The Biology of Methanogenic Bacteria

J. G. ZEIKUS

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

INTRODUCTION

My purpose here is both to describe certain features of methanogens (i.e., methane-producing bacteria) and to view the biology of this microbial group. I first heard the term methanogen used by M. P. Bryant; this term is descriptive and eliminates confusion with another microbial group, the methane-oxidizing bacteria.

The biological formation of methane (CH₄) is the result of a specific type of bacterial energy-yielding metabolism. Eucaryotic organisms and blue-green algae have not been reported to produce methane. Previous reviews on microbial methanogenesis by Barker (4), Stadtman (95), and Wolfe (112) summarized the state of knowledge on methanogenic bacteria and the biochemistry of methane synthesis through 1970; much of that material will not be presented here, except where importance dictates.

Bacterial methanogenesis is a ubiquitous process in most anaerobic environments. The association of this event with anaerobic decomposition of organic matter in microbial habitats such as sewage sludge digestors, the rumen and intestinal tract of animals, and in sediments and muds of various aquatic habitats has been recognized and documented for more than a century. Thus, gas production commonly observed in nature can often be ascribed to growth of methanogens on specific energy sources that are formed as a result of microbial decomposition of organic matter (Fig. 1A) or in association with geochemical activity (Fig. 1B). However, detailed information on the biological properties of methanogenic bacteria has only recently materialized. Paramount to an integrated understanding of these unique microorganisms was the vivid documentation of their primary chemolithotrophic metabolism by Bryant and co-workers (10, 14). Subsequent literature that will be reviewed here has provided new scientific vistas and enables a better understanding of older studies on methanogenesis.

Few natural groupings of microorganisms, such as those designated as parts in Berger's Manual of Determinative Bacteriology (17), are as morphologically diverse as the methanogenic bacteria. Nevertheless, all methanogenic species share certain unique and unifying physiological properties. Methanogens should no longer be regarded as a mysterious group of poorly studied microbes. Indeed, the present world "energy crisis" has generated a new stimulus and scientific interest to better understand bacteria that produce natural gas.
METHODS FOR STUDY

Isolation and Cultivation Techniques

Methanogens are perhaps the most strictly anaerobic bacteria known, and detailed studies require the use of stringent procedures that ensure culture in the absence of oxygen. Procedures developed by Hungate have proved most successful for cultivation of fastidious anaerobes. One should refer to a recent article by Hungate (46) for a complete description of this method. Modifications of the Hungate technique described by Bryant (12) have been most widely used for the study of methanogens. This method utilizes glass test tubes (Bellco Glass, Inc., anaerobic culture tubes) that are tightly

Fig. 1. (A) Ignited methane gas emanating from a hollow increment-borer bit drilled 20 cm into a cottonwood tree located on the shore of Lake Wingra, Wis. Photograph is from a time exposure taken at night (from reference 117). (B) Ebullition of gas bubbles that contained mainly CH₄ and CO₂ from a thermal spring in Yellowstone National Park. Methanogens were isolated from this spring, which contained geothermal hydrogen (unpublished observation).
sealed with rubber stoppers during concomitant removal of gassing cannulae. Neoprene, butyl, or synthetic, but not gum, rubber are suitable as culture tube enclosures. Gases that enter the cannula (a presterilized, cotton-filled 5-ml glass syringe, fitted with a bent 2-inch [ca. 5.08 cm], 18-gauge needle) are scrubbed free of oxygen traces by passage through heated copper filings. A gas mixture of 80% H₂ and 20% CO₂ can be routinely used for culture of methanogens. However, a 50:50 mixture of H₂-CO₂ is preferred by some investigators (12), because this gas mixture is more dense and not as easily displaced by air when culture containers are opened. Media or substrate addition and transfer or inoculation of tubes are each accomplished by pipetting while the tubes are being gassed.

Alternatively, "Hungate type" tubes (Bellco) that are screw-capped and sealed with flanged rubber stoppers can be used. Addition and transfer to these tubes utilizes the syringe methods described by Macy et al. (59). Recently, Miller and Wolin (65) have described procedures that employ serum bottles (Wheaton, Industrial Glass Division) or various glass containers fitted with serum bottle necks. Culture containers are closed with butyl rubber serum-stoppers and secured with a crimped metal seal. All inoculations are performed with a hypodermic syringe and needle. These procedures require less manipulation. The use of metal-secured containers for growth of some methanogens is advantageous because nonsecured stoppers are often blown out of culture tubes as the result of active methanol fermentation. Serum bottles are also more easily handled and less prone to breakage than test tubes and are ideal for ecological studies in the field. A new approach for cultivation of H₂-oxidizing methanogens has been described by Balch and Wolfe (1). These procedures allow for good growth of methanogens under high gas pressures (2 to 4 atmospheres of H₂-CO₂) without the need for repeated culture gassing. The authors described procedures for preparation and use of a specialized gassing manifold, glass culture tubes for liquid cultures, and anaerobic incubators for agar plate cultures. The Hungate culture technique, with its modifications, has proven to be an excellent method for isolating fastidious anaerobes and maintaining small quantities of cells. The methods described by Bryant et al. (14) for culturing larger quantities of cells have proven acceptable for most methanogenic species (for a modification of these procedures, see Daniels and Zeikus [28]).

Edwards and McBride (32) have described procedures for isolation and growth of methanogens that utilize a Freter-type anaerobic glove box equipped with an inner ultralow oxygen chamber. Cultures are plated in the outer anaerobic glove box and immediately placed into the inner ultralow oxygen chamber, which is periodically flushed with H₂-CO₂ (80:20). The inner chamber allows for growth on hydrogen and maintains the redox potential necessary for growth of methanogens. This method is considerably more expensive than Hungate procedures; however, it offers unique advantages. For example, it requires less skill and manual dexterity, and it allows for routine genetic procedures such as replica plating. In lieu of an inner chamber, plates can be incubated in specially modified pressure-cooker containers for growth under higher atmospheric pressures of H₂-CO₂ (1).

These authors (32) also described a novel screening procedure for identification of methanogenic colonies. Presumptive identification is based on the detection of fluorescent colonies when streak plates are exposed to long-wave ultraviolet light. Small colonies (less than 0.5 mm) usually do not fluoresce. All methanogens that have been examined contain a unique pigment, Factor F₄₅₀ (F₄₅₀), which fluoresces when the oxidized form is excited by long-wave ultraviolet light. During active growth, F₄₅₀ exists in a partially oxidized state (32). In addition, methanogenic bacteria will brightly fluoresce when observed by ultraviolet microscopy, although this fluorescence is short-lived (65a). Additional procedures used for identification of methanogens are based on demonstration of active methane production by isolated cultures.

**Gas Chromatographic Analysis**

During the course of physiological and ecological investigations of methanogens, it is often necessary to monitor utilized or produced metabolic gases. The energy-yielding metabolism of methane-producing bacteria often involves the oxidation of hydrogen with the concomitant reduction of carbon dioxide. Nelson and Zeikus (68) have described a gas chromatographic procedure for analysis of ^14^C-labeled and unlabeled metabolic gases from microbial methanogenic systems that is more rapid, sensitive, and convenient than gas chromatography-liquid scintillation techniques (61). This method identifies and quantifies gases by thermal conductivity detection and directly channels the gas chromatograph detector effluent into a gas proportional counter for radioactivity measurement. These procedures have been em-
employed for ecological (110, 111, 123) and physiological (122) studies of methanogens.

Thermal conductivity detection is often more useful than flame ionization detection because H₂, CH₄, and CO₂ can be accurately quantified on the same column. Although flame ionization detection is more sensitive than thermal conductivity detection, and is the preferred method for methane analysis, it is limited to CH-containing molecules and is, therefore, unsuitable for H₂ and CO₂.

GENERAL PROPERTIES

Species Characteristics

Table 1 describes features of the taxonomically identified species of methanogens that are maintained in pure culture in several research laboratories. Other taxonomically described methanogenic species (13) that are presently not in culture include: Methanococcus mazei, Methanobacterium soehngenii, and Methanosarcina methanica. These species have been lost and/or were never obtained in well-documented pure culture. Numerous strains of methanogens that have been isolated by various investigators (14, 22, 71, 90, 123, 124) remain to be described in more detail before taxonomic assignment is established. Most notably these strains include Methanobacterium strain MOH (60), isolated from the Methanobacillus omelianskii symbiosis, and a recently obtained Methanobacterium strain (22) that metabolizes acetate in a complex medium.

All methanogenic bacteria can use hydrogen as a sole source of reducing power for methanogenesis (i.e., as an energy source) and for cell synthesis; several species utilize formate, and one species, Methanosarcinaarkeri, can use methanol. Another metabolic feature shared by several species is the ability to synthesize all cellular carbon from CO₂ while growing at the expense of hydrogen oxidation. However, autotrophy has been difficult to document in some species because of very slow growth in the absence of certain organic compounds.

It has been well established that acetate is the major methanogenic precursor in several anaerobic ecosystems (see below, Ecological Aspects). However, acetate has not been demonstrated to serve as the sole electron donor for growth and methanogenesis in pure cultures. Previous isotopic studies of Stadtman and Barker (92, 93) that demonstrated very slow (15 weeks) acetate fermentation were performed with "highly purified" cultures of M. barkeri and Methanococcus species. These cultures contained more than one distinct morphological type. Methanosarcina species are difficult to isolate and maintain in pure culture. The work of Pine and Barker (73) and Pine and Vishniac (74) used crude enrichment cultures to demonstrate that the intact methyl group of acetate was fermented to methane. Conservation of the protons in the methyl moiety strongly suggests that CH₄ production from acetate was attained via a single reductive step by a single organism.

Isotopic tracer studies of Zeikus et al. (122) showed that hydrogen was required for the metabolism of acetate to methane by several pure

<table>
<thead>
<tr>
<th>Species name</th>
<th>Substrates that serve as sole electron donor for both methanogenesis and growth</th>
<th>Autotrophic growth</th>
<th>Taxonomic description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanobacterium* arbophilicum</td>
<td>Hydrogen</td>
<td>Yes</td>
<td>Zeikus and Henning (119)</td>
</tr>
<tr>
<td>Methanobacterium formicium</td>
<td>Hydrogen or formate</td>
<td>Yes</td>
<td>Schnellen (88)</td>
</tr>
<tr>
<td>Methanobacterium ruminantium</td>
<td>Hydrogen or formate</td>
<td>No</td>
<td>Smith and Hungate (91)</td>
</tr>
<tr>
<td>Methanobacterium mobile</td>
<td>Hydrogen or formate</td>
<td>No</td>
<td>Paynter and Hungate (72)</td>
</tr>
<tr>
<td>Methanobacterium* thermoautotrophicum</td>
<td>Hydrogen</td>
<td>Yes</td>
<td>Zeikus and Wolfe (116)</td>
</tr>
<tr>
<td>Methanococcus van nieli</td>
<td>Hydrogen or formate</td>
<td>Not determined*</td>
<td>Stadtman and Barker (94)</td>
</tr>
<tr>
<td>Methanosarcina barkeri</td>
<td>Hydrogen or methanol</td>
<td>Yes</td>
<td>Schnellen (88)</td>
</tr>
<tr>
<td>Methanospirillum* hungatii</td>
<td>Hydrogen or formate</td>
<td>Not determined*</td>
<td>Ferry et al. (33)</td>
</tr>
</tbody>
</table>

* Type strain deposited in American Type Culture Collection.

* Growth occurred in mineral salts medium that contained H₂ or formate and an organic reducing agent (cysteine or sodium thioglycolate). These species may be capable of autotrophy.
cultures. One strain of *M. barkeri* and one of *Methanobacterium thermoautotrophicum* produced methane in a mineral salts medium that contained acetate (1% final concentration) as the sole organic addition and an H₂-N₂ gas phase. Under these conditions, *M. thermoautotrophicum* converted approximately 5% of the acetate to methane in 150 h, and both the methyl and carboxyl moieties of acetate appeared to be reduced. Acetate conversion to CH₄ by these strains was dependent on H₂, CO₂, and acetate concentrations. Methane was not formed after prolonged incubation times (6 weeks) of either species in an acetate-mineral salts medium with an N₂ or CO₂ gas phase.

Growth of methanogens on acetate-mineral salts medium was only observed in the presence of an H₂-CO₂ gas phase. Under these conditions, most of the methane formed by *M. thermoautotrophicum* originated from CO₂ reduction. Thus, the amount of acetate converted to methane greatly depends upon the cultural conditions. The minor conversion of acetate to methane demonstrated by these pure culture studies does not account for how acetate is converted to CH₄ either in nature or in mixed cultures.

Cappenberg (22) has shown that an undescribed *Methanobacterium* species converted acetate to methane in a complex medium, although tracer studies were not used. Growth under these conditions in continuous culture was slow (65-h doubling time). When this methanogenic species was grown in continuous-mixed culture with a sulfate-reducing species, acetate utilization was greatly enhanced. This *Methanobacterium* species can also use H₂-CO₂ as methanogenic substrates. Preliminary studies of Mah (Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, Q27–29, p. 195) indicate that acetate dissimilation by a strain of *M. barkeri* in a complex medium was not dependent on H₂, although acetate was utilized more rapidly in the presence of H₂. This strain also uses H₂-CO₂ as methanogenic substrates. Factors affecting the rate of acetate conversion in a methanogenic enrichment culture were reported by Van den Berg et al. (106). The mixed culture did not use H₂ or HCOOH, and methanogenesis from acetate was greatly influenced by the redox and ionic strength of the synthetic medium. Thus, acetate conversion to methane has been demonstrated in both pure and mixed cultures, although more quantitative and detailed studies are needed both to document that pure cultures of methane-producing bacteria oxidize acetate in a facile manner and to establish the significance of acetate as an energy source for growth.

Table 2 compares the free energy available from the metabolism of various methanogenic substrates. It is clear from these calculations that H₂ (equation 1) and formate (equation 2) are nearly equivalent energy sources for methanogenesis. Less energy is available from methanol fermentation (equation 3), and limited energy is obtained by acetate fermentation (equation 4). An analysis of energy formed per mole of methane produced by these reactions demonstrates that approximately four times as much energy is available from respiration of hydrogen than from acetate fermentation. These thermodynamic calculations do not rule out the possibility of acetate-fermenting methanogens because equation 4 is exergonic. However, an acetate-fermenting methanogen would be inherently slow growing, and it would be questionable if fermentation of acetate alone would sustain the energy requirements for growth. Decker et al. (30) suggest that degradation of a substrate beyond acetate is feasible for energy production only if the oxidation of reduced coenzymes can be linked to electron transport phosphorylation. Values reported for the free energy of adenosine 5'-triphosphate (ATP) hydrolysis have been estimated to range from \(-8.2\) kcal/mol (ca. \(-34.325\) kJ/mol) to \(-12.5\) kcal/mol (ca. \(-52.325\) kJ/mol) at physiological conditions. Normal efficiencies of energy transfer in bacteria are 30 to 50% (30, 64). Thus, reactions (e.g., equation 4) that yield less than \(-11.7\) kcal/mol (ca. \(-48.976\) kJ/mol) (at standard conditions) make the formation of an energy-rich compound via substrate level phosphorylation unlikely and are insufficient for cell growth (30). It should be noted that equation 4 describes an intramolecular redox proc-

<table>
<thead>
<tr>
<th>Equation</th>
<th>(\Delta G^\circ) (kcal/mol of reaction)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 4H₂ + HCOO⁻ + H⁺ → CH₄ + 3H₂O ....... (-32.7)</td>
<td></td>
</tr>
<tr>
<td>2. 4HCOO⁻ + 4H⁺ → CH₄ + 3CO₂ + 2H₂O ....... (-34.7)</td>
<td></td>
</tr>
<tr>
<td>3. 4CH₃OH → 3CH₄ + CO₂ + 2H₂O ........... (-76.4)</td>
<td></td>
</tr>
<tr>
<td>4. CH₄COO⁻ + H⁺ → CH₄ + CO₂ ....... (-8.6)</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated from published values (107) for the free energy of formation of equation reactants and products at standard conditions (25°C, 1 atmosphere, and equal molar concentrations of reactants and products). Values for the free energy of formation of H₂, CH₄, and CO₂ represented as gases; HCOO⁻, HCO₂⁻, and CH₂CO₂⁻ represented as aqueous ions; and H⁺, H₂O, and CH₃OH represented as aqueous.
ess, and, thus, energy should not be obtained by this reaction from electron transport phosphorylation (102a). Mechanistically, it is not known how energy can be obtained from acetate metabolism by methanogens.

Previous isotopic tracer and deuterium incorporation studies (73, 74, 92, 93) proposed that acetate was metabolized by the following reaction:

\[ \text{CH}_3\text{COOH} \rightarrow \text{CH}_4^- + \text{CO}_2 \]

It is important to note that this mechanism would not allow for microbial growth on acetate alone. Microbial growth on acetate as the sole reducing source would only occur if the C2 of acetate were oxidized to generate reducing equivalents.

Electrons generated in this manner could then be used to convert acetate to cell material. If this occurred, significant amounts of \(^{14}\text{CO}_2\) would be formed from the C2 of acetate. Alternatively, acetate fermentation could be the major source of energy, but other substrates (H\(_2\) or organic compounds) would be needed to provide reducing equivalents or precursors for cell carbon synthesis. However, any alternative electron donor present in an acetate medium may also serve as an energy source for growth. Also, the repeated inability to isolate and grow acetate-fermenting methanogens on acetate alone as the sole electron donor for growth and methanogenesis (34, 66, 75, 123) may be the result of improper cultivation conditions.

Carbon monoxide can also be used as a substrate by methanogens. Kluyver and Schnellen (52) demonstrated that cell suspensions of \(M.\) \(barkeri\) and \(Methanobacterium\) \(formicicum\) converted CO to \(\text{CO}_2\) and \(\text{CH}_4\). Thus, while methane can be produced from carbon monoxide by some methanogens, its use as an energy source for growth is not documented.

Three methanogenic bacteria have been described since 1970 and include \(M.\) \(thermoautotrophicum\), \(Methanobacterium\) \(arbophilicum\), and \(Methanosporillum\) \(hungatii\). \(M.\) \(thermoautotrophicum\) type strain \(\Delta H\) was first isolated from a sewage sludge digestor in Urbana, Ill., and has an optimum temperature for growth between 65 and 70°C (116). Other thermophilic isolates that appear similar to this species have been obtained from thermophilic manure digestors, thermal springs, and decomposing algal mats associated with thermal spring effluents (Yellowstone National Park, U.S.A.). \(M.\) \(thermoautotrophicum\) strain AO, isolated from a sewage sludge digestor in Madison, Wis., appears identical to the type strain in morphology, nutrition, and guanine plus cytosine (G+C) content.

\(M.\) \(thermoautotrophicum\) is an obligately chemolithotrophic autotroph, and growth of this species is not stimulated by organic additions (116). This methanogen apparently lacks the methanol dehydrogenase and formate dehydrogenase found in some other species. \(M.\) \(thermoautotrophicum\) can proliferate in 12-liter fermentor culture, with a doubling time of less than 3 h in a totally inorganic mineral salts medium, and has the shortest doubling time of the methanogens described to date. It appears that this species is the "organism of choice" for mass culturing of methane bacteria. Also, the appearance of contaminants in large fermentors is not a problem when inorganic media and high temperatures are used.

\(M.\) \(arbophilicum\) type strain DH1 was isolated from wetwood of living trees (119). Other strains with nutritional and morphological properties identical to \(M.\) \(arbophilicum\) have been obtained from freshwater sediments and soil. Like \(M.\) \(thermoautotrophicum\), this species is an obligate chemolithotroph, although growth of \(M.\) \(arbophilicum\) is greatly stimulated by organic additions. This species will grow with a doubling time of 10 h in 12-liter fermentor culture on a mineral salts medium that contains vitamins and \(\text{H}_2\)-\(\text{CO}_2\).

The general features of \(M.\) \(hungatii\) type strain JF1, isolated from sewage sludge, were recently described by Ferry et al. (22). \(M.\) \(hungatii\) can utilize hydrogen or formate as electron donors for methanogenesis. Growth of this methanogen was stimulated by organic supplements, and a mean doubling time of 17 h was obtained on complex medium in a 12-liter fermentor (J. G. Ferry, personal communication). A different strain of \(M.\) \(hungatii\) has recently been characterized (71). This strain differs somewhat from the type strain in temperature requirements, colony morphology, and deoxyribonucleic acid (DNA) base composition. Acetate metabolism was demonstrated, but its specific contributions to cell carbon and to methane formation were not established.

Detailed nutritional studies of methanogens have been limited to relatively few species. Table 3 summarizes certain nutritional features of the best characterized strains, all of which utilize \(\text{H}_2\) and \(\text{CO}_2\) for energy and cell carbon syntheses. These methanogens do not use amino acids or \(\text{N}_2\) as nitrogen sources; and no other nitrogenous compounds have been shown to replace the obligate NH\(_4^+\) requirement for growth. \(Methanobacterium\) \(ruminantium\) strain PS, \(M.\) \(thermoautotrophicum\) strain \(\Delta H\), and \(M.\) \(arbophilicum\) strain DH1 require sulfide as a sulfur source. \(Methanobacterium\) strain MOH can use \(\text{H}_2\text{S}\) or cysteine (11). The specific vitamin requirements for growth of
most strains are not known, except that one or more of a complex vitamin mixture may be required or highly stimulatory. *M. thermoaototrophicum* strain ΔH (116) and *M. hungatii* strain JF1 (J. G. Ferry, personal communication) do not require vitamins for culture, although vitamin addition stimulates growth of *M. hungatii* but not *M. thermoaototrophicum*. *M. arbophilicum* has an obligate requirement of one or more vitamins for growth (119). Bryant et al. (11) demonstrated that *M. ruminantium* strain M1 (rumen isolate) would not grow in a complex medium unless rumen fluid were added. A cofactor present in rumen fluid essential for growth of this strain has been identified as coenzyme M (2-mercaptoethanesulfonic acid) by Taylor et al. (101).

Methanogens vary considerably with regard to specific carbon requirements and growth response to organic additions. *M. ruminantium* strain M1 (rumen isolate) requires acetate, 2-methylbutyrate, and amino acids. Isotopic incorporation studies (11) demonstrated that *M. ruminantium* strain M1 preferentially synthesized about 60% of cell carbon from acetate during growth on complex medium with H2-CO2. Acetate carbon was incorporated into protein, nucleic acid, and lipid fractions. 2-Methylbutyric acid is required for isoleucine biosynthesis (85). Amino acids are essential for growth of this strain, and they have not been reported as effective carbon sources (11). Methanogenic species other than *M. ruminantium* and *Methanobacterium mobile* (72) do not have specific organic requirements and can grow in the absence of volatile fatty acid and amino acid mixtures. However, *M. thermoaototrophicum* is the only species whose growth is not greatly enhanced by organic supplements. High cell densities in cultures of species examined, other than *M. thermoaototrophicum*, are best achieved in complex media (14, 121) that contain materials such as mineral salts, vitamins, yeast extract, acetate, Trypticase, and H2-CO2.

### Selective Enrichment and Isolation of Methanogens

Many excellent procedures for enumeration and isolation of methanogenic genera and species have been published (33, 72, 90, 91). The protocol described below has been used in my laboratory for successful enrichment and isolation of methanogenic species from diverse environments. The intent here is only to provide some insight into how methanogens, differing in generic designation and sources of metabolic energy, can be obtained from nature. The addition of either 0.5 to 1% (final concentration) sodium formate or methanol, or an 80% H2-20% CO2 gas phase to the basal medium (LPBM) described in Table 4 provides a useful culture medium for enrichment and growth of methanogens that possess minimal nutrient requirements. Pure cultures are obtained from repeatedly transferred active enrichments. Purification is achieved by performing an agar roll-tube

<table>
<thead>
<tr>
<th>Nutritional feature</th>
<th>Methanobacterium ruminantium strain M1*</th>
<th>Methanobacterium ruminantium strain PS*</th>
<th>Methanobacterium strain MOH*</th>
<th>Methanobacterium thermoaototrophicum strain ΔH*</th>
<th>Methanobacterium arbophilicum strain DH*</th>
<th>Methanospirillum hungatii strain JF1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen source</td>
<td>NH4+</td>
<td>NH4+</td>
<td>NH4+</td>
<td>NH4+</td>
<td>NH4+</td>
<td>NH4+</td>
</tr>
<tr>
<td>Vitamins</td>
<td>Mixture required or stimulatory</td>
<td>Mixture required or stimulatory</td>
<td>Mixture not required or stimulatory</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Carbon additions</td>
<td>Acetate, 2-methylbutyrate, amino acids</td>
<td>Acetate, 2-methylbutyrate, amino acids</td>
<td>Acetate</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>required for growth</td>
<td>Not determined</td>
<td>Not determined</td>
<td>H2S or cysteine</td>
<td>H2S</td>
<td>H2S</td>
<td>H2S</td>
</tr>
<tr>
<td>Sulfur source</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>H2S Slight</td>
<td>Not determined No</td>
</tr>
<tr>
<td>Growth stimulation</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>by acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth stimulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by amino acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data of Bryant et al. (11).
* Data of Zeikus and Wolfe (116).
* Data of Zeikus and Henning (119).
* J. G. Ferry, personal communication.
* High cell densities only achieved in complex medium.
TABLE 4. Composition of basal medium (LPBM) used for selective enrichment and growth of some methanogenic species

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.75 g</td>
</tr>
<tr>
<td>K₂HPO₄·3H₂O</td>
<td>1.45 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.9 g</td>
</tr>
<tr>
<td>MgCl₂·H₂O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na₂S·9H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Trace mineral solution</td>
<td>9 ml</td>
</tr>
<tr>
<td>Vitamin solution</td>
<td>5 ml</td>
</tr>
<tr>
<td>Resazurin solution (0.2%)</td>
<td>1 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>

* Prepared anaerobically under a 95% N₂-5% CO₂ gas atmosphere. Medium adjusted to pH 7.4 prior to autoclaving. Basal medium requires the addition of an electron donor (H₂, formate, or methanol) for enrichment or growth of methanogens.

* Sulfide solution added after sterilization.

* Contains, in grams per liter of distilled water (pH 7.0 to 7.5 with KOH): nitroltriacetic acid, 4.5; FeCl₂·4H₂O, 0.4; MnCl₂·4H₂O, 0.1; CoCl₂·6H₂O, 0.17; ZnCl₂, 0.1; CaCl₂, 0.02; H₂BO₃, 0.019; and sodium molybdate, 0.01.

* Optional, some methanogens require a vitamin mixture (11, 113).

* Optional, an oxidation-reduction indicator.

Dilution series in anaerobic culture tubes that contain LPBM, 2% agar (Difco, purified), and an appropriate energy source. Well-isolated colonies are picked and used to inoculate liquid cultures. The omission of sulfate lessens the growth of sulfate-reducing bacteria, whereas omission of organic components (yeast extract, Trypticase) suppresses heterotrophic contaminants. These procedures are of limited use for the determination of the total number of methanogens in natural materials because of the specific organic growth requirements of certain species.

Methanobacterium species are most numerous in lake sediments (123) and sewage sludge digestors (90). Sediment or sludge is added to anaerobic culture tubes that contain LPBM and an H₂-CO₂ gas phase. Incubation of enrichments at 25°C and repeated weekly transfer in the same medium result in a mesophilic population of methanogenic rods. An active H₂-oxidizing enrichment develops a strong negative pressure. Incubation of sludge enrichments at 65°C and repeated transfer in the same medium result in selection of thermophilic species. After six to nine successive transfers, thermophilic enrichments usually contain only methanogenic rods. Formate-degrading rods can be enriched by adding sludge or sediment to anaerobic culture tubes that contain LPBM and 0.5 to 1% sodium formate. This enrichment and successive transfers should be incubated at 44°C. An active formate fermentation will increase the culture pH.

Inoculation of anaerobic culture tubes that contain LPBM and 0.5 to 1% methanol with sludge or sediment will select for Methanosarcina species. The enrichments should be maintained at 30 to 40°C, and the culture tubes should be wired to hold the bungs in place, or the culture procedures of Miller and Wolin (65) should be used. One must exercise caution during culture transfer, since methanol utilization results in the development of high gas pressure. At present, other methanogenic genera have not been isolated from methanol enrichments with these conditions.

Selective enrichments of Methanococcus and Methanospirillum species are not as easily obtained as those for Methanobacterium and Methanosarcina species. Methanococcus species can be enriched by inoculation of anaerobic culture tubes that contain LPBM and 0.5 to 1% sodium formate with mud or lake sediment. Enrichment cultures should be incubated at 20°C, and a 15% inoculum should be used for successive culture transfers.

Methanospirillum is often associated in high numbers with Thiopedia "blooms" in the surface muds of shallow eutrophic ponds (personal observation). Inoculation of culture tubes that contain LPBM and 0.5 to 1% sodium formate with this mud (or sewage sludge), followed by incubation at 32°C and repeated transfer, will often result in enrichment of Methanospirillum.

**Morphological Variation**

Four distinct morphological types of methanogenic bacteria (121) are illustrated in Fig. 2A through D. These different cell morphologies include: sarcina, rods, spheres, and spirals. Considerable variations in cell dimension and organization as well as regularity of cell shape have been observed for individual species that are included within three of these four major cell types (Fig. 2E through H).

Sarcina-type cells (Fig. 2A and E) proliferate as irregular-sized cells that tend to clump and form sandlike aggregates. Methanosarcina species often appear more like coccolid cells of Geodermatophilus species (49) than true Sarcina species (42) and may vary considerably in size. Methanosarcina strain UBS (Fig. 2E) forms larger and more irregular cell packets than *M. barkeri* (Fig. 2A). This strain also displays a distinctive cell life cycle that involves the formation of large, irregularly sized packets of cells from initially symmetrical cocci.
Fig. 2. Phase-contrast photomicrographs of M. barkeri strain PS (A), M. thermoautotrophicum strain AO (B), M. ruminantium strain M1 (C), M. hungatii strain 3PS (D), Methanosarcina strain UBS (E), M. arbothicum strain DH1 (F), M. thermoautotrophicum strain ΔH (G), and a species of Methanococcus (H). Bar indicates 5 μm (A-D) and 8 μm (E-H). Figure 2A-D from reference 120.
Rod-shaped cells are most often crooked (Fig. 2B) and can appear in chains or long filaments (Fig. 2G). *M. arbofaculum* (Fig. 2F) is an example of a short, straight rod that does not display filamentous growth in liquid culture. Coccal-shaped cells vary in morphology from regular to ellipsoidal spheres arranged in pairs or chains. *M. ruminantium*, illustrated in Fig. 2C, is morphologically similar to "streptococci" in shape (91) and cytology of cell division (55). *Methanococcus vanneili* (94) varies in morphology from roughly spherical cells of differing diameters to pear-shaped cells. Figure 2H illustrates the appearance of an undescribed *Methanococcus* species cultured in my laboratory. This organism appears to divide by budding and is morphologically similar to *M. vanneili* (T. C. Stadtman, personal communication) but nonmotile. The cell wall of this organism and of *M. vanneili* is very sensitive to osmotic shock, and the cells readily lyse. Cell walls of most other methanogenic species are very resistant to osmotic, ultrasonic, mechanical, and enzymatic procedures commonly used to disrupt cells (94).

Spirillum-type cells grow as regularly curved rods that form continuous helical filaments (33). The morphological features of *M. hungatii* (Fig. 2D) appear unique (120). Single cells have blunt ends and are motile, but they are not spiral shaped. Cell growth results in long, nonmotile helical filaments (Fig. 2D). The construction of the cell wall and its behavior in cell division of this species (120) differ from *Spirillum* species and other helically curved microorganisms described in *Bergey's Manual of Determinative Bacteriology* (17).

**Fine Structure**

The methanogenic bacteria are structurally diverse and display no unique features by which all species can be characterized. The wall architecture of each cell type differs significantly, although all species examined (55, 120, 124) have a gram-positive-type cell envelope structure. This is interesting since many species have been reported (13) as gram negative or gram variable. However, only Methanobacterium species have a typical gram-positive cell wall.

**Coccus-type cells.** The fine structure of *M. ruminantium* is very similar to that reported for streptococci (39, 63). The cell envelope of *M. ruminantium* has a distinctive triple-layered appearance (Fig. 3). The wall consists of an inner electron-dense layer, closely adjoined to the plasma membrane, followed by a thicker, more electron-transparent middle layer, and a rough, irregular outer layer. Cells contain the membranous cytoplasmic bodies often associated with cell division. Cells of *M. ruminantium* appear to be undergoing constant cell division; before one cross wall is completed, a second division is initiated.

The ultrastructure of other coccal methanogens has not been published. Preliminary studies of the cell structure of *M. vanneili* have been reported (J. J. Jones et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, 155, p. 120). This species was not sensitive to several antibiotics (penicillin, vancomycin, cycloserine) that inhibit cell wall synthesis. Freeze-fracture replicas indicated a typical cell wall that appeared as one thin uniform layer in thin sections. However, the appearance of the cell wall was not similar to that of other methanogens described (12).

**Sarcina-type cells.** The general appearance of *M. barkeri* in thin section is shown in Fig. 4 through 6. The amorphous outer layer of the wall often appears as laminations (Fig. 4), which diffusely attach to a more electron-dense layer that is closely apposed to the plasma membrane (Fig. 6). The very thick outer wall of *M. barkeri* bears some resemblance to that observed for *Sarcina ventricula*, which is composed of cellulose (43). Cells examined from exponential-phase cultures (Fig. 4) contain numerous electron-dense granules of unknown composition. Cytoplasmic regions of low electron density, with circular to oval profiles, are often present in older cells (Fig. 5). These inclusion bodies do not appear to have limiting membranes (Fig. 6) and may contain storage material. One outstanding feature of sarcina-type cells is the unusual character of cell division. Cells divide in different planes with the formation of unequal daughter cells that share a common outer wall. Thus, mature aggregates of *Methanosarcina* appear as unevenly segmented cocci (Fig. 5). The fine structure of *M. barkeri* differs from that reported for a *Methanosarcina* species by Zhilina (124). The cell wall of this organism was triple-layered, and the cytoplasm contained numerous gas vesicles arranged in packets. Different growth conditions may influence the ultrastructural features observed.

**Rod-type cells.** The fine structure of bacillus-type cells is illustrated in Fig. 7 through 10. All Methanobacterium species examined (117, 121) have a sharply defined, smooth, gram-positive wall and contain intracytoplasmic membranes. Internal membrane inclusions are often observed in negatively stained preparations (Fig. 10) and were first described by Langen-
Fig. 3. Longitudinal section illustrating the general appearance of M. ruminantium. The cell is undergoing cell division, and the arrow shows membrane association with the second division site. Bar indicates 0.32 μm (from reference 120).

Fig. 4. Thin section of M. barkeri illustrating a multilayered outer wall (black arrows) and numerous dense cytoplasmic granules (white arrow). Bar indicates 0.45 μm (from reference 120).

berg et al. (55). Internal membranes are most numerous in fast-growing species such as M. formicicum (Fig. 8) and M. thermoautotrophicum (Fig. 9). Intracytoplasmic membranes of methanogens have been shown to consist of closely apposed unit membranes formed from
invagination of the plasma membrane (117). The function of these internal membranes has not been demonstrated; they do not appear associated with cell division (Fig. 8 and 10). The energy-yielding activity of methanogenic bacteria may require coupling of enzymatic systems that are membrane bound. Perhaps more membrane surface area is required to maintain the rapid growth rates of certain Methanobacterium species grown on hydrogen and CO₂.
Spirillum-type cells. The ultrastructural features of *M. hungatii* are shown in Fig. 11 through 16. The fine structure of this species differs dramatically from other methanogenic cell types (121) and other spiral-shaped bacteria (27, 56, 67, 83). In many respects, *Methanospirillum* has an ultrastructure that appears to be unique in the microbial world (120). The unusual nature of the cell ends and the outer cell envelope of *M. hungatii* are illustrated in phosphotungstic acid preparations (Fig. 11 through 13). Cell ends are squared-off and contain an unusually structured end component. The end component does not appear to contain cytoplasm and is bounded by structural elements that form the outer wall envelope, separating the end component from the inner wall, membrane, and cytoplasm of the cell. The outer cell envelope is composed of subunits arranged in stacked bands. The outer envelope may be a brittle structure because breaks are often observed between subunit bands. Individual cells appear as long, slender, curved rods (Fig. 14). Cells divide to form spiral filaments, which are connected by structures (cell spacers) that separate individual cells within the filaments (Fig. 16). The cell spacer is bounded by the outer wall and by widely separated septa (structural elements) continuous with the inner wall. These structural elements appear to have a subunit composition similar to that of the outer wall and probably function in support. Cell spacers appear fragile, and filamentous cells tend to break apart in this area of the filament (Fig. 14). The wall of *M. hungatii* consists of an outer layer (average thickness of 95 nm) composed of subunits and a more electron-dense layer (average thickness of 13.6 nm), which presumably contains peptidoglycan. The inner wall layer completely encompasses individual cells within...
Fig. 10. Negatively stained (2% phosphotungstate) cell of *M. thermoautotrophicum* showing cell wall topography and numerous internal structures (arrow). Bar represents 0.14 μm.

THE BIOLOGY OF METHANOGENIC BACTERIA

the filament. Numerous granular inclusions of low electron density that probably contain reserve material are visible in thin sections. The mechanism of cell division in *M. hungatii* (Fig. 15) is not typical of gram-positive bacteria. Division involves the invagination of the inner wall and plasma membrane with the formation of daughter cells that are connected by a cell spacer. The outer wall does not invaginate but remains continuous and appears to maintain the integrity of the growing filament. An interpretation of the structural features observed in *Methanospirillum* is illustrated in Fig. 17.

Taxonomy

A recent taxonomic description of methane-producing bacteria has been provided by Bryant (13). Cell shape is used as a primary property for taxonomic assignment of methanogenic genera. Physiological and nutritional properties are the basis for species designation. Schnellen's (88) cultures of *M. formicicum* and *M. barkeri* were lost; hence, the complete identity of these species in culture, at present, cannot be established. Bryant's strain of *M. formicicum* (55, 121) and *M. barkeri* strain PS (121) have been suggested as neotype strains (M. P. Bryant, personal communication). Type or neotype strains of other methanogenic species (Table 1) are presently in culture. Strains of several methanogenic species have been submitted to the American Type Culture Collection and the Deutsche Sammlung von Mikroorganismen. Hopefully, all taxonomically described species will be made available through these agencies in the future.

Table 5 provides comparative data on the DNA base composition analysis of various methanogenic bacteria. These results indicate that the moles percent of G+C of methanogen DNA varies greatly, ranging from 52% for *M. thermoautotrophicum* to 27.5% for *M. arbophilicum*. The specific epithet of *Methanobacterium* strain MOH has not been described, although this strain is considered similar to *M. formicicum* (13). By observing the range in DNA G+C contents of *Methanobacterium* species, it is obvious that morphology and energy sources for growth alone cannot be used as a basis for species designation. Furthermore, the similarity in DNA G+C contents of *Methanosarcina* species, *Methanobacterium* species, and *Methanospirillum* species indicates that DNA G+C contents cannot be used as a criterion for generic designation.

The differences in morphology, the dissimi-
larity of ultrastructural organization, the variation in nutritional properties, and the wide range of DNA G+C values observed in this microbial group may suggest that methanogens are of diverse origin and have merely exploited a common mode of energy-yielding metabolism. Thus, the diversity observed among methane-producing bacteria parallels that described for
FIG. 14. Grazing section of Methanospirillum showing spiral cell and broken filaments (arrows). Bar indicates 0.71 μm.

Fig. 15. Thin section revealing the cell division process in Methanospirillum. Only the inner wall (IW) and cytoplasmic membranes (arrows) invaginate during cell fission. Note the continuity of the outer wall (OW) and the presence of granular inclusions (G) in the cytoplasm. Bar indicates 0.10 μm (from reference 119).

Fig. 16. Thin section showing the separation of two Methanospirillum cells in a filament by a spacer (CS). Arrows point to structural elements within the spacer. Bar indicates 0.14 μm (from reference 119).
other bacterial groups that are distinguished on the basis of energy-yielding metabolism, such as the phototrophic bacteria (17).

PHYSIOLOGICAL ASPECTS

Intermediary Metabolism

The metabolic feature that unites the rather diverse species of methanogenic bacteria is the capacity to couple hydrogen oxidation with the concomitant reduction of carbon dioxide. Furthermore, the ability of many species to grow autotrophically indicates the enormous biosynthetic capabilities of these microbes. Methanogens differ from other autotrophs (organisms that proliferate with \( \text{CO}_2 \) as the sole carbon source) in that their \( \text{CO}_2 \) metabolism involves both fixation to cell carbon and reduction to methane. At present, little is known about the initial reactions involved in \( \text{CO}_2 \) reduction to methane and \( \text{CO}_2 \) fixation into cellular intermediate. It has not been determined whether the initial reductive steps in \( \text{CO}_2 \) fixation to cell carbon and methane involve a similar or dissimilar pathway. The mechanism coupling methane production and ATP synthesis also remains a mystery.

Unique biochemical components. Recent discoveries (23, 61), in the laboratory of R. S. Wolfe, of two biochemical components that seem unique to methanogens have provided excitement and new investigatory direction toward understanding the mechanism of hydrogen oxidation and carbon dioxide reduction. Coenzyme M (CoM), a new methyl transfer coenzyme, was discovered by McBride and Wolfe (61). CoM was first isolated as a dialyzable, heat-stable cofactor in \textit{Methanobacterium} strain MOH and has been found in other methanogens thus far examined (101). This cofactor was neither detected in \textit{Clostridium thermoceticum}, \textit{Clostridium sticklandii}, \textit{Desulfovibrio vulgarus}, nor in heart, liver, spinach, or yeast extract (61). Microbiological assays with \textit{M. ruminantium} (rumen strain) showed that CoM was also not present in \textit{Escherichia coli} or in a number of species of anaerobic rumen bacteria (11). CoM \((\text{HSCH}_2\text{CH}_2\text{SO}_3)\) was structurally identified and chemically synthesized by Taylor and Wolfe (100). This cofactor was
initially isolated as 2,2′-dithiodiethanesulfonic acid, and the active form was described as 2-mercaptoethanesulfonic acid. These investigators also described a simplified assay for CoM (102) and related the enzymatic conditions necessary for the methylation and demethylation of this cofactor.

A low-molecular-weight, fluorescent compound, F₄₂₀, appears to be present in all methanogenic bacteria examined. Cheeseman et al. (23) first described the properties of this pigment and its involvement in hydrogen metabolism of Methanobacterium strain MOH. The structure of F₄₂₀ has not yet been identified. It displays a conspicuous absorption peak at 420 nm when oxidized, and loses both its absorption at 420 nm and its fluorescence when reduced. Cheeseman et al. (23) suggest that the extreme oxygen sensitivity of methanogens may be associated with F₄₂₀ oxidation. They propose that enzyme denaturation occurs in air, because, when reduced, enzymes are associated with F₄₂₀ and stable; whereas, when oxidized, enzymes are disassociated from F₄₂₀ and labile. Pyrimidine nucleotide reduction (i.e., nicotinamide adenine dinucleotide phosphate [NADP]) in methanogens has been shown to be F₄₂₀ linked by Tzeng et al. (104, 105). Oxidation of formate and hydrogen in M. ruminantium are mediated via F₄₂₀ and coupled to reduction of NADP. NAD cannot substitute for NADP in the following reaction scheme proposed by Tzeng et al. (104):

\[
\begin{align*}
\text{HCOOH} & \quad \text{F}_{420} \quad \text{NADPH} \\
\text{CO}_2 & \quad \text{F}_{420} \quad \text{NADP}
\end{align*}
\]

Hydrogen oxidation by Methanobacterium strain MOH was also shown by these workers (105) to be linked to F₄₂₀ as the first low-molecular-weight or anionic compound.

It is interesting that F₄₂₀ and CoM appear to be unique to methanogenic bacteria. The functions demonstrated for these cofactors are as a primary electron carrier (F₄₂₀) and as the active methyl carrier for methanogenesis (CoM). More detailed studies may substantiate other functions for these unique biochemical components. One should now recognize that primary metabolism in methanogens may not necessarily involve mechanisms similar to those found in other C₁-utilizing organisms (77). For example, an F₄₂₀-associated electron carrier protein could be utilized instead of ferredoxin. Ferredoxin has not been isolated and identified from pure cultures of methanogens. Tzeng et al. (105) have reported that Clostridium pasteurianum ferredoxin does not replace F₄₂₀ in F₄₂₀-mediated reactions, and that F₄₂₀ or crude extracts of Methanobacterium strain MOH or M. ruminantium will not replace ferredoxin in the ferredoxin-free C. pasteurianum pyruvate-ferredoxin oxidoreductase reaction. Likewise, no evidence has been presented that substantiates an active involvement of methylcobalamin during methane formation by hydrogen-grown methanogens. It would also be of interest to document the function of CoM with moieties other than methyl.

It is worth noting that cytochromes of the b- or c-type have not yet been observed in any methanogenic bacteria, and M. thermautotrophicum does not appear to have menaquinone (A. Kroger, personal communication). This is of importance for understanding energy metabolism in methanogens, as all organisms so far documented that have electron transport phosphorylation contain quinones and/or cytochromes. However, the mechanism or occurrence of oxidative phosphorylation or substrate level phosphorylation has not been established in methanogenic bacteria.

**Methane synthesis.** Methanogenic bacteria have been shown (117, 122) to utilize H₂, CO₂, formate, methanol, and acetate as substrates for methanogenesis. The exact mechanism by which any of these substrates are converted to methane remains to be elucidated. Barker (4) proposed a unifying mechanism to account for methanogenesis from these substrates (Fig. 18). This scheme suggests that methanogenic substrates are bound to one or more unidentified carriers and are eventually reduced to methane with the regeneration of carriers. The discovery of CoM (61, 100) helps to verify this scheme. The work of Wolfe and co-workers (23, 61, 100–102, 104, 105) on CO₂ reduction in Methanobacterium species provides a basis for understanding the terminal reaction (i.e., reduction of "active methyl" to methane). The initial reductive steps from CO₂ to CH₄ would at first seem to be mediated via folate enzymes as suggested by Barker (5, 6) and Stadtman (95). However, the preliminary studies of Ferry et al. (35) suggest that several species of methanogens contain varying levels of formyltetrahydrofolate synthetase and methylenetetrahydrofolate dehydrogenase, but that the levels of these enzymes detected were not reproducible.
or were too low to postulate their involvement in a major metabolic pathway.

A modification of Barker's scheme (4) for the reduction of CO₂ (Fig. 19) was presented by Gunsalus et al. (36). This speculative scheme was based on preliminary findings and indicates the possible role of C₁-S-CoM compounds in the activation and reduction of CO₂ to CH₃-S-CoM. Evidence was presented by these investigators that H-C-S-CoM was biologically active and reduced to methane. It is of interest to note that CH₃-S-CoM was reported (61) as the major nonvolatile labeled product of whole cells of Methanobacterium strain MOH that were pulsed with ^14CO₂.

The terminal reductive step of methane formation in Methanobacterium strain MOH has been demonstrated (61, 100) to occur as follows:

\[
\text{CH}_3\text{-S-CoM} \xrightarrow{\text{H}_2, \text{Mg}^{2+}\text{ATP}, \text{Methyl reductase}} \text{CH}_4 + \text{H-S-CoM}
\]

The role of ATP was shown to be that of an activator (112), and CoM has been shown to be one of the unknown carriers postulated by Barker.

Detailed biochemical studies on methanogenesis from methanol or acetate have not been reported since the last review (117). Metabolism of formate appears to be equivalent to that of H₂ and CO₂ after an initial oxidation via formate dehydrogenase (104). Methylcobalamin has been postulated by Blaylock and Stadtman (7, 8) to be a methyl carrier in methanol-grown cells of \textit{M. barkerii}. It has not yet been documented that methyl B₁₂ is the natural methyl donor for CoM in species that grow on H₂-CO₂ or methanol as the methanogenic substrate.

**Nature of Autotrophic Growth in \textit{M. thermoautotrophicum}**

Studies (117, 122) on the effects of organic and inorganic substrates on growth and methanogenesis demonstrate that \textit{M. thermoautotrophicum} is a chemolithotrophic, autotrophic organism. This species uses molecular hydrogen to generate reducing equivalents and has an obligate CO₂ requirement for growth. Unlike other methanogenic species examined, growth of \textit{M. thermoautotrophicum} is not stimulated by organic additions. However, acetate can be slowly metabolized in the presence of H₂ and CO₂ by this species, and this feature argues against calling it an "obligate autotroph" (84).

The biochemical mechanisms that account for cell carbon synthesis in methane-producing bacteria have not been determined. It is very possible that autotrophic methanogens, like methane-oxidizing bacteria (77), can employ more than one pathway for cell carbon synthesis. Recently, attempts have been made to elucidate the biochemical basis for autotrophy in \textit{M. thermoautotrophicum}. Preliminary studies of Daniels and Zeikus (Abstr. Ann. Meet. Am. Soc. Microbiol. 1974, P136, p. 197) demonstrated that ribulose 1,5-bisphosphate carboxylase activity was not present in cell-free extracts of \textit{M. thermoautotrophicum}, although an active phosphoenolpyruvate carboxylase was present. Analysis of the short-term ^14CO₂ fixation products of whole cells pulsed with labeled carbonate in the presence of H₂-CO₂ was reported by Daniels and Zeikus (Abstr. Ann. Meet. Am. Soc. Microbiol. 1976, 158, p. 121). These preliminary studies showed that amino acids (mainly

**Fig. 18. Possible pathway of methane formation proposed by Barker (2).**

\[
\text{CO}_2 + \text{XH} \rightarrow \text{XCOOH} \quad \text{+2H} \\
\downarrow \\
\text{XCHO} \quad \text{-H₂O} \\
\downarrow \\
\text{XCH₂OH} \quad \text{+2H} \\
\downarrow \\
\text{XCH₃} \quad \text{-H₂O} \\
\downarrow \\
\text{XH + CH₄}
\]

**Fig. 19. Possible role of CoM (HS-CoM) in the reduction of CO₂. Proposed by R. Gunsalus et al. (36).**
aspartate, glutamate, and alanine) and non-phosphorylated compounds accounted for the majority of non-methanogenic precursors labeled at early times. Label was not detected in 3-phosphoglyceric acid at 2 or 60 s. These findings do not suggest that the Calvin cycle is not operative in _M. thermautotrophicum_. The labeling patterns observed cannot rule out the operation of the reductive carboxylic acid cycle (16), a total synthesis of acetate (57), or an unknown cell carbon synthesis mechanism.

Preliminary studies on the intermediary metabolism of _M. thermautotrophicum_ have also been presented by Taylor et al. (99), who used enzymatic assays and analyzed the distribution of label in cell fractions of _M. thermautotrophicum_ after long-term incorporation of various ¹⁴C-labeled compounds. On the basis of labeling patterns observed in amino acids and nucleic acids, and the absence of detectable levels of ribulose 1,5-bisphosphate carboxylase, hexulose phosphate synthetase, and hydroxy-pyruvate reductase, they concluded that it was doubtful whether the Calvin cycle, the serine or hexulose pathway (77), or the total acetate synthesis path (57) exists in *Methanobacterium*.

Autotrophy is often defined on the basis of ribulose 1,5-bisphosphate carboxylase (28, 84). If certain methanogenic species use a non-Calvin CO₂ fixation path, this may represent substantial proof that other mechanisms of autotrophy exist in the microbial world. However, documentation of the autotrophic path of _M. thermautotrophicum_ is inherently a difficult task, since the majority of the CO₂ fixed (>90%) goes into CH₄ intermediates.

**ECOLOGICAL ASPECTS**

**Activities in Nature**

Methanogens have been detected in numerous primarily organotrophic ecosystems that include: the rumen and gastrointestinal tract of animals; mud, sediment, and flooded soil of marine and freshwater environments; and various waste-processing digesters. Hydrogen-oxidizing methanogens have been shown to predominate in these ecosystems (90, 123). The activity of methanogens in the rumen and sewage sludge digesters has been examined in detail, and this literature has been reviewed (40, 41, 46). Methane occasionally has been either reported or inferred to be part of the composition of flammable gases that are sometimes trapped within the trunks of living trees. Bushong (18) reported the first analysis of gases drawn from a tree and demonstrated the presence of flammable gases in a large cottonwood tree. Visibly sound hardwood trees, including elms, poplars, willows, oaks, and maples, can contain high pressures of methane (Fig. 1A). Methane formation in these trees was demonstrated to have a microbial origin and was associated with an abnormal condition of heartwood (dead xylem tissue) known as wetwood (118).

Wetwood differs from normal tree tissues because it is infested with anaerobic bacteria, is alkaline in pH, is devoid of O₂, and has a high moisture content with characteristic fetid odor. One methanogenic species, _M. arbofolicum_, was isolated from wetwood of several cottonwood trees (119). Only one strain of _M. arbofolicum_ (type strain DHI) was described (119) from trees that contained wetwood. Other methanogenic genera were not observed by microscopic observation of wetwood enrichment cultures. Inside living trees, anaerobic decomposition of nonligninaceous dead xylem tissue or tree nutrients results in the formation of H₂ required for proliferation of methanogens. The establishment of a bacterial population in trees probably results from root injury, which provides a path of entry for indigenous soil microorganisms.

The evolution of methane bubbles from the bottoms of shallow ponds or the edges of lakes is a commonplace event. However, relatively little is known about either the environmental factors that influence methanogenesis, or the in situ microbial activities responsible for this process in aquatic sediments. Most investigations of methanogenesis in both marine (26, 70, 82) and freshwater sediments (24, 32, 43) have been concerned with detection and quantification of methane evolution from sediments. Methane formed in aquatic sediments escapes into the overlying waters, where it is metabolized by other microorganisms to CO₂ (86) or diffuses into the atmosphere. Analysis (123) of the activity of methanogens in sediments of Lake Mendota, Wis., revealed that methanogenesis was severely temperature limited and that the rate of methanogenesis varied seasonally. The maximum in situ temperature (23°C) attained during seasonal change was far below the temperature optimum (35 to 42°C) observed for in vitro methanogenesis. The increased rate of methanogenesis that was associated with seasonal change correlated with increased numbers of methanogens and increased rates of metabolic activity, when sediment temperature more closely approximated the optimum temperature for methanogenesis. The predominant methanogenic population was metabolically active between 4 and 45°C. All known methanogenic genera were found in Lake Mendota sedi-
ments, and *Methanobacterium* species predominated. Thus, methanogen diversity in aquatic sediments approximates that found in sewage sludge digestors. Methanogenic isolates obtained from sediments grew when either H₂, formate, or methanol was added as the sole electron donor to a mineral salts medium. Methanogens were not obtained in pure or mixed cultures that could be maintained on acetate-mineral salts medium. It is possible that the medium used inhibited (e.g., high sulfide content) acetate-fermenting methanogens, or that other supplements (e.g., high levels of yeast extract) were required to support their growth. One species isolated metabolizes H₂-CO₂ and acetate (127).

Cappenberg (19–22) has examined the associated activities of methanogens and sulfate-reducing bacteria in Lake Vechten and in mixed culture experiments. These studies reported that lactate metabolism of sulfate reducers in the upper sulfate-containing sediment layers provides the main energy source for acetate-fermenting methanogens located lower in the sediment and that sulfide production was toxic to methanogens. The turnover rate of lactate in sediments was 28.9 μg of lactate per g of mud per h. The rate of acetate disappearance was 1.99 μg of acetate per g of mud per h. The rate of acetate disappearance was approximately 70% of the observed rate of methanogenesis. Mixed-culture chemostat studies (27), using a sulfate reducer and a methanogen isolated from Lake Vechten, demonstrated that acetate metabolism by the sulfate reducer provided acetate for the methanogens, but it also produced toxic H₂S. Thus, a commensal relationship between these two microbial groups was proposed. However, the ecological significance of sulfate reducer-methanogen interrelationships is probably more complex and not yet fully understood.

The addition of sulfate and other compounds (e.g., nitrate, nitrite, acetylene) to sediments has been shown to inhibit methanogenesis (9, 20, 24, 58, 78). These inhibition phenomena may be either a result of channeling normal electron flow from reduction of methane carbon precursors to reduction of these alternative electron acceptors by nonmethanogens, or a direct inhibition of methanogens by the compound or a metabolite of it.

Balderston and Payne (3) have shown that addition of nitrate, nitrite, nitric oxide, nitrous oxide, or sulfite inhibited H₂-dependent evolution of methane from salt marsh sediments and whole-cell suspensions of methanogens. Their results suggest that inhibition of methane formation by these additions was not due to a change in the redox potential of the system or to substrate competition by nonmethanogens. Some compounds shown to inhibit methanogenesis in natural environments may not inhibit methanogenic bacteria. For example, methanogens in pure culture grow well even in the presence of high sulfate concentrations. Martsens and Berner (60) have demonstrated that methanogenesis in marine sediments is not initiated until sulfate is depleted from interstitial water. Cappenberg (19) reported that sulfate was depleted in actively methanogenic freshwater sediments. He proposed (22) that inhibition of methanogenesis by added sulfate (0.1%) was from the production of toxic H₂S.

Winfrey and Zeikus (119) have shown that sulfate inhibits methanogenesis in freshwater sediments by altering normal carbon and electron flow during anaerobic mineralization. Sulfate inhibition of methanogenesis in Lake Mendota sediments was reversed by prolonged incubation or by the addition of H₂ or acetate. Radioactive tracer studies demonstrated that ¹⁴C-labeled acetate was converted to ¹⁴CH₄ and ¹⁴CO₂ in the absence of added sulfate, whereas only ¹⁴CO₂ was found in sediments with sulfate. The addition of sulfate to sediments did not result in significant accumulation of H₂S in interstitial water. It was proposed that sulfate-reducing organisms assume the role of methanogens in sulfate-containing sediments by utilization of methanogenic precursors. Documentation of acetate-respiring, sulfate-reducing bacteria in pure culture will be of great interest. Recently, a *Desulfotomaculum* species has been described that respires acetate (109a). It would appear that sulfate-reducing bacteria-methanogen interrelationships in nature would be highly dependent on the sulfate concentration. At high sulfate concentrations, as exist in marine environments (26, 60), a competitive relationship might occur. In sulfate-depleted freshwater sediments (111), sulfate-reducing bacteria may not be active, or synergistic relationships might occur. For example, Bryant (Abstr. Annu. Meet. Am. Chem. Soc. 1969, MICE 18) presented evidence for a symbiotic association between sulfate-reducing and methane-producing bacteria. He was able to grow *Desulfovibrio* on lactate in the absence of sulfate in a defined-mixed culture with *Methano bacterium*. The methanogen functions as an alternative electron sink (in lieu of sulfate) and enables the catabolism of lactate by the sulfate reducers. However, knowledge of interspecies hydrogen transfer interactions (to be discussed more fully below) would suggest that lactate might not be an important metabolite in methanogenic environments.
Acetate and H₂-CO₂ are the major in situ substrates for methanogenesis. Methanol has not been demonstrated to be a significant product of anaerobic decomposition. Formate, a product of many fermentations, is not considered to be a major substrate because it is readily cleaved into H₂ and CO₂. In the rumen ecosystem, Hungate et al. (47) concluded that formate was largely utilized by non-methanogenic microbes, although the H₂ and CO₂ derived from formate metabolism accounted for 18% of the total methane produced. The relative importance of acetate and H₂-CO₂ as methanogenic precursors in organotrophic ecosystems depends largely on environmental conditions. Hungate (45) demonstrated that H₂-CO₂ accounts for most of the methane produced in the rumen. Oppermann et al. (69) showed that only 2 to 5.5% of the rumen methane was derived from acetate in vitro. The rumen ecosystem functions essentially as a chemostat, where volatile fatty acid concentrations are kept low because they are absorbed through the rumen wall. Also, the detention time for organic matter (1 to 3 days) may be too slow to allow for effective acetate conversion to methane (44).

Jeris and McCarty (50) reported that 70% of the methane from sewage sludge was derived from acetate. Similarly, Smith and Mah (89) determined that 73% of the methane came from acetate in sludge. Cappenberg and Prins (21) calculated that approximately 70% of the methane was derived from acetate in freshwater sediments. Russian investigators (2) have studied the relative importance of acetate and H₂-CO₂ as methane precursors in various lakes and reported that 32 to 98% of the methane in sediments was formed through microbiological reduction of carbon dioxide by hydrogen. Thus, although acetate is the major methane precursor during anaerobic decomposition of organic matter in sediments and sludge digestors, the reported percentages derived from it may be subject to some discussion because of environmental differences.

Noticeable environmental differences exist between the sludge digestor and sediment ecosystems. Sludge digestors are less nutrient limited, and the detention time for organic matter is usually 10 to 30 days. Thus, these long detention times appear sufficient for conversion of substantial amounts of volatile fatty acids, including acetate, to CH₄ and CO₂ (51, 97). The turnover rates for acetate in lake sediments are relatively rapid (21), and methanogenesis in this ecosystem may be substrate limited. Winfrey and Zeikus (109; Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, 1103, p. 128) reported that methanogenesis in Lake Mendota was limited by the small amounts of available H₂ and acetate in sediments. These investigators also demonstrated that addition of H₂ or acetate greatly stimulated methanogenesis and that the concentrations of these substrates greatly influenced their conversion. As the partial pressure of hydrogen was increased in sediments, carbonate was the preferred substrate for methanogenesis, whereas acetate was the preferred substrate at low partial pressures of hydrogen.

Microbial methanogenesis can also be detected in association with primarily chemolithotrophic ecosystems (i.e., habitats where the environmental hydrogen present was formed as a result of geochemical processes). Work in Lake Kivu, where hydrogen of volcanic origin exists in the environment, suggests that microbial reduction of CO₂ by volcanic hydrogen accounts for the major portion of methane formed in this African rift lake (31). Geochemists (37) have measured high concentrations of methane (>20%) and hydrogen (>8%) in the dissolved gases of certain thermal features in Yellowstone National Park, U.S.A. Methanogens have been isolated from several thermal springs (Fig. 1B) where geothermal hydrogen was present. Russian investigators (53) suggest that microbial reduction of geochemical hydrogen is associated with methanogenesis in petroleum, natural gas, and coal deposits.

**Microbial Interactions**

The methanogenic bacteria as a group offer the unique opportunity to study trophic interrelationships in anaerobic ecosystems. In anaerobic habitats where decomposition of organic matter is occurring, methanogens are the terminal organisms in the microbial food chain. The outstanding feature of this decomposition process is that its successful operation depends on the interaction of metabolically different bacteria. Detailed studies on interactions between methanogens and organotrophic anaerobes were initiated as a result of understanding the *M. omelianskii* symbiosis. Bryant et al. (10) established that ethanol fermentation by this mixed culture required a symbiotic metabolic interaction between a methanogen and an "S" organism. Analysis (79) of the metabolic coupling between the S organism and *M. ruminantium* grown on pyruvate revealed that methanogens alter fermentative metabolism in addition to displacing unfavorable reaction equilibria caused by high partial pressures of H₂. Further detailed studies of Wolin, Bryant, and coworkers (11, 48, 65, 80, 81, 97, 115) have resulted in formation of a unified concept that
describes primary metabolic interactions of methanogens and nonmethanogens known as interspecies hydrogen transfer.

Disposal of electrons from reduced NAD (NADH) generated in glycolysis in order to regenerate NAD is accomplished in all fermentative bacteria, mainly via production of various reduced products such as H₂, ethanol, lactate, formate, or propionate. In the rumen ecosystem, analysis of end products of carbohydrate-degrading anaerobes grown in the presence and absence of methanogens (114, 115) demonstrates how methanogens alter electron flow during fermentation. Growth of various carbohydrate fermenters (in pure culture) in the absence of methanogens results in formation of, mainly, H₂, CO₂, formate, acetate, succinate, lactate, and ethanol, whereas growth in the presence of methanogens results mainly in production of methane, acetate, and CO₂. In the presence of methanogens, products such as lactate and ethanol are produced in very small amounts and acetate increases. Weimer and Zeikus have reported (108) similar results when Clostridium thermocellum and M. thermoautotrophicum were grown on cellulose. Fermentation end products of C. thermocellum are mainly H₂, CO₂, ethanol, and acetate. The mixed culture formed CH₄, CO₂, and increased amounts of acetate as the main fermentation products. However, when grown on cellobiose, the methanogen caused only slight changes in the fermentation balance of the Clostridium, and free H₂ was produced. That significant metabolic interactions between the Clostridium and the methanogen were not observed during growth on cellobiose (a soluble substrate) may be of general ecological importance. The decomposition of organic matter in nature may be limited by the rate at which insoluble biopolymers are decomposed (109). Methanogens may function as "electron sinks" (15, 44) during organic decomposition in organotrophic ecosystems by altering electron flow in the direction of hydrogen production. In theory (114, 115), the altered electron flow or interspecies hydrogen transfer that occurs during coupled growth of methanogens and nonmethanogens results in: (i) increased substrate utilization, (ii) different proportions of reduced end products, (iii) more ATP synthesized by the nonmethogens, (iv) increased growth of both organisms, and (v) displacement of unfavorable reaction equilibria.

It seems obvious that metabolic interactions in the form of interspecies hydrogen transfer are operative in anaerobic environments and hold ecological significance. In the rumen and sediment ecosystem, where interspecies H₂ transfer appears to occur (44, 110, 115), the hydrogen partial pressure is extremely low (10⁻⁶ M). In addition, failure to detect significant concentrations of H₂ in various organotrophic, methanogenic niches (44, 51, 110, 118) is probably the consequence of rapid hydrogen oxidation by methanogens that results, in part, from interspecies-hydrogen-transfer reactions. Electron-sink products of "normal" fermentations (ethanol, lactate) may be of little consequence in the metabolism of carbon compounds in anaerobic ecosystems. In this regard, Hungate (44) suggests that ethanol and lactate are not important decomposition intermediates in the rumen because electrons are diverted to methane production.

Two general categories of interspecies-hydrogen-transfer interactions have been demonstrated. One category involves interactions between methanogens and carbohydrate and similar fermentative bacteria in which H₂ utilized is beneficial but not essential (25, 48, 87). The second category describes interactions between methanogens and nonmethanogens in which H₂ utilization is essential (8). This category of interspecies-hydrogen-transfer interactions may be involved in mixed methanogenic cultures that ferment various volatile fatty acids (4) or benzoate (34). However, the free energy of formation for fermentation of propionate (+17.8 kcal/mol [ca. +74.51 kJ/mol]) to H₂ and acetate is even more unfavorable than that of the ethanol fermentation (+1.5 kcal/mol [ca. +6.279 kJ/mol]) by the S organism (30). It is not known that removal of hydrogen alone would render the propionate fermentation a favorable energy-yielding reaction for the decomposing organism. Therefore, additional factors may be involved in certain fatty acid degradations by mixed-methanogenic-cultures. Perry and Wolfe (34) demonstrated that a stable microbial consortium could degrade benzoate to methane. Acetate, formate, H₂, and CO₂ were identified as intermediates in the conversion of benzoate to methane. The organism responsible for cleavage of the benzoate ring was not isolated. The species responsible for fermenting acetate to methane was not isolated, but it was reported to be similar to the rod observed by Pretorius (75) and by Mylroie and Hungate (66). It is of interest to note that Pretorius (75) was unable to maintain a sludge enrichment that contained this rod with acetate alone. However, the addition of formate and acetate maintained this methanogenic-mixed culture.

Relatively little is known about the chemical and mechanistic limitations of anaerobic decomposition of organic matter in nature. Although certain aromatic compounds (9) are me-
to be degradable. Hackett et al. (38) reported that specifically labeled synthetic \(^1^4\)C-lignins were not decomposed to gaseous products during anaerobic incubation in rumen contents or lake sediments. The absence of lignin biodegradation in anaerobic ecosystems may have profound environmental implications. The gradual accumulation of lignin and lignin-derived materials over extended periods of time might form the basis for peat and coal deposits (96).

ACKNOWLEDGMENTS

I am indebted to my colleagues J. Ward, P. Weimer, W. Hackett, L. Daniels, D. Nelson, V. Bowen, M. Winfrey, and B. Kenealy for their significant contributions in the research program, to D. Henning and S. Klevisickia for technical assistance, and to G. Fuchs and R. Thauer for discussions concerning this manuscript. It is a pleasure to thank two former mentors, T. D. Brock for stimulating my interest in microorganisms, and R. S. Wolfe for directing my attention to methanogens.

For data presented here, the research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by grant DEB 83-01511 from the National Science Foundation.

LITERATURE CITED


