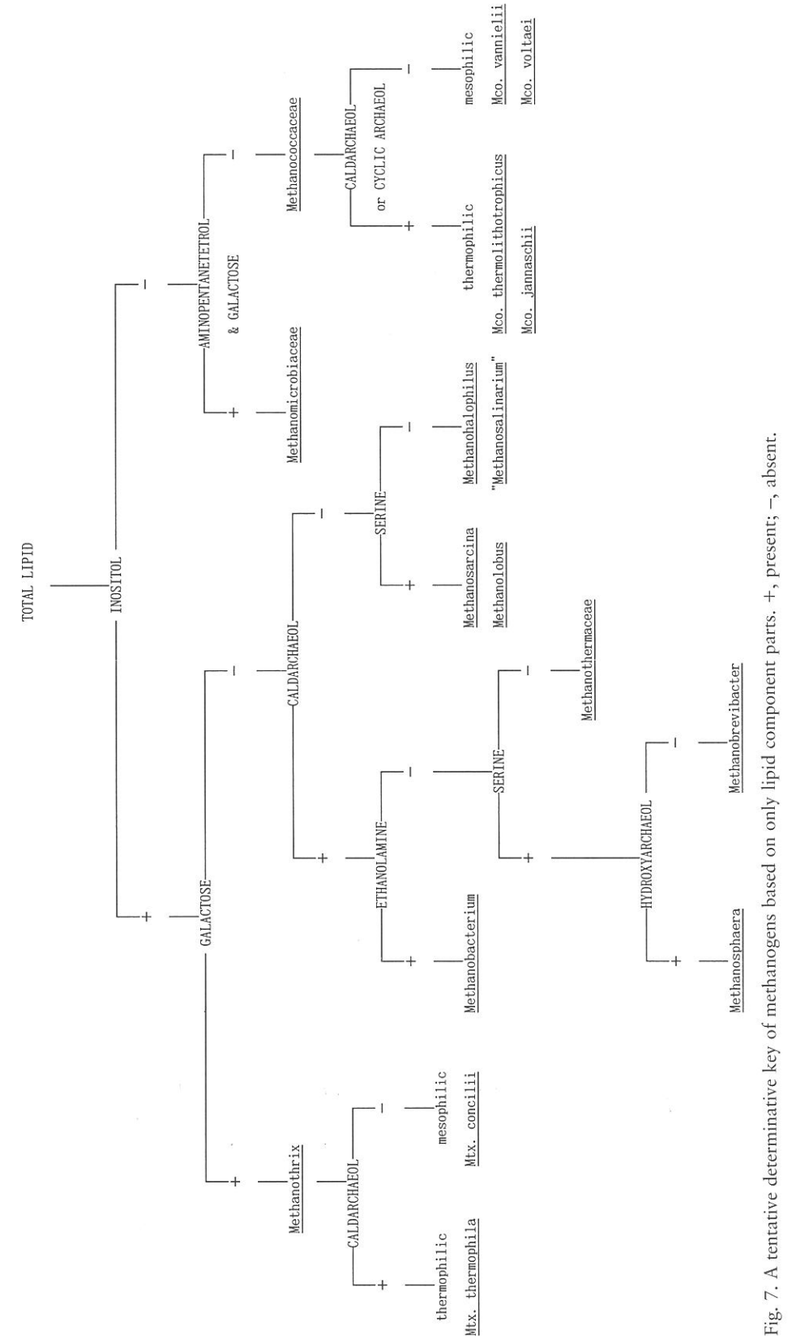

Fig. 7. A tentative determinative key of methanogens based on only lipid component parts. +, present; −, absent.

"methanogenium" group) based on a detailed phylogenetic tree constructed by the small subunit rRNA sequence analysis. The present analysis of polar lipids phenotypically confirmed the phylogenetic grouping of the members of the order *Methanomicrobiales*.

Methanogens of the family *Methanococcaceae* are, at present, classified as a single genus. Our analysis, however, revealed diversity in core lipid composition and the occurrence of ethanolamine between the mesophilic and thermophilic methanococci. Throughout the methanogen species examined so far, there is only one other example (*Methanothrix*) in which core lipid composition differs from species to species in one genus. The 16S rRNA sequence comparison showed also a significant difference in *Methanococcus* species (*Woese* et al., 1991). Therefore, there is a possibility that the genus *Methanococcus* may be divided into at least two genera.

Non-alkylated glycerol was found as a phospholipid-polar head group in most species of the order *Methanomicrobiales* (*Zellner* et al., 1989b; *Blotevogel* et al., 1991). The occurrence of glycerol as the polar head group in the extreme halophiles and these methanogens confirmed the specific relatedness between *Methanomicrobiales* and the extreme halophiles repeatedly suggested by the sequencing of 16S and 23S rRNA (*Woese* and *Olsen*, 1986; *Burggraf* et al., 1991).

It has been suggested that halophilic methanogens belong to the family *Methanosarcinaceae*, like other methylotrophic methanogens (*Sowers* et al., 1984; *Boone* and *Mah*, 1989). Nucleotide sequencing of the 16S rRNA has provided evidence for the affiliation of *Methanohalophilus zhilinaeae* (*Mathrani* et al., 1988) and *Methanohalophilus mahii* (*Oyaizu* et al., 1990) to the family. Lipid component parts analysis presented in this study phenotypically confirmed this implication.

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References

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., Wolfe, R. S.: Methanogens: reevaluation of a unique biological group. Microbiol. Rev. *43*, 260–296 (1979)

Blotevogel, K.-H., Gahl-Janssen, R., Jannsen, S., Fischer, U., Pilz, F., Auling, G., Macario, A. J. L., Tindall, B. J.: Isolation and characterization of a novel mesophilic, freshwater methanogen from river sediment *Methanoculleus oldenburgenesis* sp. nov. Arch. Microbiol. *157*, 54–59 (1991

Boone, D. R., Mah, R. A.: Family II. *Methanosarcinaceae*. p. 2198. In: Bergey's manual of systemic bacteriology (*J. T. Staley, M. P. Bryant, N. Pfennig, J. G. Holt*, eds.) Vol. 3. Baltimore, Williams and Wilkins 1989

Burggraf, S., Ching, A., Stetter, K. O., Woese, C. R.: The sequence of *Methanospirillum hungatei* 23S rRNA confirms the specific relationship between the extreme halophiles and the *Methanomicrobiales*. System. Appl. Microbiol. *14*, 358–363 (1991)

Dittmer, J. C., Wells, M. A.: Quantitative and qualitative analysis of lipids and lipid components, pp. 482–530. In: Methods in Enzymology (*J. M. Lowenstein*, ed.) Vol. 14. New York, Academic Press 1969

Ferrante, G., Ekiel, I., Sprott, G. D.: Structural characterization of the lipids of *Methanococcus voltae*, including a novel *N*-acetylglucosamine 1-phosphate diether. J. Biol. Chem. *261*, 17062–17066 (1986)

Ferrante, G., Ekiel, I., Sprott, G. D.: Structures of diether lipids of *Methanospirillum hungatei* containing novel head groups *N,N*-dimethylamino- and *N,N,N*-trimethylamino pentanetetrol. Biochim. Biophys. Acta *921*, 281–291 (1987)

Ferrante, G., Ekiel, I., Patel, G. B., Sprott, G. D.: Structure of the major polar lipids isolated from the aceticlastic methanogen, *Methanothrix concilii* GP6. Biochim. Biophys. Acta *963*, 162–172 (1988a)

Ferrante, G., Ekiel, I., Patel, G. B., Sprott, G. D.: A novel core lipid isolated from the aceticlastic methanogen, *Methanothrix concilii* GP6. Biochim. Biophys. Acta *963*, 173–182 (1988b)

Ferrante, G., Brisson, J.-R., Patel, G. B., Ekiel, I., Sprott, G. D.: Structures of minor ether lipids isolated from the aceticlastic methanogen, *Methanothrix concilii* GP6. J. Lipid Res. *30*, 1601–1609 (1989)

Ferrante, G., Richards, J. C., Sprott, G. D.: Structures of polar lipids from the thermophilic, deep-sea archaebacterium *Methanococcus jannaschii*. Biochem. Cell Biol. *68*, 274–283 (1990)

Grant, W. D., Pinch, G., Harris, J. E., de Rosa, M., Gambacorta, A.: Polar lipids in methanogen taxonomy. J. Gen. Microbiol. *131*, 3277–3286 (1985)

Koga, Y., Ohga, M., Nishihara, M., Morii, H.: Distribution of diphytanyl ether analog of phosphatidylserine and an ethanolamine-containing tetraether lipid in methanogenic bacteria. System. Appl. Microbiol. *9*, 176–182 (1987)

Kurr, M., Huber, R., König, H., Jannasch, H. W., Fricke, H., Trincone, A., Kristjanson, J. K., Stetter, K. O.: Methanopyrus kandleri, gen. and sp. nov. representing a novel group of hyperthermophilic methanogens, growing at 110 °C. Arch. Microbiol. *156*, 239–247 (1991)

Kushwaha, S. C., Kates, M., Sprott, G. D., Smith, I. C. P.: Novel polar lipids from the methanogen *Methanospirillum hungatei* GP1. Biochim. Biophys. Acta *664*, 156–173 (1981)

Langworthy, T. A.: Lipids of archaebacteria, pp. 459–497. In: The bacteria (*C. R. Woese, R. S. Wolfe*, eds.) vol. 8. Orlando, Academic Press 1985

Mathrani, I. M., Boone, D. R., Mah, R. A., Fox, G. E., Lau, P. P.: Methanohalophilus zhilinae sp. nov., an alkaliphilic, halophilic, methylotrophic methanogen. Int. J. System. Bact. *38*, 139–142 (1988)

Miller, T. L., Wolin, M. J.: Methanosphaera stadtmaniae gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. Arch. Microbiol. *141*, 116–122 (1985)

Morii, H., Nishihara, M., Ohga, M., Koga, Y.: A diphytanyl ether analog of phosphatidylserine from a methanogenic bacterium, *Methanobrevibacter arboriphilus*. J. Lipid Res. *27*, 724–730 (1986)

Morii, H., Nishihara, M., Koga, Y.: Composition of polar lipids of *Methanobrevibacter arboriphilicus* and structure determination of the signature phosphoglycolipid of *Methanobacteriaceae*. Agr. Biol. Chem. *52*, 3149–3156 (1988)

Nakatsugawa, N.: Novel methanogenic archaebacteria which grow in extreme environments, pp. 212–220. In: Superbugs (*K. Horikoshi, W. D. Grant*, eds.). Tokyo, Japan Scientific Societies Press 1991

Nishihara, M., Koga, Y.: Extraction and composition of polar lipids from the archaebacterium, *Methanobacterium thermoautotrophicum:* effective extraction of tetraether lipids by an acidified solvent. J. Biochem. *101*, 997–1005 (1987)

Nishihara, M., Morii, H., Koga, Y.: Structure determination of a quartet of novel tetraether lipids from *Methanobacterium thermoautotrophicum*. J. Biochem. *101*, 1007–1015 (1987)

Nishihara, M., Koga, Y.: Quantitative conversion of diether and tetraether phospholipids to glycerophosphoesters by dealkylation with boron trichloride: a tool for structural analysis of archaebacterial lipids. J. Lipid Res. *29*, 384–388 (1988)

Nishihara, M., Morii, H., Koga, Y.: Heptads of polar ether lipids of an archaebacterium, *Methanobacterium thermoautotrophicum:* Structure and biosynthetic relationship. Biochemistry *28*, 95–102 (1989)

Nishihara, M., Koga, Y.: Hydroxyarchaetidylserine and hydroxyarchaetidyl-*myo*-inositol in *Methanosarcina barkeri:* polar lipids with a new ether core portion. Biochim. Biophys. Acta *1082*, 211–217 (1991)

Nishihara, M., Utagawa, M., Akutsu, H., Koga, Y.: Archaea contain a novel diether phosphoglycolipid with a polar head group identical to the conserved core of eucaryal glycosyl phosphatidylinositol. J. Biol. Chem. *267*, 12432–12435 (1992).

Ohtsubo, S., Miyahara, H., Demizu, K., Kohno, S., Miura, I.: Isolation and characterization of new *Methanothrix* strains. Int. J. System. Bact. *41*, 358–362 (1991)

Oyaizu, H., Endo, G., Kawasaki, H., Nakamura, K., Koga, Y., Morinaga, T., Yamasato, K.: Sixteen S ribosomal RNA partial sequencing of methanogenic bacteria. Abstracts of IUMS congress Osaka, p. 105 (1990)

Rouwiere, P., Mandelco, L., Winker, S., Woese, C. R.: A detailed phylogeny for the *Methanomicrobiales*. System. Appl. Microbiol. *15*, 363–371 (1992)

Sowers, K. R., Johnson, J. L., Ferry, J. G.: Phylogenetic relationship among the methylotrophic methane-producing bacteria and emendation of the family *Methanosarcinaceae*. Int. J. System. Bact. *34*, 444–450 (1984)

Sprott, G. D., Ekiel, I., Dicaire, C.: Novel acid-labile, hydroxydiether lipid cores in methanogenic bacteria. J. Biol. Chem. *265*, 13735–13740 (1990)

Trincone, A., Nicolaus, B., Palmieri, G., de Rosa, M., Huber, R., Huber, G., Stetter, K. O., Gambacorta, A.: Distribution of complex and core lipids within new hyperthermophilic members of the *Archaea* domain. System. Appl. Microbiol. *15*, 11–17 (1992)

Vorbeck, M. L., Marinetti, G. V.: Intracellular distribution and characterization of the lipids of *Streptococcus faecalis* (ATCC 9790). Biochemistry *4*, 296–305 (1965)

Woese, C. R., Olsen, G. J.: Archaebacterial phylogeny: perspectives on urkingdoms. System. Appl. Microbiol. *7*, 161–177 (1986)

Woese, C. R., Achenbach, L., Rouwiere, P., Mandelco, L.: Archaeal phylogeny-reexamination of the phylogenetic position of *Archaeoglobus fulgidus* in light of certain composition-induced artifacts. System. Appl. Microbiol. *14*, 364–371 (1991)

Zellner, G., Bleicher, K., Braun, E., Kneifel, H., Tindall, B. J., Conway de Macario, E., Winter, J.: Characterization of a new mesophilic, secondary alcohol-utilizing methanogen, *Methanobacterium palustre* spec. nov. from peat bog. Arch. Microbiol. *151*, 1–9 (1989a)

Zellner, G., Messner, P., Kneifel, H., Tindall, B. J., Winter, J., Stackebrandt, E.: Methanolacinia gen. nov., incorporating *Methanomicrobium paynteri* as *Methanolacinia paynteri* comb. nov. J. Gen. Appl. Microbiol. *35*, 185–202 (1989b)

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