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CHAPTER 10

The Order Methanomicrobiales

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Characteristics of Methanomicrobiales

Although their morphology is diverse, Methanomicrobiales can be distinguished from other methanogens by growth properties, cell wall and lipid composition, and rDNA sequence. All Methanomicrobiales can use $H_2 + CO_2$ as a substrate for methanogenesis, most species can utilize formate, and many species utilize alcohols (Table 1). They cannot use acetate and methylated C-1 compounds such as methanol, methylamines and methyl sulfides as substrates for methanogenesis, and this property distinguishes them from the *Methanosarcinales*. Notably, even though the Methanomicrobiales cannot use acetate as a substrate for methanogenesis, many species require acetate as a carbon source. The absence of hydroxyarchaeol in their lipids further distinguishes them from the *Methanosarcinales* (Koga et al., 1998). In addition, all of the Methanomicrobiales so far examined contain aminopentanetetrols, galactose and glycerol in their lipids, and aminopentanetetrols are unique to this taxon. The absence of pseudomurein further distinguishes the Methanomicrobiales from both the *Methanobacteriales* and *Methanopyrales*. Additional distinctive features are listed in Table 1. Based upon the phylogeny of the 16S rRNA genes as well as phenotypic and genotypic characteristics, the order Methanomicrobiales has been divided into three families and nine genera of hydrogenotrophic methanogens (Boone et al., 1993; Rouvière et al., 1992; Garcia et al., 2000; Tables 2 and 3). Twenty-four species have been described so far within this order (Tables 3 and 4). Because some species epithets are not in agreement with the International Nomenclature Code for Taxonomy, proposed changes for the original spelling are shown in brackets after the current name (Euzéby, 1997; Chong and Boone, 2004; Ferry and Boone, 2004).

Habitats of Methanomicrobiales

Like methanogens belonging to other orders, Methanomicrobiales inhabit diverse anaerobic habitats comprising marine and fresh water sediments, anaerobic digesters and the rumen. Although based upon classical microbiological characterizations, this conclusion has been well supported recently by a wide variety of molecular and immunological studies (Kudo et al., 1997; Munson et al., 1997; Grossköpf et al., 1998; Sekiguchi et al., 1999). Moreover, a hybridization probe specific to the three families belonging to this order has been designed and characterized for environmental and determinative microbiological studies (Raskin et al., 1994).

Eight of the 24 validly published species within the order Methanomicrobiales including species of the genera *Methanoculleus*, *Methanofollis*, and *Methanocorpusculum* have been isolated from anaerobic digestors or sewage sludge (Whitehead and Cotta, 1999). *Methanoculleus bourgense* was isolated from a tannery-byproducts enrichment-culture inoculated with sewage sludge (Ollivier et al., 1986), whereas *Methanoculleus palmolei* was obtained from a digester treating wastewater from a palm oil mill in Indonesia (Zellner et al., 1998). *Methanoculleus* sp. was shown to be involved in the syntrophic oxidation of acetate in digesters containing high concentrations of ammonium salts and volatile fatty acids (Schnurer et al., 1999). *Methanofollis liminatans* was isolated from the effluent of an anaerobic reactor treating industrial wastewater in Germany (Zellner et al., 1990). *Methanocorpusculum aggregans* originated from a municipal sewage sludge digester in France (Ollivier et al., 1985). Subsequently, a similar organism, *Methanocorpusculum parvum*, was enriched from an anaerobic sour whey digester inoculated with sewage sludge (Zellner et al., 1987). Thereafter, *Methanocorpusculum bavaricum* and *Methanocorpusculum sinense* were isolated from a wastewater pond of a sugar factory in Germany and from a pilot plant for treatment of distillery wastewater in China, respectively (Zellner et al., 1989b). Ecological

Table 1. Distinguishing features of the Methanomicrobiales.

Feature	*Methanomicrobiales*	*Methanococcales*	*Methanobacteriales*	*Methanosarcinales*	*Methanopyrales*
Morphology	Small rod, plate, irregular coccus, curved rod	Irregular coccus	Rod, short rod, coccus	Irregular coccus sometimes in aggregates, rod, flat polygon	Rod
Motility	+/–	+	–	–	+
Substrates[a]	H_2 (for, alc)	H_2 (for)	H_2 (for, alc)	(me, H_2, ace)	H_2
Temperature range, °C	0–60	18–94	20–97	2–70	84–110
Cell wall	Glycoprotein (sheath)	Protein	Pseudomurein	Glycoprotein (methanochondrotin, sheath)	Pseudomurein
Distinctive lipid components	Galactose, aminopentanetetrols, glycerol	Serine	*myo*-Inositol, serine	Hydroxyarchaeol, *myo*-inositol, ethanolamine, glycerol	Galactose, mannose
Mol% G+C	38–61	30–33	26–61	36–54	60

Symbols: +, present in most or all taxa; –, present in a few or no taxa; and +/–, present in many but not all taxa.
Abbreviations: for, formate; alc, alcohols; me, methylamines; and ace, acetate.
[a]Substrates (in parentheses) are utilized by many but not all taxa.

Table 2. Some characteristics of the Methanomicrobiales families.

Family	Characteristics
Methanomicrobiaceae	Small rod, plate, or irregular coccus; substrates for methanogenesis are $H_2 + CO_2$, frequently formate, and sometimes alcohols; Gram negative; motile or nonmotile; G+C content, 39–50 mol%.
Methanocorpusculaceae	Small, irregular coccus, substrates for methanogenesis are $H_2 + CO_2$, formate, and sometimes alcohols; Gram negative; motile or nonmotile; G+C content, 48–52 mol%.
Methanospirillaceae	Curved rods; substrates for methanogenesis are $H_2 + CO_2$, formate, and sometimes alcohols; Gram negative; motile; G+C content, 45–49 mol%.

Table 3. Taxonomic groups of order Methanomicrobiales.

Family	Genus	Species
Methanomicrobiaceae[T]	*Methanomicrobium*[T]	*M. mobile*[T]
	Methanolacinia	*M. paynteri*[T]
	Methanogenium	*M. cariaci*[T] (*cariacoense*)[a]
		M. frigidum
		M. frittonii
		M. organophilum (*organiphilum*)[a]
	Methanoculleus	*M. bourgense*[T] (*bourgensis*)[b]
		M. olentangyi (*olentangyense*)[a]
		M. marisnigri (*marinigri*)[a]
		M. oldenburgensis
		M. palmolei
		M. thermophilicum (*thermophilicus*)[b]
	Methanoplanus	*M. endosymbiosus*
		M. limicola[T]
		M. petrolearius
	Methanofollis	*M. liminatans*
		M. tationis[T] (*tatioense*)[a]
Methanocorpusculaceae	*Methanocorpusculum*[T]	*M. bavaricum*
		M. labreanum
		M. parvum[T]
		M. aggregans
		M. sinense
Methanospirillaceae	*Methanospirillum*[T]	*M. hungatei*[T] (*hungateii*)[c]
Genus incertae sedis	*Methanocalculus*	*M. halotolerans*[T]
		M. pumilus

Abbreviation: [T], type family of the order, type genus of the family or type species of the genus.
[a]Proposed change for the original spelling (Euzéby, 1997).
[b]Proposed change for the original spelling (S. C. Chong and D. R. Boone, in press).
[c]Proposed change for the original spelling (J. G. Ferry and D. R. Boone, in press).

Table 4. Some characteristics of methanogenic archaea of the order Methanomicrobiales.

	Optimum growth conditions						
Organism	Dimensions (μm)	Flagella	pH	Temp. (°C)	NaCl (M)	Required organic growth factors[b]	G+C content mol%
Family *Methanomicrobiaceae*							
Methanomicrobium							
mobile	0.7 × 1.5–2	One polar	6.1–6.9	40	nd	Complex	49
Methanolacinia							
paynteri	0.6 × 1.5–2.5	Flagellated[a]	7	40	0.15	ac	38
Methanogerium							
cariaci	ϕ 1.3–2.6	Peritrichous	6.8–7.3	20–25	0.5	ac, YE	52
frigidum	ϕ 1.2–2.5	None	7.5–7.9	15	0.3–0.6	ac	52
frittonii	ϕ 1–2.5	None	7–7.5	57	0	None	49
organophilum	ϕ 0.5–1.5	None	6.4–7.3	30–35	0.3	ac, PABA, biotin, B_{12}, tung	47
Methanoculleus							
bourgense	ϕ 1–2	None	6.7	35–40	0.18	ac	59
= *olentangyi*	ϕ 1–1.5	None	nd	37	0.2	ac	54
marisnigri	ϕ <1.3	Peritrichous	6.2–6.6	20–25	0.1	Peptones	61
oldenburgensis	ϕ 0.5–1.5	None	7.5–8	45	<0.25	ac	49
palmolei	ϕ 1.25–2	Flagellated[a]	6.9–7.5	40	nd	ac	59–60
thermophilicum	ϕ 0.7–1.8	Single	6.5–7.2	55–60	0.3	ac, peptones, vit	56–60
Methanoplanus							
endosymbiosus	0.5–1 × 1.6–3	Peritrichous	6.8–7.3	32	0.25	*p*-Cresol, tung	39
limicola	0.1–0.3 × 1.5–2.8	Polar tuft	7	40	0.17	ac	48
petrolearius	ϕ 1–3	None	7	37	0.1–0.5	ac	50
Methanofollis							
liminatans	ϕ 1.5	Flagellated[a]	7	40	0–0.6	ac	60
tationis	ϕ 3	Peritrichous	7	37–40	0.1–0.2	ac, YE, peptones, tung	54
Family *Methanocorpusculaceae*							
Methanocorpusculum							
bavaricum	ϕ <1	Flagellated[a]	7	37	nd	RF	51
labreanum	ϕ 0.4–2	None	7	37	0–0.2	YE, peptones	50
parvum	ϕ 0.5–1	Single	6.8–7.5	37	0	ac, YE, tung	49
= *aggregans*	ϕ 0.5–2	None	6.6	35	<0.18	ac, YE/peptones	52
sinense	ϕ <1	Flagellated[a]	7	30	0	RF	50
Family *Methanospirillaceae*							
Methanospirillum							
hungatei	0.5 × 7.4–10	Polar tuft	6.6–7.4	30–45	nd	(ac)	47–50
Genus insertae sedis							
Methanocalculus							
halotolerans	ϕ 0.8–1	Peritrichous	7.6	38	0.8	ac	55
pumilus	ϕ 0.8–1	None	7	35	0.17	ac	52

Abbreviations: nd, not determined; =, indicates synonyms; ϕ, diameter; ac, acetate; (ac), acetate required or stimulatory depending on the strain; Complex, a combination of acetate, isobutyrate, isovalerate, 2-methylbutyrate, tryptophan (or indole), pyridoxine, thiamine, biotin, vitamin B_{12}, 4-aminobenzoate and 7-mercaptoheptanoylthreonine; PABA, 4-aminobenzoate; tung, tungsten; YE, yeast extract; vit, vitamins; and RF, rumen fluid.

[a]Type not described.

References: Blotevogel et al., 1991; Corder et al., 1983; Franzmann et al., 1997; Ferry et al., 1974; Harris et al., 1984; Maestrojuan et al., 1990; Mori et al., 2000; Ollivier et al., 1985, 1986, 1997, 1998; Paynter and Hungate, 1968; Rivard and Smith, 1982; Rivard et al., 1983; Romesser et al., 1979; Tanner and Wolfe, 1988; van Bruggen et al., 1986; Widdel et al., 1988; Wildgruber et al., 1982; Xun et al., 1989; Zabel et al., 1984, 1985; Zellner et al., 1987, 1989a,b, 1990, 1998, 1999; and Zhao et al., 1989.

studies of a number of bioreactors have found varying contributions of the Methanomicrobiales to the methanogenic microflora. In a wood-fermenting bioreactor, *Methanomicrobium mobile* was the predominant methanogen, representing nearly 90% of all methanogens detectable by the immunological methods used (Macario et al., 1991). Such predominance of a single methanogen subpopulation has not been reported in other bioreactors so far and might be specific to wood-fermenting bioreactors. Nevertheless, in the 21 anaerobic sewage sludge digesters studied by Raskin et al. (1995), Methanomicrobiales together with *Methanosarcinales* represented the majority of the methanogens detected with oligonucleotide probes. In contrast, the Methanomicrobiales represented only a small fraction of the methanogens found in the granular consortia formed in an upflow anaerobic sludge blanket reactor (Visser et al., 1991).

Among the order Methanomicrobiales, six species belonging to genera *Methanolacinia*, *Methanogenium* and *Methanoculleus* were recovered from marine sediments and water. *Methanolacinia paynteri* was isolated from the edges of the mosquito control canals within mangrove swamps located in the Cayman Islands, British West Indies (Rivard et al., 1983). *Methanogenium cariaci*, *M. marisnigri* and *M. organophilum* were found in sediments from Cariaco Trench, the Black Sea, and near Venice (Italy), respectively (Romesser et al., 1979; Widdel, 1986; Widdel et al., 1988). The psychrophilic *Methanogenium frigidum* was isolated from the perennially cold, anoxic hypolimnion of a seawater-derived Antarctic meromictic lake (Franzmann et al., 1997), and the thermophilic *Methanoculleus thermophilicum* originated from sediments underlying the high temperature effluent channel of a nuclear power plant in Florida (Rivard and Smith, 1982). Moreover, examination of 16S rDNA libraries created with archaea-specific primers found sequences closely related to those of *Methanoculleus* and *Methanogenium* spp. from coastal salt-marsh sediment samples (Munson et al., 1997).

Members of the order Methanomicrobiales also were recovered from fresh water sediments. *Methanogenium frittonii* inhabits a freshwater lake in England (Harris et al., 1984). *Methanoculleus olentangyi* (Corder et al., 1983) and *Methanoculleus oldenburgensis* (Blotevogel et al., 1991) were isolated from river sediments in the United States and Germany, respectively. Finally, *Methanocorpusculum labreanum* was isolated from the surface sediments of Tar Pit Lake in Los Angeles (in the United States; Zhao et al., 1989). Likewise, small numbers of methanogens immunologically crossreactive with *Methanoculleus marisnigri* and *Methanogenium cariaci* have been detected in other river and lake sediments (Cairœt al., 1991).

Members of the order Methanomicrobiales have also been found (using in situ hybridization with a domain-specific oligonucleotide probe) on the surfaces of rice roots and in the surrounding paddy soil (Grossköpf et al., 1998). An irregular coccus phylogenetically related to *Methanoculleus marisnigri* was also isolated from a French ricefield soil (Joulian et al., 1998). Using molecular techniques, *Methanogenium*- and *Methanoculleus*-like organisms were shown to be also present in diverse ricefield soils in Japan (Kudo et al., 1997).

Subterrestrial ecosystems such as oil reservoirs and groundwaters are suitable habitats for Methanomicrobiales. *Methanoplanus petrolearius* and *Methanocalculus halotolerans* were isolated from an African offshore oil field (Ollivier et al., 1997) and from a French oil field (Ollivier et al., 1998), respectively. Isolates affiliated to *Methanoculleus* species have been recently obtained from Californian oil reservoirs (Orphan et al., 2000). Members of the genus *Methanomicrobium* have been identified in granitic groundwater (Pedersen, 1997). Other habitats for Methanomicrobiales include a swamp of drilling waste in Italy (*Methanoplanus limicola*; Wildgruber et al., 1982) and the mud of a small, moderately thermophilic solfatara pool at an altitude of 4,750 m in Chile (*Methanofollis tationis*; Zabel et al., 1984). Indirect immunofluorescence has also detected cells related to *Methanoculleus thermophilicum* in an alkaline Icelandic hot spring (Sonnehansen and Ahring, 1997).

Large quantities of methane are produced during the rumen fermentation. Species of the genus *Methanobrevibacter*, order *Methanobacteriales*, are the predominant methanogens in bovine and caprine rumina, whereas Methanomicrobiales are the predominant methanogens in the ovine rumen (Lin et al., 1997). *Methanomicrobium mobile* has also been found in high numbers in the bovine rumen and is probably a significant contributor to the total amount of methanogenesis (Paynter and Hungate, 1968; Jarvis et al., 2000). This conclusion is supported by molecular ecological studies that indicate Methanomicrobiales are the second most abundant methanogens in the bovine rumen and in model rumen systems, where they account for 12% of the total small subunit (SSU) rRNA in rumen fluid (Sharp et al., 1998). In addition, there is one report that *Methanogenium* spp. are the predominant methanogens in the gastrointestinal tracts of chickens and turkeys (Miller and Wolin, 1986).

Termites harbor symbiotic methanogens in their gut. Nucleotide sequences of PCR-amplified 16S ribosomal RNA genes from four termite species demonstrated that Methanomicrobiales species distinct from known species were present and constitute a unique phylogenetic group (Ohkuma et al., 1999).

Methanoarchaea ascribable to the genus *Methanogenium* also were recovered in low numbers from vegetables, meat, fish and cheese where they are thought to be chance contaminants (Brusa et al., 1998).

Although the Methanomicrobiales are generally free-living, some species are endosymbionts of anaerobic protozoa. Within the protozoa, they are associated with the hydrogenosome, suggesting that the symbiosis is based upon interspecies H_2 transfer from the protozoan to the methanogen (Berger and Lynn, 1992; Embley et al., 1992). For instance, *Methanoplanus endosymbiosus* is an endosymbiont of the sapropelic marine ciliate *Metopus contortus* (van Bruggen et al., 1986). Endosymbionts closely related to *Methanocorpusculum* and *Methanoplanus* spp. also have been identified within the anaerobic protozoa *Metopus*, *Trimyema* and *Pelomyxa*. These endosymbionts appear to be different from their free-living phylogenetic relatives, suggesting that some species may have adapted specifically to this lifestyle (Embley and Finlay, 1993; Finlay et al., 1993).

Isolation

ENRICHMENT MEDIA FOR GROWTH OF METHANOMICROBIALES Numerous types of media are appropriate for the growth of Methanomicrobiales. Representative media for isolating and culturing these microorganisms will be described in this section. All Methanomicrobiales are hydrogen-oxidizing organisms; they can grow on a mixture of molecular hydrogen (H_2) and carbon dioxide (CO_2; or bicarbonate [HCO_3^-]) according the following reaction:

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$$
$$\Delta G^{o\prime} = -135\text{kJ/mol of } CH_4$$

All except *Methanolacinia paynteri* can also use formate to produce methane (CH_4):

$$4HCO_2^- + H^+ + H_2O \rightarrow CH_4 + 3HCO_3^-$$
$$\Delta G^{o\prime} = -145\text{kJ/mol of } CH_4$$

Some species can also use secondary alcohols and CO_2 to produce methane and the organic acid or ketone:

$$2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O \quad \Delta G^{o\prime} = -116\text{kJ/mol of } CH_4$$

The composition of growth medium will depend on the type of methanogen sought. Although most Methanomicrobiales will grow in one of these media described below, optimal growth may require 1) modification in the concentration of the mineral or organic components or 2) additional nutrients.

Standard Growth Medium 1

Values are per liter of medium.

K_2HPO_4	0.3 g
KH_2PO_4	0.3 g
$(NH_4)_2SO_4$	0.3 g
NaCl	0.6 g
$MgSO_4 \cdot 7H_2O$	0.13 g
$CaCl_2 \cdot 2H_2O$	8 mg
$FeSO_4 \cdot 7H_2O$	2 mg
Sodium acetate	0.5 g
Yeast extract	0.5 g
Trypticase	0.5 g
Cysteine-HCl	0.5 g
Trace minerals solution (see below)	10 ml
Resazurin	1 mg

This medium, adapted from that described by Balch et al. (1979), is recommended for isolation of species from freshwater sediments, sewage sludge or digesters.

The trace minerals solution (Balch et al., 1979) is composed of (per liter):

Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot 2H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoSO_4$ or $CoCl_2$	0.1 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$ZnSO_4$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$AlK(SO_4)_2$	0.01 g
H_3BO_3	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g

To prepare the trace minerals solution, dissolve the nitrilotriacetic acid in 800 ml of water and adjust the pH to 6.5 with KOH. Then dissolve the minerals in order, adjust the pH to 7.0, and bring the volume to 1 liter.

The vitamin solution (Balch et al., 1979) is composed of (per liter):

Biotin	2 mg
Folic acid	2 mg
Pyridoxine hydrochloride	10 mg
Thiamine hydrochloride	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
DL-Calcium pantothenate	5 mg
Vitamin B_{12}	0.1 mg
p-Aminobenzoic acid	5 mg
Lipoic acid	5 mg

Standard Growth Medium 2

Values are per liter of medium:

K_2HPO_4	0.14 g
NH_4Cl	0.25 g
KCl	0.34 g

$MgSO_4 \cdot 7H_2O$	0.35 g
$CaCl_2 \cdot 2H_2O$	0.14 g
$MgCl_2 \cdot 2H_2O$	2.75 g
NaCl	18 g
$Fe(NH_4)_2(SO_4)_2 \cdot 7H_2O$	2 mg
Sodium acetate	1.0 g
Yeast extract	0.5 g
Trypticase	0.5 g
Cysteine-HCl	0.5 g
Trace minerals solution (see above)	10 ml
Vitamin solution (see above)	10 ml
Resazurin	1 mg

This medium, also adapted from that of Balch et al. (1979), is recommended for growth of marine species.

Medium 3 is for moderately halophilic Methanomicrobiales (from saline to hypersaline ecosystems). It contains the same components as Medium 1, but the saline (NaCl) concentration will vary depending on the (slight, moderate or extreme) salinity of the ecosystem studied. Among the species described so far, the upper NaCl limit for growth of any hydrogenotrophic methanogen belonging to the order Methanomicrobiales is 125 g/liter (Ollivier et al., 1998).

Medium 4 is for growth of species from the rumen. For enriching and isolating *Methanomicrobium* species from the rumen, clarified rumen fluid (rumen contents centrifuged at 25,000 × g for 15 min) is added to Medium 1. For *Methanomicrobium mobile*, the requirement for rumen fluid can be replaced by a complex mixture containing acetate, volatile fatty acids, amino acids, vitamins, and 7-mercaptoheptanoylthreonine phosphate (Kuhner et al., 1991; Tanner and Wolfe, 1988). Growth of *Methanoplanus endosymbiosus* is also dependent on rumen fluid, which can be replaced by the eluate of a rumen-derived anaerobic digester (Poirot et al., 1991). Para-cresol was identified as a growth-stimulatory component, and 50 nM of it supported the half-maximal growth.

All four media may be prepared anaerobically as follows. The pH of the medium is adjusted to 7.0 with 10 mM KOH. The medium is boiled under a stream of oxygen (O_2)-free nitrogen (N_2) gas and cooled to room temperature. It is then dispensed into Hungate tubes, serum bottles or flasks under a stream of $N_2 + CO_2$ (80:20, v/v) at atmospheric pressure. Vessels are autoclaved, and prior to culture inoculations, $Na_2S \cdot 9H_2O$ and $NaHCO_3$ are added from sterile anaerobic solutions to final concentrations of 0.04% and 0.2% (v/v), respectively. Sodium formate, added from a sterile anaerobic solution to a final concentration of 40 mM, or $H_2 + CO_2$ (80/20%; 2 bars) serves as energy source. Growth on alcohol (2-propanol, 1-propanol, ethanol, 2-butanol, 1-butanol or cyclopentanol) is tested at a final concentration of 20–30 mM (Widdel, 1986). Because gas is produced from formate and the alcohols, care must be taken to avoid exceeding the pressure limit of the culture vessels during growth. Moreover, during growth on $H_2 + CO_2$, methanogens consume 5 moles of gas for every mole of CH_4 produced, and the vessel will quickly develop a negative pressure. A low internal pressure increases the possibility of contamination by air. In addition, the decrease in partial pressure of CO_2 renders the culture medium strongly alkaline, thus causing growth inhibition and cell lysis. To minimize these problems, the volume of the headspace should be at least five times the volume of the medium, and the culture vessels should be pressurized periodically throughout the growth period. The headspace also may be repressurized with $H_2 + CO_2$ to fully replenish the CO_2 consumed. For the thermophilic species, attention must also be given to the expansion of gas at high temperature.

Solid medium is prepared by the addition of 1.5–2% agar during the medium preparation. For the roll tube technique (Hungate, 1969), Hungate tubes are placed into a 45°C water bath after sterilization to prevent solidification of the molten agar. After inoculation, the tubes are rolled and rapidly cooled with an ice cube before incubation at the appropriate temperature. Petri plates may also be prepared in an anaerobic glove box and incubated in pressure cylinders (Balch et al., 1979) before removal from the chamber.

To measure the growth of methanogens, Hungate tubes with liquid cultures are directly inserted into a spectrophotometer, and the optical density at 580 nm is recorded.

Isolation and Characterization For isolating Methanomicrobiales, enrichment cultures are initiated by inoculating the sample (10%) into serum bottles containing basal medium and $H_2 + CO_2$ or sodium formate as growth substrate. The inoculated serum bottles are incubated without shaking, except when H_2 is used as the energy source. Pure cultures are obtained by the repeated use of the Hungate roll tube technique (Hungate, 1969) using basal growth medium solidified with 1.5% (w/v) Noble agar (Difco). For thermophilic enrichments, the agar concentration is increased up to 2%. Characterization of the axenic strain should follow the guidelines of the International Committee on Systematic Bacteriology (ICSB) Subcommittee for the Taxonomy of Methanogens (Boone and Whitman, 1988). Growth rates at various pH values, temperatures and salt concentrations are determined in Hungate tubes of basal growth medium containing 40 mM sodium formate with a $N_2 + CO_2$ (80:20, v/v) atmosphere or in the absence of sodium formate with a $H_2 + CO_2$ (80:20, v/v) atmosphere. The pH is adjusted to the desired

value by injecting appropriate volumes of anaerobic sterile 10% (v/v) $NaHCO_3$ or Na_2CO_3 stock solutions. Both the initial and final pH must be recorded because the pH can change rapidly during growth. To determine the salt requirement for growth, NaCl is weighed directly in Hungate tubes, and the medium is subsequently dispensed as described above. The strain is subcultured at least once at each salt concentration prior to measurement of the growth rate.

To test substrate utilization, substrates are added from sterile stock solutions to the basal medium at a final concentration of 20 mM (ethanol, 1-propanol, 2-propanol, 1-butanol or isobutanol) or 40 mM (formate). The gas phase is N_2 + CO_2 (80:20, 200 kPa). Hydrogen oxidation is tested using H_2 + CO_2 (80:20, 200 kPa) in the gas phase. All experiments must be performed in duplicate. Also, susceptibility to lysis by detergents and hypotonic solutions, Gram staining, and motility should be determined. Phase contrast, fluorescence and electron microscopy are also used for phenotype characterization. Methane is quantified by gas chromatography to verify that it is the major catabolic product. If possible, it is also extremely desirable to determine the nutritional requirements for isolates obtained from complex medium.

The G+C content of DNA is determined by using high-performance liquid chromatography (HPLC), as described by Mesbah et al. (1989), after isolation and purification of the DNA by chromatography on hydroxyapatite. Nonmethylated lambda DNA (Sigma) is used as the standard. Sequencing of the gene for the 16S rRNA is also very desirable and may be necessary for final placement of an isolate with the Methanomicrobiales. A primer pair, designated "FARCH-9" (5′-CTGGTTGATCCTGCCAG-3′) and "Rd1" (5′-AAGGAGGTGATCCAG CC-3′), is used to amplify the 16S rRNA gene from genomic DNA of Methanomicrobiales (Ollivier et al., 1998). The amplified product is purified (Andrews and Patel, 1996) and the sequence determined with an ABI automated DNA sequencer in conjunction with a Prism dideoxy terminator cycle sequencing kit and the protocol recommended by the manufacturer (Applied Biosystems Inc.). The primers used for sequencing are F2 (5′-CAGGATTAGATACC CTGGTAG-3′), R2 (5′-GTATTACCGCGGCT GCTG-3′), R4 (5′-CCGTCAATTCCTTTGAG TTT-3′) and the two amplification primers FARCH9 and Rd1 described above. The 16S rRNA gene sequence is manually aligned with reference sequences of various members of the domain *Archaea* by using the alignment editor "ae2" (Maidak et al., 2000). Reference sequences are obtained from the Ribosomal Database Project (Maidak et al., 2000). Positions of sequence and alignment uncertainty are omitted from the analysis. Pairwise evolutionary distances based on a number of unambiguous nucleotides are computed using the method of Jukes and Cantor (1969) and dendrograms are constructed from these distances using the neighbor-joining method. Both programs form part of the PHYLIP package (Felsenstein, 1993).

The Family Methanomicrobiaceae

Methanomicrobiaceae comprises seven genera with morphologies ranging from rods to highly irregular cocci or plane-shaped cells. The cell walls are proteinaceous and the lipids include both C_{20} and C_{40} isopranyl glycerol ethers. The sugars in glycolipids include glucose and galactose. The polar head groups contain aminopentanetetrols and glycerol. The mol% G+C for members of this family ranges from 38 to 62. Almost all strains can use formate. The use of secondary alcohols may be observed.

Methanomicrobium Genus *Methanomicrobium*

is represented by a single mesophilic species, *Methanomicrobium mobile*.

Methanomicrobium mobile. These Gram-negative, slightly curved, short rods (0.7 × 1.5–2 μm; Fig. 1) are sluggishly motile (with one polar flagellum) and subject to frequent lysis. Colonies are round, 1 μm in diameter, convex, smooth, translucent, with entire edges. Methane is produced from H_2 + CO_2 or from formate. Growth requires acetate and rumen fluid or a mixture of volatile fatty acids (VFA: isobutyrate, isovalerate,

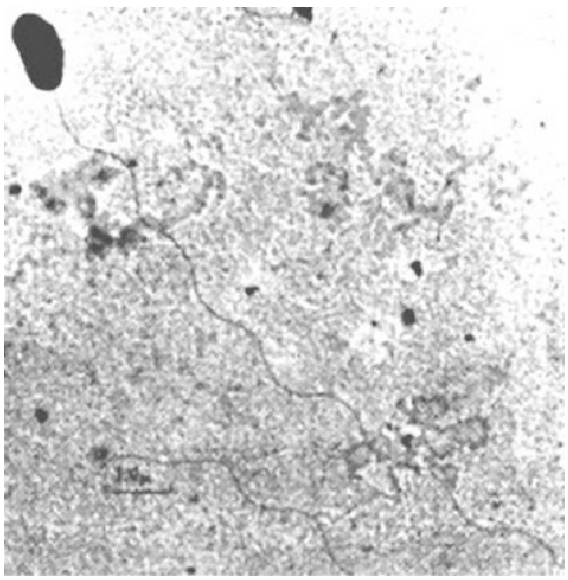

Fig. 1. Electron micrograph of *Methanomicrobium mobile* showing the single polar flagellum. Bar = 1 μm. (From Paynter and Hungate, 1968.)

and 2-methylbutyrate), amino acids (tryptophan or indole) and vitamins (pyridoxine, thiamine, biotin, vitamin B_{12}, and *p*-aminobenzoic acid), and 7-mercaptoheptanoylthreonine phosphate (component B). This latter compound can be replaced by 7-mercaptoheptanoate if the medium is also supplemented with an unidentified growth factor found in rumen fluid and extracts of *Methanobacterium thermoautotrophicum*. Optimum temperature and pH for growth are 40°C (range 30–45°C) and 6.1–6.9 (range 5.9–7.7), respectively. This organism was isolated from rumen fluid. Its DNA G+C content is 49 mol%. Its GenBank 16S rRNA sequence accession number is M59142. It is the type (and only) species of this genus; the type strain is DSM 1539 (≡ ATCC 35094). Refer to Paynter and Hungate (1968); Tanner and Wolfe (1988); and Kuhner et al. (1991).

Methanolacinia

Genus *Methanolacinia* has only one representative, *Methanolacinia paynteri* (formerly *Methanomicrobium paynteri*).

Methanolacinia paynteri. These Gram-negative, pleomorphic, short and highly irregular coccoid to lobe-shaped cells (about 1.5–2.0 μm in diameter; Fig. 2) usually occur singly and are flagellated but only weakly motile or nonmotile. Cells lyse in detergents. The cell envelope consists of a hexagonally arranged S-layer with a glycoprotein of Mr 155,000 and a lattice constant of 15.3 nm (Fig. 3). Colonies are circular, 1–2 μm in diameter, off-white, with entire edges. Methane is produced from H_2 + CO_2, 2-propanol + CO_2, 2-butanol + CO_2, or cyclopentanol + CO_2. No growth or methane production is detected on formate, acetate, methylamines, ethanol, 1-propanol, 1-butanol, and cyclohexanol. Growth requires acetate. Polar lipids consist of di- and tetraether lipids and include phosphatidyl glycerol diether (PG), phosphatidyltrimethylaminopentanetetrol diether (PPTAD), diglycosyldiether, presumptive phosphatidylaminopentanetetrol diether (PPAD) and the corresponding phosphoglycolipid ethers. Putrescine and spermidine are the only polyamines. Optimum growth temperature and pH are 40°C (range 20–45°C) and 7.0 (range 6.6–7.3), respectively. Growth is optimal in 0.15 M NaCl (range 0–0.8 M). Minimum generation time is 4.8 h. The G+C content of DNA is 44 mol% (by buoyant density; BD) and 38 mol% (by thermal denaturation; Tm). This organism was isolated from marine sediments. *Methanolacinia paynteri* is the type species of the genus; its type strain is G-2000 (≡ DSM 2545 or ATCC 33997). Refer to Rivard et al. (1983) and Zellner et al. (1989a).

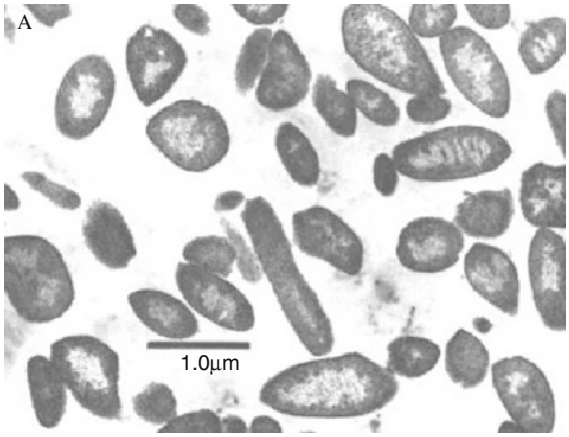

Fig. 2. Electron photomicrograph of a group of typical cells of *Methanolacinia paynteri*. (From Rivard et al., 1983.)

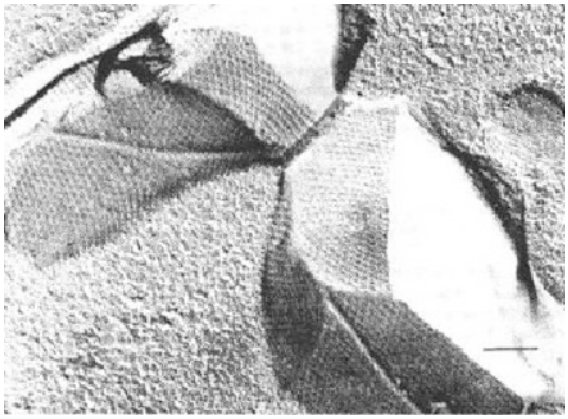

Fig. 3. Electron microphotograph of a freeze-etched preparation of *Methanolacinia paynteri*; bar = 0.1 μm. (From Zellner et al., 1989a.)

Methanogenium

Genus *Methanogenium* comprises four species. These highly irregular cocci are Gram negative. Although motility has not been observed, flagella are sometimes present. The cell wall is composed of regular glycoprotein subunits. Cells require growth factors, use formate, and are readily lysed in dilute detergents. The G+C content varies from 47 to 52 mol%. Two species were shown to use both CO_2 and secondary alcohols to form methane. *Methanogenium frittonii* is a thermophilic species (optimum temperature for growth, 57°C), whereas *M. frigidum* is psychrophilic (optimum temperature for growth, 15°C).

Methanogenium cariaci (*cariacoense*). Though motility has never been observed, these highly irregular cocci (1.3–2.6 μm in diameter) have peritrichous thin flagella up to 18 μm long and long thin pili about 4 nm in diameter. Though the cell is Gram negative, its cell wall lacks an outer

membrane and has a periodic surface pattern consisting of structural units about 14 nm in diameter; these particles seem to be composed of several subunits (Fig. 4). The cells are sensitive and 1% SDS. Colonies are circular, umbonate, greenish yellow with entire edges, shiny, 0.5 μm in diameter after two weeks of incubation, and 4 μm after 14 weeks. Methane is produced from H_2 + CO_2 or from formate. Growth requires yeast extract and acetate. Optimum temperature and pH for growth are 20–25°C (range 15–35°C) and 6.8–7.3 (range 6–7.6), respectively. Isolated from marine sediments, these organisms grow optimally in 0.54 M NaCl (range 0.2–0.8 M). The G+C content of DNA is 52 mol% (BD). The GenBank 16S rRNA sequence accession number is M59130. *Methanogenium cariaci* is the type species of its genus; the type strain is JR1 (≡ DSM 1497, ATCC 35093 or OCM 155). Refer to Romesser et al. (1979).

Methanogenium frigidum. These Gram-negative, irregular cocci (1.2–2.5 μm in diameter) are nonmotile and sensitive to 0.1% of SDS; cells occur singly without flagella or pili. The cell wall has an S-layer exterior to the plasma membrane and a fibrous coat exterior to the S-layer. Colonies are circular, smooth, green-yellow with entire edges. Methane is produced from H_2 + CO_2 or from formate. No growth or methane production is detected on acetate, trimethylamine or methanol. Growth requires acetate and is stimulated by yeast extract and peptones. Sulfide can serve as a sole sulfur source, and ammonia serves as a sole nitrogen source. An obligate psychrophile, this species grows optimally at 15°C (range 0–17°C). Optimal growth occurs at pH 7.5–7.9 (range 6.3–8.0) and in 0.35–0.6 M NaCl (range >0.1–<0.85 M). The minimum generation time is 2.9 days. The G+C content of DNA is 52 mol%; the GenBank 16S rRNA sequence accession number is AF009219. This species was isolated from the perennially cold, anoxic hypolimnion of Ace Lake in Antarctica. The type strain is Ace-2T (≡ SMCC 459W or OCM 469). Refer to Franzmann et al. (1997).

Fig. 4. Electron microphotograph of a freeze-etched preparation of (A) *Methanogenium cariaci* and (B) *Methanogenium marisnigri* showing particulate nature of the cell walls. (From Romesser et al., 1979.)

Methanogenium frittonii. These irregular cocci (1–2.5 μm in diameter) are nonmotile, fimbriated, and occur singly or in pairs. Cells are surrounded by a protein envelope (20 nm thick) and are disrupted by pronase and trypsin digestion, glass-distilled water and 1% SDS. Colonies (1–2 μm in diameter) are circular, convex, shiny, dark yellow with entire edges. Methane is produced from H_2 + CO_2 or from formate. Growth is stimulated by yeast extract, tryptone, and casamino acids. Optimum temperature for growth is 57°C (range 26–62°C); optimum pH is 7.0–7.5 (range 6.0–8.25). NaCl is not required and is inhibitory above 2%. The G+C content of DNA is 49.2 mol% (BD). This organism was isolated from lake sediment. The type strain is FR-4 (≡ DSM 2832 or OCM 200). Refer to Harris et al. (1984).

Methanogenium organophilum (*organiphilum*). These irregular cocci (0.5–1.5 μm in diameter) are nonmotile and subject to detergent lysis, e.g., by 0.01% or less SDS. Colonies are smooth and yellowish. Methane is produced from H_2 + CO_2, formate, 2-propanol + CO_2, 2-butanol + CO_2, ethanol + CO_2 and 1-propanol + CO_2. No growth or methane production is detected on acetate or methanol. Secondary alcohols are oxidized to ketones, and primary alcohols are oxidized to monocarboxylic acids. Acetate has to be added as a carbon source for growth on substrates other than ethanol. 4-Aminobenzoate, biotin, vitamin B_{12}, and tungstate are required for growth. Optimum temperature for growth is 30–35°C (maximum 39°C); optimum pH is 6.4–7.3; growth is optimal with 20 g/liter of NaCl and 3 g/liter of $MgCl_2$. Minimum generation time is 6 h on H_2 and 11 h on 2-propanol + CO_2. The G+C content of DNA is 46.7 mol% (Tm); the GenBank 16S rRNA sequence accession number is M59131. This species was isolated from marine mud. The type strain is CV (≡ DSM 3596 or OCM 72). Refer to Widdel et al. (1988).

Methanoculleus

The genus *Methanoculleus* (Maestrojuan et al., 1990) consists of five mesophilic species (including one subject synonym) and one thermophilic species of Gram-negative, highly irregular cocci. Motility has not been observed, although flagella are present in some species. Formate is used by five species. The G+C content range is between 49 and 62 mol%. The type species is *Methanoculleus bourgense*. However, DNA hybridization and rRNA sequence similarity suggest that this

species may be a subjective synonym of *Methanoculleus olentangyi* (Rouvière et al., 1992; Boone et al., 1993). If this proposal is borne out by further investigations, strains of *M. bourgense* will be reclassified as *M. olentangyi* because it is the senior synonym, and the genus *Methanoculleus* will become invalid without action by the ICSB.

Methanoculleus bourgense (*bourgensis*). These Gram-negative, irregular cocci (1–2 μm in diameter) are nonmotile and subject to lysis by 0.02% SDS. Colonies are circular, convex and white to yellowish. Methane is produced from $H_2 + CO_2$ or from formate. Acetate is required for growth. Growth is stimulated by yeast extract and trypticase. Optimum temperature for growth is 35–40°C (range 30–50°C); optimum pH is 6.7 (range 5.5–8.0); and growth is optimal with 10 g/liter of NaCl (range 0–20 g/liter). The G+C content of DNA is 59 mol% (BD). The GenBank 16S rRNA sequence accession number is AF095269. The organism was isolated from a tannery-byproduct enrichment-culture inoculated with sewage sludge. It is the type species of genus *Methanoculleus*; the type strain is MS2 (≡ DSM 3045, ATCC 43281 or OCM 15). Refer to Ollivier et al. (1986) and Maestrojuan et al. (1990).

Methanoculleus marisnigri (*marinigri*). These Gram-negative, irregular cocci (1.3 μm in diameter) have peritrichous flagella (13 μm long). The cell wall has a periodic surface pattern of 14 nm in diameter. Cells are sensitive to lysis by 1% SDS. Colonies are circular, convex, yellow, shiny and have entire edges. Methane is produced from $H_2 + CO_2$, formate, 2-propanol + CO_2, or 2-butanol + CO_2. No growth or methane production is detected on acetate or methanol. Growth requires trypticase. Optimum temperature for growth is 20–25°C (range 15–45°C); optimum pH is 6.2–6.6 (range 5.7–7.6); growth is optimal in 0.1 M NaCl (range 0–0.7 M). The G+C content of DNA is 61.2 mol% (BD); the GenBank 16S rRNA sequence accession number is M59134. This organism was isolated from marine sediments. Its type strain is JR1 (≡ DSM 1498 or ATCC 35101). Refer to Romesser et al. (1979) and Maestrojuan et al. (1990).

Methanoculleus oldenburgensis. These irregular cocci (0.5–1.5 μm in diameter) are nonmotile, fimbriated single cells, seldom in pairs, and highly sensitive to detergents, e.g., 0.01% Triton X-100 or SDS. The cell envelope consists of a protein S-layer. The cells contain putrescine, sym-homospermidine and spermine as typical polyamines. Methane is produced from $H_2 + CO_2$ or from formate. Growth requires acetate. Optimum temperature for growth is 45°C (range 25–50°C); optimum pH is 7.5–8.0 (range 6.5–8.5); NaCl is not required and inhibitory at levels exceeding 1.5%. The G+C content of DNA is 48.6 ± 1 mol% (Tm). This organism was isolated from river sediments. Its type strain is CB-1 (≡ DSM 6216). Refer to Blotevogel et al. (1991).

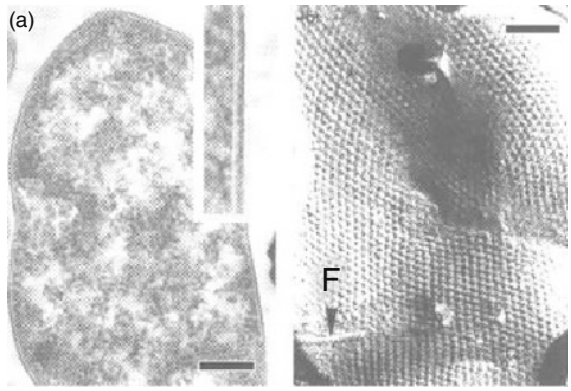

Fig. 5. (a) Electron micrographs of a thin section of a cell of *Methanocelleus palmolei*; bar = 0.1 μm. (b) Electron micrograph of a freeze-etched preparation of *Methanocelleus palmolei* showing the hexagonal arrangement of the S-layer glycoproteins and a piece of a flagellum (marked F); bar = 0.1 μm. (From Zellner et al., 1998.)

Methanoculleus olentangyi (*olentangyense*). These irregular cocci (1–4.5 μm in diameter) are nonmotile and subject to lysis by 0.001% SDS and distilled water. Colonies are circular, convex, mucoid, shiny and yellow. Methane is produced from $H_2 + CO_2$ or from formate. No growth or methane production is detected on methanol and methylamines. Growth requires acetate. Optimum temperature for growth is 37°C (range 30–45°C); growth is optimal in 1% NaCl (range 0–3%). The G+C content of DNA is 54.4 mol% (BD). The GenBank 16S rRNA sequence accession number is AF095270. This organism was isolated from river sediments. Its type strain is RC/ER (= DSM 2772, ATCC 35293 or OCM 52). It is a subjective synonym of *M. bourgense*. Refer to Corder et al. (1983) and Maestrojuan et al. (1990).

Methanoculleus palmolei. The motility of these Gram-negative, highly irregular cocci (1.25–2 μm in diameter), though flagellated, has not been observed. Cell walls have S-layers of hexagonally arranged glycoprotein subunits (Mr 120,000; Fig. 5). The cells contain putrescine, sym-homospermidine and spermine as typical polyamines. Methane is produced from $H_2 + CO_2$, formate, 2-propanol + CO_2, 2-butanol + CO_2, or cyclopentanol + CO_2. No growth or methane production is detected on acetate, methanol, ethanol, 1-propanol, 2-pentanol + CO_2, 2,3-butanediol, dimethylamine and lactate. Growth requires acetate and is stimulated by potassium and tungstate ions. Optimum temperature for growth is 40°C

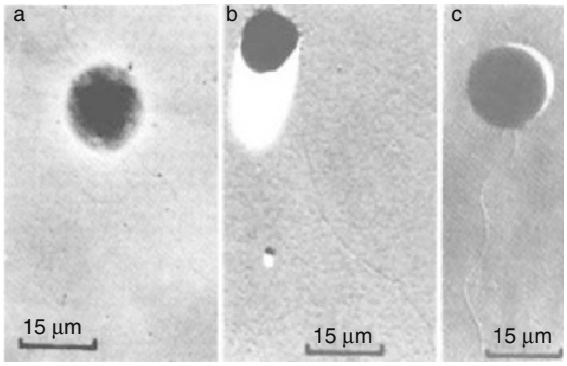

Fig. 6. Platinum-iridium shadowed cells of *Methanoculleus thermophilicus* (a) strain Ratisboa, (b) DSM 2373 and (c) strain Los Angeles. (From Zabel et al., 1985.)

(range 22–50°C); optimum pH is 6.9–7.5 (range 6.5–8.0); minimum generation time is 13.5 h. The G+C content of DNA is 59–59.5 mol% (Tm, HPLC); the GenBank 16S rRNA sequence accession number is Y16382. This organism was isolated from a digester treating wastewater of a palm oil mill in Indonesia. The type strain is INSLUZ T (≡ DSM 4273T). Refer to Zellner et al. (1998).

Methanoculleus thermophilicum (*thermophilicus*). These Gram-negative, irregular cocci to coccobacilli (0.7–1.8 µm in diameter; Fig. 6) occur singly or in pairs and have occasional internal membrane components. Cell envelope is composed of protein subunits and contains a glycoprotein (130 kD). One flagellum and pili are present; motility is not observed or weak. Colonies are circular, beige, with entire edges. Methane is produced from H_2 + CO_2 or from formate. No growth or methane production is detected on acetate, methanol, ethanol, propionate, pyruvate, dimethylamine and trimethylamine. Growth requires acetate, a trace vitamin solution and trypticase (or peptone or yeast extract). Optimum temperature for growth is 55–60°C; optimum pH is 6.7–7.2; optimum NaCl concentration is 0–0.3 M. The G+C content of DNA is 56–60 mol% (Tm); the GenBank 16S rRNA sequence accession number is M59129. This organism was isolated from digesters and marine sediments. The type strain is CR-1 (≡ DSM 2373, ATCC 33837 or OCM 174). Refer to Rivard and Smith (1982), Zabel et al. (1985), and Maestrojuan et al. (1990).

Methanoplanus

The genus *Methanoplanus* comprises three species of plane-shaped organisms. The cell walls contain at least one major glycoprotein. Formate is used for methanogenesis. One species is an endosymbiont of marine ciliates and is found in close association with microbodies that are thought to provide H_2 to the methanogen. The methanogen functions as an electron sink in the oxidation steps of the carbon flow in the ciliates. This symbiotic relationship is thought to be responsible for a total conversion of metabolites to CO_2 and CH_4 in marine sediments. The G+C range of the genus is 39 to 50 mol%.

Methanoplanus endosymbiosus. These Gram-negative, irregular discs with a diameter of 1.6–3.4 µm occur singly and are nonmotile despite the presence of peritrichous flagella or pili. The cell envelope shows a regular hexagonal surface pattern and consists of proteins. Cells are lysed by 0.001% SDS or 0.01% Triton X-100 in 2% NaCl. Colonies are circular with entire margins, shiny, convex and whitish yellow. Methane is produced from H_2 + CO_2 or from formate. No growth or methane production is detected on acetate, methanol or methylamine. Tungsten (0.1 mM) is required for growth, which is stimulated by yeast extract, tryptone or rumen fluid. *p*-Cresol replaces the requirement for rumen fluid. Optimum temperature for growth is 32°C (range 16–36°C); optimum pH is 6.8–7.3 (range 6.3–7.8); and optimum NaCl concentration is 0.25 M (range 0–0.75M). Minimum generation time is 7–12 h. The G+C content of DNA is 38.7 mol% (Tm). The GenBank 16S rRNA sequence accession number is Z29435. This organism was found as an endosymbiont in the marine ciliate *Metopus contortus*. The type strain is DSM 3599. Refer to Van Bruggen et al. (1986) and Poirot et al. (1991).

Methanoplanus limicola. These Gram-negative, angular, crystal-like plates with sharp edges (0.07–0.3 µm thick, 1.6–2.8 µm long, 1.5 µm wide) occur singly (Fig. 7). The cells are sometimes branched, without septa, and contain electron-dense round inclusions. The cell envelope shows a hexagonal surface pattern and contains a dominant glycoprotein. A polar tuft of flagella can be seen, and the cells are weakly motile. Cells are lysed by 2% SDS. Colonies on polysilicate are round, smooth, bright, ochre-colored, and flat (about 2 µm in diameter). Cells are resistant to vancomycin, penicillin, kanamycin and tetracycline. Methane is produced from H_2 + CO_2 or from formate. No growth or methane production is detected on acetate, methanol or methylamines. Acetate (0.1%) is required for growth, which is stimulated by yeast extract (or peptones and vitamins). Optimum temperature for growth is 40°C (range 17–41°C); optimum pH is 7 (range 6.5–7.5); and optimum NaCl concentration is 1% (range 0.4–5.4%). The G+C content of DNA is 47.5 mol% (Tm). This organism was isolated from a swamp of drilling waste in Italy. Its GenBank 16S rRNA sequence

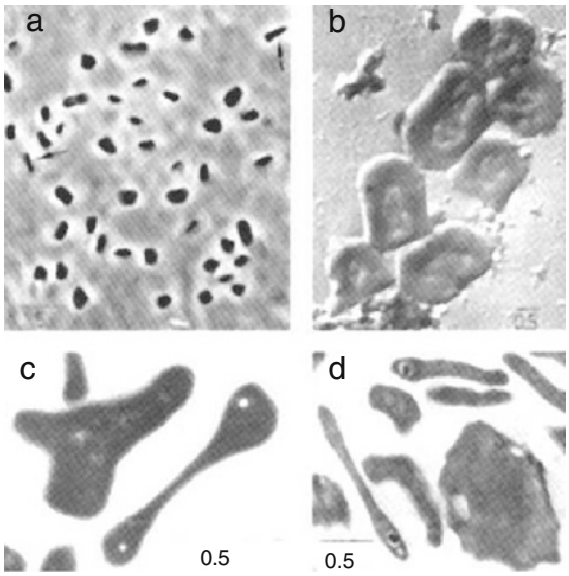

Fig. 7. Light and electron micrographs of *Methanoplanus limicola*. (a) Phase contrast of exponentially growing cells; arrows indicate bacteria appearing in profile; (b) platinum shadowed; (c, d) thin sections. The bars are of indicated size in μm. (From Wildgruber et al., 1982.)

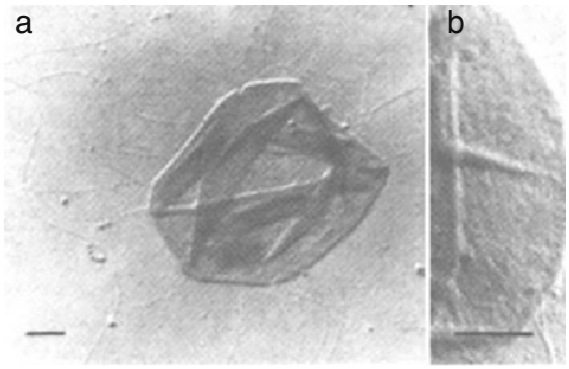

Fig. 8. Electron photomicrographs of platinum-iridium shadowed cells of *Methanofollis tationis*: (a) whole cells with peritrichous flagellation; (b) cell surface showing the hexagonal subunit structure; both bars = 0.5 μm. (From Zabel et al., 1984.)

accession number is M59143. *Methanoplanus limicola* is the type species of its genus. The type strain is M3 (≡ DSM 2279,ATCC 35062 or OCM 101). Refer to Wildgruber et al. (1982).

Methanoplanus petrolearius. These irregular disc-shaped cells (1–3 μm in diameter) occur singly or in pairs and are nonmotile. Colonies are round (1–2 μm in diameter). Methane is produced from H_2 + CO_2, formate or 2-propanol + CO_2. No growth or methane production is detected on acetate, methanol, trimethylamine, lactate, glucose, 1-propanol, 1-butanol, or isobutanol. Acetate is required for growth, which is stimulated by yeast extract. Optimum temperature for growth is 37°C (range >25–<45°C); optimum pH is 7 (range 5.3–8.4); optimum NaCl concentration is 1–3% (range 0–5%). Minimum generation time is 10 h. The G+C content of DNA is 50 mol% (HPLC). This organism was isolated from an oil-producing well. The GenBank 16S rRNA sequence accession number is U76631. The type strain is SEBR 4847T (≡ DSM 11571 or OCM 486). Refer to Ollivier et al. (1997).

Methanofollis

This genus has been proposed to reclassify *Methanogenium tationis* and *M. liminatans*. These two species use formate and have a G+C content of 54–61 mol%.

Methanofollis liminatans. These irregular cocci (1.5 μm in diameter) are flagellated and motile. The cell envelope consists of hexagonally arranged glycoproteins. Cells grow in a synthetic, acetate-containing low-salt medium with substrates H_2 + CO_2, formate, 2-propanol + CO_2, 2-butanol + CO_2, or cyclopentanol + CO_2. No growth or methane production occurs with acetate, methanol, ethanol or dimethylamine. Tungstate (1–2 mM) stimulates growth. Optimum temperature for growth is 40°C (range >25–<45°C); optimum pH is 7; optimum NaCl concentration is 0–0.6 M (range 0–<0.8 M). Minimum generation time is 7.5–8.5 h. The G+C content of DNA is 59.3 mol% (Tm) and 60.5 mol% (HPLC). This organism was isolated from effluent of a digester for the treatment of industrial waste water. The GenBank 16S rRNA sequence accession number is AF095271. The type strain is GKZPZ (≡ DSM 4140). Refer to Zellner et al. (1990) and Zellner et al. (1999).

Methanofollis tationis (*tatioense*). These Gram-negative, regular or highly irregular cells, depending on the salt concentration in the medium, approximate 3 μm in diameter (Fig. 8), have peritrichous flagella, and lyse in 1% SDS. The cell envelope is composed of hexagonal protein subunits, which seem to contain a glycoprotein. The cytoplasm has numerous polyphosphate inclusions. Methane is produced from H_2 + CO_2 or from formate. No growth or methane production is detected on acetate, methanol, ethanol, ethyl acetate, dimethylamine or trimethylamine. Yeast extract, peptone and acetate are required for optimal growth. Yeast extract and peptone can partially be replaced by a heavy metal solution. Optimum temperature for

growth is 37–40°C (range 25–45°C); optimum pH is 7 (range 6.3–8.8); optimum NaCl concentration is 0.8–1.2% (range 0–7%). Minimum generation time is 12 h at 40°C. The G+C content of DNA is 54 mol% (Tm). This organism was isolated from solfataric pools at an altitude of 4,750 m. The GenBank 16S rRNA sequence accession number is AF095272. *Methanofollis tationis* is the type species of this genus. The type strain is DSM 2702. Refer to Zabel et al. (1984) and Zellner et al. (1999).

The Family Methanocorpusculaceae

Methanocorpusculum

The family *Methanocorpusculaceae* (Zellner et al., 1989b) comprises one genus, *Methanocorpusculum*, and five mesophilic species (including one subjective synonym) of irregular cocci. They all use H_2 + CO_2 and formate, and some species can use 2-propanol + CO_2. The type species is *Methanocorpusculum parvum* (Zellner et al., 1987). However, *Methanocorpusculum aggregans* (formerly *Methanogenium aggregans*) was isolated prior to *M. parvum* and has been shown to be closely related, with about 70% DNA reassociation (Ollivier et al., 1985; Xun et al., 1989). The high level of DNA reassociation suggests that *M. parvum* and *M. aggregans* may be subjective synonyms (Boone et al., 1993). If further investigations support this conclusion, *M. aggregans* would have precedence over *M. parvum*, which would then have to be reclassified as *M. aggregans* subspecies *parvum*. This reclassification would then invalidate the genus *Methanocorpusculum* as well as the family *Methanocorpusculaceae*. *Methanocorpusculum parvum* is of special interest because it requires high levels of tungstate for growth (Zellner et al., 1987). It is also the first hydrogenotrophic methanogen found to possess cytochromes. Presumably, these cytochromes are involved in the oxidation of 2-propanol. The mol% G+C of this genus ranges from 48 to 52.

Methanocorpusculum aggregans. These Gram-negative, irregular cocci (0.5–2 μm in diameter) occur singly or in aggregates (Fig. 9) and are nonmotile and sensitive to 0.03% SDS. Colonies (3–4 μm in diameter) are round, convex, and white to yellow. Methane is produced from H_2 + CO_2 or from formate. No growth or methane production is detected on acetate, methanol or methylamines. Acetate and yeast extract or trypticase are required for growth. Optimum temperature for growth is 35°C (range 27–38°C); optimum pH is 6.6 (range 6.2–7.5); optimum NaCl concentration is 0% (range 0–2%). Minimum generation time is 8 h. The G+C content of DNA is 52 mol% (BD). This organism was isolated from a sewage sludge digestor. The type strain is Mst (=DSM 3027 or OCM 21). It is possibly a subjective synonym of *M. parvum*. Refer to Ollivier et al. (1985) and Xun et al. (1989).

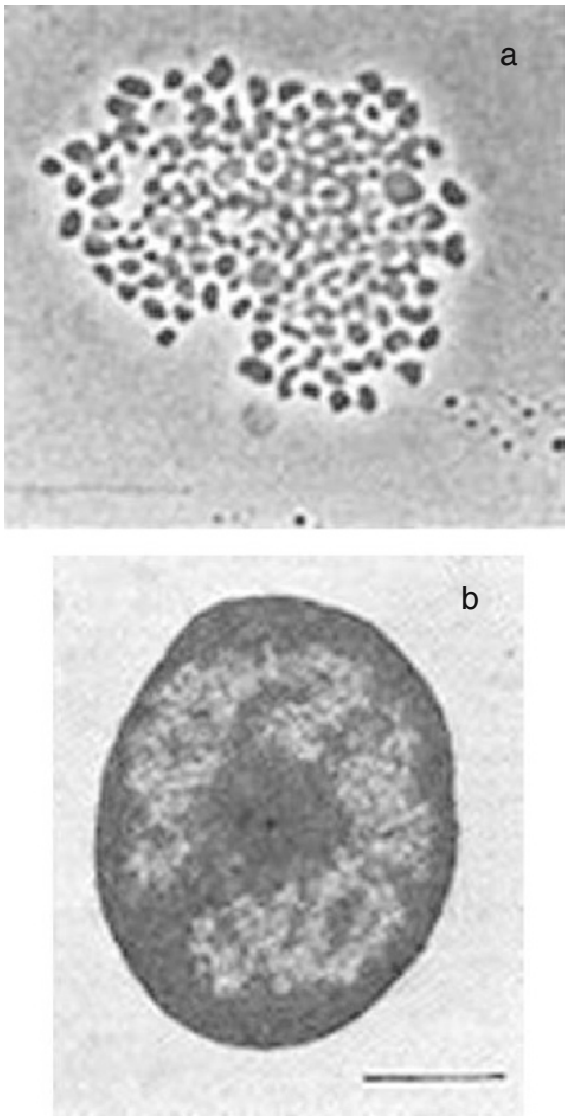

Fig. 9. (a) Phase-contrast photomicrograph of aggregates of *Methanocorpusculum aggregans*; bar = 10 μm; (b) thin-section electron photomicrograph of an individual cell of *Methanocorpusculum aggregans*; bar = 0.5 μm. (From Ollivier et al., 1985.)

Methanocorpusculum bavaricum. These Gram-negative, small, irregular cocci (<1 μm in diameter) occur singly and are flagellated and weakly motile. The cell envelope consists of a hexagonally arranged S-layer with a center-to-center spacing of the glycoprotein subunits of 16 nm (Fig. 10). Methane is produced from H_2 +

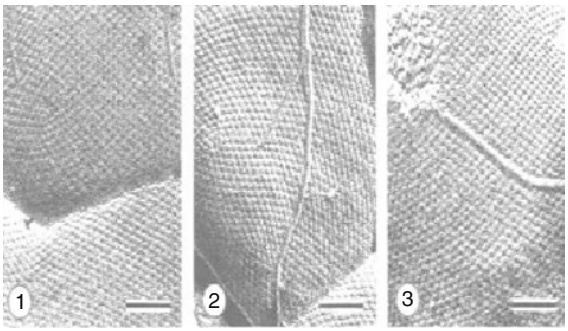

Fig. 10. Electron micrograph of a freeze-etched preparation of (1) *Methanocorpusculum parvum*; (2) *M. sinense*; (3) *M. bavaricum*; bar = 0.1 μm. (From Zellner et al., 1989b.)

CO_2, formate, 2-propanol or 2-butanol. No growth or methane production is detected on acetate, methanol, methylamines, ethanol, 1-propanol, 1-butanol or cyclohexanol. Rumen fluid is required for growth, which is stimulated by tungstate (1 mM). Optimum temperature for growth is 37°C (range 15–45°C); optimum pH is 7. The G+C content of DNA is 47.7 mol% (Tm), 51% (HPLC). This organism was isolated from anaerobic sediment of a sugar factory's wastewater treatment pond. Its GenBank 16S rRNA sequence accession number is AF042197. The type strain is SZSXXZ (≡ DSM 4179 or OCM 127). Refer to Zellner et al. (1989b).

Methanocorpusculum labreanum. These Gram-negative, irregular cocci are 0.4–2 μm in diameter. The degree of irregularity is dependent on the physiological state of the cells and on the ionic strength of the medium. The cells are nonmotile, sensitive to 0.02% SDS, and have a protein cell wall. Colonies are circular (0.5 μm in diameter), clear, and convex with entire edges. Methane is produced from $H_2 + CO_2$ or formate. No growth or methane production is detected on acetate, propionate, methanol, trimethylamine or ethanol. Trypticase, peptone or yeast extract is required for growth, which is stimulated by acetate. Optimum temperature for growth is 37°C (range 25–40°C); optimum pH is 7 (range 6.5–7.5); optimum NaCl concentration is 0–1.5% (range 0–<3%). The G+C content of DNA is 50 mol% (BD). This organism was isolated from surface sediments of Tar Pit Lake in Los Angeles, California. The GenBank 16S rRNA sequence accession number is AF095267. The type strain is ZT (≡ DSM 4855, ATCC 43576, or OCM 1). Refer to Zhao et al. (1989).

Methanocorpusculum parvum. These Gram-negative, irregular cocci (= 1 μm in diameter) occur singly or in pairs. Each cell has one flagellum and is weakly motile. The cell envelope consists of hexagonally arranged protein substructures (Fig. 10). Complete lysis occurs with 1% SDS. Methane is produced from $H_2 + CO_2$, formate or 2-propanol + CO_2. No growth or methane production is detected on acetate, propionate, methanol, methylamines, ethanol, pyruvate, lactate, butyrate, 1-butanol, 2-pentanol, formaldehyde, L-alanine, L-lysine or L-leucine. Yeast extract, acetate and tungsten (1 mM) are required for growth, which is stimulated by clarified rumen fluid. Optimum temperature for growth is 37°C (range 20–40°C); optimum pH is 6.8–7.5; optimum NaCl concentration is 0% (range 0–5%). Minimum generation time is 8 h. The G+C content of DNA is 48.5 mol% (Tm).

This organism was isolated from an anaerobic sour whey digester inoculated with sewage sludge. The GenBank 16S rRNA sequence accession number is M59147.

Methanocorpusculum parvum is the type species of this genus and possibly a subjective synonym of *M. aggregans*. The type strain is XII (≡ DSM 3823, ATCC 43721 or OCM 63). Refer to Zellner et al. (1987).

Methanocorpusculum sinense. These Gram-negative organisms occur singly as flagellated, weakly motile, small, irregular cocci (<1 μm in diameter). The cell envelope consists of a hexagonally arranged S-layer with a center-to-center 15.8-nm spacing of the glycoprotein subunits (Fig. 10). Colonies are circular, convex with entire edges. Methane is produced from $H_2 + CO_2$ or from formate. No growth or methane production is detected on acetate, methanol, methylamines, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol or cyclohexanol. Clarified rumen fluid is required for growth. Optimum temperature for growth is 30°C (range 15–45°C); optimum pH is 7. The G+C content of DNA is 52 mol% (Tm) and 50 mol% (HPLC). This organism was isolated from an anaerobic biogas plant treating distillery wastewater. The GenBank 16S rRNA sequence accession number is AF095268. The type strain is CHINAZ (≡ DSM 4274 or OCM 128). Refer to Zellner et al. (1989b).

The Family Methanospirillaceae

Methanospirillum

Based upon its unique morphology and low 16S rRNA sequence similarity to the other Methanomicrobiales, the family *Methanospirillaceae* was created to include the single genus *Methanospirillum* (Boone et al., 1993). Members of the genus are mesophilic and have been reported from a wide range of habitats. However, only one species, *Methanospirillum hungatei*, has been described so far.

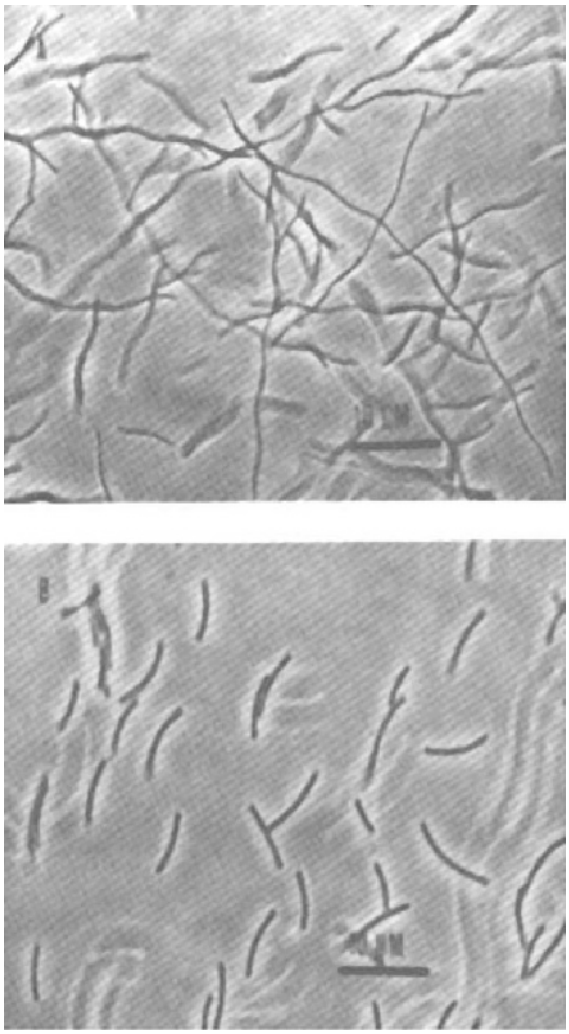

Fig. 11. Phase-contrast photomicrograph of (a) long, wavy filament and (b) individual cells of *Methanospirillum hungatei*. Both bars = 10 μm. (From Mah and Smith, 1981.)

Methanospirillum hungatei (*hungateii*). These Gram-negative, curved rods (7.4 μm × 0.5 μm) form filaments from 15 μm often to several hundred μm in length (Fig. 11). Possessing polar, tufted flagella and sheaths, cells exhibit weak motility and progressive movement. The cell envelope and sheath have been extensively studied (see below). Cells are striated on the surface and resistant to lysozyme. Colonies are circular, <3 μm in diameter, light yellow, convex with lobate margins, uniquely striated when observed under low-power magnification. The type species forms methane from $H_2 + CO_2$ or from formate, and some strains (GP1 and SK) are able to use 2-propanol + CO_2 or 2-butanol + CO_2 (Widdel, 1986; Widdel et al., 1988). No growth or methane production is detected on acetate, methanol, ethanol, pyruvate or benzoate.

Methanospirillum hungatei has a positive chemotactic response to acetate (Migas et al., 1989). Yeast extract and trypticase are stimulatory for growth. Optimum temperature for growth is 30–37°C; optimum pH is 6.6–7.4. The G+C content is 45–49 mol% (BD). This hydrogenotrophic methanogen shows a high affinity for hydrogen and is often utilized for isolation of syntrophic bacteria in place of sulfate-reducing bacteria when sulfate is not available in the culture medium. This organism was isolated from various anaerobic environments. Its GenBank 16S rRNA sequence accession number is M60880. It is the type species of genus *Methanospirillum*. Its type strain is JF1 (≡ DSM 864, ATCC 27890 or OCM 16). Refer to Ferry et al. (1974), Patel et al. (1976), Ferry and Wolfe (1977), and Widdel et al. (1988).

Genus *insertae sedis*

Methanocalculus

It is a newly described genus that encompasses two irregular cocci, *Methanocalculus halotolerans*, an isolate from an offshore oil well, and *M. pumilus*, isolated from a waste-disposal site. *Methanocalculus halotolerans* is a hydrogenotrophic halotolerant methanogen. Further investigation may lead to the reclassification of this genus within the family *Methanocorpusculaceae*.

Methanocalculus halotolerans. These irregular cocci (0.8–1 μm in diameter; Fig. 12) occur singly or in pairs and possess 2–3 peritrichous flagella. Colonies are round, reaching 1 μm in diameter after 10 weeks incubation at 37°C. Methane is produced from $H_2 + CO_2$ or from formate. No growth or methane production is detected on acetate, methanol, trimethylamine, lactate, glucose, 1-propanol, 2-propanol, 1-butanol or 2-butanol. Acetate is required for growth stimulated by yeast extract. For growth, the optimum temperature is 38°C (range >24–<50°C); optimum pH is 7.6 (range 7–8.4); optimum NaCl concentration is 5% (range 0–12.5%; the widest reported range to date for any hydrogenotrophic methanogen, including members of the orders *Methanobacteriales*, *Methanococcales* and Methanomicrobiales). Minimum generation time is 12 h. The G+C content of DNA is 55 mol% (HPLC). This organism was isolated from an oil-producing well. Its GenBank 16S rRNA sequence accession number is AF033672. It is the type species of genus:

Methanocalculus. The type strain is SEBR 4845^T (OCM 470^T). Refer to Ollivier et al. (1998).

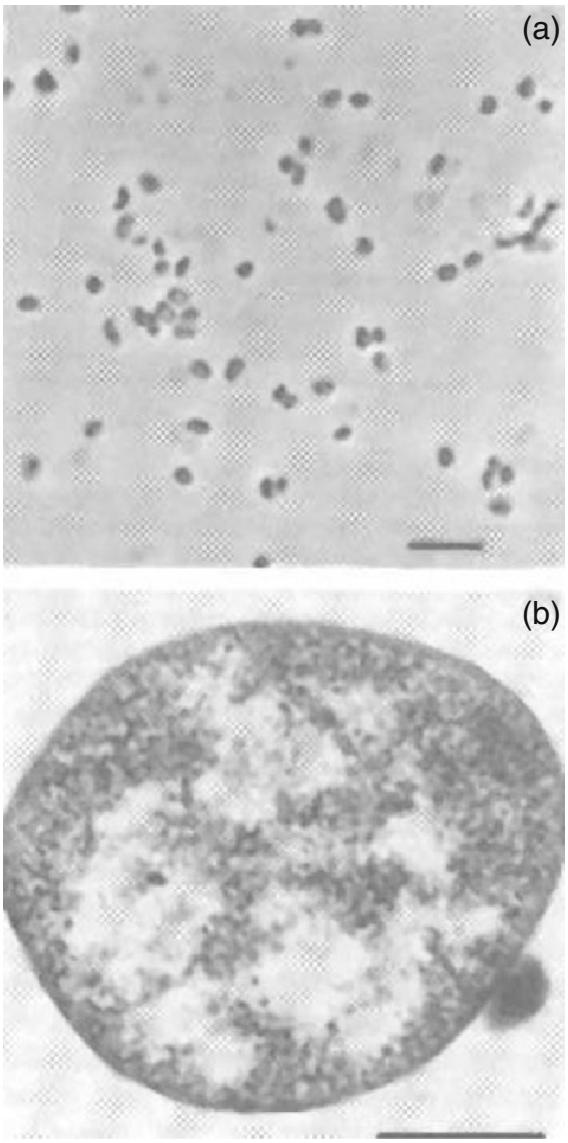

Fig. 12. (a) Phase-contrast micrograph of *Methanocalculus halotolerans* showing irregular coccoid cells; bar = 5 μm; (b) electron micrograph of an ultrathin section showing the cell wall structure; bar = 0.2 μm. (From Ollivier et al., 1998.)

Methanocalculus pumilus. These irregular cocci (0.8–1 μm in diameter) occur singly or in pairs and are nonmotile. They lyse in 0.01% SDS and under hypotonic conditions. Methane is produced from H_2 + CO_2 or from formate. No growth or methane production is detected on acetate, methanol, trimethylamine, lactate, glucose, 1-propanol, 2-propanol, 1-butanol or 2-butanol. Acetate is required for growth stimulated by yeast extract. Cells are tolerant to heavy metals. For growth, optimum temperature is 35°C (range 24–45°C); optimum pH is 7 (range 5.5–9); and optimum NaCl concentration is 1% (range 0–7%). The minimum generation time is 12 h. The G+C content of DNA is 51.9 mol% (HPLC). This organism was isolated from a waste-disposal site. Its GenBank 16S rRNA sequence accession number is AB008853. Its type strain is MHT-1^T (≡ DSM 12632^T or JCM 10627^T). Refer to Mori et al. (2000).

Biochemical and Physiological Properties

Coenzymes and Enzymes Within the Methanomicrobiales

In those Methanomicrobiales that have been tested, the activities of methane biosynthesis enzymes are comparable to those found in other methanogens (Schwörer and Thauer, 1991; Raemakers-Franken et al., 1990; Berk and Thauer, 1997). Specifically, high levels of formylmethanofuran dehydrogenase, coenzyme F_{420}-dependent methylenetetrahydro-methanopterin dehydrogenase, methylenetetrahydromethanopterin reductase, and heterodisulfide reductase have been found. The proton-reducing methylenetetrahydro-methanopterin dehydrogenase was not found. The Methanomicrobiales also contain high levels of the methyl coenzyme M reductase (Rouvière and Wolfe, 1987). Methanopterin and sarcinapterin are common C-1 carriers in most methanogens. However, the Methanomicrobiales contain three unique pterins in place of methanopterin (Gorris and van der Drift, 1994). Two novel pterins, called "tatiopterin-O" and "tatiopterin-I," have been isolated and characterized from *Methanofollis tationis* (Raemakers-Franken et al., 1991b). Tatiopterin-I (a methanopterin-like compound) lacks the characteristic methyl group on the 7-position of the pterin and has additional aspartyl and glutamyl residues on the side chain. Tatiopterin-O is similar to tatiopterin-I, except the glutamyl residue is missing from the side chain. In addition, *Methanoculleus thermophilicum* contains a pterin similar to tatiopterin-O, except the aniline moiety contains two hydroxyl residues (Raemakers-Franken et al., 1991a). Interestingly, extracts of *M. hungatei* contain serine hydroxymethyltransferase activity that is dependent on tetrahydrofolate, but not on tetrahydromethanopterin and tetrahydrosarcinopterin (Lin and Sparling, 1998). This observation may indicate that folates are also present in these methanogens. Alternatively, a tatiopterin-dependent enzyme may be present whose substrate specificity includes folate.

The Methanomicrobiales also contain high levels of coenzyme F_{420} and vitamin B_{12} (Gorris and van der Drift, 1994). The predominant coenzymes $F_{420\text{-}2}$ and $F_{420\text{-}3}$ have two and three

glutamyl residues on their side chains, respectively. Coenzyme F_{420-4} and coenzyme F_{420-5}, which are abundant in the *Methanosarcinales*, are either present at much lower concentrations or absent. Like most methanogens, the Methanomicrobiales contain high levels of the vitamin B_{12} compound, 5-hydroxybenzimidazoylcobamide. The ability to utilize alcohols as electron donors for methanogenesis is widely distributed among the Methanomicrobiales (Widdel, 1986; Zellner and Winter, 1987; Widdel et al., 1988). The secondary alcohol dehydrogenases responsible for these activities are either coenzyme F_{420}- or $NADP^+$-dependent (Bleicher et al., 1989; Frimmer and Widdel, 1989). The coenzyme F_{420}-dependent enzymes have been purified from *M. thermophilicum* and *M. liminatans* (Widdel and Wolfe, 1989; Bleicher and Winter, 1991). In both cases, the enzymes were composed of subunits with an Mr of about 39,000 and were unstable during storage. The enzyme from *M. liminatans* had a broad substrate specificity that included 2-propanol, R(-)-2-butanol, S(+)-2-butanol, 2 pentanol, cyclopentanol, and the corresponding ketones. Like other coenzyme F_{420}-dependent enzymes, the *M. thermophilicum* enzyme possesses Si-face stereospecificity with respect to the C-5 position of coenzyme F_{420} (Klein et al., 1996). The ethanol dehydrogenase activity in extracts of *M. organophilum* has also been characterized (Frimmer and Widdel, 1989). This activity is $NADP^+$-dependent and has a pH optimum of 10. It also has a Re-face stereospecificity at the C-4 position of $NADP^+$ (Berk et al., 1996). Organisms which utilize the $NADP^+$-dependent alcohol dehydrogenase also possess high levels of coenzyme F_{420}-dependent $NADP^+$ reductase (Berk and Thauer, 1997). This enzyme is necessary to couple alcohol oxidation with the coenzyme F_{420}-dependent steps in methanogenesis. The enzyme from *M. organophilum* has been purified and found to be similar in molecular weight and other properties to the enzymes from other methanogens (Berk and Thauer, 1997).

The bioenergetics of the Methanomicrobiales have not been studied in detail. In *Methanospirillum hungatei*, low affinity potassium transport and Na^+/H^+ antiport systems have been described (Sprott et al., 1985; Rusnák et al., 1992).

Cell Envelope Structure

With the exception of *Methanospirillum hungatei*, the cell envelopes of the Methanomicrobiales are composed of a regularly structured protein layer (S-layer) and a cytoplasmic membrane (Sleytr et al., 1986; Sprott and Beveridge, 1993). The S-layer forms a paracrystalline array, usually with hexagonal (p6) symmetry and center-to-center spacing of 14–16 nm (Table 5). The molecular weight and antigenicity of the S-layer proteins vary between species and genera. Except for *Methanogenium cariaci* and *Methanospirillum hungatei*, the S-layer proteins are generally glycosylated. Unlike cells containing pseudomurein in their envelopes, cells where the envelope is composed entirely of a S-layer are frequently sensitive to detergents. The S-layers of *Methanoculleus marisnigri* and *Methanoplanus limicola* have been analyzed in some detail. In *M. marisnigri*, the S-layer-membrane complex forms a tight but noncovalent association that is deformable and not rigid (Bayley and Koval,

Table 5. Envelopes of Methanomicrobiales.

		S-layer				
		Monomer				
Envelope type	Species	kDa	Glycosylation	Lattice	Spacing (nm)	Refs
1. RS-layer + CM	*Methanocorpusculum parvum*	90	G	H	14.3	1
	Methanocorpusculum sinense	92	G	H	15.8	1
	Methanocorpusculum bavaricum	94	G	H	16.0	1
	Methanoplanus limicola	135	G	H	14.7	2
	Methanoculleus thermophilicum	130	G	H	—	3
	Methanoculleus marisnigri	138	G	H	14	3,4
	Methanoculleus cariaci	117	NG	H	14	3,4
	Methanoculleus palmolei	120	G	H	14	6
	Methanofollis liminatans	118	G	H	15.4	6
	Methanofollis tationis	120	G	H	—	7
	Methanolacinia paynteri	155	G	H	15.3	8
2. Sheath + S-layer + CM	*Methanospirillum hungatei* GP1	110	NG	H	15	9

Abbreviations: RS, regularly structured; CM, cytoplasmic membrane; G, glycosylated; NG, nonglycosylated; and H, hoop.
References: 1, Zellner et al., 1989b; 2, Cheong et al., 1991; 3, Zabel et al., 1985; 4, Romesser et al., 1979; 5, Zellner et al., 1998; 6, Zellner et al., 1990; 7, Zabel et al., 1984; 8, Zellner et al., 1989a; and 9, Firtel et al., 1993.
Data from Sprott and Beveridge, 1993.

1994). In *M. limicola*, the molecular architecture of the S-layer was reconstructed from a tilt series of negatively stained preparations and surface relief reconstructions of metal-shadowed preparations as well as from scanning tunneling microscopy (Cheong et al., 1991; Cheong et al., 1993). The surface layer has hexagonal or p6 symmetry, a lattice constant of 14.7 nm, and thickness of approximately 6.5 nm. The S-layer protein has an apparent molecular weight of 135 kDa, shifting to 115 kDa after removal of the carbohydrate components with anhydrous trifluoromethanesulfonic acid. In addition, quantitative estimations revealed a total neutral sugar content of 240 mg/g of polypeptide. To form an irregular coccus (as is common in the Methanomicrobiales), the S-layer must incorporate local faults or edge dislocations in the paracrystalline structure (Pum et al., 1991). Likewise, edge dislocations are required for invagination of the envelope during cell division.

The cell envelope of *Methanospirillum hungatei* has an unique ultrastructure. Cells contain an S-layer similar to that found in other Methanomicrobiales (Firtel et al., 1993; Table 5). Individual cells or chains of cells are then further enclosed by a sheath. Within the chains, cells are separated by a spacer region composed of multiple lamellae or spacer plugs. The sheath is extremely resilient to denaturants, salts, proteases, and other enzymes (Beveridge et al., 1985), but it can be disassociated into hoop structures and free polypeptides by treatment with sulfhydryl reagents (Sprott et al., 1986; Southam and Beveridge, 1991). In addition, about 20% of the mass of the sheath is made up of phenol-soluble proteins that confer rigidity (Southam and Beveridge, 1992). The intact sheath is a paracrystalline array of 2.8-nm particles and is composed of stacked hoops (Stewart et al., 1985; Beveridge et al., 1990; Blackford et al., 1994), and the tight packing of the sheath particles produces a barrier of low porosity that limits the movement of even small molecules (Beveridge et al., 1991). Measurements of the elasticity of the sheath by atomic force microscopy indicate that it can withstand pressures in the range of 300–400 atm (Xu et al., 1996). It is hypothesized that the accumulation of intracellular CH_4 to high pressures causes expansion of the sheath, which opens pores in the sheath and allows the release of CH_4 and uptake of H_2 and CO_2. In this way, the sheath is envisioned to act as a pressure regulator (Xu et al., 1996). In contrast to the sheath, the spacer plugs at the ends of the cell are highly permeable (Beveridge et al., 1991). These plugs are proteinaceous disks that span the sheath and consist of two types of paracrystalline layers sandwiched between amorphous layers of unknown composition (Firtel et al., 1994). One type of paracrystalline layer is composed of 14-nm particles with 18-nm interparticle spacing to produce a highly porous structure. The second layer is net-like with large, 12.5-nm pores. These layers are attached to each other and the cell by intervening amorphous layers.

Cell Envelope Lipids

Similarly to other methanogens, the cell membranes of Methanomicrobiales are characterized by many unusual lipids. These lipids are polar, either archaeol (diphytanyl-glycerol-diethers) or caldarchaeol (dibiphytanyl-diglycerol-tetraethers). Caldarchaeol appears to span the membrane, with the polar head groups at opposite sides. Therefore, the membranes are probably arranged in a monolayer with bilayer regions resulting from interspersed diethers (Whitman et al., 1992). These lipids are then present as phospho- or glyco-derivatives. Among the Methanomicrobiales, the major sugars in the glycolipids are glucose (glc) and galactose (gal; Koga et al., 1998). The major polar head groups in the phospholipids are aminopentanetetrols and glycerol. In *Methanospirillum hungatei*, four of the polar lipids had as one head group either α-glc(p)-(1-2)-β-gal(f)- or β-gal(f)-(1-6)-β-gal(f)- in glycosidic linkage to the first glycerol of the lipid backbone and either a *N*,*N*-dimethylaminopentanetetrol or a *N*,*N*-trimethylaminopentanetetrol moiety in phosphodiester linkage to the second glycerol of the backbone (Sprott et al., 1994). A fifth lipid was a tetraether structure with carbohydrate moieties at both head group positions, namely α-glc(p)-(1-2)-gal(f) and β-gal(f)-. Two other lipids, a diether and a tetraether, had a single head group consisting of α-glc(p)-(1-2)-β-gal(f)- modified by O-acetylation of the gal(f) residue at C-6. Lastly, the diether and tetraether analogs of phosphatidylglycerol were found.

Motility

Flagella are observed in species of the order Methanomicrobiales. In *Methanospirillum hungatei* mono- or bipolar flagella are inserted through the end plugs of the filaments (Cruden et al., 1989; Southam et al., 1990). Filaments, with a tuft of flagella at each of the terminal cells on opposite ends of the filament, are able to coordinate flagellar rotation so that propulsion is achieved (Sprott and Beveridge, 1993). Isolated flagellar filaments from *Methanospirillum hungatei* can be dissociated by low concentrations (0.5% [v/v]) of Triton X-100 (Faguy et al., 1992). The filaments are composed of multiple glycosylated flagellins, where the flagellins represent distinct gene products rather than differentially glycosylated forms of the same protein. Similarly,

the purified flagellar filaments isolated from *Methanoculleus marisnigri* were shown to be composed of two flagellins (Kalmokoff et al., 1992). Lastly, the flagellar filaments have a simple knob basal structure with no apparent ring or hook structures such as found in bacteria (Faguy et al., 1994). When cultivated at 37°C, *Methanospirillum hungatei* grows as single cells or short chains of cells (typically 10–30 μm long), and both forms are motile. When grown in low Ca^{2+} concentrations or with the divalent cation chelator EDTA, nonflagellated filaments (up to 900 μm long) are produced. Likewise, at suboptimal growth temperatures, the cells form short filaments that do not possess flagella. The amount of flagellin present appears to be equal in both nonflagellated and flagellated cultures (Faguy et al., 1993).

Compatible Solutes

Methanogens respond to osmotic stress by accumulating a series of organic molecules which function as compatible solutes. Four key organic solutes were observed in *Methanogenium cariaci*. They include L-α-glutamate, β-glutamate, N(e)-acetyl-β-lysine and betaine. Though L-α-glutamate, β-glutamate and N(e)-acetyl-β-lysine are synthesized de novo, betaine is preferentially assimilated from the medium (Robertson et al., 1992). In the absence of betaine, N(e)-acetyl-β-lysine is the dominant osmolyte.

Miscellaneous

Methanomicrobium mobile is light sensitive. Growth is inhibited by light in the blue end of the visible spectrum (370 to 430 nm; Olson et al., 1991).

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