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Polar Lipids in Methanogen Taxonomy

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Polar lipid patterns of representative methanogens were recorded by two-dimensional thin-layer chromatography. Phenotypically similar *Methanobacterium* spp., *Methanobrevibacter* spp. and *Methanomicrobium* spp. could readily be distinguished from each other. Similarly, *Methanogenium* spp. and phenotypically similar *Methanococcus* spp. had different polar lipid patterns. Single examples from the monospecific genera *Methanospirillum*, *Methanoplanus* and *Methanothermus* had distinctive polar lipid patterns, but *Methanolobus tindarius* had a similar pattern to *Methanosarcina* spp. The isopranoid ether lipid cores from the polar lipids were identical for those species within any one genus. Novel core lipids were identified in examples from the genera *Methanomicrobium*, *Methanosarcina* and *Methanolobus*.

INTRODUCTION

The methane-producing archaebacteria comprise a physiologically similar but genotypically diverse group of prokaryotes (Archer & Harris, 1985; Balch *et al.*, 1979). Taxonomic relationships are primarily determined by comparative cataloguing of 16S rRNA (Balch *et al.*, 1979). The 16S rRNA groups are consistent with those groups derived by nucleic acid hybridization (Tu *et al.*, 1982), the immunological comparison of ribosomal proteins (Schmid & Bock, 1981) and total sequence comparisons of 5S rRNA (Fox *et al.*, 1982). All of these techniques are technically demanding and unsuitable for the rapid identification of new isolates. Complex immunological procedures for the identification of new isolates are available (Macario & Conway de Macario, 1983), but these methods rely on the availability of a bank of appropriate antisera and it is reported that only two isolates per week may be processed.

Phenotypic characters are often of limited use in the identification of new isolates in view of the relative uniformity of substrate utilization in methanogenesis (Archer & Harris, 1985). The morphology of the cell may be of considerable use where an isolate has a distinct and characteristic cell shape and cell wall (e.g. *Methanosarcina* or *Methanospirillum* spp.) but often isolates are rather pleomorphic and difficult to assign readily to any group. In this respect we have found the genera *Methanococcus* and *Methanogenium* particularly difficult to distinguish. Similarly, rod-shaped cells are often difficult to assign to the genera *Methanobacterium*, *Methanobrevibacter* or *Methanomicrobium* because of variation in cell length and filamentation dependent on growth conditions.

Chemotaxonomic procedures such as cell wall analyses have not been widely used in the identification of methanogens. Cell wall composition (pseudomurein, protein, heteropolysaccharide) can be used to assign an isolate to family and in some cases genus level (Balch *et al.*, 1979), but a more detailed analysis of each cell wall type would be required for this approach to be of general use at genus and species level. Polyamine distribution is consistent with family relationships derived from 16S rRNA cataloguing (Scherer & Kneifel, 1983), but of little use in distinguishing genera and species.

The analysis of polar lipid composition by two-dimensional thin-layer chromatography (TLC) has proved to be a rapid and simple technique for classifying halophilic archaebacteria, polar lipid groups being consistent with groups derived by 16S rRNA/DNA hybridizations (Ross & Grant, 1985). Preliminary results have also indicated that the technique is of use in the identification of different methanogen genera, and might have considerable potential for species differentiation in view of the extremely complex lipid patterns observed (Ross *et al.*, 1985). The lipophilic core components of the polar lipids of archaebacteria are saturated isopranoid ethers, typical types being diphytanylglycerol diether (abbreviated as $C_{20}C_{20}$; Fig. 1*a*), 2-*O*-sesterterpanyl-3-*O*-phytanylglycerol diether (abbreviated as $C_{20}C_{25}$; Fig. 1*b*) and bidiphytanyldiglycerol tetraether (abbreviated as $C_{40}C_{40}$; Fig. 1*c*). $C_{40}C_{40}$ core components may be partly cyclized (Langworthy *et al.*, 1982; De Rosa *et al.*, 1982). In the present study, thin-layer chromatograms of polar lipids and their isopranoid ether lipid cores have been determined for a comprehensive range of methanogens.

METHODS

Strains and culture conditions. Details of strains, substrates and growth conditions are shown in Table 1. Cultures (400 ml) were grown in 1 litre bottles on either methanol (1%, v/v) or H_2/CO_2 (4:1, v/v) as substrate as described previously (Ross *et al.*, 1985). Cells were harvested at the stationary phase and freeze-dried.

Lipid analyses. Polar lipids were extracted by the procedure of Bligh & Dyer (1959) as modified by Minnikin *et al.* (1979) and analysed by two-dimensional TLC (Ross *et al.*, 1985). To determine the type of lipids present on the chromatograms a variety of differential stains were used. Total lipids were detected by charring with ethanolic deca-molybdophosphoric acid (Ross *et al.*, 1981), phospholipids were identified by the Zinzadze reagent of Dittmer & Lester (1964), glycolipids were revealed with the α-naphthol–sulphuric acid reagent of Jacin & Mishkin (1965) and aminolipids were shown by spraying with 0·2% (w/v) ninhydrin in water-saturated butanol followed by heating at 105 °C for 10 min.

Diether and tetraether core lipids were extracted after acid methanolysis (Ross *et al.*, 1981) and analysed by TLC (Ross *et al.*, 1981, 1985).

RESULTS AND DISCUSSION

Analysis of polar lipids

Polar lipid patterns derived from single examples from each of four genera are shown in Fig. 2. *Methanothermus*, *Methanospirillum* and *Methanoplanus* are presently monospecific genera and we have examined only one of the two *Methanomicrobium* spp.; it is not possible therefore to be certain that an overall generic pattern exists for each of these genera. However, the four isolates were distinguished both in terms of the overall patterns and the presence or absence of particular classes of lipid. Kushwaha *et al.* (1981) have analysed the main lipids of *Methanospirillum hungatii* and we have speculated on the identity of the major spots shown here (Fig. 2*a*) for this isolate (Ross *et al.*, 1985); to date this is the only methanogen to be examined in detail.

The polar lipid patterns shown in Fig. 2 are also clearly distinguishable from those of a representative number (at least half the named species) of members of six other genera, *Methanobacterium*, *Methanobrevibacter*, *Methanococcus*, *Methanogenium*, *Methanosarcina* and *Methanolobus* (Figs 3–5). Overall generic markers can be defined from comparisons of several species of each of these genera. Three *Methanobacterium* spp. are compared with three *Methanobrevibacter* spp. in Fig. 3. *Methanobrevibacter* spp. are characterized by three aminophospholipids not present in *Methanobacterium* spp., whereas *Methanobacterium* spp. have characteristic aminoglycophospholipids and two aminophospholipids not present in *Methanobrevibacter* spp. Despite sharing a common generic pattern, the patterns obtained for different species are sufficiently different from each other to allow positive identification.

Methanogenium spp. are compared with *Methanococcus* spp. in Fig. 4. The *Methanococcus* patterns are all rather similar and very characteristic. Marker features for the genus include the presence of two major glycolipids and a group of two or three phospholipids in the upper centre of the chromatogram. *Methanogenium* spp. have quite different patterns, being considerably more complex. Although *Methanogenium thermophilicum* and *Methanogenium marisnigri* have some similarities, *Methanogenium cariaci* appears quite different and it is difficult to define any clear generic markers. 16S rRNA cataloguing studies indicate that these *Methanogenium* spp.

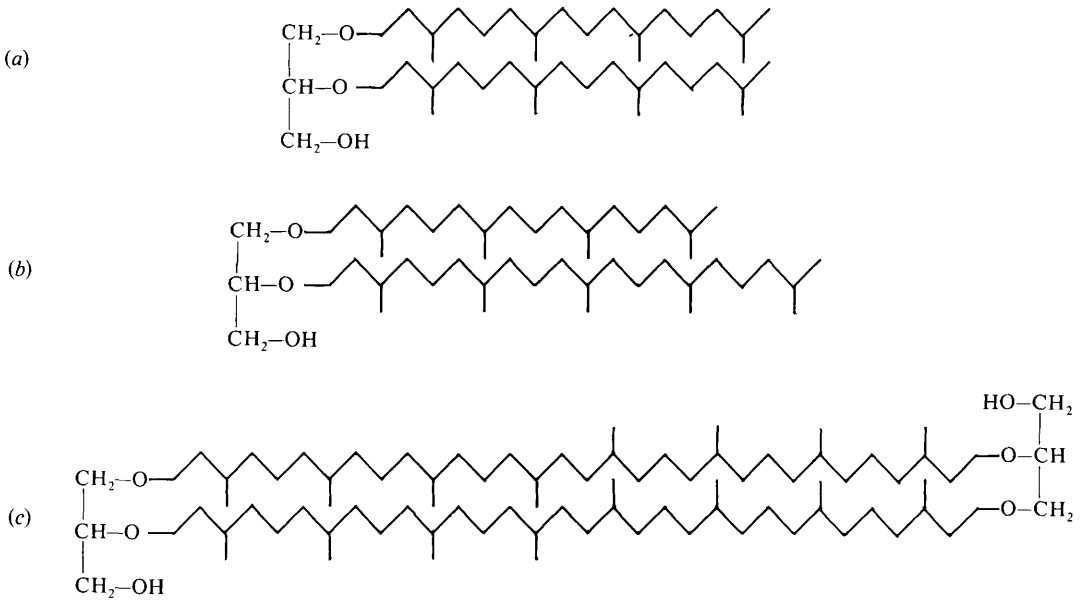

Fig. 1. Core glycerol ether lipids of archaebacteria: (*a*) diphytanylglycerol diether ($C_{20}C_{20}$); (*b*) 2-*O*-sesterterpanyl-3-*O*-phytanylglycerol diether ($C_{20}C_{25}$); (*c*) bidiphytanyldiglycerol tetraether ($C_{40}C_{40}$).

Table 1. *Strains and growth conditions*

Species	Strain no.	Growth temp. (°C)	Basic medium*	Substrate
Methanobacterium bryantii	DSM 862	37	M1	H_2/CO_2
Methanobacterium formicicum	DSM 1535	37	M1	H_2/CO_2
Methanobacterium thermoautotrophicum	DSM 1053	65	M1	H_2/CO_2
Methanobrevibacter smithii	DSM 861	37	M1	H_2/CO_2
Methanobrevibacter ruminantium	DSM 1093	37	M1†	H_2/CO_2
Methanobrevibacter arboriphilicus	DSM 744	25	M1	H_2/CO_2
Methanothermus fervidus	DSM 2088	85	M1†	H_2/CO_2
Methanococcus vannielii	DSM 1224	37	M3	H_2/CO_2
Methanococcus voltae	DSM 1537	37	M3	H_2/CO_2
Methanococcus thermolithotrophicus	DSM 2095	65	M3	H_2/CO_2
Methanomicrobium mobile	DSM 1539	37	MMO	H_2/CO_2
Methanogenium cariaci	DSM 1497	25	M3	H_2/CO_2
Methanogenium marisnigri	DSM 1498	25	M3	H_2/CO_2
Methanogenium thermophilicum	DSM 2373	55	M3	H_2/CO_2
Methanospirillum hungatii	DSM 864	37	M1	H_2/CO_2
Methanoplanus limicola	DSM 2279	37	M3	H_2/CO_2
Methanosarcina barkeri	DSM 800	37	M1	CH_3OH
Methanosarcina mazei	DSM 2053	37	M1	CH_3OH
Methanolobus tindarius	DSM 2278	25	M3	CH_3OH

DSM, Deutsche Sammlung von Mikroorganismen, Munich, FRG.

* M3 is medium 3 of Balch *et al.* (1979) for marine isolates, supplemented with NiCl (0·01 g l^{-1}) and Na_2SeO_3 (0·01 g l^{-1}). M1 is the low salt modification of M3 as described by Ross *et al.* (1985). MMO is as M3 except that Casamino acids (0·6 g l^{-1}), tryptone (0·6 g l^{-1}), vitamin solution (10 ml l^{-1}) (Balch *et al.*, 1979), clarified rumen fluid (30%, v/v) and 2% (v/v) fatty acid solution are added before autoclaving. Composition of fatty acid solution (g l^{-1}): valeric acid, 0·5; isovaleric acid, 0·5; methylbutyric acid, 0·5; isobutyric acid, 0·5.

† Coenzyme M (2-mercaptoethanesulphonate) added at 0·1 g ml^{-1}.

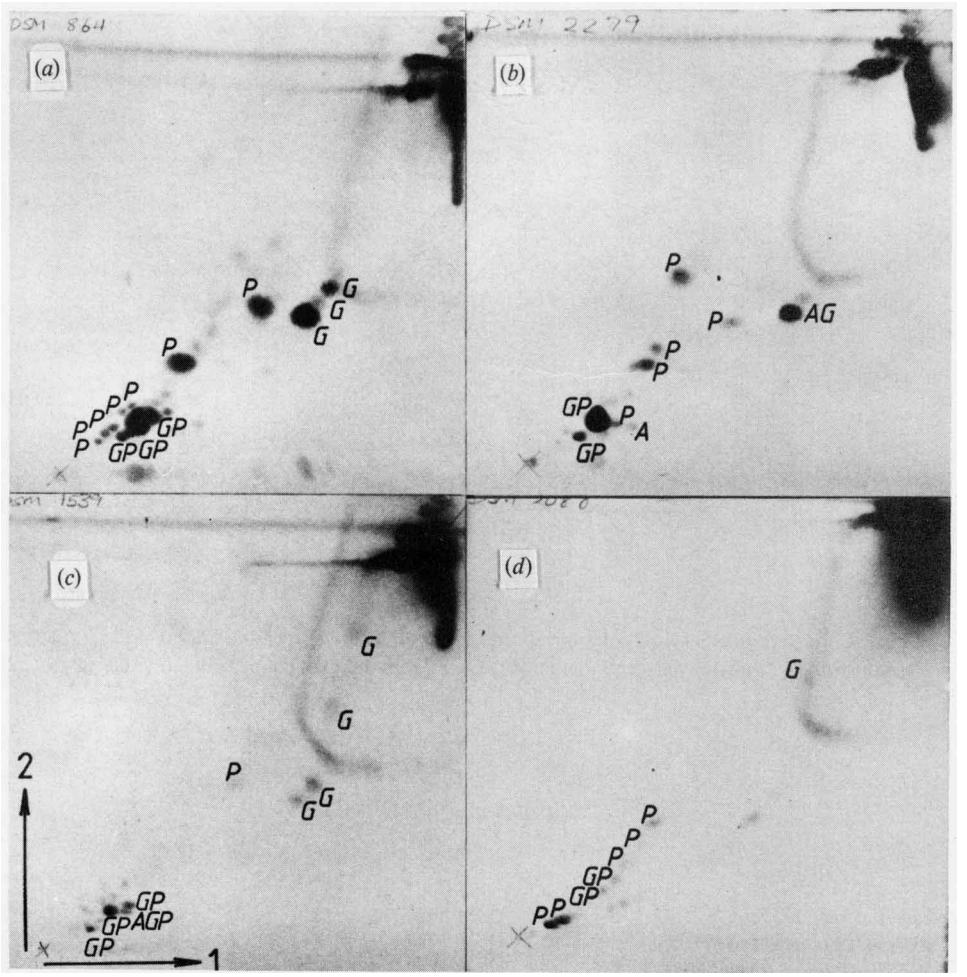

Fig. 2. Polar lipid patterns of species from different methanogen genera: (*a*) *Methanospirillum hungatii*; (*b*) *Methanoplanus limicola*; (*c*) *Methanomicrobium mobile*; (*d*) *Methanothermus fervidus*. Chloroform/methanol/water (65:25:4, by vol.) was used in the first dimension followed by chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) in the second dimension. Abbreviations: A, aminolipid; G, glycolipid; P, phospholipid.

are as closely related to each other as the *Methanococcus* spp. (Balch *et al.*, 1979; Jones *et al.*, 1983), so the lack of a clear generic polar lipid pattern here is unexpected.

Polar lipid patterns of *Methanosarcina* spp. and *Methanolobus tindarius* are shown in Fig. 5. These patterns are extremely similar and characterized by the total lack of glycolipids. Although both genera are methylotrophic (Table 1), *Methanolobus tindarius* does not possess the heteropolysaccharide cell wall characteristic of *Methanosarcina* spp. *Methanolobus tindarius* awaits 16S RNA oligonucleotide analysis, but rRNA and DNA homology studies suggest that it should be assigned to the family *Methanosarcinaceae* (Sowers *et al.*, 1984).

No attempt has been made to identify the polar lipid components in Figs 2–5, characterization being limited to their response to the various TLC spray reagents. There are certain apparent discrepancies between some of the patterns shown here and those shown in the preliminary study of Ross *et al.* (1985). An incomplete range of spray reagents was used in the earlier study, hence a number of spots previously identified as glycolipids are shown here to be glycophospholipids. Additionally, an aminophospholipid in *Methanosarcina barkeri* and a

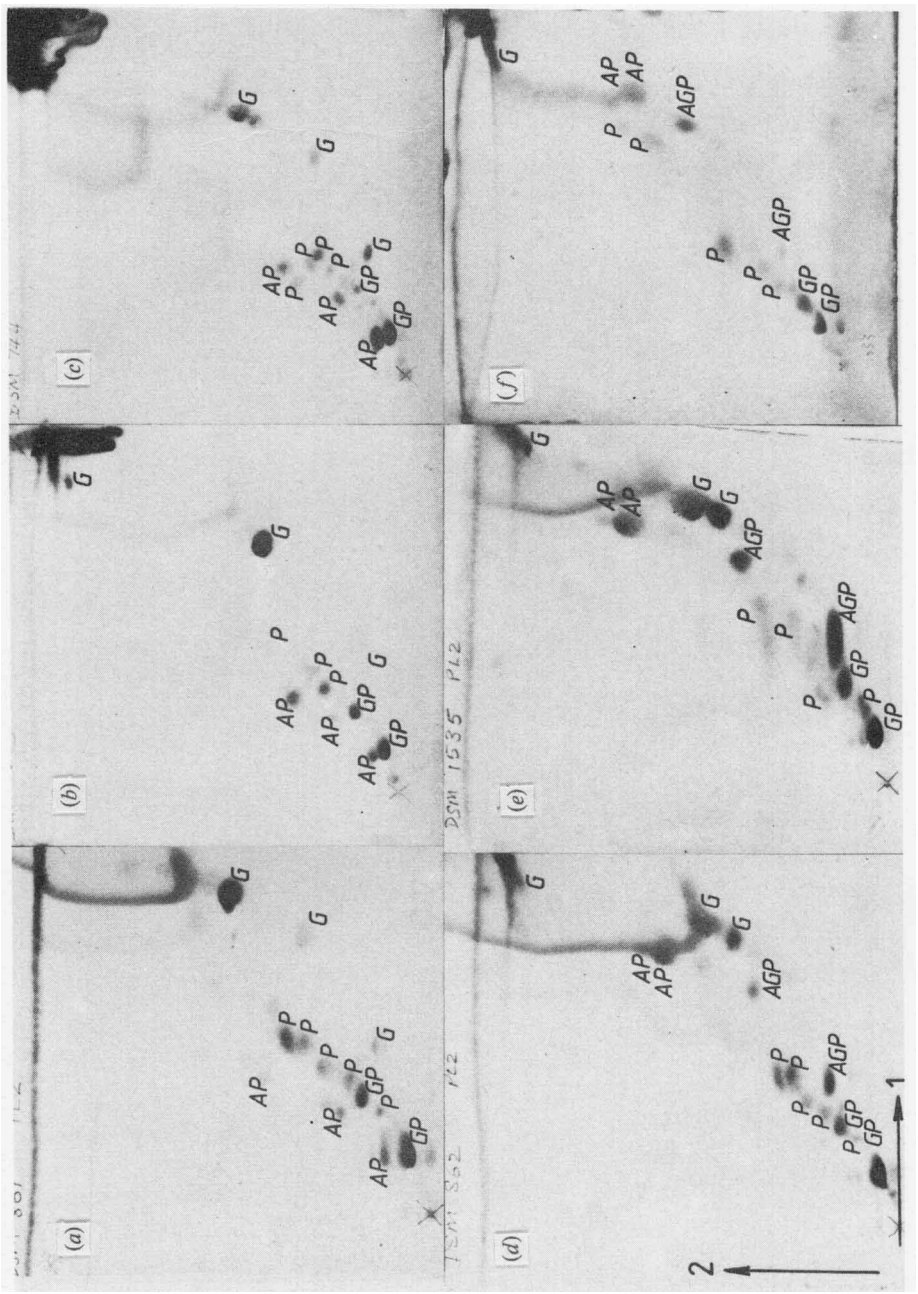

Fig. 3. Polar lipid patterns of *Methanobacterium* and *Methanobrevibacter* spp.: (*a*) *Methanobrevibacter smithii*; (*b*) *Methanobrevibacter ruminantium*; (*c*) *Methanobrevibacter arboriphilicus*; (*d*) *Methanobacterium bryantii*; (*e*) *Methanobacterium formicicum*; (*f*) *Methanobacterium thermoautotrophicum*. See Fig. 2 for developing system and abbreviations.

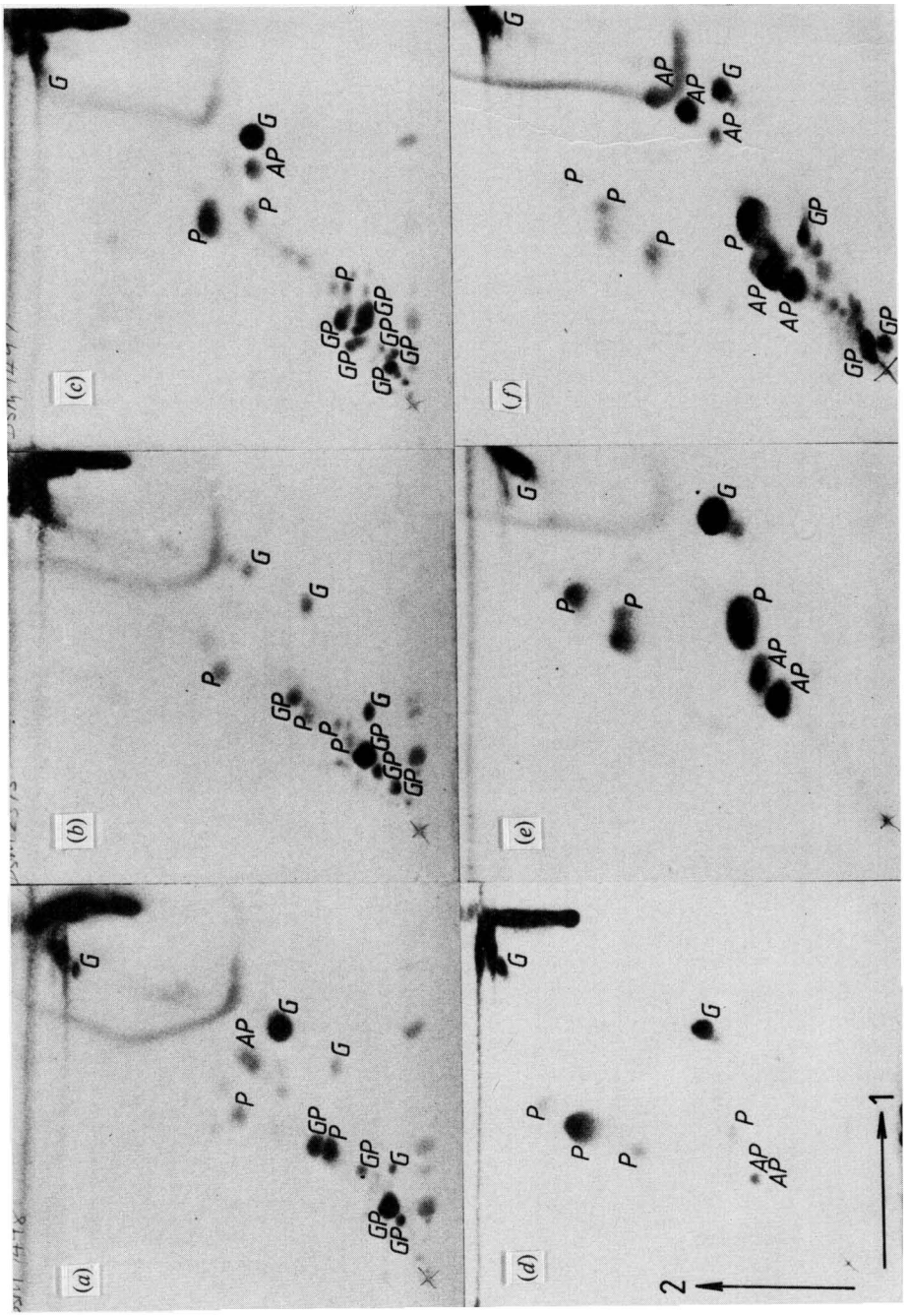

Fig. 4. Polar lipid patterns of *Methanogenium* and *Methanococcus* spp.: (*a*) *Methanogenium marisnigri*; (*b*) *Methanogenium thermophilicum*; (*c*) *Methanogenium cariaci*; (*d*) *Methanococcus voltae*; (*e*) *Methanococcus vannielii*; (*f*) *Methanococcus thermolithotrophicus*. See Fig. 2 for developing system and abbreviations.

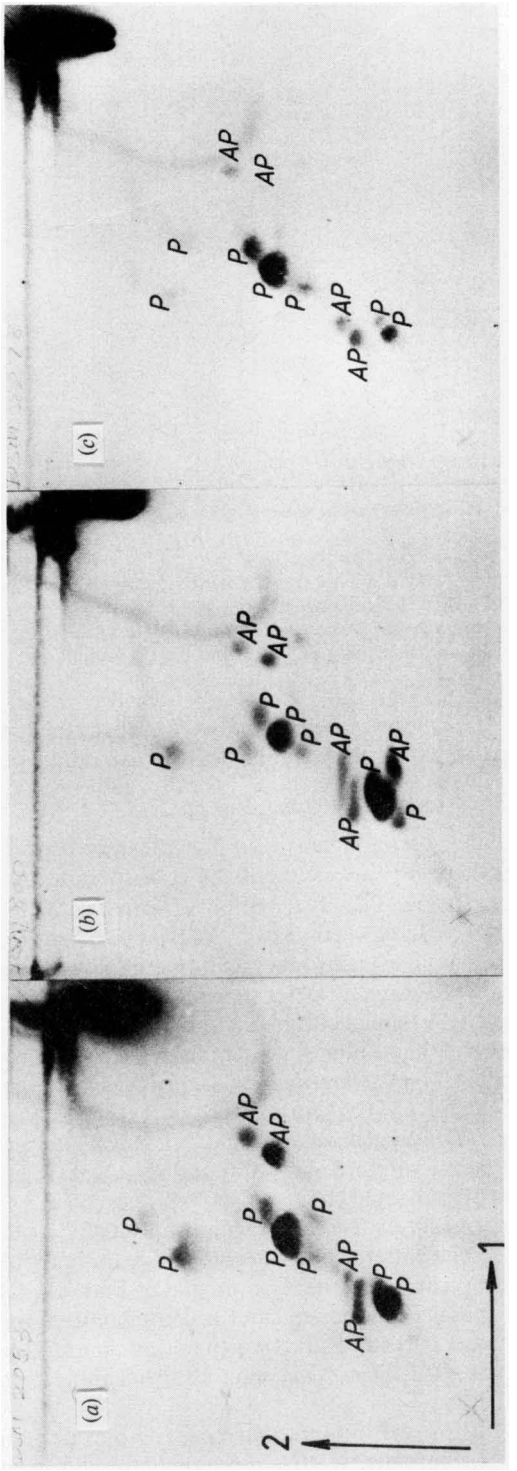

Fig. 5. Polar lipid patterns of *Methanosarcina* spp. and *Methanolobus tindarius*: (*a*) *Methanosarcina mazei*; (*b*) *Methanosarcina barkeri*; (*c*) *Methanolobus tindarius*. See Fig. 2 for developing system and abbreviations.

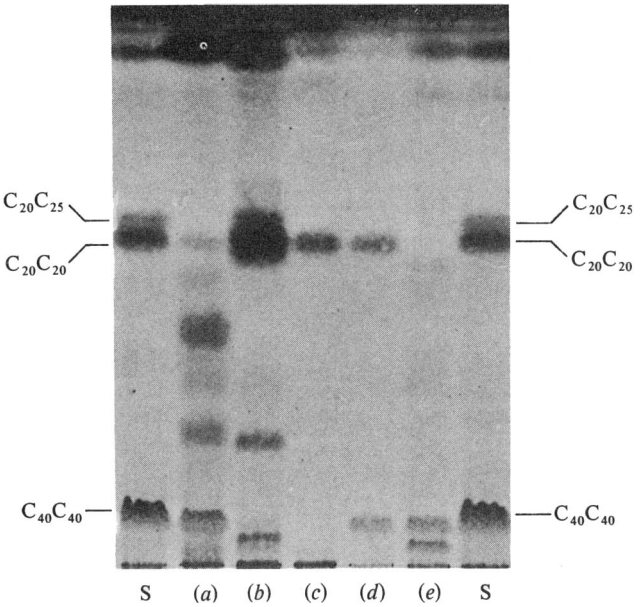

Fig. 6. TLC of core ether lipids using a double development system with light petroleum (b.p. 60–80 °C)/acetone (95:5, v/v) followed by toluene/acetone (97:3, v/v) in the same dimension. (*a*) *Methanomicrobium mobile*; (*b*) *Methanosarcina barkeri*; (*c*) *Methanococcus vannielii*; (*d*) *Methanogenium cariaci*; (*e*) *Methanothermus fervidus*; S, $C_{20}C_{20}$, $C_{20}C_{25}$ and $C_{40}C_{40}$ standards.

phospholipid shown here in *Methanospirillum hungatii* were earlier misidentified as glycolipids. However, total polar lipid patterns are very similar for the two studies.

Analysis of ether core lipids

Analyses of halophilic archaebacteria have shown that those with similar polar lipids have identical isopranoid glycerol ether core lipids, either $C_{20}C_{20}$ diethers or a mixture of $C_{20}C_{20}$ and $C_{20}C_{25}$ diethers (Ross *et al.*, 1985). The distribution of ether core lipids in methanogens analysed to date (Balch *et al.*, 1979; Langworthy *et al.*, 1982) is consistent with generic groupings derived by 16S rRNA cataloguing. Using the acid methanolysis and chromatographic procedures of Ross *et al.* (1981, 1985) we have determined the types of ether core lipids possessed by all the strains listed in Table 1. A typical chromatogram is shown in Fig. 6 and the results are summarized in Table 2. In general the results agree with those previously reported in that all the examples tested from the genera *Methanobrevibacter*, *Methanobacterium* and *Methanospirillum* possess a mixture of $C_{20}C_{20}$ and $C_{40}C_{40}$ core ethers (e.g. Fig. 6*d*) as resolved by these chromatographic procedures. *Methanogenium* spp. and *Methanoplanus* spp., which have not been analysed before, also possess a mixture of $C_{20}C_{20}$ and $C_{40}C_{40}$ core ethers. *Methanococcus* spp. (e.g. Fig. 6*c*) as previously indicated, have only $C_{20}C_{20}$ core ethers.

It has been reported that *Methanosarcina* spp. contain only $C_{20}C_{20}$ diethers (Balch *et al.*, 1979; Langworthy *et al.*, 1982). The pattern shown here (Fig. 6*b*) for both *Methanosarcina* spp. is considerably more complex, indicating the likely presence of both $C_{20}C_{20}$ and $C_{20}C_{25}$ core ethers together with a slow moving component and a component of mobility intermediate between $C_{20}C_{20}$ and $C_{40}C_{40}$ ether core lipids. It is worth noting that *Methanolobus tindarius* has an identical core lipid pattern to *Methanosarcina* spp., a further indication of a possible close phylogenetic relationship.

Methanomicrobium mobile (Fig. 6*a*) exhibits an even more complex pattern of novel core lipids as well as $C_{20}C_{20}$ and $C_{40}C_{40}$ components. *Methanothermus fervidus* is equally interesting in uniquely lacking $C_{20}C_{20}$ core ethers (Fig. 6*e*), probably having a roughly equal mixture of a $C_{40}C_{40}$ core ether and a component similar in mobility to the slow moving component of

Table 2. *Core lipids of methanogens*

Species	Core ether lipids*			
	$C_{20}C_{20}$	$C_{20}C_{25}$	$C_{40}C_{40}$	?
Methanobacterium bryantii	+	−	+	−
Methanobacterium formicicum	+	−	+	−
Methanobacterium thermoautotrophicum	+	−	+	−
Methanobrevibacter smithii	+	−	+	−
Methanobrevibacter ruminantium	+	−	+	−
Methanobrevibacter arboriphilicus	+	−	+	−
Methanothermus fervidus	−	−	+	+[a]
Methanococcus vannielii	+	−	−	−
Methanococcus voltae	+	−	−	−
Methanococcus thermolithotrophicus	+	−	−	−
Methanomicrobium mobile	+	−	+	+[b]
Methanogenium cariaci	+	−	+	−
Methanogenium marisnigri	+	−	+	−
Methanogenium thermophilicum	+	−	+	−
Methanospirillum hungatii	+	−	+	−
Methanoplanus limicola	+	−	+	−
Methanosarcina barkeri	+	+	(+)	+[c]
Methanosarcina mazei	+	+	(+)	+[d]
Methanolobus tindarius	+	+	(+)	+[d]

* (+), Trace amount; [a], see Fig. 6(*e*); [b], see Fig. 6(*a*); [c], see Fig. 6(*b*); [d], as Fig. 6(*b*).

Methanosarcina spp. The characteristic nature of these novel core ether lipids may offer a further means to rapid identification for these genera, although other isolates have yet to be screened.

Definitive classification of a new isolate may in the end depend on nucleic acid analyses in view of the relative physiological uniformity of the group, but our results have shown that the procedures described here provide a useful and rapid indication of the likely taxonomic status of a new isolate.

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