Prokaryotes (2006) 3:165–207
DOI: 10.1007/0-387-30743-5_9

CHAPTER 9

The Methanogenic Bacteria

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The methanogenic bacteria are a large and diverse group that is united by three features: 1) They form large quantities of methane as the major product of their energy metabolism. 2) They are strict anaerobes. 3) They are members of the domain Archaea, or archaebacteria, (see Chapter 1) and only distantly related to the more familiar classical bacteria or eubacteria. Like the photosynthetic eubacteria, the methanogenic bacteria are related to each other primarily by their mode of energy metabolism but are very diverse with respect to their other properties.

Methanogenic bacteria obtain their energy for growth from the conversion of a limited number of substrates to methane gas. The major substrates are H_2 + CO_2, formate, and acetate. In addition, some other C-1 compounds such as methanol, trimethylamine, and dimethylsulfide and some alcohols such as isopropanol, isobutanol, cyclopentanol and ethanol are substrates for some methanogens. All of these substrates are converted stoichiometrically to methane. In this regard, the metabolism of the methanogens is strikingly different from that of the so-called "minimethane" producers, which are other anaerobic microorganisms that produce very small amounts of methane as a consequence of side reactions of their normal metabolism (for an overview of the minimethane producers, see Rimbault et al., 1988). Another feature that distinguishes the methanogens from the minimethane producers is that the methanogens are obligate methane-producers, that is, they only grow under conditions where methane is formed.

The list of substrates for growth of methanogens may be divided into three groups (Table 1). In the first group, the energy substrate (electron donor) is H_2, formate, or certain alcohols and the electron acceptor is CO_2, which is reduced to methane. The ability to utilize H_2 as an electron donor for CO_2 reduction is almost universal among methanogens. Likewise, many methanogens also utilize formate, but the ability to utilize alcohols is less common (Bleicher et al., 1989; Zellner and Winter, 1987a). Some methanogens also utilize carbon monoxide as an electron donor, but growth is very slow (Daniels et al., 1977). CO_2 reduction is the major source of methane in certain habitats such as the rumen. In other environments, such as the sediments of freshwater lakes and certain bioreactors, only about one-third of the methane is formed from CO_2 reduction. However, this reaction is still very important for maintaining the very low concentrations of H_2 and formate typical of these anaerobic habitats and facilitating the process of interspecies electron transfer.

In the second group, the energy substrate is one of a variety of methyl-containing C-1 compounds, which can serve as substrates for a few taxa of methanogens. Usually these compounds are disproportionated. Some molecules of the substrate are oxidized to CO_2. The electron acceptors are the remaining methyl groups, which are reduced directly to methane (Table 1). Although dimethylselenide and methane thiol also serve as substrates for methanogenesis, these substrates do not support growth (Kiene et al., 1986). Methanogenesis from C-1 compounds is common where methyl-containing C-1 compounds are abundant. In marine sediments, trimethylamine may be formed from choline, glycine betaine, or trimethylamine oxide. In the large intestine of mammals, methanol may be formed from the anaerobic transformation of the methoxy groups of pectin. Dimethylsulfide is also common in anaerobic environments where it is formed from both methionine and the osmoregulant dimethylsulfoniopropionate.

In the third group, acetate is the major source of methane, but the ability to catabolize this substrate is limited to species of *Methanosarcina* and *Methanosaeta ("Methanothrix").* Acetate is present in many environments, and methane synthesis proceeds by an aceticlastic reaction, in which the methyl carbon of acetate is reduced to methane and the carboxyl carbon is oxidized to CO_2. Methanogenesis from acetate is common in anoxic freshwater sediments where the catabolism of acetate by other anaerobes is limited by the availability of alternate electron acceptors such as sulfate or nitrate.

Methane synthesis is the major source of energy for growth of methanogens. Thus, meth-

Table 1. Reactions and standard changes in free energies for methanogenesis.[a]

Reaction	$\Delta G^{\circ\prime}$ (kJ/mol of methane)
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	–135.6
4 Formate $\rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	–130.1
4 2-Propanol $+ CO_2 \rightarrow CH_4 +4$ Acetone $+ 2\ H_2O$[b]	–36.5
2 Ethanol $+ CO_2 \rightarrow CH_4 +$ 2 Acetate[c]	–116.3
Methanol $+ H_2 \rightarrow CH_4 + H_2O$	–112.5
4 Methanol $\rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	–104.9
4 Methylamine $+ 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4^+$	–75.0
2 Dimethylamine $+ 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_4^+$	–73.2
4 Trimethylamine $+ 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_4^+$	–74.3
2 Dimethylsulfide $+ 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + H_2S$	–73.8
Acetate $\rightarrow CH_4 + CO_2$	–31.0

[a]The standard changes in free energies were calculated from the free energy of formation of the most abundant ionic species at neutral pH. Thus, "CO_2" is $HCO_3^- + H^+$ and formate is $HCOO^- + H^+$.

[b]Other secondary alcohols utilized include 2-butanol, 1,3-butanediol, and cyclopentanol.

[c]Other primary alcohols utilized include 1-propanol and 1-butanol. From Kiene et al. (1986), Nagle and Wolfe (1985), and Widdel (1986).

Table 2. Distinctive features found in various members of the domain Archaea (archaebacteria).

Capability of extreme thermophily in some groups
Lipids composed of glycerol ethers of isoprenoids and tertraethers are common
Stereochemistry of lipids is 2,3-*sn* glycerol
Cell walls composed of protein, glycoprotein, or pseudomurein; murein is absent
Antibiotic sensitivity differs from that of eubacteria
Unique modes of energy metabolism in some groups; i.e., bacteriorhodopsin-driven photosynthesis, methanogenesis

anogenesis may be viewed as a form of anaerobic respiration where CO_2, the methyl groups of C-1 compounds, or the methyl carbon of acetate is the electron acceptor. However, the standard change in free energy ($\Delta G^{\circ\prime}$) during most methanogenic reactions is very small, and the amount of ATP produced per mole of methane is probably close to or less than one (Table 1). In most natural habitats where the concentrations of H_2 and formate are very low, the change in free energy may even be much smaller.

The second distinctive feature of methanogens is their extreme sensitivity to oxygen. Thus, the methanogens are very strict anaerobes, and they are generally present in nature only in anoxic environments. For instance, the half-time for survival of one species of *Methanosarcina* is reported to be only 4 minutes in air-equilibrated medium (Zhilina, 1972). In part, some of the sensitivity of methanogens is probably due to the oxygen lability of many of the enzymes involved in methanogenesis. Other mechanisms are probably important as well. For instance, the methanococcal enzyme acetohydroxy acid synthase—an enzyme in the pathway of branched-chain amino acid biosynthesis—is unusually sensitive to oxygen (Xing and Whitman, 1987). Likewise, many methanogens are unable to utilize oxidized sulfur compounds. Because the most widely used sulfur source, sulfide, reacts chemically with oxygen, anaerobiosis is also required to protect the sulfur source in the medium. Paradoxically, when cultures of some species are washed and resuspended in unreduced medium in the absence of substrates, their viability in unaffected by atmospheric levels of oxygen for at least 30 hours (Kiener and Leisinger, 1983).

The third distinctive feature of the methanogens is that they are archaebacteria (Jones et al., 1987; Woese, 1987). Other archaebacteria include the extreme halophiles and the sulfur-dependent extreme thermophiles. Some properties the methanogens share with other archaebacteria are listed in Table 2 and are described in more detail in subsequent sections. However, the methanogens are different from other archaebacteria because they are abundant in environments of moderate temperature, pH, and salinity. Although some extremely halophilic or extremely thermophilic methanogens have been described, most species are found in moderate environments where they are often closely associated with anaerobic eubacteria and eukaryotes. Likewise, the methanogens are unlike other archaebacteria in that they contain large amounts of coenzymes essential for methane synthesis (see below). Some of these, such as coenzyme F_{420} and coenzyme M, may serve as distinctive biomarkers.

Habitats of Methanogenic Bacteria

Methanogenic bacteria are abundant in habitats where electron acceptors such as O_2, NO_3^-, Fe^{3+} and SO_4^{2-} are limiting. Common habitats for methanogens are anaerobic digestors, anoxic sediments, flooded soils, and gastrointestinal tracts. Methanogens are generally absent from the water column of unstratified lakes and rivers because convection currents rapidly aerate the deep waters. However, the diffusion of O_2 between the layers of stratified lakes is often too slow to maintain oxic conditions in the lower layers. Similarly, the physical structure of sedi-

ments limits dispersive mechanisms, so deeper sediments are often anoxic and harbor methanogens. Soil environments may be divided into the vadose zone, which is not saturated with water, and the zone below the water table, which is saturated. In the vadose zone, O_2 diffusion is rapid and anoxic vadose zones are extremely rare, although anoxic microenvironments may occur. Thus, the activity of methanogens in the vadose zone is limited. O_2 diffusion through water is slower than through soil, so the water-saturated zone is often anoxic. Because air-saturated water at 20°C contains less than 0.3 mM O_2, even small amounts of organic pollution cause groundwater to become anoxic and suitable for development of methanogenic populations.

In axenic culture, methanogenic bacteria are extremely sensitive to small amounts of O_2. However, in natural habitats, the activities of other organisms protect methanogens in apparently oxic environments. For instance, methanogens have been isolated from large dental caries and subgingival plaque in the human mouth (Belay et al., 1988; Brusa et al., 1987). Methanogens may survive in such environments because O_2-uptake by aerobic and euryoxic bacteria creates anoxic microenvironments.

In anoxic environments, the presence of NO_3^-, Fe^{3+}, and SO_4^{2-} inhibits methanogenesis by allowing other organisms to outcompete methanogens for reduced substrates. For instance, in the presence of sulfate, sulfate-reducing bacteria utilize H_2 at concentrations lower than the minimum concentration which can be utilized by methanogens (Kristjansson et al., 1982; Lovley, 1985). Presumably, the ability of the sulfate-reducing bacteria to outcompete the methanogens is a direct consequence of the more-positive reduction potential of SO_4^{2-} compared to that of CO_2.

In environments with sufficient quantities of sulfate, hydrogen sulfide is the predominant reduced product, and the major fate of biodegradable organic carbon is oxidation to CO_2. If sulfate becomes limiting, methane replaces hydrogen sulfide as the reduced product, and the organic carbon is disproportionated to CO_2 and methane. The fate of the methyl group of acetate is an indicator of whether methanogenesis is a dominant catabolic pathway in an ecosystem. If acetate is catabolized by the sulfate-reducing bacteria, CO_2 is formed from the methyl group of acetate. In a methanogenic system, acetate is catabolized by the aceticlastic reaction, which forms methane from the methyl group. Thus, methanogenic degradation of [2-^{14}C]-acetate leads to $^{14}CH_4$, whereas sulfidogenic degradation leads to $^{14}CO_2$, and the ratio of these labeled gaseous products indicates whether methanogenesis predominates (Winfrey and Zeikus, 1979). However, this test fails in some thermophilic ecosystems where acetate is oxidized to CO_2 prior to formation of methane (see below).

Sulfidogenesis normally dominates in estuarine, marine, and hypersaline sediments, where sulfate diffuses from overlying water. Beneath areas of high productivity, such as kelp forests, sulfate may be limiting in the deep organic sediments. Under these conditions, aceticlastic and H_2- or formate-using methanogens develop. Even in surface layers where sulfate is in excess, some methanogenesis may occur from methylated compounds such as trimethylamine and dimethylsulfide. Trimethylamine is often found in marine sediments, where it is formed from betaine glycine or other related osmoprotectants which are produced by marine plants and bacteria to balance the osmolarity of their cytoplasm with that of the seawater. Trimethylamine is not easily utilized by sulfidogenic bacteria, but it is rapidly fermented by methanogens to methane, CO_2, and ammonia. Thus, trimethylamine has been termed a "noncompetitive" substrate for methanogens. Trimethylamine-degrading methanogens from marine environments are all in the family Methanosarcinaceae, and all methanogens that have been isolated to date from hypersaline environments use trimethylamine as catabolic substrate.

Interspecies Electron Transfer and Obligate Syntrophy

Because of their limited substrate range, methanogens depend on fermentative bacteria to convert a wide range of organic compounds into methanogenic substrates. In environments where organic matter is completely degraded to methane and CO_2, the methanogenic precursors are predominantly acetate, formate, and H_2 + CO_2. The organic matter is initially fermented mainly to volatile organic acids, H_2, and CO_2. Methanogens can directly catabolize H_2 + CO_2, formate, and acetate, but longer-chain volatile organic acids (with three or more carbon atoms) such as propionate and butyrate must be metabolized to one or more of these methanogenic precursors by a specialized group of microbes called syntrophs. These syntrophs form methanogenic substrates only in the presence of vanishingly low concentrations of H_2 or formate (for reviews see Boone and Mah, 1988; Wolin and Miller, 1987).

During the catabolism of sugars and amino acids, acetate production is more favorable for fermentative bacteria because an extra ATP is produced from acetyl CoA, which is derived

from pyruvate oxidation. However, if acetate is formed, the cells require an alternative mechanism to reoxidize the NADH generated during the fermentation. Many fermentative bacteria have NADH-linked hydrogenases or formate dehydrogenases, but the thermodynamics for NADH oxidation are unfavorable except at very low concentrations of H_2 or formate. For instance, the H_2 concentration must be less than about 1 μM and the formate concentration less than 100 μM for these substances to be produced from NADH. Thus, in pure cultures, H_2 or formate production from NADH is inhibited by end-product accumulation. However, when these bacteria are grown in coculture with H_2 and formate-utilizing methanogens, the concentrations of H_2 and formate remain low, and they become important products for fermentative bacteria. In such methanogenic cocultures, the fermentative bacteria produce more acetate and less reduced products such as propionate, butyrate, lactate, and ethanol. Although the fermentative bacteria can generally grow without methanogens, additional energy is obtained from phosphoroclastic acetate production when methanogens are present. Because the activities of the methanogens are not required by the fermentative bacteria, this type of interspecies electron transfer is called nonobligate interspecies electron transfer.

A second type of interspecies electron transfer cannot proceed without the activities of H_2 or formate-utilizing species. A specialized group of bacteria called obligate syntrophs oxidize compounds such as propionate, longer-chain volatile organic acids, and aromatic compounds. The obligate syntrophs must dispose of the electrons by the reduction of protons to H_2 or of CO_2 to formate. They lack alternative fermentative reactions and cannot produce other reduced organic compounds. When the concentrations of H_2 and formate are high, end-product inhibition prevents the oxidation of the syntrophic substrates. Thus, propionate and butyrate oxidation are accomplished by obligate syntrophy of fatty acid-oxidizing bacteria and methanogens. The activities of CO_2-reducing methanogens keep the concentrations of H_2 and formate low, allowing the exergonic oxidation of fatty acids by the syntrophs.

Until recently it was assumed that H_2 is the major precursor for CO_2-reduction to methane, although many of the CO_2-reducing methanogens can use H_2 or formate equally well. The butyrate-oxidizing bacterium *Syntrophomonas wolfei* can produce either H_2 or formate, so the relative importance of these substances as interspecies electron carriers may depend on the enzyme kinetics of their production and degradation or the rate of diffusion between cells. Although the diffusion coefficient of formate is only one-fifth as large as that of H_2, the formate concentration may be one hundred times greater than that of H_2. Under this condition, formate may be responsible for the bulk of interspecies electron transfer in some environments (Boone et al., 1989; Thiele and Zeikus, 1988).

Habitats of Special Interest. When organic matter is completely catabolized to methane and CO_2, the major substrates of methanogens are usually acetate, formate, and $H_2 + CO_2$. However, in some environments the growth of aceticlastic methanogens and obligate syntrophs is too slow to maintain a large population in the system. For instance, in the rumen and colon, acetate accumulates to concentrations of 50 to 100 mM. Although this is well above the concentration required for aceticlastic growth of methanogens such as *Methanosarcina,* these organisms do not catabolize significant quantities of acetate because their growth rate on this substrate is too slow to maintain the population in a rapid-turnover ecosystem. However, when methylamine or methanol is present, the cell numbers of *Methanosarcina* in the rumen may reach 10^5 to 10^6 per milliliter because these substrates support a faster growth rate. Propionate and butyrate are also present in the rumen at significant concentrations, but the slowly growing propionate- and butyrate-degrading organisms are not found in abundance. In the rumen, a wide range of CO_2-reducing methanogens may be found, including *Methanobrevibacter ruminantium, Methanobacterium formicicum,* and *Methanomicrobium mobile. Methanobrevibacter* species are the most commonly found CO_2-reducing methanogen in nonruminant intestinal tracts. *Methanosphaera* species have also been isolated from colonic environments; they only grow by using H_2 to reduce methanol to methane.

Methanogenic bacteria form mutualistic associations with rumen, freshwater and marine ciliates and amoebae, growing as ecto- or endosymbionts. The physical associations of these microbes with protozoa may improve the efficiency of interspecies H_2- or formate-transfer. The methanogenic partners in the endosymbioses have been classified as *Methanoplanus endosymbiosus, Methanobrevibacter* species, and *Methanobacterium formicicum.* It is quite possible that in some habitats such as sapropel (aquatic sludge rich in organic matter), the majority of the methanogens are involved in these symbioses (Lee et al., 1987; van Bruggen et al., 1985).

In some environments, such as hot springs and solfataras (volcanic fissures that emit steam and other gases), the predominant substrate is geo-

thermal H_2 rather than decaying organic matter. In such environments, methanogens do not rely on the activities of other organisms for provision of their substrates. The waters near these sources are often thermal as well, and thermophilic methanogens have been isolated from hot springs in Yellowstone National Park (Zeikus et al., 1980) and in Iceland (Huber et al., 1982; Lauerer et al., 1986), and from submarine thermal vents (Jones et al., 1983a).

Atmospheric Methane

At its current atmospheric concentration of 1.7 ppm, methane is the second most abundant carbon-containing gas, and its atmospheric concentration is increasing at a rate of about 1% per year (for a review, see Cicerone and Oremland, 1988). Because methane is a major greenhouse gas, its sources and atmospheric chemistry are of considerable interest. Each year, about 400–640 $\times 10^{12}$ g of methane are released into the atmosphere. Estimates based upon the isotopic composition of the atmospheric methane suggest that about 74% of this methane is derived from recent microbiological activity. The total amount of methane that is produced may be far greater, and in some important habitats, microbial methane oxidation is known to be very significant. Thus, the methane-producing bacteria are an important component of the earth's carbon cycle.

The major sources of atmospheric methane include: enteric fermentations in animals; wetlands such as bogs, swamps, and rice paddies; landfills; and termites. Little methane is obtained from marine systems where competition with the sulfate-reducing bacteria and anaerobic methane-oxidizing organisms limits its production and release, respectively.

Isolation

Methanogenic bacteria are extremely sensitive to oxygen, and strict anaerobiosis and prereduced media are required for their isolation. Two general strategies are employed: 1) Enrichment techniques are generally very successful because few other microorganisms are capable of utilizing the major methanogenic substrates under anaerobic conditions. In addition, the sulfate- and sulfur-reducing bacteria, the denitrifying bacteria, and the photosynthetic bacteria may be further excluded by omitting sulfate, sulfur, and nitrate from the enrichment medium and performing the incubations in the dark. While the chemolithotrophic acetogenic bacteria may also be enriched under these conditions, they may be eliminated in subsequent steps. 2) Antibiotics can be employed as selective inhibitors of the eubacteria. Since the methanogens are archaebacteria, many of their enzyme systems are unaffected by a wide variety of common antibiotics.

Enrichments are performed in media that simulate the source of the sample and mimic the environmental pH, salt concentration, and temperature. The presence of methanogens in the enrichment is determined by gas chromatographic analysis of the headspace for methane. Consumption of $H_2 + CO_2$ or gas production from acetate or methylamines is a less reliable indicator of methanogenic activity. For example, acetogenic bacteria will also consume $H_2 + CO_2$ gas under the same conditions as the methanogens (see Chapter 21). Likewise, in enrichments containing sediments or large amounts of other organic material, gas may be produced by fermentative bacteria. If the samples are believed to contain high numbers of methanogens, they are first serially diluted in medium, and after incubation, the highest dilution with methanogenic activity is processed further. Mineral medium is used to obtain autotrophic methanogens. Although most of the heterotrophic contaminants will be eliminated upon successive transfers, low levels of some contaminants may persist due to growth on exudates of the methanogen or on lysed cells. Because many methanogens have nutritional requirements for low levels of acetate, amino acids, volatile fatty acids, or vitamins, a complex enrichment medium is often more suitable. For very fastidious methanogens, the enrichment medium may also be amended with 30% rumen fluid or sludge extract (Mah and Smith, 1981). In this case, the heterotrophic contaminants must be eliminated by colony isolation on solid medium. Antibiotics may also reduce the numbers of contaminants. However, because of the rapid selection of antibiotic resistant contaminants, they are seldom sufficient alone for the isolation of pure cultures.

Antibiotics are especially useful when used in conjunction with enrichment techniques. Because some antibiotics are toxic to certain methanogens (Böck and Kandler, 1985), it is also useful to test their effects on the enrichment culture. Success has been obtained with a variety of antibiotics, used either singly or in combination (Table 3). However, to avoid the selection of resistant contaminants, the antibiotics are only utilized for one or two transfers. In addition, the antibiotics must be omitted from the medium when determining the purity of the isolated methanogen.

When a successful enrichment has been obtained, the methanogen may be observed by fluorescence microscopy (Doddema and Vogels, 1978). For optimal fluorescence, the excitation

Table 3. The use of antibiotics for isolation of methanogens.

Antibiotic (μg/ml)	Source of sample	Kind of methanogen isolated
Vancomycin (100)	Marine sediment	*Methanosarcina acetivorans*[a]
Marine sediment		*Methanoccoccoides methylutens*[b]
Sewage sludge		*Methanosaeta soehngenii*[c]
Sewage sludge		"*Methanothrix*" sp.[d]
Rabbit feces		"*Methanosphaera cunicuh*"[e]
Vancomycin (200)	Sewage sludge	*Methanosarcina* sp.[f]
Vancomycin (500)	Sewage sludge	*M. mazei*[g]
Cephalothin (1.7) and clindamycin (6.7)	Human feces	*Methanobrevibacter smithii*[h]
Human feces		*Methanosphaera stadtmaniae*[i]
Rabbit feces		"*M. cunicuh*"[e]
Groundwater		*Methanobacterium bryantii*[j]
Cycloserine (100) and penicillin G (2,000)	Sewage sludge	*Methanosarcina thermophila*[k]
Lake sediment		*Methanocorpusculum labreanum*[l]
Penicillin G (2,000)	Sewage sludge	*Methanobacterium* sp.[m]
Cyanobacterial mat		*Methanococcus halophilus*[n]
Penicillin G (1,000 units/ml)	Protozoan coculture	*Methanoplanus endosymbiosus*[o]
Protozoan coculture		*Methanobacterium formicicum*[p]
Penicillin G (50)	Pasture mud	*M. thermoaggregans*[q]
Penicillin G (30–100) and vancomycin (50)	Marine sediment	*Methanococcus thermolithotrophicus*[r]
Marine sediment		*Methanosarcina frisia*[s]
Cattle manure		*Methanobacterium thermoalcaliphilum*[t]
Kanamycin (100)	Sewage sludge	*Methanosaeta concilii*[u]
Vancomycin (150), penicillin G (150), kanamycin (150), and tetracycline (100)	Drilling-waste swamp	*Methanoplanus limicola*[v]
Penicillin G (200), erythromycin (200), and streptomycin sulfate (200)	Salt-marsh sediment	*Methanococcus* spp.[w]

References are: [a]Sowers et al., 1984a; [b]Sowers and Feny, 1983; [c]Huser et al., 1982; [d]Ahring and Westermann, 1984; [e]Biavati et al., 1988; [f]Touzel et al., 1985; [g]Touzel and Albagnac, 1983; [h]Miller and Wolin, 1982; [i]Miller and Wolin, 1985; [j]Godsy, 1980; [k]Zinder and Mah, 1979, [l]Zhao et al., 1989; [m]Zhao et al., 1986; [n]Zhilina, 1983; [o]van Bruggen et al., 1986; [p]van Bruggen et al., 1984; [q]Blotevogel and Fischer, 1985; [r]Huber et al., 1982, [s]Blotevogel et al., 1986; [t]Blotevogel et al., 1985; [u]Patel, 1984; [v]Wildgruber et al., 1982; [w]Whitman et al., 1986.

wavelength should be between 350 and 420 nm. Under these conditions, most species autofluoresce a blue-green color due to an abundance of coenzyme F_{420}. However, the intensity of the fluorescence varies greatly, and it may not be observed following growth of *Methanosaeta* on acetate (Zehnder et al., 1980). The fluorescence may also rapidly fade within a few seconds or be absent in older and inactive cells. In spite of these limitations, fluorescence microscopy is useful for determining if more than one type of methanogen is present in an enrichment or for checking the relative purity of the culture.

The isolation of single colonies on solid medium is usually necessary for obtaining axenic cultures of methanogens. Solid medium is prepared by the roll-tube technique or in petri plates in an anaerobic glove box. One advantage of roll tubes is that colonies of methanogens may be frequently visualized through the wall of the tube by their fluorescence. In any case, the anaerobic techniques must be stringent enough to ensure that the plating efficiency is high, since, otherwise, most of the colonies may contain contaminants that are less oxygen-sensitive than the methanogen. In a minimal medium, it may not be possible to obtain isolated colonies because the methanogen may require a nutrient produced by a contaminating organism. For instance, in media lacking acetate, this nutrient may be produced by the fermentation of amino acids by a contaminating bacterium. Under such conditions, if acetate is required for growth of the methanogen, colonies will contain a mixture of both organisms.

In addition to the normal microbiological tests for axenic cultures, certain growth tests are useful to establish culture purity. Growth should not be observed in complex medium prepared without the substrates for methanogenesis. The medium is prepared with the same salt and trace nutrient composition as the enrichment medium, but 0.2–0.4% trypticase peptones or casamino acids and 0.2% glucose are added. Yeast extract, a source of B-vitamins, frequently contains small amounts of formate and acetate and should be avoided. Growth in a complex medium without a methanogenic substrate indicates the presence of a heterotrophic contaminant. The plating efficiency of the methanogenic culture in the enrichment medium should also be greater than 50%. If it is much lower, it is possible that growth is dependent upon crossfeeding between the methanogen and another organism.

Techniques for Culturing Methanogenic Bacteria

The methodology for culturing methanogenic bacteria is based upon the pioneering work of Hungate on anaerobic rumen bacteria (1969). Anaerobiosis is achieved by the replacement of air with oxygen-free gases and the addition of a reducing agent. The culture vessels are made of glass and sealed with butyl rubber stoppers. Oxygen-permeable rubber and plastic are avoided whenever possible. Media are dispensed in an anaerobic glove box, under a stream of oxygen-free gas, or with a glass syringe. Commercial gases are scrubbed to remove traces of contaminating oxygen. Details of the procedures are described in the primary literature (Balch and Wolfe, 1976; Macy et al., 1972) and in several recent reviews (Ljungdahl and Wiegel, 1986; Mah and Smith, 1981).

Enrichment Media for Growth of Methanogens

A large number of different types of media have been formulated for the growth of methanogens. Many are described in the original species descriptions cited elsewhere in this chapter. Therefore, only a few general media will be described. Although most methanogens will grow in one of these media, optimal growth may require modification of the concentrations of some of the components or special additions.

Medium 1 for Freshwater, Sewage, and Intestinal Species

Its composition is similar to media described earlier (Balch et al., 1979; Mah and Smith, 1981). Its composition is (per liter of medium):

Yeast extract	2.0 g
Trypticase peptones	2.0 g
Salt solution A (see below)	10 ml
Phosphate solution (200 g/l of $K_2HPO_4 \cdot 3H_2O$)	2 ml
Resazurin solution (0.5 g/l in water)	2 ml
Sodium acetate solution (136 g/l of Na acetate $\cdot 3H_2O$)	10 ml
Trace element solution (see below)	10 ml
Vitamin solution (see below)	10 ml
$NaHCO_3$	5.0 g
Cysteine/hydrochloride	0.5 g
Sulfide solution (see below)	20 ml

Salt solution A is composed of (per liter):

NH_4Cl	100 g
$MgCl_2 \cdot 6H_2O$	100 g
$CaCl_2 \cdot 2H_2O$	40 g

After dissolving the salts, the pH is adjusted to 4 with HCl.

The trace element solution is modified from Wolin et al. 1963 and is composed of (per liter):

Nitrilotriacetic acid	1.5 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.2 g
Na_2SeO_3	0.2 g
$CoCl_2 \cdot 6H_2O$	0.1 g
$MnSO_4 \cdot 2H_2O$	0.1 g
$Na_2MoO_4 \cdot 2H_2O$	0.1 g
$Na_2WO_4 \cdot 2H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$AlCl_3 \cdot 6H_2O$	0.04 g
$NiCl_2 \cdot 6H_2O$	0.025 g
H_3BO_3	0.01 g
$CuSO_4 \cdot 5H_2O$	0.01 g

To prepare the trace element 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 is modified from Bryant et al. 1971 and is composed of (per liter):

p-Aminobenzoic acid	10 mg
Nicotinic acid	10 mg
Calcium pantothenate	10 mg
Pyridoxine hydrochloride	10 mg
Riboflavin	10 mg
Thiamine hydrochloride	10 mg
Biotin	5 mg
Folic acid	5 mg
α-Lipoic acid	5 mg
Vitamin B_{12} (stored in dark at 5°C)	5 mg

The sodium sulfide solution is prepared in anoxic water under N_2 gas. A 10 mM solution of NaOH, 110 ml, is boiled under a stream of N_2 gas until the volume is reduced to 100 ml. It is then allowed to cool under a stream of N_2 gas in a fume hood. Because sodium sulfide is very toxic, precautions must be taken to avoid contact with the solid and the solution, as well as inhalation of the vapor. To remove sulfur oxides from the surface, a large crystal of Na_2S9H_2O (about 3 g) is washed in 50 ml of water for a few seconds. The crystal is blotted dry on a paper towel and weighed. About 2.5 g should remain and is immediately added to the anaerobic solution of NaOH. The sulfide solution is then dispensed anaerobically into tubes or bottles, pressurized with 100 kPa of N_2, and autoclaved. If an anaerobic glove box is available, the tubes or bottles and their stoppers should be placed in the glove box 24 hours before use to remove adsorbed O_2. The stoppers must fit tightly and should not be greased. They may be lubricated with anaerobic water to facilitate insertion and twisting into place. After autoclaving, the sulfide solution may be stored for up to two weeks or until the solution becomes cloudy. For organisms like Methanococcus, best results are obtained if the sulfide solution is stored in the anaerobic chamber immediately after autoclaving.

Medium 2 for Methanogens Isolated from Marine Environments

It is modified from Romesser et al. 1979, and it is composed of (per liter):

Salt solution B (see below)	500 ml
Phosphate solution (14 g/l of $K_2HPO_4 \cdot 3H_2O$)	10 ml
Trace element solution	10 ml

Vitamin solution	10 ml
Iron stock solution (see below)	5 ml
Sodium acetate solution (136 g/l of sodium acetate $\cdot 3H_2O$)	10 ml
Resazurin solution (0.5 g/l in water)	2 ml
NaCl solution (293 g/l)	75 ml
Yeast extract	2 g
$NaHCO_3$	5 g
Cysteine hydrochloride	0.5 g
Sulfide solution	20 ml

Salt solution B is composed of (per liter):

$CaCl_2 \cdot 2H_2O$	0.28 g
KCl	0.67 g
NH_4Cl	1.00 g
$MgCl_2 \cdot 6H_2O$	5.50 g
$MgSO_4 \cdot 7H_2O$	6.90 g

To prepare the iron stock solution, add 0.2 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ to a screw-top bottle. Then add 0.1 ml of concentrated HCl followed by 100 ml of H_2O. The solution should be replaced every four weeks.

Medium 3 for Moderately Halophilic Methanogens

It contains the same components as Medium 1 except that 88 g/l of NaCl is added.

As described above, the media do not contain a substrate for methanogenesis. For growth on H_2 + CO_2 (80 : 20 vol/vol), the gas mixture is added after dispensing and sterilizing the medium, and the culture tubes are pressurized to 100 kPa above atmospheric pressure. After inoculation, the culture tubes are pressurized to 240 kPa. For growth on other substrates, the gas atmosphere is 100 kPa of N_2 + CO_2 (80:20 vol/vol), and the $NaHCO_3$ concentration is reduced to 2 g/l. For growth on acetate, 50 ml of the sodium acetate solution (1 M) is added. For growth on trimethylamine/hydrochloride, 12 ml of a 1 M solution (95.5 g/l) is added. For growth on methanol, 4 ml of methanol is added per liter. For growth on 2-propanol, 1-propanol, ethanol, 2-butanol, and 1-butanol, the alcohols are added at a final concentration of 20–30 mM (Widdel, 1986). For growth on formate, 4 g/l of sodium formate is added. To control the pH in batch cultures, a formic-acid reservoir may also be added to the culture tube (Schauer and Whitman, 1989). Regardless of the substrate, care must be taken to avoid exceeding the pressure limit of the culture vessels. For instance, 10 ml of medium containing 50 mM acetate will generate nearly 15 ml of gas if the acetate is completely converted to CO_2 and CH_4. Therefore, the headspace of the culture vessel must be large enough to prevent an excessive increase in pressure, which could lead to an explosion. To minimize this danger, the culture vessels should be handled in wire baskets or metal cans. During growth on H_2 + CO_2, the opposite problem occurs. Because the methanogens consume 5 moles of gas for every mole of CH_4 produced, the vessel will quickly develop a negative pressure. Also, as the partial pressure of CO_2 decreases, the medium will become strongly alkaline, which may inhibit growth and cause 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 vessel should be pressurized periodically throughout the growth period. For better control of the pH, the headspace may also be repressurized with H_2 + CO_2 (75 : 25 vol/vol) to fully replenish the CO_2 consumed. For the thermophilic species, attention must also be given to the expansion of gas at higher temperatures.

All three media may be prepared by the same method. All the components are combined except the gaseous substrates and the reducing agents, cysteine hydrochloride and the sulfide solution. The medium is then brought to a boil under a stream of N_2 + CO_2 gas (80 : 20 vol/vol) and immediately removed from the heat. Solid cysteine hydrochloride is added, and the medium is allowed to cool under a stream of N_2 + CO_2. During this time, the oxygen indicator resazurin changes from blue to pink to colorless. The change from blue to pink occurs upon the pH-dependent formation of resorufin. The change from pink to clear occurs upon the reduction of resorufin to dihydroresorufin. When the medium has cooled to about 50°C, it can be dispensed anaerobically to the culture tubes or bottles under a stream of O_2-free N_2 + CO_2 gas or in an anaerobic glove box (Ljungdahl and Wiegel, 1986; Mah and Smith, 1981). For growth on H_2 + CO_2, the gas atmosphere of the culture tubes is exchanged before autoclaving. After autoclaving, the pH of the bicarbonate-CO_2 buffered medium is 6.8–7.0.

The media may be stored for several months in the anaerobic glove box or for about one week on the laboratory bench prior to the addition of the sulfide solution. One hour before inoculation, one part of the sterile sulfide solution is added anaerobically to 50 parts of sterile medium. Some species appear to be especially sensitive to the products of sulfide oxidation. When small inocula are used, more reproductible results are obtained by adding the sulfide solution 24 hours before the inoculation.

For growth of fastidious methanogens, the media may be amended (per liter) with 100–300 ml of clarified rumen fluid, 10 ml of a volatile fatty acid mixture, or 2 ml of boiled cell extract (BCE) of a methanogen (Mah and Smith, 1981; Tanner and Wolfe, 1988). To prepare rumen fluid, samples of the rumen contents are obtained from a fistulated animal, a mouth tube, or a slaughtered animal. The fluid is separated from the large solids by filtration through 8 layers of cheese cloth into an Erlenmeyer flask. The fluid

is then centrifuged under anoxic conditions at 10,000 × *g* for 20 minutes to remove microbial cells and small particulate matter. The supernatant is decanted and sparged slowly with CO_2 overnight. Then it is dispensed into serum bottles, flushed with CO_2, and autoclaved. The volatile fatty acid mixture is modified from Bryant et al. 1971 and is composed as follows: 46 ml of butyric, 46 ml of isobutyric, 55 ml of isovaleric, 55 ml of DL-2-methylbutyric, 37 ml of propionic, and 54 ml of valeric acids are added to 500 ml of water in a fume hood, neutralized (by litmus) with 2 M NaOH, and brought to a final volume of 1 liter.

Some methanogens require growth factors that are only produced by other methanogenic bacteria. These factors may be obtained from rumen fluid or BCE (Tanner and Wolfe, 1988). To prepare BCE, 10 g wet weight of methanogen cells are resuspended in 20 ml of anaerobic 20 mM potassium phosphate buffer, pH 7.0, and incubated under a stream of N_2 gas in a boiling water bath for one hour. After cooling, the suspension is centrifuged anaerobically at 20,000× *g* for 30 min at 4°C. The resulting supernatant, BCE, is stored anaerobically at –20°C.

Mineral media may be prepared by omitting the organic supplements: yeast extract, trypticase, acetate, and cysteine. However, cysteine also functions as a reducing agent. Therefore, the medium will remain oxidized until the sulfide solution is added. In this case, the sulfide solution should be added 24 h before inoculation.

For growth of the alkalophilic methanogens, the medium is prepared under a N_2 atmosphere. The $NaHCO_3$ concentration is increased to 10 g/l (pH 8.5) or replaced with 3 g/l of $NaHCO_3$ plus 2 g/l of Na_2CO_3 (pH 9.3; Mathrani et al., 1988; Worakit et al., 1986). If H_2 is the substrate, the culture is initially pressurized with 50 kPa H_2. CO_2 is not added initially to avoid acidification of the medium. The gas is then replenished with H_2 + CO_2 (75 : 25 vol/vol) during growth, stoichiometrically replacing the CO_2 reduced to methane. For mass culture, gas additions are controlled by a pH auxostat (Boone et al., 1987).

Solid medium is prepared by the addition of agar or Gelrite during the medium preparation. For roll tubes or bottles, the medium plus sulfide with 2% agar or 1% Gelrite is allowed to coat the walls to provide a surface for inoculation (Harris, 1985; Hermann et al., 1986; Hungate, 1969). The agar surface may be streaked or the cells may be added as a soft agar overlay. Alternatively, molten agar may be cooled to 45°C and inoculated prior to solidification. Petri plates containing 0.8–2% agar or Gelrite (Kelco Div., Merck & Co., Inc., San Diego, Calif.) may be prepared in an anaerobic glove box (Balch et al., 1979). In this case, the plates are prepared without sulfide (Jones et al., 1983c). After inoculation, the plates are pressurized with H_2 + CO_2 or N_2 + CO_2 in a cannister and removed from the chamber. An open tube containing 0.75 ml of 20% $Na_2S \cdot 9H_2O$ per liter of cannister volume is added just prior to pressurization. Because the CO_2 neutralizes the sulfide solution, volatile H_2S (about 1.5%) is generated inside the cannister. Because H_2S is toxic, the cannister must either be opened in a fume hood or be flushed with N_2 + CO_2 gas before opening in the anaerobic glove box.

Measurement of Growth

In many cases, the growth of methanogens can be measured by normal microbiological techniques. However, some organisms grow as aggregates or have very low cell yields, and it is difficult to measure growth turbidimetrically or by other common techniques. An alternative is to measure CH_4 accumulation in the headspace by gas chromatography. However, whenever product formation is used to estimate growth rate, it is important to insure that cell growth is balanced, that is, product formation is proportional to the increase in cell mass. Thus, control experiments should demonstrate a correlation between CH_4 formation and cell mass. This method is additionally complicated because upon inoculation, only cells and not the initial product are transferred (Powell, 1983). Therefore, plots of the logarithm of CH_4 accumulated versus time are nonlinear for about four generations, and the growth curves appear to be convex. This artifact results in an overestimate of the specific growth rate. To correct for this effect, the logarithm of the rate of methane formation versus time should be plotted (Powell, 1983). This replot is linear, and the specific growth rate is equal to the slope.

Maintenance of Stock Cultures

Many of the same techniques used for storage of stock cultures of other anaerobes are suitable for at least some of the methanogens (Hippe, 1984). While freezing in liquid nitrogen is a very reliable method, less-expensive alternatives may be employed for laboratory collections. Lyophilization in horse serum plus 7.5% glucose and 3 mg/ml of ferrous sulfide followed by storage at either 8° or –70°C is suitable for many species of Methanobacteriaceae and *Methanosarcina* (Hippe, 1984). However, this method is less effective for *Methanococcus* and *Methanospirillum.* Many methanogens can also be stored on agar slants at 4°C for one year if oxygen is excluded (Winter, 1983). Likewise, storage at –18°C in medium containing 50% glycerol in sealed glass ampules

is effective for 20 months. *Methanococcus* cultures can be preserved in medium containing 25% glycerol in screw-top vials at –70°C for 30 months without special anaerobic precautions (Whitman et al., 1986). *Methanobrevibacter* species and some other methanogens can be stored in biphasic cultures at –76°C for 6–12 months (Miller, 1989). Culture tubes are prepared with a slant of double-strength medium containing 3% agar and twice the usual concentration of reducing agents. One-third volume of reduced, single-strength broth is added. The tube is inoculated and allowed to grow to a heavy density. The culture is then cooled for 1 hour at 4°C prior to freezing at –76°C.

Cultures of the type strains of most species of methanogens are available through the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG) and the OGC Collection of Methanogenic Archaeobacteria (Oregon Graduate Institute, Beaverton, Oregon, USA).

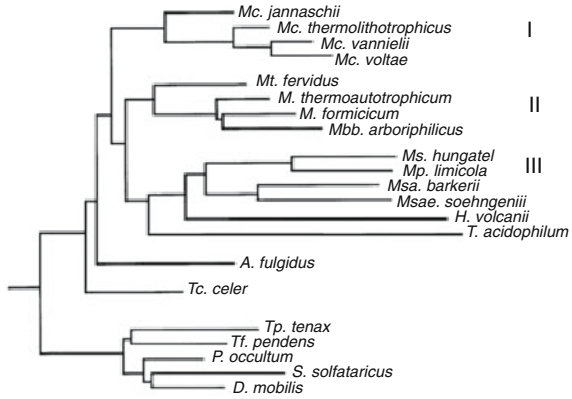


Fig. 1. Phylogenetic tree for the methanogenic bacteria and other archaebacteria based upon 16S rRNA sequences. *Mc. Methanococcus; Mt., Methanothermus; M., Methanobacterium; Mbb., Methanobrevibacter; Ms., Methanospirillum; Mp., Methanoplanus; Msa., Methanosarcina; Msae., Methanosaeta; H., Halobacterium; T., Thermoplasma; A., Archaeoglobus; Tc., Thermococcus; Tp., Thermoproteus; Tf., Thermofilum; P., Pyrodictium; S., Sulfolobus;* and *D., Desulfurococcus.* (P. E. Rouvière and C. R. Woese, personal communication.)

Identification

Three major difficulties are encountered in the identification of isolates of methanogenic bacteria: 1) Most of the species descriptions are based upon the examination of only a few strains. Therefore, it is frequently not known if the phenotypic characterization is representative of the species. 2) Probably only a fraction of the methanogens in nature have been described. Therefore, a new isolate may represent an undescribed species or subspecies. 3) Because of their restricted catabolism coupled with extreme genetic diversity, phenotypic characters alone are often insufficient to identify methanogens. For these reasons, phylogenetic methods such as DNA-DNA hybridization and ribosomal RNA sequence analysis are frequently necessary for a definitive assignment (Boone and Whitman, 1988). Other techniques that have proven useful in the identification of methanogens include analysis of their antigenic relationships, polyamine content, molecular weights of the methylreductase subunits, and molecular weights of the polar lipids. Cross-reactivity of specific immunoglobulins has proven useful for identifying methanogens at the species and genus level (Conway de Macario et al., 1981). For distinguishing among members of different families, data on the relative distribution of putrescine, spermidine, spermine, and homospermidine, as well as the molecular weight of the small subunit of component C of the methylreductase, are useful (Kneifel et al., 1986; Rouvière and Wolfe, 1987). Likewise, the distribution of polar lipids distinguishes the families of methanogens (Koga et al., 1987; Morii et al., 1988).

Nineteen genera and more than 50 species of methanogenic bacteria have been described. The current taxonomy reflects the phylogeny described by the oligonucleotide catalogs of the 16S rRNAs (Balch et al., 1979). More recent information, which includes nearly complete sequences of the 16S rRNA and many new species, has confirmed many of these earlier conclusions. The current phylogenetic tree supports the classification of the methanogens into three major groups that correspond to the orders proposed by Balch et al., 1979 (Fig. 1). The orders have been further divided into six families, and the creation of additional families may be warranted to include other deep branches of the major lineages. However, the proposal to place *Methanoplanus* in a separate family (Wildgruber et al., 1982) is not supported by the most recent information.

At present, three orders and six families are recognized within the methanogens (Tables 4 and 5). As indicated in the footnotes to Table 4, most genera have not been formally assigned to an order and family. While the present scheme is consistent with the current phylogenetic and phenotypic data, future changes are to be expected.

Two families, the Methanobacteriaceae and the Methanothermaceae, are closely related. These methanogens possess cell walls composed in part of pseudomurein (Kandler and König, 1985). The cell walls of the Methanothermaceae also contain an additional surface layer composed of protein. The family Methanothermaceae contains one genus, *Methanothermus,*

Table 4. Major taxonomic groups of methanogenic bacteria.

Order	Family	Genus
Methanobacteriales	Methanobacteriaceae (type)[a]	*Methanobacterium* (type)[a]
	″	*Methanobrevibacter*
	″	*Methanosphaera*[b]
	Methanothermaceae	*Methanothermus* (type)
Methanococcales	Methanococcaeae (type)	*Methanococcus* (type)
Methanomicrobiales	Methanosarcinaceae	*Halomethanococcus*[b]
	″	*Methanococcoides*
	″	*Methanohalobium*[b]
	″	*Methanohalophilus*[b]
	″	*Methanolobus*
	″	*Methanosarcina* (type)
	″	*Methanosaeta* (“*Methanothrix*”)
	Methanomicrobiacaea (type)	*Methanoculleus*[c]
	″	*Methanogenium*
	″	*Methanolacinia*[c]
	″	*Methanomicrobium* (type)
	″	*Methanoplanus*[b]
	″	*Methanospirillum*
	Methanocorpusculaceae	*Methanocorpusculum* (type)

[a]Type family of the order or type genus of the family.
[b]Not formally placed within the family.
[c]While not formally placed within the family, it includes species originally placed within a genus of the family.

Table 5. Some characteristics of the methanogen families.

Family	Characteristics
Methanobacteriaceae[a]	Long or short rods, which use $H_2 + CO_2$ and sometimes formate or alcohols as substrates for methanogenesis; cocci, which utilize only H_2 + methanol; mostly Gram-positive; contain pseudomurein; nonmotile; GC content, 23–61 mol%.
Methanothermaceae	Rods; substrate for methanogenesis is $H_2 + CO_2$; Gram-positive; contain pseudomurein; nonmotile; extreme thermophiles; GC content, 33–34 mol%.
Methanococcaceae	Irregular cocci; substrates for methanogenesis are $H_2 + CO_2$ and formate; Gram-negative; motile; GC content, 29–34 mol%.
Methanomicrobiaceae	Rods, spirals, plates, or irregular cocci; substrates for methanogenesis are $H_2 + CO_2$, frequently formate, and sometimes alcohols; Gram-negative; motile or nonmotile; GC content, 39–61 mol%.
Methanocorpusculaceae	Small, irregular cocci; substrates for methanogenesis are $H_2 + CO_2$, formate, and sometimes alcohols; Gram-negative; motile or nonmotile; GC content, 48–52 mol%.
Methanosarcinaceae	Pseudosarcina, irregular cocci, sheathed rods; substrates for methanogenesis are sometimes $H_2 + CO_2$, acetate, and methyl compounds; formate is never used; Gram-positive or negative; frequently nonmotile; GC content, 36–52 mol%.

[a]Including *Methanosphaera*.

both species of which are extremely thermophilic bacilli with temperature optima of 83–88°C. Like in many of the Methanobacteriaceae, the only substrate for methanogenesis is $H_2 + CO_2$. The family Methanobacteriaceae contains two genera, composed of thermophilic as well as mesophilic species. These genera, *Methanobacterium* and *Methanobrevibacter,* are bacilli that utilize either $H_2 + CO_2$ alone or $H_2 + CO_2$ and formate as substrates for methanogenesis. An additional genus, *Methanosphaera,* is closely related to the *Methanobacteriaceae,* although it has not yet been formally placed within this family. It includes two coccoid species that utilize H_2 + methanol as substrates. These organisms are unique in that they are incapable of both the reduction of CO_2 to methane and the oxidation of methanol to CO_2 (Miller and Wolin, 1983).

The family Methanococcaceae contains one genus of irregular cocci, *Methanococcus,* most species of which utilize both $H_2 + CO_2$ and formate as substrates. The six known species of *Methanococcus* contain protein cell walls and are either mesophilic or extremely thermophilic. Most species are also autotrophic. By the criterion of the 16S rRNA sequences, this genus is very diverse (Fig. 1). To maintain genera of equal phylogenetic depth, the two thermophilic species should be placed in two new genera.

The family Methanosarcinaceae contains all the methanogens capable of utilizing acetate as

a substrate for methanogenesis or oxidizing the methyl groups of C-1 compounds. Formate is not utilized by any species in this family and most species are also unable to grow on $H_2 + CO_2$. Seven genera have been described. The genus *Methanosarcina* contains six species of cocci, which may occur singly, in packets, or in large pseudoparenchyma (Zhilina, 1976). The aggregates are held together by methanochondroitin, a polymer of N-acetyl-D-galactosamine and D-glucuronic acid similar to animal chondroitin (Kreisl and Kandler, 1986). Pseudomurein is absent. Pseudoparenchyma formation is species and strain specific and varies even in strains that do form aggregates, depending upon the culture conditions and growth stage. Therefore, the absence of aggregation is not sufficient evidence to exclude an isolate from this genus. Another typical property of this genus is the ability to utilize both acetate and C-1 compounds as substrates for methanogenesis. In addition, some species also utilize $H_2 + CO_2$. Other genera in this family that contain cocci are distinctive in their ability to utilize only C-1 compounds as substrates for methanogenesis. These genera include *Methanolobus, Methanococcoides, Methanohalobium, Halomethanococcus,* and *Methanohalophilus.* The last three genera contain the moderately and extremely halophilic species of methanogens, but have not been formally placed within this family. An additional species, *Methanococcus halophilus,* should probably also be classified with these organisms on the basis of its 5S rRNA sequence (Lysenko and Zhilina, 1985). The last genus, *Methanosaeta* ("*Methanothrix*"), includes large sheathed rods that can only utilize acetate as a substrate for methanogenesis.

The family Methanomicrobiaceae contains six genera of different morphological types with similar physiology. With one exception, all species utilize both $H_2 + CO_2$ and formate as substrates. Two genera, *Methanogenium* and *Methanoculleus*, are cocci that can be distinguished from the methanococci by their slower growth rates under optimal conditions, more complex nutritional requirements, higher GC content, and lower NaCl requirements. The genus *Methanospirillum* has a distinctive spiral morphology, and the genus *Methanoplanus* contains plate-like or irregular disk-shaped species. Lastly, the genera *Methanomicrobium* and *Methanolacinia* contain rod-shaped species. These species may be distinguished from *Methanobrevibacter* because they stain Gram-negative due to the absence of pseudomurein and they have a higher GC content.

The family Methanocorpusculaceae is related to the family Methanomicrobiaceae. It contains one genus, *Methanocorpusculum,* of very small cocci. The complex nutritional requirements of the species of this family distinguishes them from species of *Methanococcus.* The absence of a requirement for NaCl for good growth distinguishes them from species of *Methanogenium* and *Methanoculleus.*

Characteristics of Methanogenic Bacteria

Some of the most important and most distinctive features of methanogenic species will be described in this section. More detailed descriptions of the various taxa of bacteria may be found in the original citations, which are included in the footnotes to Tables 6 to 16. Whenever possible, the descriptions summarize the properties of all the described strains as well

Table 6. Some characteristic properties of species of the genus.

Species	Cell width (μm)	Cell length (μm)	Temperature optimum (°C)	pH optimum	Required organic growth factors[a]	GC content (mol%)
alcaliphilum	0.5–0.6	2–25	37	8.1–9.1	Peptone	57
bryantii	0.5–1.0	10–15	37–39	6.9–7.2	None	33–38
espanolae	0.8	6	35	5.6–6.2	B-vit, (Ac)	34
formicicum	0.4–0.8	2–15	37–45	6.6–7.8	None	38–42
ivanovii	0.5–0.8	1.2	37–45	7.0–7.4	None	37
"palustre"	0.5	2.5–5	37	7.0	None	34
thermoaggregans	0.4	4–8	65	7.0–7.5	None	42
thermoalcaliphilum	0.3	3–4	58–62	7.5–8.5	YE	39
thermoautotrophicum	0.4–0.6	3–120	65–70	7.2–7.6	None	48–52
thermoformicicum	0.3–0.6	2–120	45–60	7.0–8.0	None	43
uliginosum	0.2–0.6	2–4	37–40	6.0–8.5	None	29–34
wolfei	0.4	2.5	55–65	7.0–7.7	None	61

[a]None, autotrophic growth with CO_2 as sole carbon source; YE, yeast extract; B-vit, B-vitamins are stimulatory; (Ac), acetate added to medium but a growth requirement was not reported.

References: Belyaev et al., 1983, 1986; Blotevogel and Fischer, 1985; Blotevogel et al., 1985; Boone et al., 1986; Bryant and Boone, 1987b; Bryant et al., 1971; Bryant et al., 1967; Jain et al., 1987; König, 1984; Patel et al., 1990; Winter et al., 1984; Worakit et al., 1986; Zeikus and Wolfe, 1972; Zellner et al., 1989a; Zhao et al., 1986; Zhilina and Ilarionov, 1984.

Table 7. Some characteristic properties of species of the genus *Methanobrevibacter.*

Species	Cell width (μm)	Cell length (μm)	Temperature optimum (°C)	Catabolizes formate[a]	Required or stimulatory growth factors[b]	GC content (mol%)
ruminantium	0.7	0.8–1.7	37–39	+	Ac, MB, CoM, peptone	31
arboriphilicus	0.5	1–3	30–37	–	None	28–32
smithii	0.5–0.7	1–1.5	37–39	+	Ac, Peptone, YE, B-vit	28–31

[a]+, growth; –, no growth—with formate as an electron donor for methanogenesis.
[b]None, autotrophic growth with CO_2 as a sole carbon source; Ac, acetate; B-vit, B-vitamins; CoM, coenzyme M; MB, 2-methylbutyrate; YE, yeast extract.
References: Bryant et al., 1971; Lovley et al., 1984; Miller et al., 1982, 1986; Smith and Hungate, 1958; Zeikus and Hennig 1975.

Table 8. Some characteristic properties of species of the genus *Methanosphaera.*

Species	Cell diameter (μm)	Temperature optimum (°C)	pH optimum	Stimulatory or required growth factors[a]	GC content (mol%)
stadtmaniae	1.0	37	6.5–6.9	Ac, CO_2, ile, leu, thiamin, biotin	26
"cuniculi"	0.6–1.2	35–40	6.8	Ac[b]	23

[a]Ac, acetate; ile, isoleucine; leu, leucine.
[b]The requirement for other growth factors was not tested. The medium contained trypticase and yeast extract.
References: Biavati et al., 1988; Miller and Wolin, 1985.

Table 9. Some characteristic properties of species of the genus *Methanothermus.*

Species	Cell width (μm)	Cell length (μm)	Temperature optimum (°C)	pH optimum	Required organic growth factors[a]	GC content (mol%)
fervidus	0.3–0.4	1–3	83	6.5	YE	33
sociabilis	0.3–0.4	3–5	88	6.5	None	33

[a]None, autotrophic growth with CO_2 as the sole carbon source. YE, yeast extract.
References: Lauerer et al., 1986; Stetter et al., 1981.

Table 10. Some characteristic properties of species of the genus *Methanococcus.*

Species	Cell diameter (μm)	Temperature optimum (°C)	pH optimum	Optimal salinity (M or NaCl)	Required organic growth factors[a]	GC content (mol%)
vannielii	1.3	35–40	7.0–9.0	0.1–0.4	None	33
voltae	1.5	35–40	6.5–8.0	0.2–0.4	Ac, ile, leu	29–32
maripaludis	1.0	35–40	6.5–8.0	0.2–0.4	None	33–34
thermolithotrophicus	1.0	65	7.0	0.3–0.7	None	34
jannaschii	1.0	85	6.0–7.0	0.4–0.7	None	31–33
"aeolicus"	1.7	ND[b]	ND	0.2–0.4	None	30

[a]None, autotrophic growth with CO_2 as the sole source of carbon; Ac, acetate; ile, isoleucine; leu, leucine.
[b]ND, not determined.
References: Huber et al., 1982; Jones et al., 1983a, 1983b, 1989; Stadtman and Barker, 1951; Whitman et al., 1982, 1986; Zhao et al., 1988; W. B. Whitman, unpublished observations.

as the type strain. Because many of the growth descriptions depend greatly upon the experimental conditions, some caution must be exercised in the evaluation of results from different laboratories. In particular, variation in the growth optima may occur depending on whether growth is measured by turbidity, methane formation, growth rate, or growth yield.

The Family Methanobacteriaceae

The presence of a peptidoglycan chemically different from murein (called pseudomurein) is ubiquitous among species of the family Methanobacteriaceae. This peptidoglycan, like its counterpart in eubacteria, confers shape to these rods and coccobacilli, which have a wall structure typical of Gram-positive bacteria (Kandler and König, 1985). While the cells frequently stain Gram-positive, they are not formally considered Gram-positive because the cell wall is not composed of true murein. Many of the rod-shaped methanogens are in this family, and the morphologies of representative species are shown in Fig. 2. The exceptions are a few other species that have protein cell walls and are sensitive to

Table 11. Some characteristic properties of species of the genera *Methanogenium* and *Methanoculleus*.

Species	Diameter (μm)	Flagella	Temperature optimum (°C)	Optimal salinity (M of NaCl)	Required organic growth factors[a]	GC content (mol%)
Methanogenium						
cariaci	2.6	Peritrichous	40–45	<1.0	Ac, YE	52
organophilum	0.5–1.5	None[b]	30–35	0.3	Ac, PABA, biotin, B_{12}	47
tationis	3	Peritrichous	37–40	0.1	Ac	54
"frittonii"	1.0–2.5	None	57	0.1	None	49
"liminatans"	1.5	Present	40	<0.6	Ac	60
Methanoculleus						
marisnigri	1.3	Peritrichous	40–45	0.2	Peptones	61
thermophilicum	0.7–1.8	Single	55–60	0.2	Ac	56–60
bourgense	1–2	None	35–40	0.2	Ac	59
olentangyi	1.0–1.5	None	37	0.2	Ac	54

[a]Ac, acetate; YE, yeast extract; PABA, 4-aminobenzoate.
[b]Flagella not observed.
References: Corder et al., 1983; Harris et al., 1984; Maestrojuan et al., 1990; Ollivier et al., 1986; Rivard and Smith, 1982; Romesser et al., 1979; Widdel, 1986; Widdel et al., 1988; Zabel et al., 1984, 1985; Zellner et al., 1990.

Table 12. Some characteristic properties of species of the genera *Methanolacinia*, *Methanomicrobium*, *Methanoplanus*, and *Methanospirillum*.

Species	Cellular morphology (dimensions, μm)	Flagella	Catabolizes formate[a]	Required organic growth factors[b]	GC content (mol%)
Methanomicrobium					
mobile	Rod (0.7 × 1.5)	Simple, polar	+	Complex	49
Methanoplanus					
limicola	Plate (1.5 × 1.6–2.8)	Polar tuft	+	Ac	48
endosymbiosus	Disc (1.6–3.4)	Peritrichous[d]	+	ND	39
Methanolacinia					
paynteri	Pleomorphic (0.6 × 1.5–2.5)	Flagellated[c]	–	Ac	44–45
Methanospirillum					
hungatei	Spiral (0.5 × 7.4)	Polar tuft	+	(Ac)	47–50

[a]+, Growth; –, no growth with formate as an electron donor for methanogenesis.
[b]Ac, acetate; (Ac), required or stimulatory depending on the strain; complex, includes acetate, isobutyrate, isovalerate, 2-methylbutyrate, tryptophan (or indole), pyridoxine, thiamine, biotin, vitamin B_{12}, 4-aminobenzoate, and an unidentified growth factor from methanogen cell extracts; ND, not determined.
[c]Type not described.
[d]Peritrichous flagella or pili.
References: Ferry et al., 1974; Ferry and Wolfe, 1977; Patel et al., 1976; Paynter and Hungate, 1968; Rivard et al., 1983; Tanner and Wolfe, 1988; van Bruggen et al., 1986; Widdel et al., 1988; Wildgruber et al., 1982; Zellner et al., 1989b.

Table 13. Some characteristic properties of species of the genus *Methanocorpusculum*.

Species	Cellular morphology	Flagella	Temperature optimum (°C)	Optimal salinity (M of NaCl)	Required organic growth factors[a]	GC content (mol%)
Methanocorpusculum						
parvum	Coccus	Single	37	<0.80	Ac + YE	48.5
labreanum	Coccus	None	37	<0.25	Peptone	50
aggregans	Coccus	None	35	<0.10	Ac + peptone	52
sinense	Coccus	Flagellated[b]	30	NR[c]	RF + YE	52
bavaricum	Coccus	Flagellated[b]	37	NR[c]	RF + YE	48

[a]Ac, acetate; YE, yeast extract; RF, rumen fluid.
[b]Arrangement of flagella was not specified.
[c]NaCl is not required for growth.
References: Ollivier et al., 1985; Xun et al., 1989; Zellner et al., 1987a; Zhao et al., 1989; Zellner et al., 1989c.

Table 14. Some characteristic properties of species of the genus *Methanosarcina.*

Species	Morphology	Gram reaction[a]	Catabolic substrates[b]			Temperature optimum (°C)	GC content (mol%)
			H_2	Me	Ac		
barkeri	Pseudoparenchyma	+	+	+	+	30–40	41–43
mazei	Coccus, macrocyst	–/V	+/–	+	+/–	30–40	42
thermophila	Irregular aggregate	+	+/–	+	+	50	42
acetivorans	Coccus, macrocyst	–	–	+	+	35–40	41
vacuolata	Small packet	+	+	+	+	37–40	36
frisia	Coccus	_	+	+	–	36	38

[a]+, Gram-positive; –, Gram-negative; V, Gram-variable.
[b]Me, methylated C-1 compounds and methanol; Ac, acetate; +, growth; –, no growth; +/–, very slow growth with the indicated substrates for methanogenesis.
References: Blotevogel and Fischer, 1989; Blotevogel et al., 1986; Bryant and Boone, 1987a; Liu et al., 1985; Mah, 1980; Murray and Zinder, 1985; Sowers et al., 1984a, 1984b; Touzel and Albagnac, 1983; Zhilina and Zavarzin, 1979a, 1987a; Zinder and Mah, 1979; and Zinder et al., 1985.

Table 15. Some characteristic properties of the obligately methylotrophic cocci.

Genus	Species	Cell diameter (μm)	Optimal salinity (M of NaCl)	Range of salinity (M of NaCl)	pH optimum	Required organic growth factor[a]	GC content (mol%)
Methanococcoides	*methylutens*	1	0.4	0.2–1.0	7.0–7.5	Biotin	42
Methanolobus	*tindarius*	0.8–1.3	0.5	0.05–1.3	6.5	None	40
Methanohalophilus	*oregonense*	1.0–1.5	0.5	0.1–1.6	8.6	Thiamine	41
Methanohalophilus	*zhilinae*	0.8–1.5	0.7	0.2–2.1	9.2	None	38
"Methanococcoides	*euhalobius"*	1.0–2.5	1.0	0.2–2.4	6.8–7.3	YE	43
Methanococcus	*halophilus*	0.5–2.0	1.2–1.5	0.3–2.6	6.5–7.4	None	41
Methanohalophilus	*mahii*	0.8–1.8	1.0–2.5	0.5–3.5	7.5	ND[b]	49
Halomethanococcus	*doii*	0.3–1.5	3.0	>1.8	6.8	Ac, RF	43
Methanohalobium	*evestigatum*	0.2–2.0	4.3	2.6–5.1	7.0–7.5	B-vitamins	37

[a]YE, yeast extract; Ac, acetate; RF, rumen fluid.
[b]ND, not determined.
References: König and Stetter, 1982; Liu et al., 1990; Lysenko and Zhilina, 1985; Mathrani and Boone, 1985; Mathrani et al., 1988; Obraztsova et al., 1987; Paterek and Smith, 1985, 1988; Sowers and Ferry, 1983, 1985; Stetter, 1989; Yu and Kawamura, 1987; Zhilina, 1983; Zhilina and Svetlichnaya, 1989; Zhilina and Zavarzin, 1987b.

Table 16. Some characteristic properties of species of the genus *Methanosaeta* (*"Methanothrix"*).

Species	Culture purity	Cell width (μm)	Cell length (μm)	Temperature optimum (°C)	Required organic growth factors	GC content (mol%)
"soehngenii"	–	0.8	2	37	None[a]	52
concilii	+	0.8–3.5	2–7	35–40	Vitamins	49–50
thermoacetophila	+	1.0–1.3	2–6	65	None	57
strain CALS-1	+	1.0–1.2	5	60	Biotin	ND

[a]Growth in medium containing vitamins, but a requirement has not been demonstrated.
References: Huser et al., 1982; Nozhevnikova and Chudina, 1984; Nozhevnikova and Yagodina, 1982; Patel, 1984; Patel and Sprott, 1990; Touzel et al., 1988; Zehnder et al., 1980; Zinder et al., 1987.

lysis by detergents (*Methanomicrobium*) or are enclosed within a sheath that appears to confer the shape (*Methanosaeta*). The family Methanobacteriaceae contains three genera.

Methanobacterium. The species of *Methanobacterium* vary widely in length, and filaments are common (Table 6). One species, *M. thermoaggregans,* forms large multicellular aggregates. The rod-shaped cells are often irregularly crooked. All species grow with $H_2 + CO_2$ as a substrate for methanogenesis. In addition, formate is used by *M. formicicum, "M. palustre,"* and *M. thermoformicicum.* The secondary alcohols 2-propanol and 2-butanol are also utilized by *M. bryantii, M. formicicum,* and *"M. palustre"* (Widdel et al., 1988; Zellner and Winter, 1987a). In addition, 2-propanol and 2-butanol support low levels of methane synthesis but not growth of the moderately acidophilic species *M. espanolae* (Patel et al., 1990). Most species of *Methanobacterium* are capable of autotrophic growth

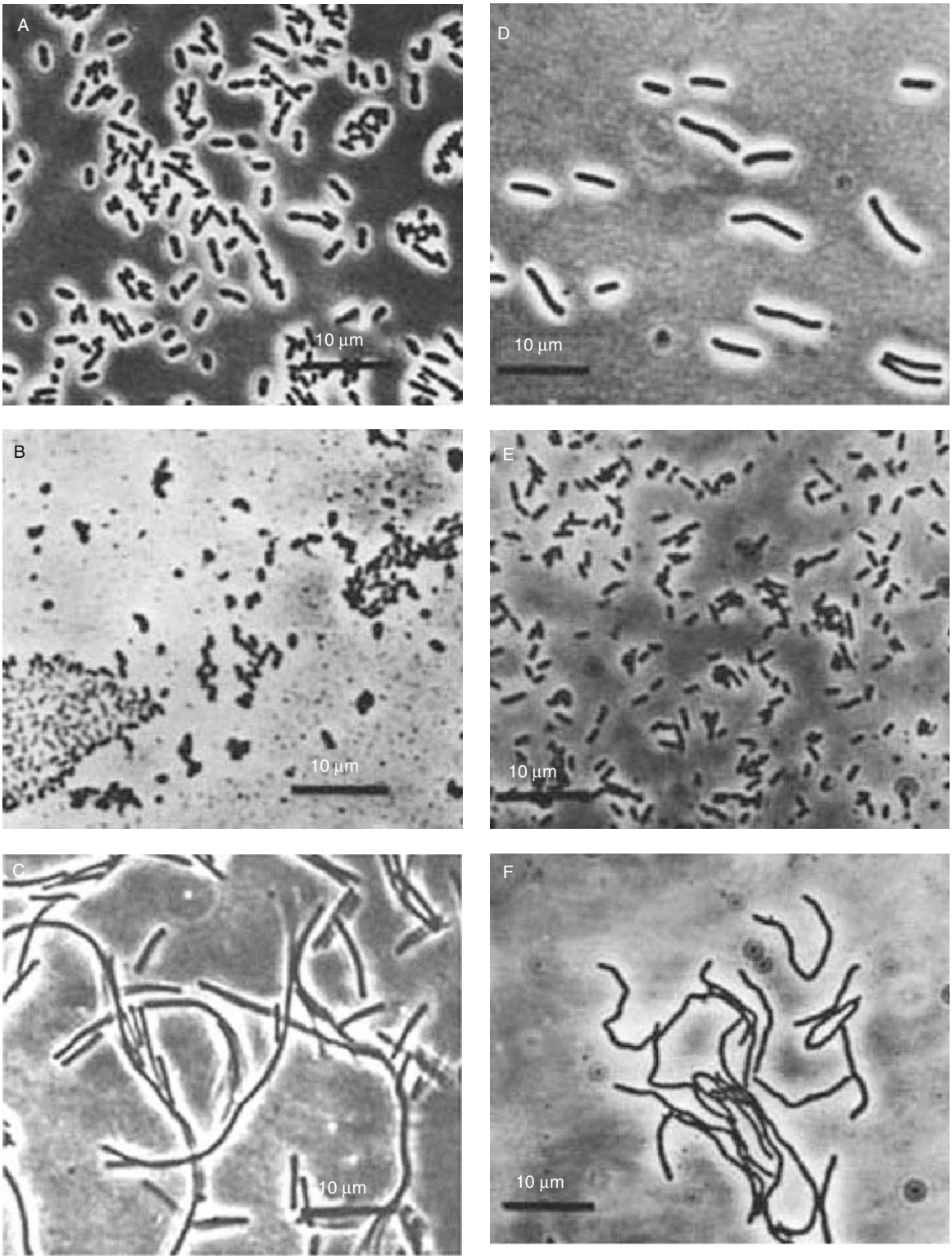


Fig. 2. Phase contrast photomicrographs of some rod-shaped methanogenic bacteria of the genera *Methanobrevibacter, Methanobacterium,* and *Methanomicrobium.* (A) *Methanobrevibacter ruminantium;* (B) *Methanomicrobium mobile* (stained preparation); (C) *Methanobacterium formicicum;* (D) *Methanobacterium bryantii;* (E) *Methanobrevibacter arboriphilicus;* (F) *Methanobacterium thermoautotrophicum.* (From Mah and Smith, 1981.)

with CO_2 as a sole carbon source. However, acetate, cysteine, and yeast extract are frequently stimulatory. *M. bryantii* is also stimulated by a mixture of B-vitamins. The alkaliphilic species, *M. alcaliphilum* and *M. thermoalcaliphilum,* both require yeast extract for growth, and their major carbon sources are not known.

While the nitrogen and sulfur sources have not been investigated systematically, all species of *Methanobacterium* that have been tested use ammonium, sulfide, and elemental sulfur. In addition, *M. ivanovii* uses glutamine as a sole nitrogen source (Bhatnagar et al., 1984) and *M. thermoautotrophicum* uses glutamine and urea. Note that at the high temperature at which the thermophile is grown glutamine decomposes rapidly in the medium (Friedmann and Thauer, 1987). *M. ivanovii, M. bryantii,* and *M. thermoautotrophicum* use cysteine as a sole sulfur source and *M. ivanovii* also uses methionine. *M. thermoautotrophicum* also uses sulfite and thiosulfate as sole sulfur sources (Daniels et al., 1986).

The species of *Methanobacterium* have been isolated from anaerobic digestors, sewage sludge, manure, groundwater, and formation water of oil-bearing rocks. In general, these habitats contain low NaCl concentrations, which are optimal for growth. Concentrations above 0.2 M are frequently inhibitory. *M. wolfei* also has a growth requirement for 8 μM tungstate, which is an unusually high concentration.

Methanobrevibacter. Species of the genus *Methanobrevibacter* are oblong cocci or very short rods (Table 7). Flagella or fimbriae are not observed. All species utilize $H_2 + CO_2$ as substrates for methanogenesis. Formate is utilized by some species. Secondary alcohols were not utilized by the species tested, *M. arboriphilicus* and *M. smithii* (Widdel et al., 1988; Zellner and Winter, 1987a). The optimum pH for growth is near 7, except for *M. arboriphilicus,* which has an optimum near 8.0. *M. ruminantium* was isolated from the rumen and has very complex nutritional requirements. These requirements may be met by rumen fluid, acetate, 2-methylbutyrate, coenzyme M, or a mixture of amino acids. In addition, several similar strains have been isolated from the rumen that do not require coenzyme M (Lovley et al., 1984; Miller et al., 1986). At present, it is not known whether these strains represent new species or atypical strains of *M. ruminantium. M. smithii* appears to be a common methanogen in the human gastrointestinal tract (Miller and Wolin, 1982; Weaver et al., 1986). Acetate, trypticase, yeast extract, and B-vitamins are required or stimulatory for growth. In contrast to *M. ruminantium* and *M. arboriphilicus,* growth of *M. smithii* is not inhibited by bile salts. *Methanobrevibacter* species have also been isolated from the feces of the horse, pig, goose, and rat (Miller and Wolin, 1986). The strain from the rat may be a new and undescribed species. On the basis of serological evidence, it has also been proposed that an additional species of *Methanobrevibacter* may predominate in some human feces (Misawa et al., 1986).

Methanosphaera. *Methanosphaera* is distinct from other Methanobacteriaceae because it is coccoid and cannot reduce CO_2 to CH_4. Cells are capable of growth only by using H_2 to reduce methanol. However, the cell walls contain pseudomurein, and sequence analysis of the 16S rRNA suggests that it is closely related to the Methanobacteriaceae. Cells are spherical and occur singly or in pairs, tetrads, or small clusters (Table 8). So far, they are found only in the mammalian colon, and they are resistant to bile salts. Cells are chemoorganotrophic, and they require CO_2, acetate, some vitamins, and amino acids for growth. In addition to the amino acids, *M. stadtmaniae* requires NH_3 as a nitrogen source. Although the two species are very similar phenotypically, they can be easily distinguished by DNA-DNA hybridization or serology.

The Family Methanothermaceae

This family contains one genus, *Methanothermus,* which is extremely thermophilic and rod-shaped (Table 9). The temperature optimum is between 80° and 90°C and no growth occurs below 60°C or above 97°C. Its habitat is geothermal (85° to 101°C) waters and muds in Iceland, and it has not been found in similar habitats in other geographical locations. $H_2 + CO_2$ is the only substrate for methanogenesis. Colonies do not grow on agar plates; therefore, polysilicate plates have been used. Unlike those of the Methanobacteriaceae, the cell wall of *Methanothermus* has an S-layer external to the pseudomurein layer. Two species of *Methanothermus* have been described: *M. fervidus* grows as dispersed cells and never forms filaments whereas *M. sociabilis* forms aggregates as large as 3 mm in diameter and contains pili-like appendages. These species also differ in their lipid and pseudomurein composition (Lauerer et al., 1986). Growth of each species is inhibited in vessels made of borosilicate glass, so soda-lime glass vessels are used.

The Family Methanococcaceae

Methanococcaceae contains one genus, *Methanococcus,* which is composed of six species of mesophilic and thermophilic organisms (Table 10). An additional species has been proposed, *M. deltae,* but it is probably a subspecies of *M. mari-*

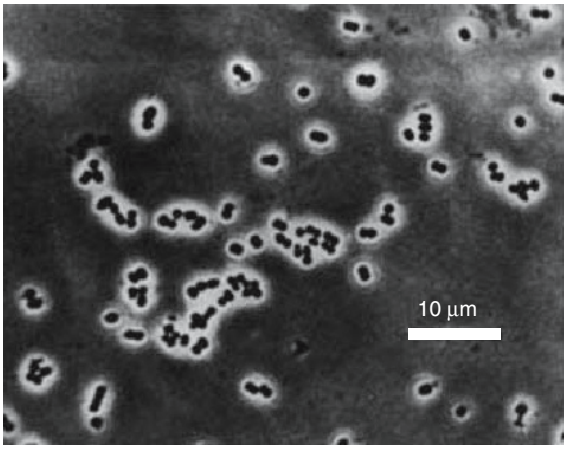


Fig. 3. Phase contrast photomicrograph of *Methanococcus voltae.* (From Mah and Smith, 1981.)

paludis (Corder et al., 1983; Whitman, 1989). The methanococci are all irregular cocci, 1–2 μm in diameter (Fig. 3). Thus, they are not easily distinguished morphologically from each other or other coccal methanogens. The cell wall is composed of an S-layer containing hexagonally arranged protein subunits (Koval and Jarrell, 1987; Nusser and König, 1987). The molecular weights of all the major wall proteins are less than 90,000, which distinguishes them from the S-layer proteins of the members of the family Methanomicrobiaceae. In addition, the S-layer proteins of the methanococci are not glycosylated. Cells lyse rapidly in 0.01% sodium dodecyl sulfate and in distilled water and they disintegrate upon heat fixation to microscope slides. The methanococci are motile, with one or two polar tufts of flagella. Isolated flagella of *M. voltae* are composed of two flagellins with molecular weights of 31,000 and 33,000 (Kalmokoff et al., 1988). The basal body is knob-like and lacks the complex ultrastructure typical of the eubacterial basal body.

The methanococci utilize $H_2 + CO_2$ as a substrate for methanogenesis. Formate is also an electron donor for all the methanococci except for two of the three described strains of *M. jannaschii.* None of the methanococci utilize acetate, methyl compounds, or alcohols as substrates for methanogenesis. Except for *M. voltae,* the methanococci are capable of rapid autotrophic growth with CO_2 as the sole carbon source. *M. voltae* requires acetate, isoleucine (or propionate or 2-methylbutyrate), and leucine (or isovalerate) as carbon sources (for a review see Jarrell and Koval, 1989). *M. voltae* grows mixotrophically, and its cellular carbon is derived from both the assimilation of exogenous acetate and autotrophic acetyl-CoA biosynthesis (Shieh et al., 1988). *M. maripaludis* also assimilates acetate, glycine, and a variety of nonpolar and basic amino acids (Whitman et al., 1987). In contrast, *M. vannielii* and *"M. aeolicus"* do not assimilate amino acids when they are provided at low concentrations, and acetate is not stimulatory to growth. Pantoyl lactone stimulates the growth of *M. voltae,* but water-soluble vitamins do not affect the growth rates of the other mesophilic methanococci.

The methanococci utilize ammonia as a sole nitrogen source. *M. maripaludis* and *M. thermolithotrophicus* can also use N_2; *M. maripaludis* can use alanine; and *M. vannielii* can use purines as sole nitrogen sources (DeMoll and Tsai, 1986). The methanococci utilize H_2S and elemental sulfur as sole sulfur sources. In addition to these, *M. thermolithotrophicus* utilizes thiosulfate, sulfite, and sulfate as sole sulfur sources (Daniels et al., 1986). The methanococci are not known to use organic sulfur sources, although *M. voltae* readily assimilates coenzyme M (Santoro and Konisky, 1987).

The methanococci appear to be restricted to marine environments. *M. vannielii* was isolated from a formate enrichment of black mud from San Francisco Bay, CA, USA. Only a single strain has been described. A number of strains of *M. voltae* and *M. maripaludis* were isolated from estuarine sediments and *Spartina alterniflora* marshes. The source of *"M. aeolicus"* has not been reported. The single described strain of *M. thermolithotrophicus* was isolated from geothermally heated sediments at Stufe di Nerone near Naples, Italy. Three strains of *M. jannaschii* were isolated from hydrothermal vents in the East Pacific Rise and the Guaymas Basin.

The Family Methanomicrobiaceae

The family Methanomicrobiaceae contains H_2-utilizing cocci (*Methanogenium* and *Methanoculleus*), as well as disc-shaped (*Methanoplanus*), rod-shaped (*Methanomicrobium* and *Methanolacinia*), and spiral-shaped (*Methanospirillum*) methanogens. The phylogentic tree is shown in Fig. 4. The coccoid Methanomicrobiaceae have protein S-layers, which cause them to stain Gram-negative and to be osmotically fragile. Cells lyse when exposed to dilute detergents or hypotonic shock. They all grow by reducing CO_2, using H_2 and formate as electron donors. Acetate is generally required as a growth factor and peptones are often required or stimulatory as well; many strains require tungstate and nickel. Motility is rare, although electron micrographs indicate that several strains have flagella.

Methanogenium and *Methanoculleus.* Nine species have been described within the genera *Methanogenium* and *Methanoculleus* (Table 11).

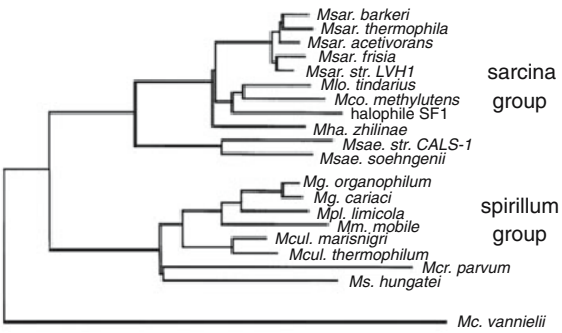


Fig. 4. Phylogenetic tree for the families *Methanomicrobiaceae* (spirillum group) and *Methanosarcinaceae* (sarcina group) based upon 16S rRNA sequences. *Msar., Methanosarcina; Mlo., Methanolobus; Mco., Methanococcoides;* SF1 is a strain closely related to *Methanohalophilus mahii; Mha., Methanohalophilus; Msae., Methanosaeta; Mg., Methanogenium; Mpl., Methanoplanus; Mm., Methanomicrobium; Mcul., Methanoculleus; Mcr., Methanocorpusculum; Ms., Methanospirillum;* and *Mc., Methanococcus.* (P. E. Rouvière and C. R. Woese, personal communication.)

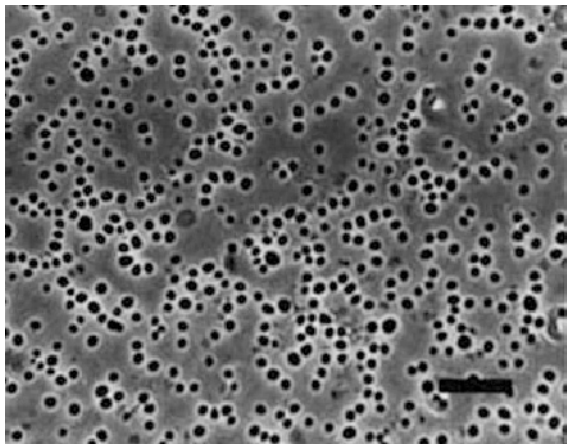


Fig. 5. *Methanogenium cariaci.* Bar = 10 μm. (Courtesy of D. Boone.)

In addition, *Methanogenium aggregans,* which is not listed, has recently been reclassified as *Methanocorpusculum* (Xun et al., 1989). Since the time when the genus *Methanogenium* was originally defined (Romesser et al., 1979), the circumscription of the genus has gradually and informally been broadened to include most coccoid, H_2- and formate-utilizing methanogens that did not fit into the genus *Methanococcus.* Recently, these organisms were shown to fall into three phylogenetic groups (Xun et al., 1989): The first group, comprised of *Methanogenium cariaci* (the type species) (Fig. 5) and *M. organophilum,* includes the two species with a requirement for concentrations of NaCl close to that found in seawater. The second group, which is the most diverse physiologically, has been reclassified as a new genus called *Methanoculleus* (Maestrojuán et al., 1990). This group includes both mesophiles and thermophiles that require low concentrations of NaCl for optimal growth but may also be halotolerant. The species in this group are *Methanoculleus marisnigri, M. bourgense, M. olentangyi,* and *M. thermophilicum. Methanogenium tationis* constitutes the third group, and, on the basis of DNA-DNA hybridization, it is not closely related to other species of *Methanogenium* or *Methanoculleus.* However, at this time, it has not been reclassified. Likewise, the phylogenetic positions of *"M. frittonii"* and *"M. liminatans"* have not been examined in detail at this time.

The substrates for methanogenesis for all nine species in this group include $H_2 + CO_2$ and formate. Four species, *M. marisnigri, M. thermophilicum* (one out of three strains), *M. organophilum,* and *"M. liminatans,"* also utilize secondary alcohols. In addition, *M. organophilum* utilizes ethanol and 1-propanol. With the exception of *"M. frittonii,"* all species require organic carbon supplements. Growth of *M. marisnigri* requires trypticase, and other peptones and yeast extract do not substitute. Peptones or yeast extract also stimulate the growth of *M. organophilum, M. thermophilicum, M. bourgense, M. tationis,* and *"M. frittonii."* For *M. tationis,* peptones can be partially replaced by a heavy metal solution. Certain strains of *M. thermophilicum* also have growth requirements for 4-aminobenzoate, biotin, nickel, molybdate, or tungstate.

Methanogenium and *Methanoculleus* species have been isolated from a variety of habitats. *M. cariaci, M. marisnigri, M. organophilum,* and *M. thermophilicum* were isolated from marine sediments. Additional strains of *M. thermophilicum* were also isolated from apple, potato, and kelp fed bioreactors. *M. bourgense* was isolated from a bioreactor fed tannery byproducts. *M. olentangyi* and *"M. frittonii"* were isolated from freshwater sediments. *M. tationis* was isolated from a moderately thermophilic solfataric pool in Chile. *"M. liminatans"* was isolated from treated industrial wastewater. Because the *Methanogenium* species grow more slowly than many other H_2- and formate-utilizing methanogens, they may be missed during viable-cell enumerations. In broth culture, they may be overgrown by *Methanococcus* species or other organisms. In roll tubes, colonies of *Methanogenium* may take several weeks to become visible. The addition of 0.05–0.20 M NaCl may improve their recovery, however.

METHANOPLANUS. The cellular morphology of *Methanoplanus* is very distinctive (Table 12): by

phase contrast microscopy, *M. limicola* forms rectangular plates, and *M. endosymbiosus* is disc shaped. As the cells rotate in solution, they can be viewed on edge and appear as very thin rods. The dimensions of *M. limicola* are 0.07–0.30 μm thick, 1.6–2.8 μm long, and 1.5 μm wide. *M. endosymbiosus* is more disc shaped, with a diameter of 1.6–3.4 μm. Both species utilize H_2 and formate as substrates for methanogenesis. Best growth is obtained at neutral pH, mesophilic temperatures, and low NaCl (0.20–0.25 M). Growth of *M. limicola* is stimulated by yeast extract or peptones plus vitamins. The nutritional requirements of *M. endosymbiosus* have not been fully investigated. The cell envelope of each species contains an S-layer composed of hexagonally arranged subunits. The major proteins stain with the periodate-Schiff reagent and have apparent molecular weights of 143,000 and 110,000 for *M. limicola* and *M. endosymbiosus,* respectively. *M. limicola* was isolated from a small swamp formed from drilling wastes. *M. endosymbiosis* was isolated from a marine sapropelic ciliate.

Methanomicrobium and *Methanolacinia*. *Methanomicrobium* cells are short rods, but unlike the Methanobacteriaceae, they contain no pseudomurein (Table 12). The physical structure of the cell is apparently conferred by a protein cell wall. Exposure to low concentrations of sodium dodecyl sulfate and other detergents causes rapid cell lysis. Cells stain Gram-negative (Fig. 2). H_2 and formate are substrates for methanogenesis. Growth is most rapid at temperatures near 40°C and between pH 6.1 to 6.9. *M. mobile* has exceptionally complex nutritional requirements and was isolated from the rumen of a fistulated heifer fed alfalfa hay, where it was very abundant.

Methanolacinia cells are pleomorphic, and they have been described both as short rods and as highly irregular and lobed cocci. Although flagella have been observed, motility has not. Until recently, the single species, *M. paynteri,* was classified in the genus *Methanomicrobium.* The single isolate utilizes H_2 and secondary alcohols as substrates for methanogenesis (Zellner and Winter, 1987a). Growth is stimulated by yeast extract, trypticase, and 0.15 M NaCl. The temperature and pH optima are 40°C and pH 7, respectively. *M. paynteri* may readily be distinguished from *Methanomicrobium mobile* by differences in its polyamine and lipid content (Zellner et al., 1989b). *Methanolacinia paynteri* was isolated from marine sediment.

Methanospirillum

Methanospirillum is a spiral-shaped cell (0.4 μm wide by 7.4–10 μm long), twisted into a gentle helix (Table 12). The helical shape may be conferred by a sheath that surrounds the cell (Patel et al., 1986). Cells may form either long chains, or filaments, encased by a continuous sheath or be single (Fig. 6; Patel et al., 1979). *M. hungatei* also lyses slowly in 1% sodium dodecyl sulfate. Colonies in solidified medium have a wavy appearance, probably conferred by the helical shape of the cells. *M. hungatei* is the only species in this genus that has been described. H_2+CO_2 and formate are substrates for methanogenesis, and some strains use 2-propanol and 2-butanol as well. The temperature optimum for growth is 35–40°C. Acetate is required or stimulatory. In addition, one strain is stimulated by peptones and B-vitamins. *M. hungatei* has been isolated from sewage sludge and a pear-waste digestor.

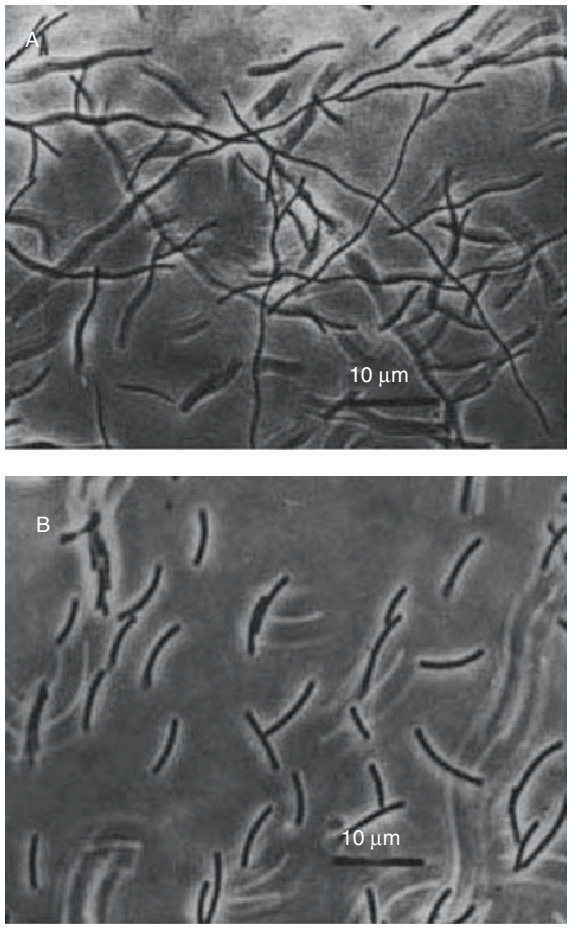


Fig. 6. *Methanospirillum* (A) Long, wavy filaments. (B) Individual cells. (From Mah and Smith, 1981.)

The Family Methanocorpusculaceae

This family contains only one genus, *Methanocorpusculum,* and is more closely related to the Methanomicrobiaceae than to any other families

of methanogens. The five species of the genus are very small, irregular cocci that utilize H_2 and formate as substrates for methanogenesis (Table 13). *M. parvum* and *M. bavaricum* also utilize secondary alcohols (Zellner and Winter, 1987a; Zellner et al., 1989c). The diameter of the cells varies somewhat with the growth conditions. For *M. parvum,* the cell diameter is less than or equal to 1 μm. The cell diameters of *M. labreanum* and *M. aggregans* vary from about 0.5–2.0 μm. *M. aggregans* also forms large, multicellular clumps. All species have complex nutritional requirements for peptones, rumen fluid, or yeast extract. In addition, *M. labreanum* is stimulated by acetate, and *M. parvum* requires 1 μM tungstate. *M. parvum* was isolated from a whey digestor and is unusually halotolerant. *M. aggregans* was isolated from a sewage digestor. *M. labreanum* was isolated from lake sediment near the La Brea Tar Pits, California. *M. sinense* was isolated from a biogas plant in Chengdu, China. *M. bavaricum* was isolated from a wastewater treatment pond in Germany.

The Family Methanosarcinaceae

All of the aceticlastic methanogens belong to this family, as well as all methanogens that disproportionate methanol, methylamines, or other methyl-containing compounds. The only other species that catabolize methanol are *Methanosphaera* species, which require H_2 to reduce it to methane. The family Methanosarcinaceae can be divided into three physiological and morphological types: 1) the genus *Methanosarcina,* which contains coccoid and pseudosarcinal cells and can disproportionate methanol and catabolize acetate and $H_2 + CO_2$; 2) the genus *Methanosaeta,* which grows only by the aceticlastic reaction; 3) the halophilic, methylotrophic organisms of the genera *Methanolobus, Methanococcoides,* and *Methanohalophilus.* Phylogenetically, only the first of these groups is composed of closely related species (Fig. 4).

METHANOSARCINA. *Methanosarcina* species disproportionate methanol and methylamines, forming CH_4 and CO_2 in a ratio of approximately 3:1 (Table 14). Most strains also use acetate, and most also use H_2 to reduce either CO_2 or methanol to CH_4. Formate is never used. Cells appear to have a protein cell wall adjacent to the cytoplasmic membrane. External to the protein wall, many strains have a layer of methanochondroitin, referred to as a matrix. Cells without the matrix are individual, irregular cocci, but when the matrix is present, packets or pseudoparenchyma are formed (Fig. 7). Within the packets, the cells are irregular in shape, the division

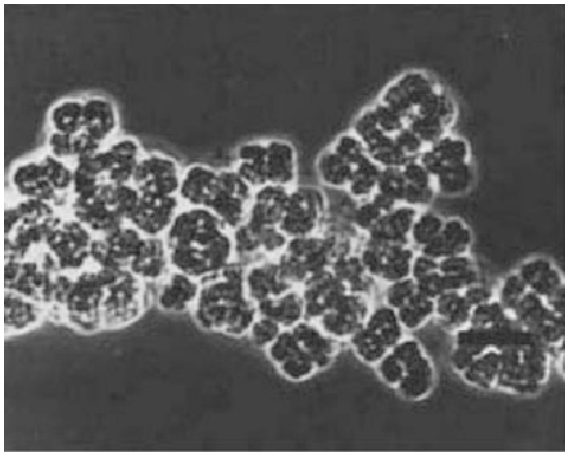


Fig. 7. *Methanosarcina barkeri* strain MS. Bar = 10 μm. (Courtesy of D. Boone.)

planes are not always perpendicular, and the volume of the daughter cells is not always equal. Three morphotypes of *Methanosarcina* have been described. Morphotype 1 includes some strains of *M. barkeri* that form aggregates of packets of cocci termed pseudoparenchyma, which may be visible to the unaided eye (Zhilina, 1976). Morphotype 2 includes *M. vacuolata,* which forms small packets and tends to remain dispersed and settles slowly in liquid medium. Morphotype 3 includes *M. mazei,* which forms single cocci, packets of pseudococci, and large macrocysts (Zhilina and Zavarzin, 1979b; Robinson, 1986).

While morphology and substrate range are important characteristics of *Methanosarcina,* they are not always reliable indicators of species differences. These characteristics frequently vary when closely related strains are tested, or the same strain may give varied results under different conditions. For example, a single enzyme may be responsible for changing large pseudoparenchymal aggregates into individual coccoid cells, and this enzyme may be produced only under certain growth conditions (Harris, 1987; Sowers and Gunsalus, 1988; Xun et al., 1988). The ability to use substrates often depends on the conditions of the inoculum (Boone et al., 1987), and the ability to form gas vacuoles is not a constant characteristic of strains (R. A. Mah, personal communication). Thus, variations in these characteristics within species may be commonplace, and they may not always be reliable for the placement of strains into species.

The diameter of the cocci or cells within the aggregates is generally 1–2 μm, although both smaller and larger cells are observed. The cells of *Methanosarcina* is nonmotile, and in those species that stain Gram negative, the cells lyse in the presence of sodium dodecyl sulfate. Cells of

M. vacuolata contain numerous gas vacuoles tightly clustered in the cytoplasm. The *Methanosarcina* do not require additional carbon sources, and species that use $H_2 + CO_2$ as a substrate for methanogenesis can grow autotrophically. However, peptones or yeast extract are stimulatory for *M. mazei, M. vacuolata,* and *M. frisia,* and riboflavin is stimulatory or required for at least one strain of *M. barkeri* (Scherer and Sahm, 1981a). The chemicals 4-aminobenzoate and calcium chloride (0.7 mM) are required for growth of *M. thermophila. M. barkeri* grows with dinitrogen as its sole nitrogen source (Bomar et al., 1985; Murray and Zinder, 1984). Methionine, cysteine, thiosulfate, and elemental sulfur are also sulfur sources for some strains of *M. barkeri* (Mazumder et al., 1986; Scherer and Sahm, 1981b).

M. barkeri, M. mazei, M. thermophila, and *M. vacuolata* were isolated from anaerobic digestors. *M. barkeri* is also found in freshwater and marine sediments, rumens of ungulates, and animal-waste lagoons. *M. mazei* has been found in garden soil, sewage sludge, and various other sources. *M. vacuolata* is common in freshwater sediments, marshes and wetlands. *M. acetivorans* and *M. frisia* were isolated from marine sediments and for optimal growth require 0.2 and 0.3–0.4 M NaCl, respectively. *M. acetivorans* also requires 0.05–0.10 M magnesium for optimal growth. In addition to the type strain of *M. thermophila,* two strains of thermophilic *Methanosarcina* have been described (Ollivier et al., 1984; Touzel et al., 1985), whose nutritional characteristics resemble those of *M. thermophila.* However, both the pseudoparenchymal and coccoid morphologies are present, and the taxonomic status of these latter strains is uncertain.

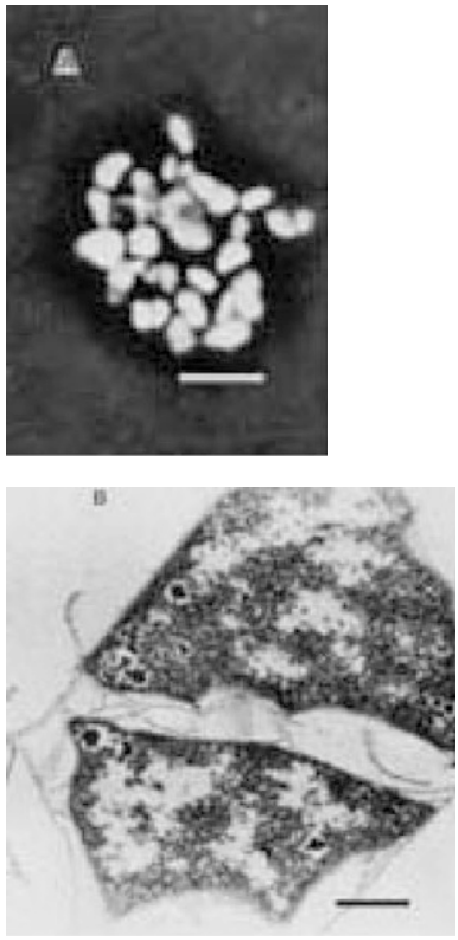


Fig. 8. *Methanohalobium evestigatum.* (A) Cells and aggregates, anoptral contrast Reichert. Bar = 5 μm. (B) Ultrathin section of late exponential phase cells. Layers of the envelope slip from the cell surface. Nuclear region and star-shaped granules (possibly glycogen) are present. Bar = 0.5 μm. (Courtesy of T. Zhilina.)

Obligately Methylotrophic Cocci. Eight species and five genera of obligately methylotrophic, irregular cocci have been described (Table 15). In addition, two species of *Methanolobus, M. siciliae* and *M. vulcani,* have been proposed, but a complete description is unavailable (Stetter, 1989). At present, the taxonomy of these organisms is uncertain. Therefore, they have been arranged in the table in the order of increasing optimal salinity for growth, which varies from marine to extremely halophilic. Phylogenetic studies by RNA sequencing, RNA-DNA hybridization and DNA-DNA hybridization suggest that *Methanococcoides methylutens, Methanolobus tindarius, Methanohalophilus zhilinae, M. mahii,* and *Methanohalobium evestigatum* probably represent five different genera rather than only four (Sowers et al., 1984b; Chumakov et al., 1987; also see Fig. 4). The placement of the three remaining species listed in Table 15 is not known.

These species grow by disproportionation of methanol, methylamines, and other C-1 compounds. H_2, formate, and acetate are not substrates. Cells are small irregular cocci, and some species form small aggregates. Cells of *M. evestigatum* are flat and polygonal, and they often occur in sheets (Fig. 8). *Methanococcus halophilus* produces slime. All the species are nonmotile, except for *Methanolobus tindarius,* which has monotrichous flagellation. With exception of *Methanohalobium evestigatum,* they stain Gram negative and lyse rapidly in dilute sodium dodecyl sulfate (or Sarkosyl for *Methanococcus halophilus*). These species are mesophilic, and their temperature optima are 30–35°C with three exceptions: the temperature optimum for *Methanolobus tindarius* is 25°C; the temperature optimum for *Methanohalophilus zhilinae* is 45°C; the temperature optimum for *Methanohalobium*

evestigatum is 50°C. *Methanohalophilus zhilinae* and *M. oregonense* are also alkalophilic.

In addition to NaCl, *M. euhalobius* and *M. mahii* require high concentrations of magnesium and calcium for good growth. *M. methylutens* and *M. oregonense* require high concentrations of magnesium. Although the organic growth factors of the type strain of *M. mahii* are not known, strain SF-1, which appears to be closely related, requires yeast extract. Vitamins stimulate the growth of *M. tindarius* and peptones that of *M. zhilinae.* In contrast, greater than 0.05% yeast extract or peptones inhibit the growth of *M. halophilus* and *M. evestigatum.*

M. tindarius and *M. methylutens* were isolated from marine sediments. *M. mahii, Halomethanococcus doii, M. zhilinae,* and *M. oregonense* were isolated from sediments of saline or saline and alkaline lakes. *M. halophilus* was isolated from a sample containing the cyanobacterial mat and associated mud of a stromatolite in Shark Bay, Australia. *M. evestigatum* was isolated from a cyanobacterial mat in a hypersaline lagoon close to Arabat, Sivash. *M. euhalobius* was isolated from the stratal liquid of an exploratory oil well.

METHANOSAETA. In contrast to the other genera of the family Methanosarcinaceae, which are coccoid, the species of *Methanosaeta* (formerly called *Methanothrix*) are sheathed rods. These organisms grow only by the aceticlastic reaction (Table 16). Acetate is the only substrate for methanogenesis, and H_2, formate, and methyl compounds are not utilized. Because of their very long generation time, 1 to 3 days under optimal conditions, pure cultures have been difficult to obtain. For instance, the original culture of *"M. soehngenii"* is not pure, and vancomycin is required to suppress the growth of contaminants (Patel and Sprott, 1990; Touzel et al., 1988). Likewise, the description of *M. thermoacetophila* is based upon a monoculture, although a pure culture has recently been obtained. Two other pure cultures have also been described. *M. concilii* appears to be closely related to the methanogen in *"M. soehngenii"* cultures, and strain CALS-1 appears to be similar to *M. thermoacetophila,* but insufficient cell mass has been obtained to complete the species description.

Methanosaeta is a short, fat, nonmotile rod with flat ends. Cells stain Gram negative and are sheathed. Except for CALS-1, stationary cultures may form long filaments with a contiguous sheath (Fig. 9). The filaments may form large bundles, mats, or flocs. In shaken cultures, the filaments tend to fragment into short filaments or individual cells. *M. thermoacetophila* and CALS-1 contain gas vacuoles and *M. concilii* produces a capsule.

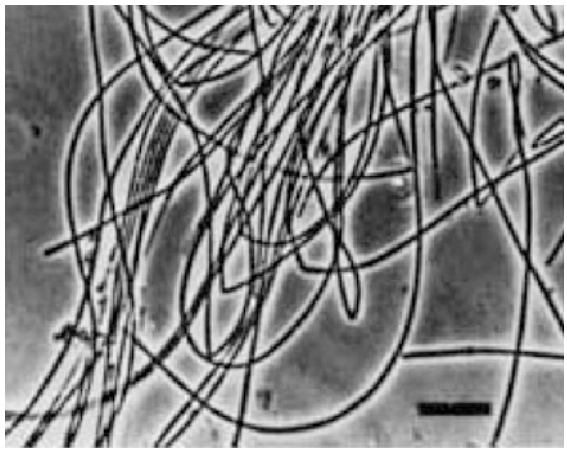


Fig. 9. *Methanosaeta concilii.* Bar = 10 μm. (Courtesy of D. Boone.)

Marine or halophilic species have not been described, and NaCl does not need to be added to the media above trace amounts. The pH optima of *"M. soehngenii," M. concilii,* and CALS-1 are 7.4–7.8, 7.1–7.5, and 6.5, respectively. Yeast extract inhibits the growth of *M. concilii,* and penicillin inhibits the growth of *"M. soehngenii"* and *M. thermoacetophila.* CALS-1 is very sensitive to the reducing agents in the medium, and 1 mM sulfide and 1 mM 2-mercaptoethanesulfonate appear to be optimal.

Methanosaeta is widely distributed in nature. Its distinctive morphology has allowed it to be identified in sewage sludge, digestors, animal wastes, and sanitary landfills. Monocultures of the thermophiles have been obtained from different types of thermophilic digestors and thermal sediments. *"M. soehngenii"* and CALS-1 were obtained from sludge digestors. *M. concilii* was obtained from a pear waste digestor. *M. thermoacetophila* was obtained from the sediments of a thermal lake.

Methanogens of Uncertain Affiliation

A thermophile, TAM, that morphologically resembles *Methanosaeta* has been isolated from a sewage sludge digestor (Ahring and Westermann, 1984, 1985). Cells are 0.8–1 μm wide and 4–5 μm in length, and they normally form short filaments. A sheath is present. Cells stain Gram positive to variable. In contrast to *Methanosaeta,* $H_2 + CO_2$ and formate are substrates for methanogenesis in addition to acetate. Growth factors are not required during growth on acetate. The pH optimum is 7.3–7.5, and the temperature optimum is 60°C. Penicillin and other typical eubacterial antibiotics are inhibitory at high concentrations.

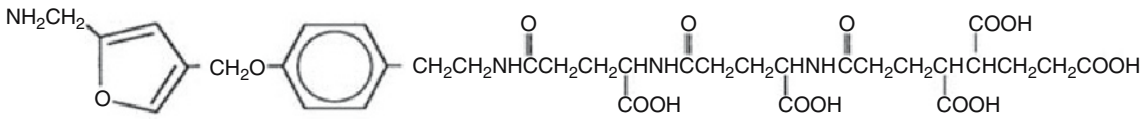
The isolation of a wall-less methanogen, *"Methanoplasma elizabethii,"* from an anaerobic chemostat fed glucose was reported but not confirmed (Rose and Pirt, 1981). *"M. elizabethii"* uses H_2 + CO_2 and formate as substrates for methanogenesis.

Biochemical and Physiological Properties

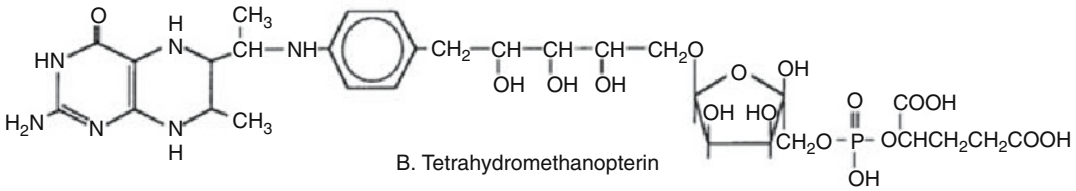
Coenzymes of Methanogenesis

Methanogens contain many novel coenzymes that are associated with the biochemistry of methane synthesis. Thus, what should be a chemically simple reduction of C-1 compounds to methane is biochemically complex. Because the structure and synthesis of many of the coenzymes have recently been reviewed (Jones et al., 1987), only the most recent citations are included below.

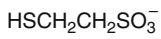
Methanofuran (MFR) (Fig. 10A) functions as a formyl carrier in methanogenesis. It is required for the initial activation of CO_2 to formyl-MFR, the first stable product of CO_2 reduction. MFR has been found in all methanogens examined at levels ranging from 0.5 to 2.5 mg per kg dry weight of cells. At least five different forms of methanofuran exist among the methanogens



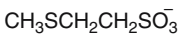
A. Methanofuran



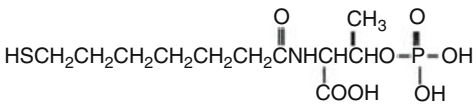
B. Tetrahydromethanopterin



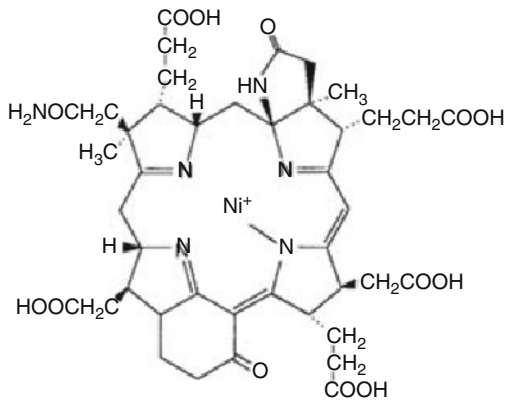
C. Coenzyme M



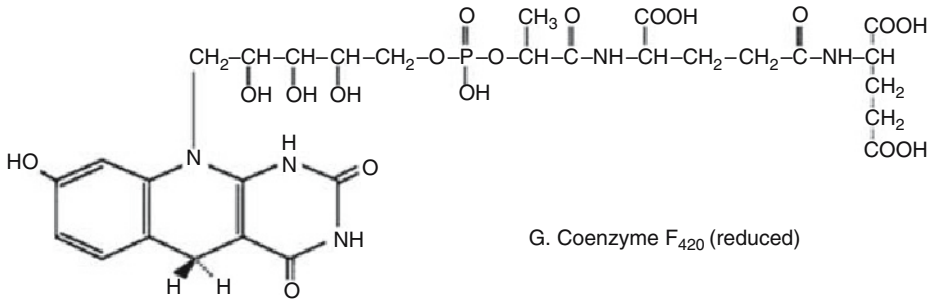
D. Methyl coenzyme M



E. 7-Mercaptoheptanoylthreonine phosphate



F. Coenzyme F_{430}



G. Coenzyme F_{420} (reduced)

Fig. 10. The seven coenzymes of methanogenesis.

(White, 1988). This coenzyme has not been detected in either nonmethanogenic archaebacteria, with the exception of *Archaeoglobus fulgidus*, or eubacteria. The 2,4-disubstituted furan moiety and the 4,5-dicarboxy octanoate moiety are unique structures in nature.

Tetrahydromethanopterin (H_4MPT) is a one-carbon carrier similar to folate in structure and function (Fig. 10B). Likewise, biosynthesis of H_4MPT is also similar to tetrahydrofolate biosynthesis in eubacteria. The coenzyme was first noticed as a yellow fluorescent compound (YFC) that was rapidly labeled by $^{14}CO_2$ in cell extracts (Daniels and Zeikus, 1978). H_4MPT is also required for several biosynthetic reactions that are folate-dependent in eubacteria, such as serine hydroxymethyltransferase.

A third one-carbon carrier unique to methanogens is coenzyme M (HS-CoM) (Fig. 10C), which chemically is 2-mercaptoethanesulfonic acid. HS-CoM serves as the terminal carbon carrier in methanogenesis where methyl coenzyme M (CH_3-S-CoM) (Fig. 10D) is reduced to CH_4. Since HS-CoM has been found in all methanogens examined, it may serve as a sensitive biomarker for the qualitative identification of methanogens in various ecological niches.

7-Mercaptoheptanoylthreonine phosphate (HS-HTP) (Fig. 10E) is a colorless coenzyme that is required for the final step of methanogenesis. This coenzyme is the electron donor for CH_3-S-CoM reduction, and it is proposed to participate in the energy-conserving step of methanogenesis (Ellermann et al., 1988). It is also required for the activation of CO_2 reduction (Bobik and Wolfe, 1988). HS-HTP is probably biosynthesized from α-ketoglutarate by repeated α-keto-acid chain elongation in a process similar to the aminoadipate pathway of lysine biosynthesis in eubacteria (White, 1989).

Coenzyme F_{430} (Fig. 10F) is a nickel-containing coenzyme required for the final step of methanogenesis, and it is tightly associated with component C of the methylreductase system. The coenzyme was first noticed in cell extracts by J. LeGall and reported by Gunsalus and Wolfe (1978). It is interesting to note that biosynthesis of coenzyme F_{430} proceeds from 5-aminolevulenate via uroporphyrinogen III, which indicates that nickel tetrapyrroles share a common biosynthetic pathway with all other porphinoid compounds (Pfaltz et al, 1987).

Autofluorescence of methanogenic bacteria is largely due to the presence of high levels of coenzyme F_{420} (Fig. 10G). Coenzyme F_{420} is ubiquitous among methanogens, and it is found at low levels in some other organisms. It is a deazaflavin and participates in two-electron transfer reactions. In this regard, it functions in a manner analogous to NADH, but its redox potential of –340 to –350 mV is lower. The coenzyme is an electron donor in methanogenesis. In addition, coenzyme F_{420} is coupled to hydrogenase, formate dehydrogenase, carbon monoxide dehydrogenase, NADP+ reductase, pyruvate synthase, and α-ketoglutarate synthase (Keltjens and van der Drift, 1986).

In addition to the novel coenzymes described above, methanogens contain a number of common vitamins (Noll and Barber, 1988). These include thiamine, riboflavin, pyridoxine, corrinoids, biotin, niacin, and pantothenate. Flavins are known to function as electron carriers for hydrogenase, NADH reductase, formate dehydrogenase, and the methylreductase system in *Methanobacterium*. Many methanogens contain abundant amounts of unusual corrinoids, with factor III or pseudovitamin B_{12} being the most predominant (Stupperich and Kräutler, 1988). Corrinoids have also been implicated in methane synthesis, where they function as methyl carriers. In contrast, folates are absent or present in very small amounts in methanogens (Worrell et al., 1988). Presumably, methanopterin substitutes for folate as a methyl carrier. Methanogens have also been found to contain ferredoxin, thioredoxin, and cytochromes *b* and *c.* Ferredoxin is abundant in the aceticlastic methanogens, where it is coupled with the catabolic carbon monoxide dehydrogenase, and has been isolated from *Methanococcus thermolithotrophicus* (Hatchikian et al., 1989; Terlesky and Ferry, 1988a). Thioredoxin was isolated from *Methanobacterium thermoautotrophicum,* but its function is unknown (Schlicht et al., 1985). Cytochromes function as electron carriers during methanogenesis from methanol, methylamines, and acetate. They are implicated in the oxidation of these substrates during their disproportionation (Terlesky and Ferry, 1988b). In support of this hypothesis, cytochromes are absent from *Methanosphaera,* which must use H_2 as a reductant during growth on methanol.

The Pathway of Methanogenesis

The pathway of methanogenesis has been extensively reviewed (Jones et al., 1987; Rouvière et al., 1988; Rouvière and Wolfe, 1988). The reduction of CO_2 to CH_4 involves seven steps. The source of electrons may be either H_2 via hydrogenase or formate via formate dehydrogenase. Formate itself does not appear to be an intermediate. Initially, CO_2 is activated to form formylmethanofuran. Next, the formyl group is transferred to H_4MPT, where it is reduced to the methylene and methyl levels. Last, the methyl group is transferred to coenzyme M and reduced to methane by the methylreductase system.

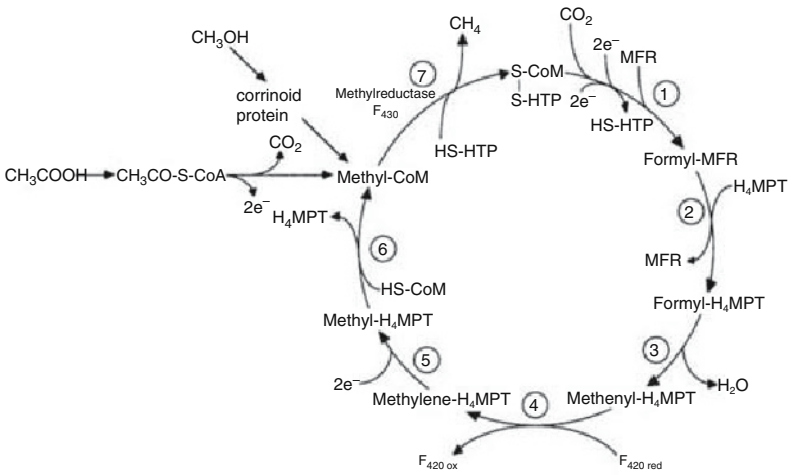


Fig. 11. The pathway of methane formation from acetate, methanol, and CO_2. Methyl-CoM is the key intermediate for the reduction of CO_2, acetate, and methanol to methane. The numbers refer to the seven steps of the cycle. (Adapted from Rouvière et al., 1988.)

The first step of CO_2 activation is not clearly understood (Fig. 11, step 1). It requires ATP, but not in stoichiometric amounts. The energy for the reaction is derived from the final step of methane formation (Fig. 11, step 7). This conclusion is supported by the observation that CO_2 activation in vitro requires small amounts of CH_3-S-CoM or a strong reductant such as titanium citrate (Bobik and Wolfe, 1989). The formyl group is transferred from MFR to H_4MPT by formyl methanofuran:tetra-hydromethanopterin formyltransferase (Fig. 11, step 2). The formyl group then undergoes sequential reduction beginning with its conversion to 5,10-methenyl-H_4MPT by the enzyme 5,10-methenyltetrahydromethanopterin cyclohydrolase (Fig. 11, step 3). The methenyl group is reduced by coenzyme F_{420} in a reaction catalyzed by methylenetetrahydromethanopterin:coenzyme F_{420} oxidoreductase to yield 5,10-methylene-H_4MPT (Fig. 11, step 4). The enzymes for steps 5 and 6 have not been purified to homogeneity. Step 5 is presumably catalyzed by methylene-H_4MPT reductase to yield methyl-H_4MPT. Conversion of methyl-H_4MPT to CH_3-S-CoM involves a corrinoid-containing methyltransferase (Kengen et al., 1988; Fig. 11, step 6).

The terminal reaction, catalyzed by the methylreductase system, represents the completion of the cycle with the release of CH_4 and the activation of CO_2 (Fig. 11, step 7). The methylreductase system catalyzes the conversion of CH_3-S-CoM and HS-HTP to CH_4 and CoM-S-S-HTP. Although the exact process is not known, methane formation is also coupled to ATP synthesis via a chemiosmotic mechanism (Blaut and Gottschalk, 1985). However, this coupling may occur during the reduction of the disulfide bond of CoM-S-S-HTP rather than during the reduction of CH_3-S-CoM (Ellermann et al., 1988). CoM-S-S-HTP is also required for the coupling of CH_3-S-CoM reduction to CO_2 activation (Bobik and Wolfe, 1988).

The process of CH_3-S-CoM reduction is complex and requires four protein components and at least three unique coenzymes. Components A1, A2, A3, and C are proteinaceous. A1 and A3 are oxygen-labile and have not been highly purified. A1 has coenzyme F_{420}-dependent hydrogenase activity and is probably required for the reduction of CoM-S-S-HTP (Rouvière et al., 1988). A3 has been resolved into two fractions: A3a and A3b. A3a is a large iron-sulfur protein, which may be involved in providing electrons for component C. A3b has methyl viologen-dependent hydrogenase activity (Rouvière and Wolfe, 1989). A2 is an oxygen-stable component and has been purified to homogeneity. A2 and A3 are probably required for the ATP-dependent reductive activation of component C (Rouvière et al., 1988). Component C is an oxygen-stable protein, which is the methyl-S-CoM methylreductase proper. It contains 2 moles of F_{430} and 2 moles of HS-CoM per mole of enzyme, and it has three types of subunits with a stoichiometry of $\alpha_2\beta_2\gamma_2$. The enzyme represents about 10% of the total cellular protein. The genes for component C are organized into a five-gene cluster, *mcrBDCGA*. The primary sequences of these genes are highly conserved in all methanogens examined. Subunits α, β, and γ are coded for by *mcrA, mcrB,* and *mcrG,* respectively. The functions of *mcrC* and *mcrD* are unknown (Allmansberger et al., 1989; Weil et al., 1989).

The methylreductase system is also required for methanogenesis from substrates other than CO_2 (Fig. 11). During the catabolism of acetate, the methyl and carboxyl groups are converted to CH_4 and CO_2, respectively, via an aceticlastic reaction. This reaction involves the initial activation of acetate as acetyl-CoA (Terlesky et al.,

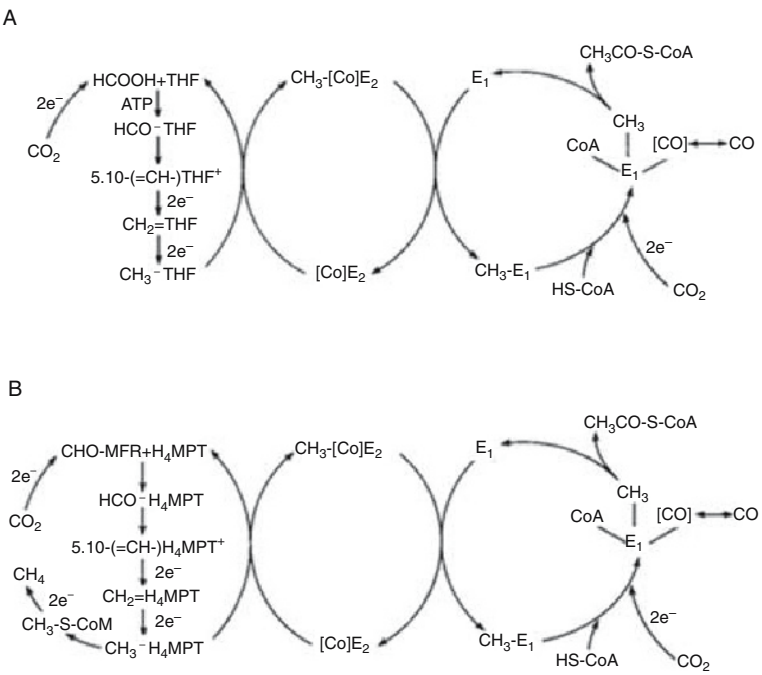


Fig. 12. The two acetogenic pathways for the autotrophic fixation of CO_2 to acetyl-CoA in (A) *Clostridium thermoaceticum* and (B) *Methanobacterium thermoautrotrophicum.* THF, tetrahydrofolate; E1, carbon monoxide dehydrogenase; E2, the corrinoid enzyme involved in methyl transfer. (Adapted from Jones et al., 1987.)

1987). The carbon-carbon bond is then cleaved by the carbon monoxide dehydrogenase system, producing HS-CoA, an enzyme-bound CO, and a methyl group. The methyl group is transferred to HS-CoM via H_4MPT with the aid of an unidentified corrinoid enzyme (Fischer and Thauer, 1989; van de Wijngaard et al., 1988). Oxidation of the enzyme-bound CO to H_2 and CO_2 provides electrons for the reduction of CH_3-S-CoM to CH_4 (Terlesky and Ferry, 1988b; Bott and Thauer, 1989).

Methane is also produced from the catabolism of methanol and methylamines. Methanogenesis from methanol can occur in two different ways depending on the electron donor: 1) if it is H_2, the methyl group of methanol is transferred to HS-CoM via two methyltransferases (MT1 and MT2). The former contains an oxygen-sensitive cobamide moiety as a methyl carrier. CH_3-S-CoM is then reduced to CH_4 by H_2. 2) When H_2 is absent, some of the methanol is oxidized in a disproportionation reaction to serve as reductant for the methylreductase system. Likewise, for methylamines the methyl group is either reduced to CH_4 or oxidized to CO_2. After growth in the presence of trimethylamine, *Methanosarcina barkeri* contains a trimethylamine:HS-coenzyme M methyltransferase similar to the methanol-induced methyltransferases.

Carbon Metabolism

Methanogens use only simple organic or inorganic carbon compounds as energy sources. About one-half of methanogens are also capable of autotrophic growth, and they obtain all their organic carbon from the assimilation of CO_2. The proposed methanogenic autotrophic pathway, the Ljungdahl-Wood pathway, is unlike classic autrotrophic CO_2 fixation (the ribulose bisphosphate pathway) since the primary intermediate is acetyl-CoA. The Ljungdahl-Wood pathway was discovered in the acetigenic clostridia and has been extensively studied in *Clostridium thermoaceticum* (Fig. 12A; Ljungdahl, 1986; Wood et al., 1986). The key enzyme is a nickel-containing carbon monoxide dehydrogenase system (CODH). In methanogens, the one-carbon carrier of the clostridial pathway, folate, is replaced by tetrahydromethanopterin (H_4MPT) (Fig. 12B; Fuchs, 1986). The pathways of autotrophy and methanogenesis are closely linked, and they share common intermediates. Thus, many of the methanogenic coenzymes serve both anabolic and catabolic functions. CO_2 is first reduced to formyl-MFR. The formyl group is then transferred to H_4MPT and reduced to a methyl group. At this point the anabolic and catabolic pathways diverge. The subsequent transfer of this methyl group to HS-CoA is believed to involve a cobamide-containing protein. The carboxyl moiety is derived from CO_2 via CODH and a carbonylation reaction. At this step the carboxyl group freely exchanges with CO in the environment. Although acetogenesis in methanogens appears similar to the pathway in eubacteria, it is distinctive due to the absence of folates and the lack of an ATP requirement for the activation of CO_2.

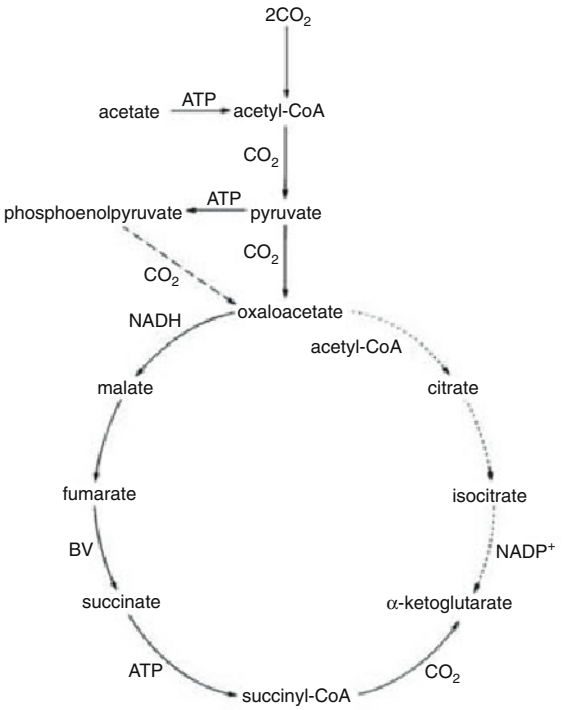


Fig. 13. The pathways of carbon assimilation in methanogens. Solid lines represent the incomplete reductive TCA cycle found in *Methanobacterium thermoautotrophicum* and *Methanococcus maripaludis.* Broken arrows represent the incomplete oxidative TCA cycle found in *Methanosarcina barkeri.* (Shieh and Whitman, 1987.)

Methanogens utilize the anabolic tricarboxylic acid (TCA) cycle to synthesize cell carbon from acetyl-CoA (Jones et al., 1987; Fig. 13). However, the exact nature of the cycle varies among different groups of methanogens. In *Methanosarcina barkeri,* α-ketoglutarate is formed by an oxidative branch. *Methanospirillum, Methanococcus,* and *Methanobacterium* utilize the reductive or reverse pathway (Fuchs and Stupperich, 1986). Hexoses are derived from acetate by gluconeogenesis. The major storage product for carbohydrates is glycogen. Very little information is available on how this polysaccharide reserve is used, since no known methanogens can catabolize exogenous sugars. *Methanosarcina thermophila* is capable of mobilizing its reserves and actually producing small amounts of acetate when starved for acetate (Murray and Zinder, 1987). Likewise, *Methanolobus* produces small amounts of methane upon mobilization of its glycogen reserves (König et al., 1985).

Heterotrophic methanogens rely on exogenous acetate for growth. An incomplete, reductive TCA cycle has been found in *Methanococcus voltae,* indicating the presence of a mechanism of anabolic carbon metabolism that is similar to the one found in autotrophic methanogens (Shieh and Whitman, 1987).

Nitrogen and Sulfur Metabolism

Ammonia serves as the sole nitrogen source during autotrophic growth, although some methanogens are capable of utilizing a variety of other nitrogenous compounds. The presence of glutamate synthase in *Methanobacterium thermoautotrophicum* enables it to use glutamate as a sole nitrogen source (Kenealy et al., 1982). Some methanogens have also been shown to utilize urea, purines, and dinitrogen (Bhatnagar et al., 1984; DeMoll and Tsai, 1986; Lobo and Zinder, 1988). Nitrogen fixation (diazotrophy) in *Methanosarcina barkeri* requires molybdenum and is inhibited by tungsten, like in the eubacteria (Lobo and Zinder, 1988). For at least one strain, vanadium will substitute for molybdenum (Scherer, 1989). The rate of biosynthesis of the nitrogenase enzyme decreases in the presence of ammonia. In addition, the decrease in growth rate suggests diazotrophy is an energetically expensive process, as it is in eubacteria. Although the enzyme has not been purified, genetic evidence suggests sequence homology between methanogen and eubacterial nitrogenase genes (Sibold et al., 1985).

Sulfide is commonly included as a reductant and a sulfur source in the cultivation of methanogens. However, several species have the ability to use other inorganic sulfur sources, including elemental sulfur, sulfate, sulfite, and thiosulfate (Daniels et al., 1986). The observed reduction of sulfate and sulfite is strictly assimilatory in methanogens. Therefore, it is unlikely that these compounds can serve as alternate electron acceptors. Organic sulfur sources include methionine and cysteine (Mazumder et al., 1986; Scherer and Sahm, 1981b).

Cell Envelope Structure

The diversity of the methanogenic bacteria is demonstrated by the large differences in cell envelope structure. These topics are reviewed in detail by König (1988). The three known types of cell walls are composed of pseudomurein, protein, and heteropolysaccharide.

The cell wall of the Methanobacteriaceae (Fig. 14A and B) is composed of pseudomurein, a peptidoglycan analogous to eubacterial murein (Fig. 15). Pseudomurein contains L-talosaminuronic acid instead of muramic acid, β(1–3) linkages instead of β(1–4) linkages in the polysaccharide backbone, and different sequences of crosslinking amino acids. When these differences are considered, it is obvious

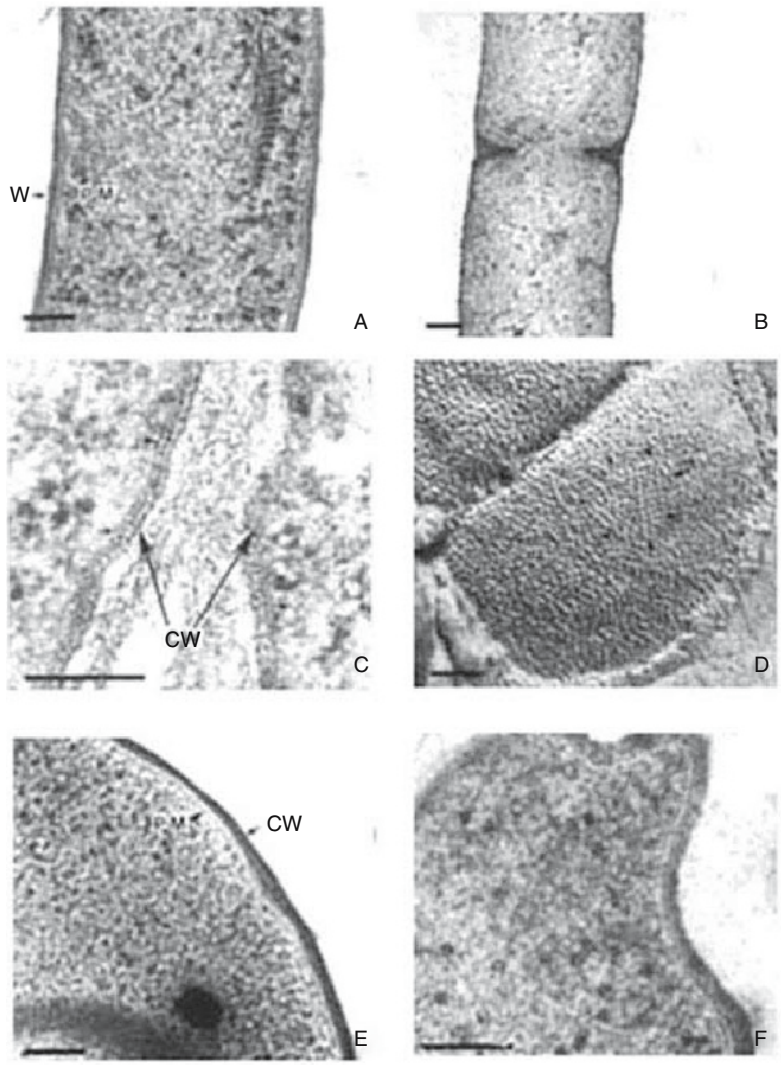


Fig. 14. (A) *Methanobacterium thermoautotrophicum* prepared by freeze-substitution in osmium-acetone. The wall (W) averages 15–16 nm in thinkness and the membrane (PM) 8 nm. (B) *M. thermoautotrophicum* prepared by chemical fixation: glutaraldehyde-formaldehyde mixture followed by osmium tetroxide. Appearance is very similar to freeze-substituted cell (A and B from Aldrich et al., 1987). (C) Chemically fixed cells of *Methanosarcina mazei.* Wall (CW) in this preparation appears expanded and fluffy. Wall thickness is 40–50 nm. Small arrows indicate plasma membrane. (D) Freeze-fractured cell of *Methanosarcina barkeri,* showing large expanse of plasma membrane. Arrows delimit areas in which 12–13-nm-diameter intramembrane particles are arranged in regular arrays. (E) Cell of *M. barkeri* freeze-substituted in osmium-acetone. Wall (CW) appears dense and compact and measures about 20 nm in thickness. Trilaminar plasma membrane (PM) measures 7 nm. (F) Freeze-substituted cell of *M. mazei.* Wall and membrane are similar in size and appearance to those shown in (E). Bars = 100 nm. (Figs. 10C–F are courtesy of H.C. Aldrich.)

why *Methanobacterium* is resistant to the β-lactam antibiotics that inhibit eubacterial cell wall biosynthesis. Variation of pseudomurein structure within the Methanobacteriaceae occurs by alteration of the amino acid residues.

In contrast is the simple protein cell wall of the Methanococcaceae and most of the Methanomicrobiaceae. It consists of a crystalline arrangement of proteins or glycoproteins called an S-layer (Fig. 16). Unlike the rigid pseudomurein, the S-layer provides only limited support; therefore, the cells are osmotically fragile. The composition of the S-layer varies between genera based on the presence of glycosylation and between species by molecular weight of the subunits and antigenicity.

Members of the Methanosarcinaceae have a third type of cell wall, referred to as a heteropolysaccharide. The cell wall of *Methanosarcina* is composed of a polysaccharide matrix (Fig. 14 C–F), which is similar in structure to eukaryotic chondroitin found in connective tissue. This polysaccharide stabilizes the cell aggregates formed by *Methanosarcina* species. The work of Aldrich et al. (1986) and Kreisl and Kandler (1986) has elucidated the chemical structure and arrangement of these polysaccharides on the cell surface as a loose matrix.

Methanosaeta (*"Methanothrix"*) and *Methanospirillum*, which are characteristically filamentous, have an outer protein sheath (Figs. 17 and 18). The cells are surrounded by an electron-dense inner wall of unknown composition and are separated by septa or plugs. The crystalline structure of the sheath has been studied in considerable detail by Beveridge et al. (1986) and Patel et al. (1986).

The cell membranes of methanogens are characterized by several unusual lipids (see reviews by De Rosa and Gambacorta, 1988; Langworthy, 1985; and Jones et al., 1987). In the majority of methanogens, the lipids are polar, either diphyt-

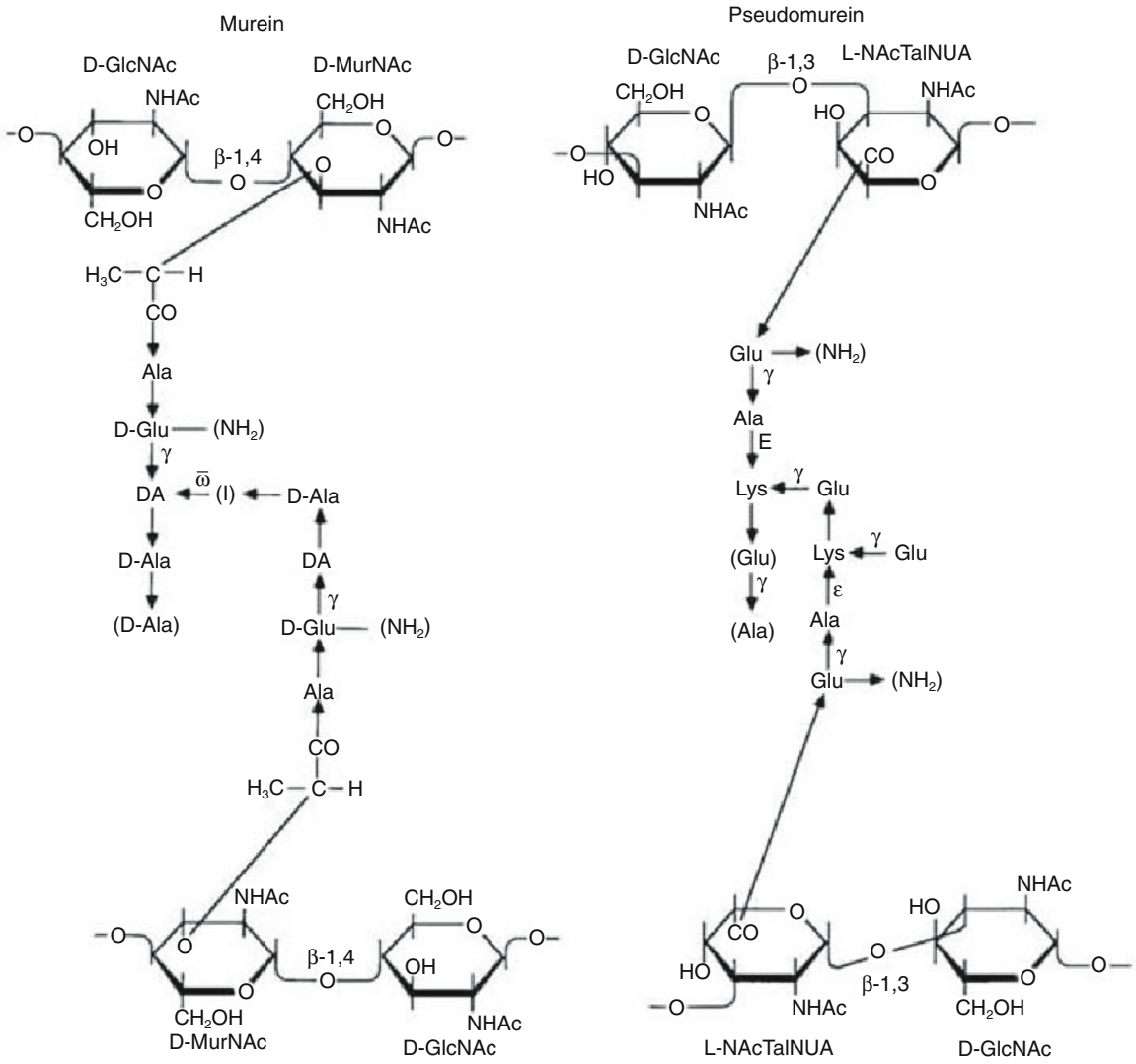


Fig. 15. Comparison of murein and pseudomurein structure. D-GlcNAc, D-N-acetylglucosamine; D-MurNAc, D-N-acetylmuramic acid; L-NAcTalNOA, L-N-acetyltalosaminuronic acid; L-DA, L-diamino acid; (I), interpeptide bridge. (Adapted from Kandler and König, 1985.)

anyl-glycerol-diethers or dibiphytanyl-diglycerol-tetraethers (Fig. 19). Thus, the membrane structure in many methanogens differs from the lipid bilayer typical of eubacteria. The tetraethers appear 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. As in eubacteria and eukaryotes, these lipids are often present as phospho- or glyco-derivatives. The major nonpolar lipids consist of isoprenoid and hydroisoprenoid hydrocarbons.

Molecular Biology of the Methanogens

Because the methanogens and other archaebacteria represent a very ancient line of descent, there has been a great deal of interest in their molecular biology. Thus, the molecular features that the methanogens share with the eubacteria and eukaryotes may have been inherited from the common ancestor of all three kingdoms. Only a brief overview of this topic is presented here, but a number of detailed reviews on different aspects are available (Brown et al., 1989; Dennis, 1986; Fox, 1985; Jones et al., 1987; Kandler and Zillig, 1986; Matheson, 1985; Thomm et al., 1986; Woese and Wolfe, 1985; Zillig et al., 1988). Furthermore, an issue of the *Canadian Journal of Microbiology* (1989; vol. 35, no. 1) is devoted to this topic.

Methanogens have some genetic characteristics of both eubacteria and eukaryotes. Sequence analysis of methanogen genes suggests that they are arranged in operons similar to those in

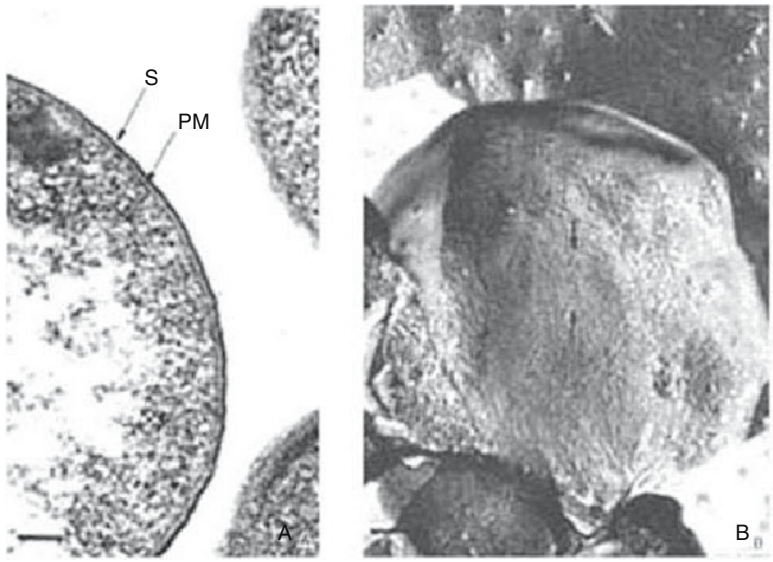


Fig. 16. (A) Thin section of *Methanococcus voltae* showing the S-layer (S) and plasma membrane (PM). (From Jarrell and Koval, 1989.) (B) Freeze-etch replica of *Methanococcus voltae* showing the hexagonal arrangement of the S-layer protein (arrows). (From Koval and Jarrell, 1987.) Bars = 100 nm.

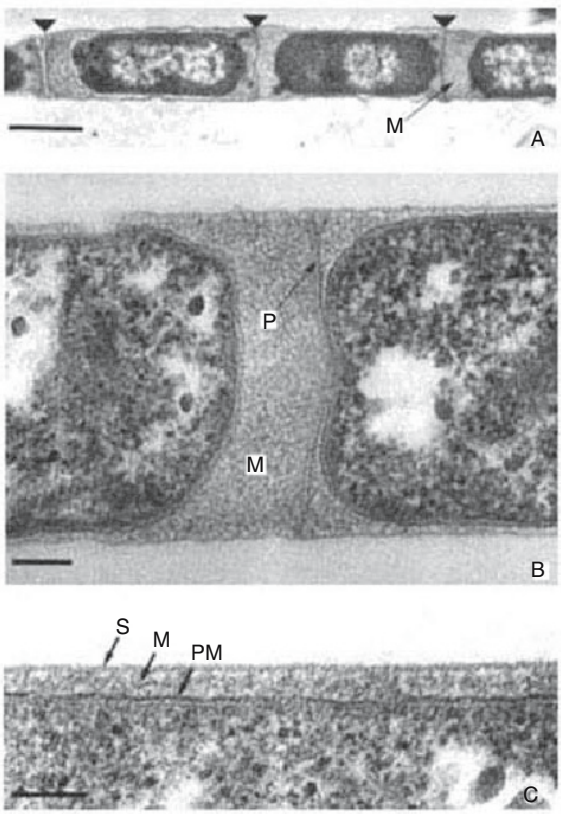


Fig. 17. (A) Thin section of a *Methanosaeta consilii* filament showing spacer plugs (arrow heads) separating the cells and the amorphous matrix (M) surrounding each cell. Bar = 1 μm. (B) Thin section showing the spacer plug (P) and the matrix (M) between two cells. Bar = 100 nm. (C) Thin section showing the sheath (S), the matrix (M), and the plasma membrane (PM). Bar = 100 nm. (From Beveridge et al., 1986.)

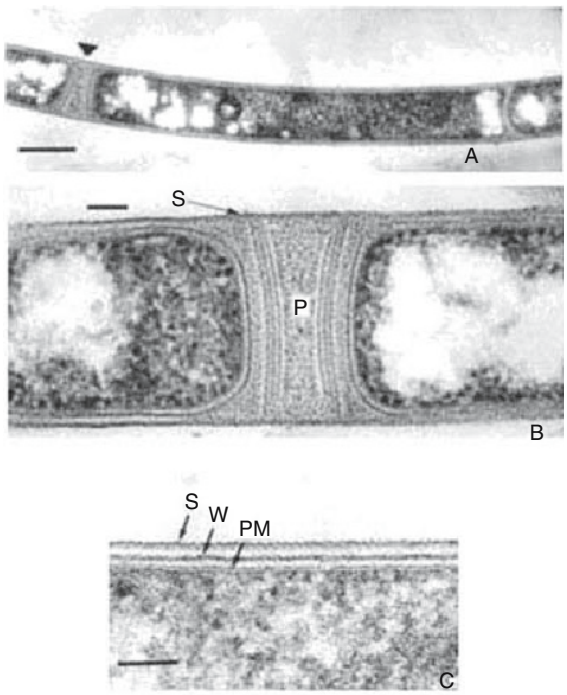


Fig. 18. (A) Thin section of a *Methanospirillum hungatei* filament showing a spacer plug (arrow head) separating two cells. Bar = 500 nm. (B) Thin section showing spacer plug (P) and sheath (S). Bar = 100 nm. (C) Thin section showing sheath (S), cell wall (W), and plasma membrane (PM). Bar = 100 nm. (From Beveridge et al., 1987.)

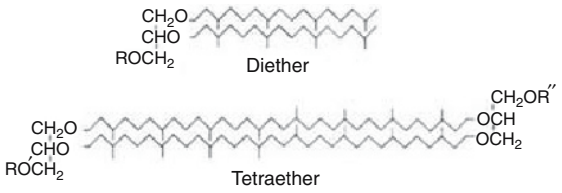


Fig. 19. Structures of a characteristic diether lipid and tetraether lipid found in the membranes of methanogenic bacteria. (Adapted from Jones et al., 1987.)

eubacteria. However, the process of transcription initiation and the termination sequences of certain genes are more similar to those found in eukaryotes. Moreover, the methanogen DNA polymerases that have been purified resemble both eubacterial and eukaryotic enzymes.

The DNA-dependent RNA polymerase of methanogens is genetically more closely related to the enzyme of eukaryotes than of eubacteria (Bernghofer et al., 1988). As a consequence, it might be expected that the methanogenic promoter sequences should also resemble eukaryotic sequences. Analysis of these sequences in numerous methanogen operons has revealed two conserved sequences. One has the consensus sequence of AAANNTTTATATA, where N represents any of the four nucleotides (Allmansberger et al., 1988). This sequence is found 25 bp upstream of the start site of transcription and resembles the "TATA" box found in eukaryotes. Binding of purified RNA polymerase to this sequence has been demonstrated in the methylreductase operon, *hisA,* and in the ribosomal RNA genes of *Methanococcus vannielii* (Thomm et al., 1989). The second conserved region, TGCAAGT, has been found at the start site of transcription of the ribosomal RNA genes. Termination of transcription has not been studied in detail. However, the gene *mcrA,* which codes for the α subunit of methylreductase, has a sequence analogous to the rho-independent terminator of eubacteria. In contrast, analysis of rRNA genes suggests a termination mechanism similar to that of eukaryotes, which use AT-rich sequences.

DNA polymerases in methanogens are heterogeneous. The enzyme purified from *Methanococcus vannielii* resembles the type A enzyme of eukaryotes in its sensitivity to the inhibitor aphidicolin (Zabel et al., 1987). In contrast, the purified enzyme of *Methanobacterium thermoautotrophicum* is resistant to aphidicolin and is similar to DNA polymerase I of *Escherichia coli* (Klimczak et al., 1986). The enzymes of *M. vannielii* and *M. thermoautotrophicum* also differ from each other in molecular weight.

The methanogen ribosome has a "typical" prokaryotic composition. The ribosome is 70S in size and dissociates into 30S and 50S subunits. The 30S subunit contains 16S rRNA, and the 50S subunit contains 5S and 23S rRNAs. A 5.8S rRNA, as found in eukaryotes, is not present. However, the structure of the methanogen ribosome differs from the eubacterial ribosome in that the 30S subunit has the characteristic archaebacterial "bill," a small protrusion for which the function is unknown. A similar "bill" is also present on the eukaryotic 40S subunit (Lake et al., 1982). Ribosomal proteins of methanogens and other archaebacteria are unusually acidic, in contrast to the basic ribosomal proteins of eubacteria and eukaryotes. However, the number of acidic ribosomal proteins correlates well with the internal K^+ concentration in the strains studied. The ribosomal proteins of *M. vannielii* are encoded by a transcriptional unit homologous in organization to the "spectinomycin operon" of *E. coli*. However, the deduced amino acid sequences of the methanogen proteins more closely resemble those of their eukaryotic homologs (Auer et al., 1989; Köpke and Wittmann-Liebold, 1989).

Sequencing of methanogen rRNAs has been instrumental in establishing their phylogenetic relationship with eubacteria and eukaryotes. The secondary structure of the methanogen 5S rRNA has characteristics of both the eubacterial and eukaryotic molecule. However, the methanogen rRNA molecule has characteristics that are unique to archaebacteria. Sequence data for the 16S rRNA support the hypothesis of the extreme diversity of the archaebacteria. The secondary structure of the methanogen 16S rRNA is more similar to that of the eubacterial structure than the eukaryotic secondary structure. Less information is available concerning the secondary structure of the 23S rRNA molecule. However, it does have characteristics of both the eubacterial and eukaryotic molecules. The genes for rRNA are arranged in a typical operon similar to that of eubacteria. The 16S, 23S, and 5S genes are closely linked and interspersed with several tRNA genes. *Methanococcus vannielii* and *M. voltae* also have unlinked 5S rRNA genes that are associated with tRNA-encoding genes.

Antibiotic Sensitivity and Resistance

The lack of sensitivity of methanogens and other archaebacteria to most antibiotics is a reflection of their unique biochemical nature and has been reviewed previously (Böck and Kandler, 1985). Methanogens are resistant to many commonly used antibiotics simply because the specific target is not present. Therefore, comparison of the antibiotic sensitivity spectrum of methanogens

Table 17. Comparison of the antibiotic sensitivity of several species of methanogens, eubacteria and eukaryotes.

		Size of zone of inhibition (mm)									
		Methanogen[a]						Eubacterium[b]		Eukaryote[c]	
Target	Antibiotic	A	B	C	D	E	F	G	H	I	J
Cell wall	Fosfomycin	—[d]	—	—	—	—	—	10	12	—	—
	D-Cycloserine	—	—	—	9	—	—	13	16	—	—
	Vancomycin	—	—	—	—	—	—	—	10	—	—
	Penicillin G	—	—	—	—	—	—	7	26	—	—
	Cephalosporin C	—	—	—	—	—	—	2	22	—	—
	Nocardicin A	—	—	—	—	—	—	2	—	—	—
	Bacitracin	40	27	40	17	—	—	—	8	—	—
	Gardimycin	30	16	6	—	—	—	—	3	—	—
	Nisin	11	8	10	3	—	—	—	3	—	—
	Enduracidin	20	16	10	5	—	8	—	10	—	—
	Flavomycin	—	—	—	—	—	—	3	16	—	—
	Subtilin	4	7	5	10	—	—	—	6	—	—
RNA polymerase	α-Amanitin	—	—	—	ND[e]	—	ND	ND	ND	ND	ND
	Rifampicin	2	—	—	2	—	—	9	22	—	—
Protein biosynthesis	Cycloheximide	—	—	—	—	—	—	—	—	23	22
	Chloramphenicol	25	13	22	28	4	40	11	10	—	—
	Virginiamycin	5	14	—	15	—	—	5	19	—	—
	Gentamycin	—	25	12	—	—	—	9	13	—	—
	Tetracycline	—	—	8	8	—	—	13	18	—	—
	Oleandomycin	—	—	—	—	—	—	—	13	—	—
	Erythromycin	—	—	—	3	—	—	3	17	—	—
	Kanamycin	—	—	—	—	—	—	9	13	—	—
Membrane	Garmicidin S	7	7	15	10	4	16	—	4	3	4
	Garmicidin D	—	—	11	—	—	—	—	4	—	—
	Polymyxin	—	—	8	—	—	—	5	—	4	3
	Amphotericin B	—	—	—	—	—	—	—	—	6	5
	Valinomycin	—	—	—	—	—	—	2	2	—	—
	Nonactin	—	—	—	—	—	—	2	5	—	—
	Monensin	—	5	25	12	15	25	—	6	—	—
	Lasalocid	22	25	42	21	40	40	—	15	—	—

[a]Species identification: A, *Methanobrevibacter smithii*; B, *Methanobacterium bryantii*; C, *M. thermoautotrophicum*; D, *Methanococcus vannielii*; E, *Methanospirillum hungatei*; F, *Methanosarcina barkeri*.
[b]G, *Escherichia coli*; H, *Staphylococcus aureus*.
[c]I, *Saccharomyces cerevisiae;* J, *Hansenula* sp.
[d]—, No inhibition.
[e]ND, not determined.
Adapted from Hilpert etal. (1981).

with those of eubacteria and eukaryotes can provide insight into their biochemical similarities and differences.

The differences in chemical structure of methanogen cell envelopes to those of eubacteria have been well established. These differences are supported by the lack of sensitivity of methanogens to many cell-wall inhibitors (Table 17). For example, *Methanobacterium thermoautotrophicum* is resistant to penicillin G, since the crosslinking of pseudomurein, which replaces peptidoglycan in the cell wall, is not inhibited by the antibiotic. However, antibiotics such as bacitracin, which inhibit the formation of lipid-bound precursors of murein in eubacteria, may inhibit formation of similar lipid-bound precursors involved in biosynthesis of lipids and glycoproteins in methanogens.

In addition to genetic homology, the antibiotic sensitivity of DNA-dependent RNA polymerase reflects the similarity of the methanogen enzyme to the eukaryotic enzyme (Table 17). Both enzymes are resistant to rifampicin, an antibiotic which inhibits RNA polymerase of eubacteria. The methanogen enzyme is also resistant to α-amanitin, which inhibits the type B and C polymerases of eukaryotes but not the type A enzyme.

Antibiotics that inhibit protein synthesis may block aminoacyl-tRNA synthesis, elongation, or translation. Pseudomonic acid is a potent inhibitor of *Methanococcus voltae* and inhibits isoleucyl-tRNA synthetase activity (Possot et al., 1988). Translation in methanogens is resistant to many of the antibiotics that inhibit translation by either the eubacterial 70S or eukaryotic 80S ribo-

somes (Böck and Kandler, 1985). While growth of the methanogens is sensitive to chloramphenicol, this inhibition is not due to an effect on protein synthesis. Instead, inhibition is probably due to the sensitivity of the particulate hydrogenase of *Methanobacterium* to this antibiotic and to other halogenated compounds. However, the elongation factor aEF-2 is sensitive to diphtheria toxin, which is also characteristic of the eukaryotic protein (Lechner et al., 1988). Sequence analysis of the genes encoding aEF-2 indicates that the methanogen enzyme is more closely related to its eukaryotic than its eubacterial homolog. In addition, all archaebacterial aEF-2 genes examined possess a histidine residue that is posttranslationally modified to diphthamide, a phenomenon analogous to that of the eukaryotic EF-2. Translation in the methanogens is also resistant to most antibiotics in the streptomycin class of aminoglycosides, as well as to tetracycline and the macrolides. However, methanogens are sensitive to deoxystreptamine aminoglycosides, such as gentamycin and neomycin, as well as to virginiamycin. Therefore, the translation process in methanogens is different from that of the eubacterial process with respect to antibiotic sensitivity.

Antibiotics that act on membrane integrity also have varying effects on methanogens, as they do with eubacteria. Ionophores such as monensin, lasalocid, and gramicidin S are very effective inhibitors of methanogens. This inhibition is presumably the result of interference with Na^+ transport and thus with methanogenesis (Perski et al., 1982). Monensin and lasalocid inhibit both growth and methanogenesis (Dellinger and Ferry, 1984). However, this inhibition depends on the culture conditions, since they both stimulate methanogenesis at low Na^+ concentrations in vivo (Schönheit and Beimborn, 1986).

Motility

Motility in methanogens has been associated with the presence of flagella. However, little is known about their structure, attachment to the cell envelope, and the energetics of motility. Kalmokoff et al. (1988) found that the flagella of *Methanococcus voltae,* consisting of two protein subunits with molecular weights of 33,000 and 31,000, have a filament diameter of 13 nm, unlike those of eubacteria which generally consist of only one monomer. The basal structure is knob-like in appearance, as opposed to the multiple ring structure found in eubacteria. This may be due to the simpler structure of the methanococcal S-layer. It will be interesting to compare the basal structures of methanogens that have more complex cell envelopes. The arrangement of flagella varies greatly among methanogen genera, as it does in eubacteria. *Methanococcus jannaschii* has lophotrichous flagella with two bundles of flagella at one pole arranged in a "corkscrew" fashion (Jones et al., 1983a). In contrast, *Methanogenium* species have peritrichous flagella (Romesser et al., 1979).

Chemotaxis in methanogens has been described in *Methanospirillum hungatei,* for which acetate is a chemoattractant (Migas et al., 1989). In addition, *Methanococcus voltae* responds to acetate, isoleucine, and leucine as attractants but not to histidine (Sment and Konisky, 1989). *M. voltae* has an absolute requirement for all these nutrients except histidine. Therefore, it is evident that some methanogens respond positively to higher nutrient concentrations. The advantage of this in certain habitats, such as marine marshes where substrate concentrations may be low, is self evident.

Applications

Methanogenic bacteria have numerous applications, especially in the degradation of wastes rich in organic matter to methane and CO_2. Methane is the major component of natural gas, a clean fuel which is already in wide use. This topic has been reviewed extensively (Daniels, 1984; Hobson et al., 1981; van den Berg, 1984; Wodzinski et al., 1987), and only a few major points will be discussed here.

Because of the narrow substrate specificity of methanogens, pure cultures have limited usefulness in waste treatment. For most applications, consortia containing both methanogenic bacteria and heterotrophic bacteria capable of converting the complex organic materials common in waste materials to methanogenic substrates are used. The consortia are usually obtained from an inoculum of manure, sewage sludge, freshwater sediment, or some other natural source, and they are formed upon enrichment with the waste material of interest under methanogenic conditions. In this manner, consortia have been obtained for the degradation of a wide variety of wastes and other materials. Moreover, thermophilic consortia have been obtained by performing the enrichments at high temperatures.

For example, methanogenic consortia that degrade whey, a major by-product in milk and cheese production, have been described (Chartrain and Zeikus, 1986a, 1986b; Wildenauer and Winter, 1985; Zellner et al., 1987b; Zellner and Winter, 1987b). These consortia were composed of sugar- and protein-fermenting strains of *Lactobacillus*, *Eubacterium*, *Clostridium*, *Klebsiella*,

and *Leuconostoc*. Their fermentation products were then metabolized by acetogenic strains of *Desulfovibrio*, *Eubacterium*, and *Clostridium*. The acetate, H_2, and formate formed were substrates in turn for the methanogens present, *Methanobacterium, Methanobrevibacter, Methanospirillum, Methanocorpusculum, Methanosarcina,* and *Methanosaeta.* These complex consortia were capable of the nearly complete conversion of whey to methane and CO_2 within 5 days.

Another common application of methanogens is in the treatment of domestic sewage. Whether it be a simple cesspool or an advanced anaerobic digestor, the methane produced from sewage can be collected and used to offset the cost of an otherwise obligatory process. In countries such as India and China, where the economic resources required for such digestors are not available, animal and human wastes are still used as a source of energy. Instead of advanced digestors, these countries have developed inexpensive small scale digestors for production of methane. In addition, the spent slurry can be used as a natural fertilizer. Obvious drawbacks of these digestors are their limited size and requirement for manual loading and emptying. The digestors are also limited to temperate climates since they must rely on the ambient temperature to eliminate the additional heating costs required to maintain an active biomass.

While consortia can be obtained for the conversion of many types of organic wastes to methane and CO_2, the growth of these consortia is usually slow when compared to growth of those composed of aerobic microorganisms. This can cause problems since high waste input can result in loss of cell biomass, and thus waste decomposition is limited by the growth rate of the bacteria. Therefore, to increase efficiency, specialized bioreactors have been designed to retain the microbial population in the bioreactor while allowing for a high input of waste. In this way, the rate of waste decomposition is not limited by the growth rate of the bacteria. The five types of bioreactors commonly used have been reviewed by van den Berg (1984) and are described below:

1. The anaerobic contact reactor uses a sludge, which settles to the bottom and contacts the raw waste. Thus, the settling ability and mixing of the sludge with the waste are important in the efficiency of bioconversion. This bioreactor type works well for particulate wastes, which settle easily and are completely biodegradable.
2. The anaerobic filter reactor retains suspended bacteria and waste in a packing material or solid support where degradation occurs. It is advantageous for treatment of dilute soluble wastes. However, it is easily plugged by suspended particulate waste.
3. The upflow anaerobic sludge bed reactor avoids plugging by reducing the volume of the packing material and using a gas collector to encourage settling of the sludge. High concentrations of microbial biomass are needed in addition to proper development of a granular sludge to promote settling.
4. The anaerobic fluidized or expanded bed reactors allow growth of biomass on inert particles, which settle and are suspended by a rapid flow of waste.
5. The downflow stationary fixed-film reactor avoids plugging of the anaerobic filter by forming an active biomass film. This reactor can accommodate a wide variety of wastes.

Another area of potential applications of methanogenic consortia is in degradation of xenobiotics (for reviews see Berry et al., 1987; Young, 1984). Especially important is the degradation of halogenated aromatic and aliphatic compounds, which are common pollutants. For instance, tetrachloroethylene is a common pollutant in ground waters due to improper disposal in landfills (Fatherpure and Boyd, 1988). In the presence of sewage sludge or pure cultures of methanogens, it is reductively dehalogenated to trichloroethylene. In contrast, tetrachloroethylene is not rapidly degraded under aerobic conditions. Likewise, low concentrations of chloroform, carbon tetrachloride, and 1,2-dichloroethane are almost completely oxidized to CO_2 by methanogenic consortia (Bouwer and McCarty, 1983). Haloaromatic compounds are also reductively dehalogenated by methanogenic consortia (Suflita et al., 1983). Thus, chlorinated phenols and chlorinated benzoates are converted to phenol and benzoate, respectively. Phenol may then be carboxylated to form benzoate (Knoll and Winter, 1989). Benzoate is then converted to methane and CO_2, presumably by a reductive route via cyclohexane carboxylate.

Methanogens have also been implicated in anaerobic biocorrosion, which is a significant economic problem. By the process of cathodic depolarization, elemental iron is oxidized and protons in water are reduced to H_2 (Daniels et al., 1987). Methanogens accelerate this process by maintaining a very low partial pressure of H_2. The mechanism is similar to that proposed for sulfate-reducing bacteria, which also consume H_2. Protons from water combine with electrons from iron to form hydrogen, which is utilized by the hydrogenase enzyme.

Acknowledgements. The authors wish to thank Drs. Terry Miller, Josef Winter, and Tatjana

N. Zhilina for their critical reading of this manuscript.

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