Physiological and Phylogenetic Studies of Marine Methanogenic Bacteria

by

Kevin R. Sowers

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APPROVED:

James G. Ferry, Chairman

R. E. Benoit

E. M. Gregory

S. G. Horner

W. E. C. Moore

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Blacksburg, Virginia

FORWARD

This dissertation contains twelve sections. An introduction (Section I) and a literature review (Section II) serve as an introduction to the research problem. Sections III through IX are written as manuscripts for publication and have been included in their entirety. A discussion (Section X) summarizes the work. Literature cited in Sections I, II and X is included in Section XI; all other cited literature is listed at the end of the respective sections.

The following are the titles of the manuscripts that have been accepted for publication:

Section III: Sowers, K. S., and J. G. Ferry. 1984. Characterization of a marine methanogenic consortium. Proceedings of the 1984 International Gas Research Conference, in press.

Section IV: Sowers, K. R., and J. G. Ferry. 1983. Isolation and characterization of a methylotrophic marine methanogen, Methanococcoides methylutens gen. nov., sp. nov. Appl. Environ. Microbiol. 45:684-690.

Section VII: Sowers, K. R., S. F. Baron, and J. G. Ferry. 1984. Methanosarcina acetivorans sp. nov., an acetotrophic methane-producing bacterium isolated from marine sediments. Appl. Environ. Microbiol. 47:971-978.

Section VIII. Sowers, K. R., M. J. Nelson, and J. G. Ferry. 1984. Growth of acetotrophic, methane-producing bacteria in a pH-auxostat. Curr. Microbiol., in press.

Section IX: Sowers, K. R., J. L. Johnson, and J. G. Ferry. 1984. Phylogenetic relationships among the methylotrophic methane-producing bacteria and emendation of the family Methanosarcinaceae. Int. J. Syst. Bacteriol. 34:440-450.

The following manuscript has been submitted for publication:

Section V: Sowers, K. S., and J. G. Ferry. Trace element and vitamin requirements of Methanococcoides methylutens grown with trimethylamine (manuscript submitted to Arch. Microbiol.).

Dedicated to the memory of George Dewey Sowers (1898-1984)

...grandfather, friend and mentor.

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SECTION I. INTRODUCTION

Research on marine methanogenesis has been sparse because sulfate reduction is thought to displace methanogenesis as the predominant terminal process of anaerobic degradation. However, ecological niches exist in marine habitats where degradation is dependent on methanogenesis as the predominant terminal process. Despite a recent interest in marine hydrogen-utilizing methane-producing bacteria (96,115,117,118, 227, 228,232,310,312) attention has not focused on marine methylotrophic and acetotrophic methanogenic organisms and their function in polymer-degrading marine methanogenic consortia. The purpose of this research project is to study organisms of methanogenic food chains from marine sediments and to characterize and compare these consortia with those described from non-marine environments. This includes isolation and physiological and phylogenetic characterization of acetotrophic and methylotrophic methanogenic bacteria that have not been previously described.

SECTION II. LITERATURE REVIEW

History of Research on Methanogenesis

Biologically produced methane was first described by the physicist Alessandro Voltae in 1776 as "combustible air" formed from the decay of vegetation in the sediments of lakes, ponds and streams (73). In the second half of the 19th century Béchamp, Tappeiner, Popoff, Hoppe-Seyler and others used undefined enrichment cultures to demonstrate that methanogenesis is a microbial process (12). In 1906 Söhngen first described the morphologies of some methane bacteria and established that acetate, formate and H_2-CO_2 were substrates for methanogenesis in selective enrichment cultures (197). Subsequent investigators used enrichment cultures to demonstrate methanogenesis from substrates such as ethanol (159), acetone (138), butanol (61), short chain fatty acids (32), and proteinaceous material (2). The limitation of the research up to this point was that investigators were not using pure cultures and they were unable to distinguish between those substrates that were used directly by the methanogenic bacteria and those that required cocultures. In 1940 Barker (11) described "Methanobacillus omelianskii" that he isolated from Delft canal mud. This "isolate" could convert ethanol to methane and carbon dioxide, but three decades later Bryant et al. (25) discovered that this 'pure' culture was actually a coculture that contained an ethanol-degrading hydrogen-producer designated the "S" organism and a hydrogen-utilizing methane producer later designated Methanobacterium bryantii strain M.o.H. (3). It was not until 1947 when Kluyver and Schnellen (108) described the isolation of

Methanobacterium formicicum and Methanosarcina barkeri that pure cultures of methanogenic bacteria were available for physiological research.

Anaerobic culture techniques developed by Hungate (76) enabled investigators to maintain and culture methanogenic bacteria for physiological studies (17,22,204,206,245). Throughout this period of time a few new species were isolated by the dilution technique including Methanococcus vannielii (206), Methanobrevibacter ruminantium (3,194), and Methanomicrobium mobilis (163). It was not until Hungate (78) developed the roll tube technique that a large number of methanogenic bacteria were isolated and the extent of their morphological and physiological diversity was realized. Table 1 is a list of known species along with their morphologies and growth substrates. Subsequent refinements in anaerobic culture techniques by Bryant (18) in addition to techniques for transfer by syringe (130), culturing on agar plates (39), mass-culturing of H_2-utilizers (22) and formate-utilizers (178) and the use of pressurized culture vessels for H_2-CO_2 utilizing methanogens (5,146), have resulted in a proliferation of information concerning the physiology and biochemistry of the methane bacteria in recent years.

In 1977 Balch et al. (4) reported that methanogenic bacteria were phylogenetically distinct from other procaryotes based on a new technique called comparative 16S oligonucleotide sequencing. It was proposed that the methanogenic bacteria along with the extreme halophiles and the extreme thermophiles represent a third kingdom or line of decent called the Archaeobacteria. This group has several unique

Table 3. Equations and free energy changes[a] for the oxidation of propionate, butyrate, benzoate and ethanol with and without hydrogen utilizing methanogenic or sulfate-reducing bacteria.

		$\Delta G^{\circ\prime}$ kJ/reaction
I	Proton-reducing acetogenic bacteria	
	1) $CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H$	+ 48.1
	2) $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+ 76.1
	3) $C_7H_5O_2^- + 7H_2O \rightarrow 3CH_3COO^- + 3H_2 + HCO_3^- + 3H^+$	+ 89.7
	4) $CH_3OH + 2H_2O \rightarrow CH_3COO^- + H_2 + H^+$	+ 9.6
II	H_2-utilizing methanogenic and sulfate-reducing bacteria	
	5) $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6
	6) $4H_2 + SO_4^= + H^+ \rightarrow HS^- + 4H_2O$	-151.9
III	Cocultures of I and II	
	7) $2CH_3CH_2CH_2COO^- + HCO_3^- + H_2O \rightarrow 4CH_3COO^- + H^+ + CH_4$	- 39.4
	8) $2CH_3CH_2CH_2COO^- + SO_4^= \rightarrow 4CH_3COO^- + H^+ + HS^-$	- 55.7
	9) $4CH_3CH_2COO^- + 12H_2 \rightarrow 4CH_3COO^- + HCO_3^- + H^+ + 3CH_4$	-102.4
	10) $4CH_3CH_2COO^- + 3SO_4^= \rightarrow 4CH_3COO^- + 4HCO_3^- + H^+ + 3H5$	-151.3
	11) $4C_7H_5O_2^- + 19H_2O \rightarrow 12CH_3COO^- + HCO_3^- + 9H^+ + 3CH_4$	- 48.2
	12) $4C_7H_5O_2^- + 16H_2O + 3CO_4^= \rightarrow 12CH_3COO^- + 4HCO_3^- + 9H^+ + 3HS^=$	- 96.90
	13) $4CH_3OH + 5H_2O + HCO_3^= \rightarrow 4CH_3COO^- + 3H^+ + CH_4$	- 97.20
	14) $4CH_3OH + 4H_2O + SO_4^= \rightarrow 4CH_3COO^- + 3H^+ + HS^=$	-113.5

[a] based on data from Thauer et al. (217)

features that distinguish them from both the eucaryotes and other procaryotes (239). The most recent review articles on the methanogenic bacteria are by Mah and Smith (132), Balch et al. (3), Mah et al. (134), and Zeikus (254).

Phylogeny of the Methanogenic Bacteria

The methanogenic bacteria belong to a phylogenetic group called the archaeobacteria that differ from the eubacteria and eucaryotes based on comparative analysis of 16S ribosomal RNA oligonucleotide sequences (3,4,54,239). The inclusion of methanogenic bacteria in a coherent group that includes the extremely halophilic bacteria (3) and the thermoacidophiles (221,258) is supported by several unique characteristics.

DNA-dependent RNA polymerase in Methanobacterium thermoautotrophicum differs from eubacterial RNA polymerases in several respects. This polymerase has a 5 subunit and a 6 subunit form which do not resemble the configuration of eubacterial RNA polymerases (209,259). However, the complexity, composition and subunits of the RNA polymerase from the methanogenic bacterium resembles those of other archaeobacteria and eucaryotes, especially yeast RNA polymerase A (258,260,261). Immunological cross-reaction studies indicate that DNA-dependent RNA polymerase of archaeobacteria and eucaryotes are a common type (5). In addition, the RNA polymerase of Methanobacterium thermoautotrophicum and other archaeobacteria are stimulated by the alkaloid silybin which enhances transcription by eucaryotic RNA polymerase (186). Other unique characteristics of archaeobacterial polymerases include oxygen

sensitivity and resistance to the eubacterial RNA polymerase inhibitors rifampin and streptolydigin, and the eucaryotic RNA polymerase inhibitor α-amanitin. Despite the similarities among archaeobacterial RNA polymerases immunological cross-reaction studies indicate that some differences exist (278).

The structure of tRNA molecules from methanogenic bacteria do not closely resemble tRNA structures from either procaryotes or eucaryotes (17,38,83). The modified bases ribothymidine and 7-methylguanosine do not occur in the tRNA of any methanogenic bacteria tested. The ribosomes from methanogenic bacteria are a 30s plus 50s configuration like those of the eubacteria (56). However, electrophoretic separations of ribosomal proteins indicate that those molecules from methanogenic bacteria are more acidic than eubacterial ribosomes. Immunological studies show that ribosomal proteins from Methanobacterium bryantii do not cross react with those from eubacteria or yeast (248). The amino acid sequence of ribosomal protein 'A' from Methanobacterium thermoautotrophicum is different from the equivalent region in eubacteria but more closely resembles the corresponding 'A' sequence from cytoplasmic components of eucaryotes (79,180). Ribosomal elongation factor from Methanococcus vannielli is also unlike that from eubacteria (164) and diptheria toxin can ADP-ribosylate an elongation factor from Methanobacterium thermoautotrophicum which indicates a closer relationship with eucaryotes. Although chloramphenicol inhibits the growth of methanogenic bacteria (221) it does not inhibit protein synthesis in vitro which indicates that the mechanism of inhibition is different from that of the eubacteria (15).

A variety of cell wall structures exists among the methanogenic bacteria, but one characteristic feature distinguishes this group and all the other archaeobacteria from the eubacteria; they lack peptidoglycan (93,95). The genera Methanobacterium and Methanobrevibacter possess a unique cell wall polymer, pseudomurien, that consists of glycan strands cross-linked by short peptide chains analogous to eubacterial cell walls. Unlike the cell walls of the eubacteria, L-talosaminuronic acid is substituted for muramic acid in the glycan strand and the amino sugars are β-1,3 glycosidically linked instead of β-1,4 linked (110,111). In addition, the peptide chains contain only L-amino acids in sequences that are different from those of typical peptidoglycan polymers. Several other genera of archaeobacteria possess a non-rigid glycoprotein or protein cell envelope (93) and the genus Methanosarcina has a thick heteropolysaccharide cell wall (94).

Another unique characteristic of the archaeobacteria is that they possess isoprenyl ether lipids in their membrane fractions instead of fatty acid ester lipids found in eubacteria (120,219). Methanogenic bacteria contain C_{20} diphytanylglycerol diether or both the diether and C_{40} dibiphytanyl glycerol tetraether. Isoprenoid and hydroisoprenoid hydrocarbons are the predominant neutral lipids (120).

The methanogenic bacteria not only appear to use novel pathways for the synthesis of cell walls and lipids, but they also have unique pathways for the synthesis of methane from hydrogen, formate, acetate, methanol, and methylated amines. These pathways share several unique cofactors that have only been found in methanogenic species and include coenzymes M (216), F_{420} (41) and F_{430} (99). Despite the similarities

among the methanogenic bacteria, species in this group are just as diverse as species among the eubacteria and include various cell wall types and a species without cell walls (174), various morphologies, thermophiles and mesophiles, nonhalophiles and halophiles, motile and nonmotile species, autotrophs and heterotrophs (3). This diversity suggests that divergence from the eubacteria occurred early in evolutionary time.

Taxonomic divisions among the archaeobacteria (Table 2) have been proposed by Balch et al. (3) based on 16S rRNA oligonucleotide sequencing and are supported by phenotypic differences such as lipid content, cell wall composition, and immunological cross-reactivity of whole cells (30,129). The divisions were further elaborated by Stackebrandt et al. (202) and Tu et al. (221). Methanogenic species are included in the orders Methanobacteriales, Methanococcales and Methanomicrobiales; extremely halophilic species are included in the order Halobacteriales; and thermoacidophilic species are included in the orders Thermoplasmales, Sulfolobus and Thermoproteales. Studies on RNA polymerase structure suggest that the orders of archaeobacteria are divided into two branches; sulfur-reducing thermoacidophiles and non-sulfur-reducing methanogens and halophiles (258). However, this "metabolic division" is inconsistent because Thermoplasmales is included in the non-sulfur-reducing branch (258) and catabolic reduction of sulfur by methanogenic species has recently been described (208; J. G. Ferry, personal communication). The phylogenetic positions of several recently described genera have not yet been determined (see Table 1).

Table 2. Taxonomic divisions of[a] the archaeobacteria based on phylogenetic relationships.

ORDER	FAMILY	GENUS
Methanobacteriales	Methanobacteriaceae	Methanobacterium
		Methanobrevibacter
	Methanothermaceae	Methanothermus
Methanococcales	Methanococcaceae	Methanococcus
Methanomicrobiales	Methanomicrobiaceae	Methanomicrobium
		Methanogenium
		Methanospirillum
	Methanosarcinaceae	Methanosarcina
		Methanothrix
Halobacteriales		Halobacterium
		Halococcus
Thermoplasmales		Thermoplasma
Sulfolobales		Sulfolobus
Thermoproteales	Thermoproteaceae	Thermoproteus
	Desulfurococcaceae	Desulfurococcus
	Thermococcaceae	Thermococcus

[a] based on data by (3,133,202,258).

Ecology of Methanogenesis

The terminal electron acceptors in methanogenic environments are CO_2 and the methyl moiety of acetate and methylotrophic compounds which are generated from the degradation of organic substrates. Methanogenesis is inhibited in environments where alternate electron acceptors such as oxygen or nitrate are readily available, and it is greatly reduced in the presence of sulfate (6,20,236). Methanogenesis is optimal in anaerobic environments that are provided with large amounts of organic material such as aquatic sediments, sewage digesters, ruminants, human large intestines and decaying heartwood (19,134,166,244, 250). The combination of these sources contributes an estimated 90% of the atmospheric methane, which is equivalent to 5 to 8 x 10^8 tons of biologically generated methane annually (227). This represents as much as 0.5 percent of the total annual primary production of dry organic matter conserved in the form of methane (40). Estimates may be less than the actual total production due to the loss of some methane by bacterial oxidation (167,168). The rumen and sewage are the most thoroughly studied methanogenic habitats and represent two distinct types of methanogenic consortia.

A. Methanogenesis in the rumen.

Anaerobic fermentations occur in two stages in the rumen, cecum, and large intestine (20,72,77). The fermentative bacteria constitute an acid forming stage that hydrolyzes polymers and monomers to organic acid, alcohols, hydrogen, carbon dioxide, ammonia, and sulfide. Figure 1 is a composite of pathways for the fermentation of carbohydrates (20,72,125). Some fermentative species excrete extracel-

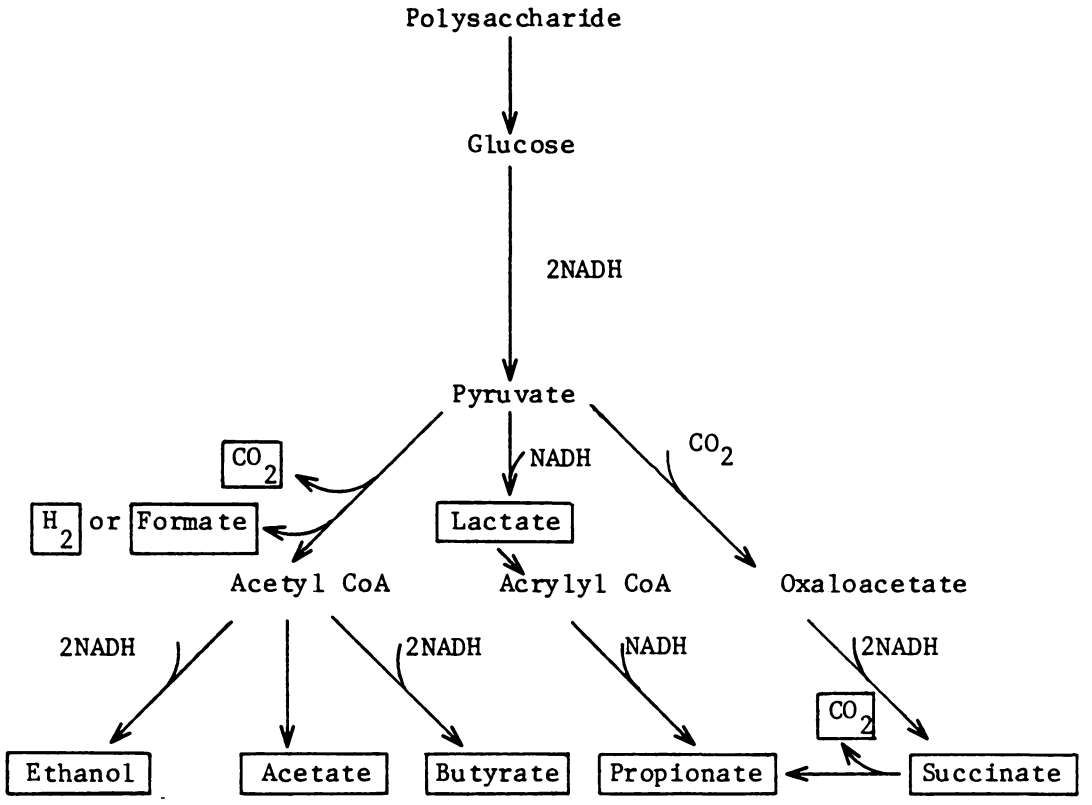

Figure 1. Pathways of carbohydrate fermentation by the fermentative bacteria. Products are enclosed in boxes.

lular enzymes that hydrolyze polymers such as polysaccharides (72), proteins (44,136), and lipids (29), to monomers that can be transported into the cell and metabolized. In many fermentative bacteria carbohydrates are converted to pyruvate by the Embden-Meyerhof-Parnas (EMP) pathway (Figure 1). The pyruvate is then catabolized to various end products depending on the individual organism and the growth conditions. In all the pathways except for the acetate pathway, pyruvate or the products of pyruvate are electron sinks for the electrons produced by the EMP pathway. Generally, pyruvate is metabolized in three ways (20): 1) pyruvate is decarboxylated and oxidized to acetyl CoA which is subsequently metabolized to acetate or condensed and reduced to higher fatty acids and alcohols; (2) pyruvate is reduced to lactate which may be dehydrated and oxidized to acrylyl CoA, and subsequently reduced to propionate; (3) pyruvate is carboxylated to oxaloacetic acid and subsequently reduced to succinate. In some cases the succinate is decarboxylated to propionate (181). Acetate, propionate and butyrate are the major fatty acids produced in the rumen along with lower concentrations of caproate, valerate, succinate, lactate and formate (Figure 2). These fatty acids are absorbed into the bloodstream and provide energy for the ruminant.

The methanogenic bacteria constitute the second stage and obtain energy by catabolizing formate, hydrogen and carbon dioxide formed from the first stage to methane. The ability of the methanogenic bacteria to catabolize hydrogen provides an important regulatory effect on the fermentative bacteria. Pure cultures of fermentative rumen bacteria form end products that are more reduced than those normally identified

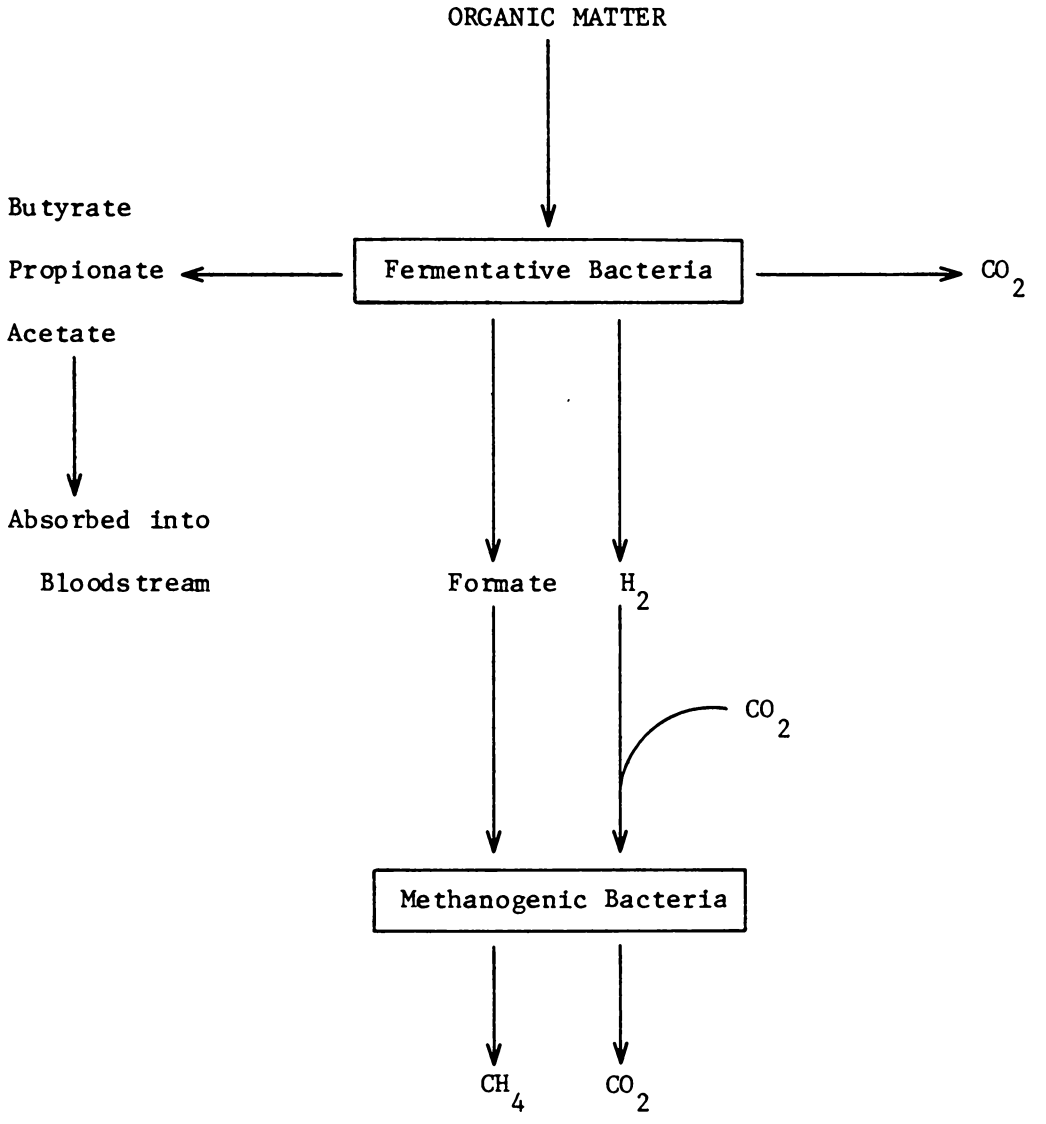

Figure 2. A simplified representation of the degradation of organic matter by a methanogenic consortium in the rumen.

in the rumen. This observation led Hungate (78) to propose the concept of interspecies hydrogen exchange; a process in which hydrogen utilization by methanogenic bacteria exerts a direct effect on the metabolic activities of fermentative bacteria by diverting electrons from the role of a reductant in fermentative metabolism to methane formation (242). The partial pressure of hydrogen has an important role in controlling the type and proportions of end products produced (24,77, 217,243). The mechanism of this control is NAD-linked hydrogenase. In the presence of methanogenic bacteria a low partial pressure of H_2 is maintained causing a shift in the equilibrium of NADH oxidation from product reduction to proton reduction. The reaction:

$$NADH + H^+ \qquad NAD^+ + H_2 \qquad G^{o'} = +18.0 \text{ kJ}$$

is thermodynamically unfavorable at standard conditions but it becomes favorable if hydrogen is removed (20). This shift in electron flow causes more of the substrate to be fermented to the methanogenic substrates acetate, hydrogen, and carbon dioxide without major production of highly reduced products. In the absence of the methanogenic bacteria or when there is stress on the consortium caused by a low retention time or overloading with substrate, electrons are shifted away from hydrogen production. This results in a build up of fatty acids or alcohols which could prevent complete degradation by inhibiting growth. The effect of hydrogen-utilizing methanogenic bacteria on the production of end products by fermentative bacteria has been described for the degradation of amino acids (154), cellulose (121,230), glucose (174,192,237,238), and pectin (184).

B. Methanogenesis in freshwater sediments and sewage.

Before many strains of methanogenic bacteria were available in pure culture their range of substrates was not known and it was proposed that non-rumen methanogenic fermentations also occurred in two stages with fermentative and methanogenic bacteria (12,218). As a result of research with pure cultures it was apparent that the range of substrates for methanogenic bacteria was more limited than the range of substrates used by methanogenic enrichment cultures (see Table 1). Bryant (19) explained this anomaly by proposing an intermediate stage between fermentative and methanogenic bacteria: the hydrogen-producing acetogenic bacteria.

This intermediate group in the methanogenic consortium consists of species that are responsible for the following reactions: (1) oxidation of alcohols to their corresponding carboxylic acid and hydrogen (25); (2) ß-oxidation of even-numbered carbon fatty acids to acetate and odd-numbered carbon fatty acids to acetate, propionate and hydrogen (142,143); (3) decarboxylation of propionate to acetate, carbon dioxide and hydrogen (16); cleavage of aromatic ring structures (52,70,152); (4) and decarboxylation of dicarboxylic acids (25,220). This group of bacteria catabolically oxidize substrates without light or electron acceptors such as sulfate, nitrate, or carbon dioxide. They are referred to as obligate proton reducers since their only mechanism for disposal of electrons is the reduction of protons to form hydrogen. Because their only electron acceptor is a proton they require the presence of hydrogen-utilizing bacteria to maintain a low hydrogen partial pressure (217,250). Low hydrogen partial pressure is required

for growth because the thermodynamics of the reactions are unfavorable under standard conditions (Table 3). However, under conditions of low hydrogen concentration the thermodynamics are favorable and the organism can obtain energy for growth.

Three species of obligate proton-reducing acetogenic bacteria have been isolated in coculture with either a methanogenic or sulfate-reducing species: (i) Syntrophomonas wolfei which oxidizes butyrate and higher molecular weight volatile fatty acids (142); (ii) Syntrophobacter wolinii which oxidizes propionate (16); and (iii) a benzoate-degrading rod (152). The 'S' organism isolated from the coculture "Methanobacterium omelianski" oxidizes ethanol in pure culture but growth is stimulated in coculture with a hydrogen-utilizing methanogenic species (25). Other bacteria that can oxidize volatile fatty acids by proton reduction are the sulfate-reducing bacteria (21,141,220) when grown with hydrogen-utilizing methane bacteria in the absence of sulfate. Although this symbiotic role between methanogenic and sulfate-reducing bacteria has been demonstrated in pure cocultures it has not been shown to occur in sediments. The physiological pathways of the obligate proton-reducing acetogenic bacteria have not been studied because of the present inability to grow pure cultures (16,142, 152).

The three stage methanogenic consortium found in freshwater sediments and sewage is shown in Figure 3. Complex organic matter is converted to methane by the interaction of the three metabolically distinct groups of bacteria: (i) the fermentative bacteria that degrade polymers and organic monomers to fatty acids, hydrogen, and carbon dioxide; (ii) the acetogenic bacteria that convert high molecular weight

Table 1. Morphologies and growth substrates of described methanogenic species.

Species	Morphology	Substrates	Reference
Methanobacterium formicicum	rod	H_2, formate	153
Methanobacterium bryantii	rod	H_2	25
Methanobacterium thermoautotrophicum	rod	H_2	256
Methanobrevibacter ruminantium	short rod	H_2, formate	194
Methanobrevibacter smithii	short rod	H_2, formate	193
Methanobrevibacter arboriphilus	short rod	H_2	225
Methanococcus vannielii	irregular coccus	H_2, formate	206
Methanococcus thermolithotrophicus	irregular occcus	H_2, formate	74
Methanococcus voltae	irregular coccus	H_2, formate	233
Methanococcus deltae	irregular coccus	H_2, formate	32
Methanococcus jannaschii	irregular coccus	H_2	87
Methanococcus maripaludis	irregular coccus	H_2	89
Methanococcus olentangyi	irregular coccus	H_2	32
Methanomicrobium mobile	slightly curved rod	H_2, formate	163
Methanomicrobium paynteri	irregular rod	H_2	170
Methanogenium cariaci	irregular coccus	H_2, formate	173
Methanogenium marisnigri	irregular coccus	H_2, formate	173
Methanogenium thermophilicum	irregular coccus	H_2, formate	171
Methanospirillum hungatei	spirillum	H_2, formate	51
Methanoplanus limicola	disc-shaped	H_2, formate	234
Methanothermus fervidus	rod	H_2	207
"*Methanplasma elizabethii*"	pleomorphic	H_2	174
Methanosarcina barkeri	packets of irregular cocci	H_2, acetate, methanol, methylated amines	71,108
"*Methanosarcina thermophila*"	packets of irregular cocci	H_2, acetate, methanol, methylated amines	262
Methanosarcina mazei	pleomorphic cocci	H_2, acetate, methanol, methylated amines	131
Methanolobus tindarius	irregular cocci	methanol, methylated amines	112
Methanothrix soehngenii	rod	acetate	79

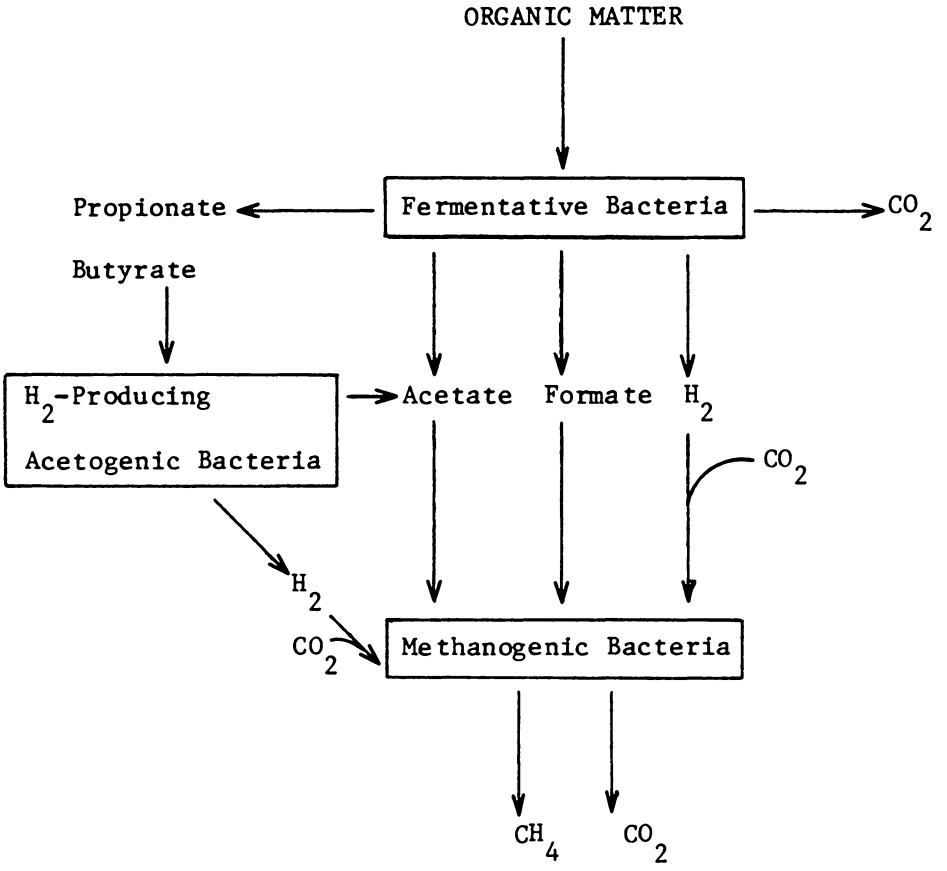

Figure 3. A simplified representation of the degradation of organic matter by methanogenic consortia in freshwater sediments and sewage.

fatty acids to acetate, hydrogen and carbon dioxide; and (iii) the methanogenic bacteria that convert products of the first two groups to methane and carbon dioxide. The efficiency of biomass conversion to methane is directly dependent on the interaction of the three metabolic groups as carbon flow is directed through high molecular weight fatty acids to acetate, formate and carbon dioxide, and electrons are directed to hydrogen gas: all are substrates for methanogenesis. The direction of carbon flow is controlled by the maintenance of low hydrogen partial pressure which results from interspecies hydrogen exchange with the methanogenic bacteria (20,24,243). Low hydrogen partial pressure results in a shift to more oxidized products by the fermentative bacteria and also causes oxidation of fatty acids by the acetogenic bacteria to be thermodynamically favorable (16,142,143). In the absence of interspecies hydrogen exchange the fermentative bacteria shift to production of reduced products such as alcohols and high molecular weight fatty acids and the acetogenic bacteria are inhibited. This would result in a lower yield of methane from the substrate and could result in complete inhibition as acids accumulated and lowered the pH (28,97). The methanogenic bacteria have three critical functions in the consortium: 1) they remove formate and acetate that could accumulate and inhibit degradation of polymers by fermentative bacteria; 2) they maintain low hydrogen partial pressure necessary for the conversion of volatile fatty acids to methanogenic substrates by the acetogenic bacteria; 3) they produce methane and carbon dioxide as the terminal carbon forms of degradation which are readily returned to the carbon cycle.

C. Methanogenesis in marine sediments

The predominant terminal process responsible for the anaerobic degradation of organic matter in eutrophic freshwater sediments is methanogenesis (148,193,210). The sulfate reducing bacteria, which compete with methanogenic bacteria for the same substrates, account for only 15% of the substrate used (125,249) when sulfate is added to eutrophic sediments (236). However, sulfate reduction is the predominant terminal process in oligotrophic freshwater sediments where organic nutrients are limiting (126). In marine sediments sulfate is replenished by the high concentrations present in seawater and sulfate reduction is the predominant terminal process in the anaerobic degradation of organic matter in the absence of alternative electron acceptors such as nitrate (118, 190,198). However, in certain ecological niches methanogenesis is the predominant terminal process. These niches include marine canyons (86,223), the lower depths of marine sediments (135), sediments that receive large amounts of organic matter (151), and the elevated intertidal portions of marshes (1,6,107); all environments where sulfate is depleted and is not rapidly replenished.

Studies on the activities of methanogenic and sulfate reducing bacteria in sediment profiles show that methanogenesis is excluded in the upper sediments where the sulfate concentration is greatest and increases with depth as sulfate is depleted (144,150,151,175,190,235). Jones and Paynter (88) showed that populations of methanogenic bacteria were greatest in sulfate depleted sediment layers. Methanogenesis is stimulated in the presence of sulfate by the addition of substrate (1,108) or by adding molybdate which inhibits sulfate reducing activity

(7). The mechanism of this apparent inhibition of methanogenesis is kinetic competition by bacteria for substrates which has been demonstrated in sediments (123,125) and in pure cultures (113,188). The apparent inhibition results from the ability of the sulfate bacteria to use common substrates at concentrations that are below those required by the methanogenic bacteria; that is, the sulfate reducing bacteria have a lower K_s for uptake of substrate than the methanogenic bacteria.

Marine methanogenesis may also occur with non-competitive substrates such as methylated amines (161) that are available from several sources in the marine environment. Marine animals contain trimethylamine oxide that is introduced to sediments by excretion or decomposition and is subsequently reduced to trimethylamine by bacterial anaerobic respiration (62,105,211,247,248). Trimethylamine and dimethylamine also occur in marine algae (97). Choline, a component of plant and animal tissues, may be fermented to trimethylamine by various bacteria. Oremland (160) demonstrated that the addition of sulfate did not affect methane production from trimethylamine in enrichment cultures and sulfate reduction was not stimulated by the presence of trimethylamine. Oremland et al. (160,161) have proposed that trimethylamine and methanol are non-competitive substrates for methanogenesis in salt marsh soils. Senior et al. (190) suggested that an unknown substrate may be more important than hydrogen for methanogenesis in saltmarsh sediments and Winfrey and Ward (235) have suggested that this unknown substrate(s) may be methylated amines. However, King et al. (106) have conducted labelling studies in intertidal sediments and have shown that methylated amines are not exclusively metabolized to methane. Further

studies are needed to determine the contribution of methylated amines to methanogenesis in marine sediments and to identify microorganisms that are responsible for this process.

Growth Requirements of the Methanogenic Bacteria

The methanogenic bacteria are fastidous anaerobes that require a highly reduced environment below -330 mV (104,193) for growth, a condition that has been recognized since early attempts to isolate pure strains (10). The optimum pH for growth of described isolates ranges between 6.5 and 7.5 although exceptions have been reported such as Methanococcus vannielli which grows optimally at pH 8.0 to 8.5 (206). Optimum growth temperatures range from 25°C for Methanolobus tindarius (112) to 83°C for Methanothermus fervidus (207). The range of substrates used by the methanogenic bacteria is limited as shown in Table 4. Two additional substrates that have been reported are carbon monoxide (33,108) and dimethylethylamine (48,71) but their occurrence has not yet been documented in methanogenic habitats.

All described methanogenic bacteria can grow in mineral medium but various organic growth factors may be required (3,132). Some species are autotrophic and only require hydrogen and carbon dioxide as the sole sources of carbon and energy. Autotrophic species include Methanobacterium thermoautotrophicum (57,256), Methanosarcina barkeri (231), Methanospirillum hungatei (53), Methanococcus thermolithotrophicus (74), Methanococcus jannaschii (87) and Methanococcus maripaludis (89). Other species such as Methanogenium cariaci (173), Methanobrevibacter ruminantium (23), Methanoplanus limicola (234), and Methanomicrobium

Table 4. Equations and free energy changes for the oxidation of substrates used by the methanogenic bacteria.[a]

	$\Delta G°'$:kJ/rx	kJ/mol CH_4
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6	-135.6
$4HCOOH + H_2O \rightarrow CH_4 + 3HCO_3^- + 3H^+$	-130.4	-130.4
$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H^+ + H_2O$	-310.6	-102.5
$4CH_3NH_3^+ + 3H_2O \rightarrow 3CH_4 + HCO_3^- + 4NH_4^+ + H^+$	-230.0	- 76.6
$2(CH_3)_2NH_2^+ + 3H_2O \rightarrow 3CH_4 + HCO_3^- + 2NH_4^+ + H^+$	-216.1	- 72.0
$4(CH_3)_3NH^+ + 9H_2O \rightarrow 9CH_4 - 3HCO_3^- + 4NH_4^+ + 3H^+$	-658.0	- 63.1
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	- 28.0	- 28.0

[a]data from Patterson and Hespell (163) and Thauer et al. (217).

paynteri (170) assimilate acetate for cell carbon. Cell fractionation studies indicate that M. barkeri (237), Methanothrix soehngenii (78,252) and Methanobacterium thermoautotrophicum (254) assimilate acetate and carbon dioxide simultaneously. Ammonium salts are used as a nitrogen source by all species tested (254) although nitrogen fixation has recently been observed in some species (S. Zinder, personal communication). Sulfide is the sulfur source for most species but organic sources have also been reported (253,254).

Trace organic supplements are also required by some species. The vitamins thiamine, pyridoxine and p-aminobenzoic acid are required by Methanomicribum mobile (R. S. Tanner and R. S. Wolfe. Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I90, p. 109), pantothenic acid stimulates the growth of Methanococcus voltae (233), and riboflavin stimulates the growth of Methanosarcina barkeri (183). The amino acids leucine and isoleucine are required for growth of Methanococcus voltae (233), Methanomicrobium mobile requires tryptophan (R. S. Tanner and R. S. Wolfe. Abstr. Annu. Meet. Am. Soc. Microbiol. 1982 , I90, p. 109), and Methanobrevibacter arboriphilus requires cysteine (253). The fatty acids 2-methylbutyrate and isovalerate are required by Methanococcus voltae in the absence of leucine and isoleucine (233), 2-methylbutyrate and isobutyrate are required by Methanomicrobium mobile (R. S. Tanner and R. S. Wolfe. Abstr. Annu. Meet. Am. Soc. Microbiol., 1982, I90, p. 109), and Methanobrevibacter ruminantium requires 2-methylbutyrate (23). Coenzyme M, a novel coenzyme present in all methanogenic bacteria is required by Methanobrevibacter ruminantium for growth (214). Unidentified growth factors are used by many species that

require yeast extract, Trypticase, casamino acids, rumen fluid, vitamin mixtures and other undefined supplements (132). Strains that require no organic growth supplements also have been described (3,74,87,89,256).

Trace metal requirements have been reported for some species. Jones and Stadtman (90) reported that Methanococcus vanniellii requires selenium and tungsten. Schonheit et al. (187) showed that Methanobacterium thermoautotrophicum requires nickel, cobalt and molybdenum, and Taylor and Pirt (215) showed a requirement for iron. Methanococcus voltae requires nickel and iron, while selenium and cobalt are stimulatory (233). Growth of Methanosarcina barkeri is stimulated by cobalt, molybdenum, nickel and selenium (183). Studies with plasma emission spectrometry suggest that zinc and copper may have a physiological role (182). Sodium chloride, magnesium and calcium are required in relatively high proportions for growth of marine species (3,74,87,89, 112,170,171,173,233,234).

Physiology of Methanogenic Bacteria

The metabolic pathways by which the methanogenic substrates hydrogen, formate, acetate, methanol and methylated amines are converted to CH_4 have not been completely elucidated. However, refinements in mass culturing and anaerobic biochemical techniques have enabled investigators to make significant progress.

A. Hydrogen and formate metabolism.

In 1956 Barker (12) proposed a unifying pathway by which methanogenic substrates were reduced in successive steps to methane via one or more unidentified carrier molecules (Figure 4). It was not

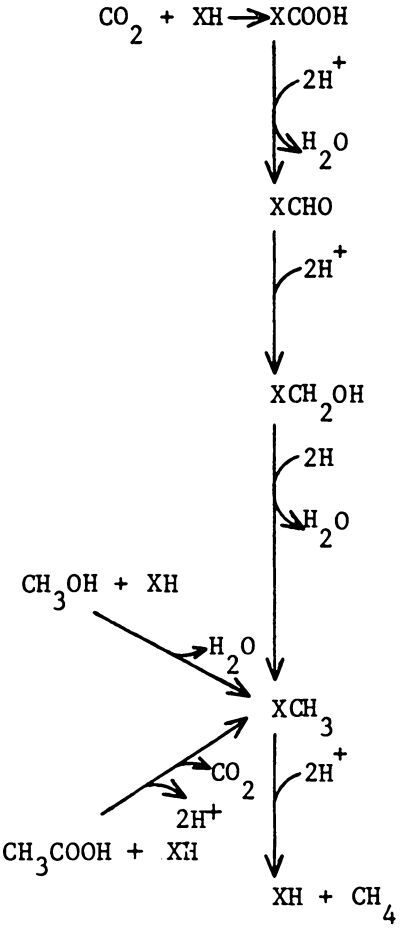

Figure 4. Pathways of methane formation proposed by Barker (12).

until 1971 that one of these carrier molecules, a novel coenzyme termed Co M, was discovered in Methanobacterium bryantii and was shown to be required for methanogenesis in dialized cell extracts with the addition of methylcobalamin (139). The structure of CoM: $HSCH_2CH_2SO_3^-$ was elucidated and the methylated form (CH_3-S-CoM) was shown to be an intermediate in methanogenesis (216). Methylated CoM stimulates the reduction of carbon dioxide to methane 30-fold in cell extract with magnesium cations, hydrogen and catalytic amounts of ATP (64,65). The methylreductase system of Methanobacterium thermoautotrophicum was later resolved into three fractions (66). Component A is a hydrogenase that consists of three protein fractions and FAD (155), component B is a heat-stable oxygen-labile low molecular weight cofactor (66), and component C is methylreductase that has a prosthetic group consisting of CoM (103) and cofactor F_{430} (43). This methylreductase system is the ultimate reduction step by which a C_1 unit at the methyl level is reduced to methane. Terminal enzymes involved in the methylreductase system have been described and include hydrogenase (60,80,85,145,221, 223) and formate dehydrogenase (92,179,222).

The initial reductive steps of carbon dioxide for methane formation were at first suggested to be mediated by folate enzymes (203,240), but Ferry et al. (50) reported that the levels of formyltetrahydrofolate synthetase and methylene tetrahydrofolate dehydrogenase were too low to be involved in catabolic carbon dioxide reduction. When cell extracts are passed through a molecular sieve gel to remove low molecular weight factors they can produce methane from CH_3-S-CoM if component B of the methylreductase system is added (172). When the fraction

containing low moleculer weight factors is added back to the cell extract with component B methane is formed from carbon dioxide. This soluble fraction has been separated into two components (122). Short term labelling experiments show that $^{14}CO_2$ is incorporated into a yellow fluorescent compound (34) in the soluble fraction which was identified as methanopterin (101,102). This pterin is found in all methanogenic bacteria assayed (W. J. Jones, J. C. Escalante-Semerena, and R. S. Wolfe. Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I24, p. 125) and it has been shown to be the C-carrier at the formyl, methylene and methyl levels of oxidation (45,47). The other component, designated carbon dioxide reduction (CDR) factor, has been purified and a structure has been proposed (46). Figure 5 is a modification of Barker's scheme (Figure 4) with the recent data incorporated. Wolfe (241) has proposed a scheme in which energy from the terminal reduction step for methane production is conserved to activate carbon dioxide for the stepwise reduction via the pterin intermediates.

The bioenergetics of the methane bacteria are poorly understood. Synthesis of ATP is presumably linked to methane formation although the mechanism of energy coupling is not known (98,129). An artificially imposed proton gradient results in net synthesis of ATP in intact cells which is consistent with membrane-dependent proton gradients (26,36,82, 83,84,189). In addition several investigators have reported ATP synthesis sites in internal membrane vesicles (35,37,176,177,201) which supports Mitchell's chemiosmotic principle (147). Electron and proton transport molecules have been identified in methanogenic bacteria. Coenzyme F_{430} is a prosthetic group in component C of the methyl-

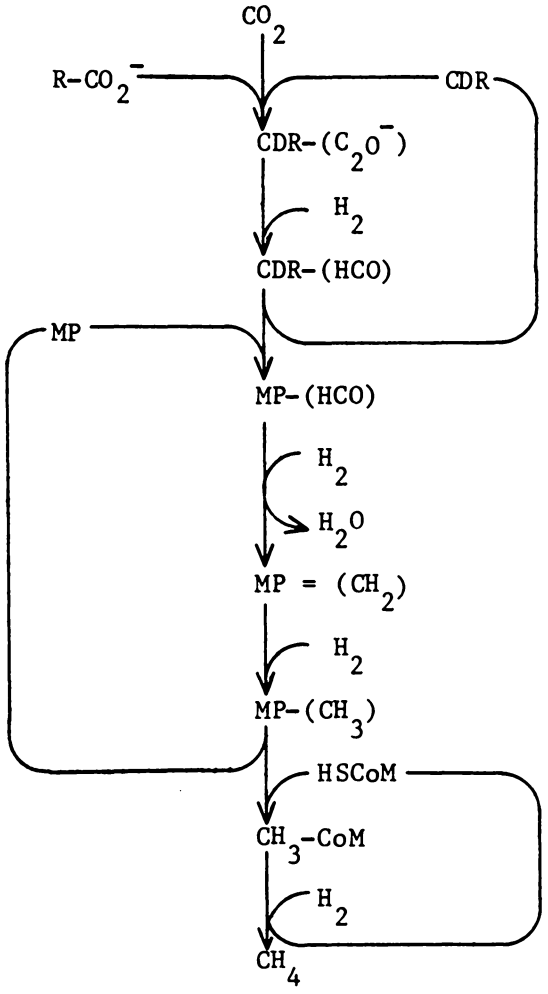

Figure 5. Pathway of methane formation proposed by Escalante-Semerena, Leigh and Wolfe (46) incorporating carbon dioxide reduction factor (CDR), methanopterin (MP), and coenzyme M (HSCoM).

reductase system (43). Coenzyme F_{420} is an electron acceptor for hydrogenase and formate dehydrogenase (180,222,223). Recent evidence suggests that FAD is required for the intramolecular electron transport of an electron pair for the reduction of the obligate two-electron acceptor coenzyme F_{420} (180,155,156). $NADP^+$ and NAD^+ reducing systems have also been described (91,144,245).

The assimilatory pathways for carbon dioxide and acetate have been described in Methanobacterium thermoautotrophicum. Autotrophic carbon dioxide fixation proceeds by synthesis of acetyl CoA from two-carbon dioxide molecules in a reaction involving carbon monoxide dehydrogenase, followed by a reductive carboxylation of acetyl CoA to pyruvate (57,212,213). Acetate is assimilated by synthesis of acetyl CoA via acetate thiokinase (158). All cell components are then synthesized from pyruvate and acetyl CoA via an incomplete reductive carboxylic acid cycle (42,55,56). Gluconeogenesis proceeds via pyruvate and aldol condensation of triose phosphates (59,81). Amino acid synthesis pathways have been reported for Methanobacterium thermoautotrophicum and Methanospirillum hungatei (42,55). The phytanyl chains of lipids in Methanospirillum hungatei are synthesized from acetate via mevalonic acid (42).

B. Acetotrophic metabolism.

The lack of information concerning methanogenesis from acetate can be largely attributed to the difficulty in obtaining active cell-free extracts. However, Baresi (8) recently reported that he has obtained methanogenesis from acetate with cell lysates of acetate-grown Methanosarcina barkeri at rates similar to those of whole cells. Co-

enzyme M and methyl coenzyme M methylreductase, which are components of the pathway for methanogenesis from hydrogen and formate (Figure 5), occur in hydrogen- and acetate-grown Methanosarcina barkeri at similar concentrations (9). Whole cell studies with ^{13}C-acetate (127) and deuterated acetate (165) indicate that the methyl carbon of acetate is transfered intact to methane via HS-CoM. In addition, cell-free extracts of acetate-grown Methanosarcina barkeri convert the methyl derivative of tetrahydromethanopterin to methane (W. J. Jones, J. C. Escalante-Semerena, R. S. Wolfe. Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I24, p. 125). It has been proposed that the methyl moiety of acetate enters the pathway shown in Figure 5 at the methyl level of methanopterin, but a pathway from the demethylation of acetate to methylation of HS-CoM has not been elucidated.

Growth of Methanosarcina barkeri on mixtures of acetate and trimethylamine, methanol or hydrogen-carbon dioxide is biphasic; the methyl moiety of acetate is not cleaved to methane until the other substrates are depleted (14,49,133,196). However, a small amount of the methyl moiety is oxidized in the presence of methanol (133). Methanogenesis from acetate in cell lysates from hydrogen-grown Methanosarcina barkeri is not detectable after repeated transfers in medium without acetate (8). This result suggests that a portion(s) of the acetotrophic pathway is different from the hydrogen and methylotrophic pathways. Methanosarcina barkeri and Methanothrix soehgenii contain carbon monoxide dehydrogenase (109,114). Methanosarcina sp. strain TM-1 grown on acetate contains 20-fold greater carbon monoxide-dependent methyl coenzyme M methylreductase activity than cells grown with meth-

anol (157). A pathway has been proposed which involves a carbon monoxide-dependent methylreductase system (Figure 6). Hydrogen production also has been reported during growth of Methanosarcina sp. strain TM-1 and Methanosarcina acetivorans, a species described later in this report, on acetate but a physiological role for its production has not been determined (124).

The means of energy production from acetotrophic metabolism has not been determined. A hypothesis for energy production in the absence of electron transport or substrate level phosphorylation has been proposed (251). However, cytochromes b and c which were not found in hydrogen utilizing species (115) do occur in Methanosarcina barkeri when it is grown on acetate (115,116,117). Ferredoxin (69) and coenzyme F_{420} also occur in Methanosarcina barkeri grown on acetate.

Acetate assimilation occurs by synthesis of acetyl CoA via acetate thiokinase (109,232) and pathways for the synthesis of some amino acids by Methanosarcina barkeri have been described (232).

C. Methylotrophic metabolism.

Methanol, methylamine, dimethylamine, trimethylamine and dimethylethylamine are methylotrophic substrates for methanogenesis and all acetotrophic methanogens, except Methanothrix soehngeii, can use these substrates (229). Strictly methylotrophic species which cannot use H_2 or acetate have also been reported (112,200,257) indicating that acetotrophic and methylotrophic pathways are different. In methylotrophic metabolism the methyl group of methanol is reduced intact to CH_4 via coenzyme M (191). Van der Meijden (224,225,226) has described a two component system for methanogenesis from methanol which consists of

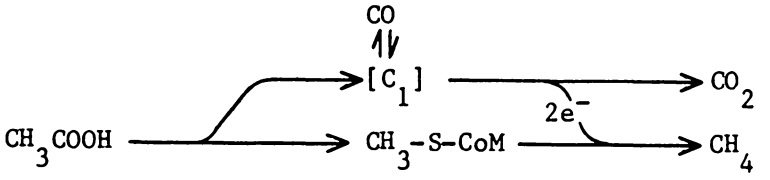

Figure 6. Pathway of methanogenesis from acetate proposed by Nelson and Ferry (157).

methanol:5-hydroxybenzimidazolylcobamine methyltransferase and Co-methyl-5-hydroxybenzimidazolylcobamide:HS-CoM methyltransferase. The methyl group of trimethylamine is also reduced intact to methane via coenzyme M (191,228) but the pathway(s) for transfer of the methyl moieties from methylated amines to methyl coenzyme M has not been determined. During growth on trimethylamine cultures of Methanosarcina barkeri (71) and Methanococcoides methylutens, a species described later in this report, accumulate dimethylamine and methylamine until trimethylamine is depleted (Figure 7). These results suggest that the enzyme(s) responsible for demethylation of dimethylamine and methylamine are repressed by trimethylamine. When Methanosarcina barkeri is grown on mixtures of trimethylamine and hydrogen-carbon dioxide or methanol the substrates are used simultaneously (49,133). However, the methyl moiety of methanol is entirely reduced to methane as a result of hydrogen oxidation (133).

Coenzymes F_{420} and F_{430} have been detected in cells grown with methanol and trimethylamine (9,99,200). Cytochromes also occur at levels slightly lower than those found in acetate-grown cells (117), but their physiological role has not been determined.

The recent description of the methane producing pathway should facilitate efforts to describe the initial methyl transfer reactions of the methylotrophic pathways.

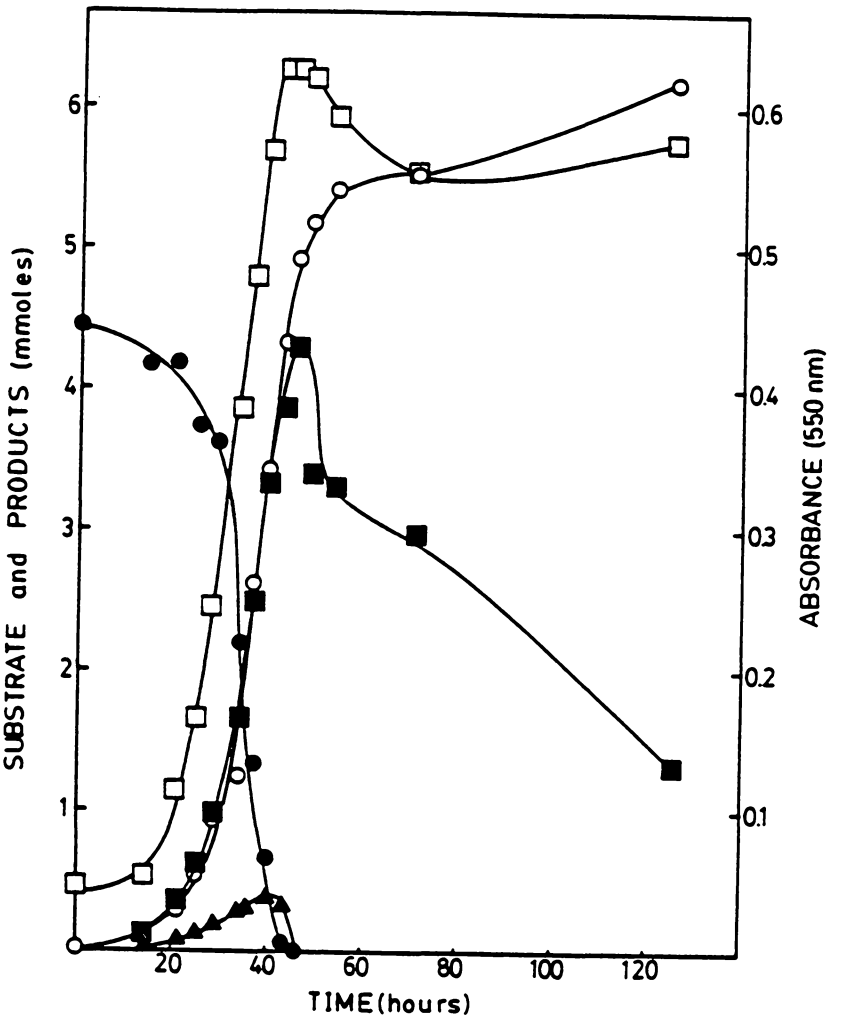

Figure 7. Growth of Methanococcoides methylutens on trimethylamine. The experiment was carried out in 160 ml serum vials that contained 100 ml of medium. Values are corrected for the decrease in culture volume from withdrawal of samples. ●, trimethylamine; ▲, dimethylamine; ■, methylamine; □, absorbance at 550 nm; ○, methane.

SECTION III. CHARACTERIZATION OF A MARINE METHANOGENIC CONSORTIUM

SUMMARY

A consortium of bacteria that degraded kelp to methane was obtained from a marine canyon which contained large deposits of kelp and sea grass. The methane profile in the water column above the canyon ranged from 4.7 nM at the surface to 870 nM at 23 meters. Enrichment cultures were initiated in an artificial seawater medium prepared without sulfate and containing kelp (Macrocystis pyrifera) as the sole substrate. Intermediates formed during degradation included hydrogen, formate, acetate, propionate, butyrate, succinate and ethanol. Enrichment cultures were begun for methanogenic bacteria with the substrates hydrogen, formate and acetate; and for acetogenic hydrogen-producers with the substrates propionate and butyrate. Hydrogen and formate enrichments yielded two "plate"-shaped isolates, differentiated by morphology, which are similar to Methanoplanus limicola and a long helical rod which is similar to Methanospirillum hungatei. Acetate enrichments yielded irregularly shaped cocci, differentiated by size, which are similar to Methanosarcina acetivorans; and large rods similar to Methanothrix soehngenii. Propionate and butyrate enrichments yielded non-UV-fluorescing rods which occurred in long chains in close association with the UV-fluorescing methanogenic bacteria described above. Results show that a complete methanogenic consortium consisting of a close association of fermentatives, acetogenic hydrogen-producers and a diversity of methane-producers was obtained from this specific marine habitat.

INTRODUCTION

Biosynthesis of CH_4 by anaerobic fermentation of biomass is a potential renewable resource to supplement present energy sources. This naturally occurring process, which results from the activities of an interacting consortium of bacteria, is an important degradative process in the global carbon cycle. Methanogenesis from biomass offers several advantages: (i) up to 90% of biomass energy may be recovered as CH_4; (ii) the low solubility of CH_4 facilitates collection; and (iii) CH_4 is a readily usable and transportable fuel.

MATERIALS AND METHODS

Methane profile. Samples were recovered with 100 ml syringes fitted with gas tight teflon valves. The diver withdrew approximately 80 ml of seawater at various depths then sealed the syringe before surfacing. When the syringes were returned to the lab, 4 ml of nitrogen gas was drawn into each and the dissolved gas was allowed to equilibrate with the headspace by shaking 10 times in 10 seconds for a total of ten treatments in 1 hour. 3-500 µl subsamples of the gas phase were then analyzed for methane. Equilibrium concentrations were based on calculations by Yamamoto et al. (47).

Enrichment medium. A marine methanogenic food chain was obtained in enrichment culture using Macrocystis pyrifera as the substrate. Sediments were obtained from Scripps Canyon by a diver assisted by SCUBA. Three 6-liter fermenter vessels that contained 2.7 liters of medium were each inoculated with 300 ml of sediment. Sterile medium was prepared anaerobically under a O_2-free N_2-CO_2 (80:20) atmosphere as

described by Hungate (14). The enrichment medium was a mixture of 20% (vol/vol) demineralized water and 80% (vol/vol) artificial seawater (23) from which Na_2SO_4 was omitted. The medium also contained 0.05% (vol/vol) cysteine-sulfide reducing agent (5), 0.1% (wt/vol) Na_2CO_3, 0.001% (wt/vol) resazurin, 0.035% (wt/vol) Na_2HPO_4, and 0.05% (wt/vol) NH_4Cl. Frozen M. pyrifera was chopped into fine particles (< 2 mm) and added to the medium at a final concentration of 0.4% (dry wt). Cultures were maintained by semi-continous feed in which kelp was replenished when CH_4 production subsided. 20% of the medium was also replaced at this time to prevent accumulation of salts from the kelp.

Isolation method. Enrichment cultures were established for (i) mannitol and sodium alginate degrading fermentative bacteria, (ii) sodium butyrate and sodium propionate degrading acetogenic bacteria, and (iii) sodium acetate, sodium formate and H_2-CO_2 degrading methanogenic bacteria. Enrichment cultures were started by inoculating kelp enrichment culture (10 ml) into serum vials that contained enrichment medium (40 ml) and substrate. The H_2-CO_2 enrichment medium was made by substituting H_2-CO_2 (80:20) for N_2-CO_2 in the headspace. All other media were prepared by adding the appropriate substrates to a final concentration of 20 mM. After CH_4 production subsided, 10% of the culture was transferred to new medium and this procedure was repeated for 2 to 5 subsequent transfers. Isolates were obtained by serial dilution in roll tubes that contained enrichment medium, substrate, and 1% purified agar as previously described (14). Enrichment cultures (1 ml) were inoculated into molten agar medium (9 ml) in an anaerobic culture tube (25 x 150 mm), then rolled until it solidified. After

incubation for several weeks colonies were picked and transferred to agar slants.

Analytical methods. Methane was assayed with a gas chromatograph as previously described (42). Dissolved hydrogen was extracted by the procedure of Robinson et al. (35) and assayed with a thermal conductivity gas chromatograph (Dohrman, model 1503). The stainless steel column (0.32 x 305 cm) was packed with Carbosieve B (120/140 mesh, Supelco) and was operated at 120°C with an N_2 carrier gas. Samples were injected into a glass precolumn (0.32 x 3.8 cm) packed with $CaSO_4$ (Drierite) that was maintained at room temperature to remove water vapor. Short chain fatty acids were assayed by gas chromatography after derivatization to p-bromophenacyl esters as described by Barcelona (3). Dicarboxylic fatty acids were methylated with methanol that contained 14% boron trifluoride and assayed by gas chromatography as described by Metcalfe (28). Alcohols were assayed with a gas chromatograph equipped with a flame ionization detector (GowMac, series 750). Aqueous samples (2 µl) were injected onto a stainless steel column (0.32 by 182.88 cm) packed with Chromosorb 101 (Supelco) and operated at 110°C with an N_2 carrier gas. Mannitol was assayed by the phenylhydrazine-ferricyanide colorimetric method of Baily (1). Alginate was assayed by the phenol-sulfuric acid method as described by Handa (12). Results represent the mean values obtained from three fermenters.

RESULTS AND DISCUSSION

METHANOGENESIS FROM MARINE BIOMASS

Macrocystis pyrifera as a Substrate for Methanogenic Fermentation

The oceans represent an untapped source of solar energy in the form of plant biomass (6,7,11). Approximately 43 percent of the total gross primary production occurs at depths of up to 100 meters (33). Investigators have discussed the feasibility of using plant biomass as a source of methane production (13). Giant Pacific kelp, Macrocystis pyrifera, is a preferred substrate for several reasons: (i) the high ratio of readily degradable organics to recalcitrant materials such as lignin and cellulose results in a high yield of CH_4 per unit mass; (ii) the soluble nature of the major organic constituents, mannitol and alginate, renders it available to degradation, thereby decreasing the retention time; (iii) the occurrence of methanogenesis in kelp sediments enables the selection of a natural food chain.

Methanogenesis in Marine Sediments

In marine sediments, which contain high concentrations of sulfate from seawater, sulfate reduction is the predominant terminal process in the degradative food chain (39,40). Although low rates of methanogenesis occur concurrently with sulfate reduction the sulfate reducing bacteria have a greater affinity (lower K_s) for hydrogen and acetate than the methane producing bacteria (19,21,22,36). In marine environments where sulfate is rapidly depleted, methanogenesis becomes the predominant terminal process. These environments include the lower depths of sediments (25), tidal marine marshes (18), and sediments that frequently receive large amounts of organic matter (30). One such

methanogenic environment is Scripps Canyon, a submarine canyon located near LaJolla, California. This canyon receives large deposits of kelp that are deposited by longshore currents. To determine dissolved methane in the water column above the canyon samples were collected at various depths by a diver equipped with SCUBA. The subsurface methane concentration was 4.5 nM and increased logrithmically with depth to 32 nM at 14 meters which was the approximate point of a sharp thermocline (Figure 1). Below this point methane concentrations increase at a greater rate with depth reaching 873 nM at the lowest depth assayed. Values observed by other investigators include 1.78 to 0.27 nM in the north Pacific (20), 1.5 to 0.76 nM in the north Atlantic (37), 4 to 879 nM in Walvis Bay (38) and 2.19 to 35874 nM in the Cariaco Trench (20). Scripps Canyon and Walvis Bay represent coastal areas with high organic loading and have similar methane profiles. It is likely that methane concentrations would continue to increase with the full depth of Scripps canyon as observed in the Cariaco Trench. Determinations may be an underestimate of the potential methane produced in the sediments, representing only 1-10% of the upward methane evolution due to anaerobic oxidation (34). Although an anaerobic methane oxidizing bacterium has not been isolated methane has been associated with sulfate reduction in marine sediments (16,34) and methane oxidation has been observed in enrichments cultures with sulfate as the sole terminal electron acceptor (31).

Intermediates of Kelp Degradation

Figure 2 shows the time course of fermentation in a kelp-degrading enrichment culture. In the initial 48 hours after feeding the concen-

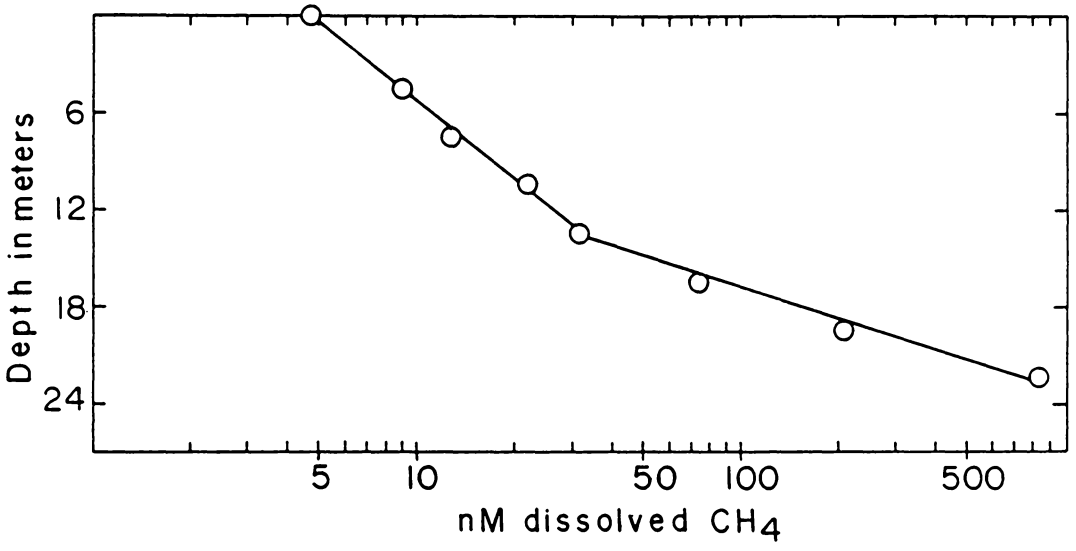

FIGURE 1. Dissolved CH_4 profile in the water column above Scripps Canyon.

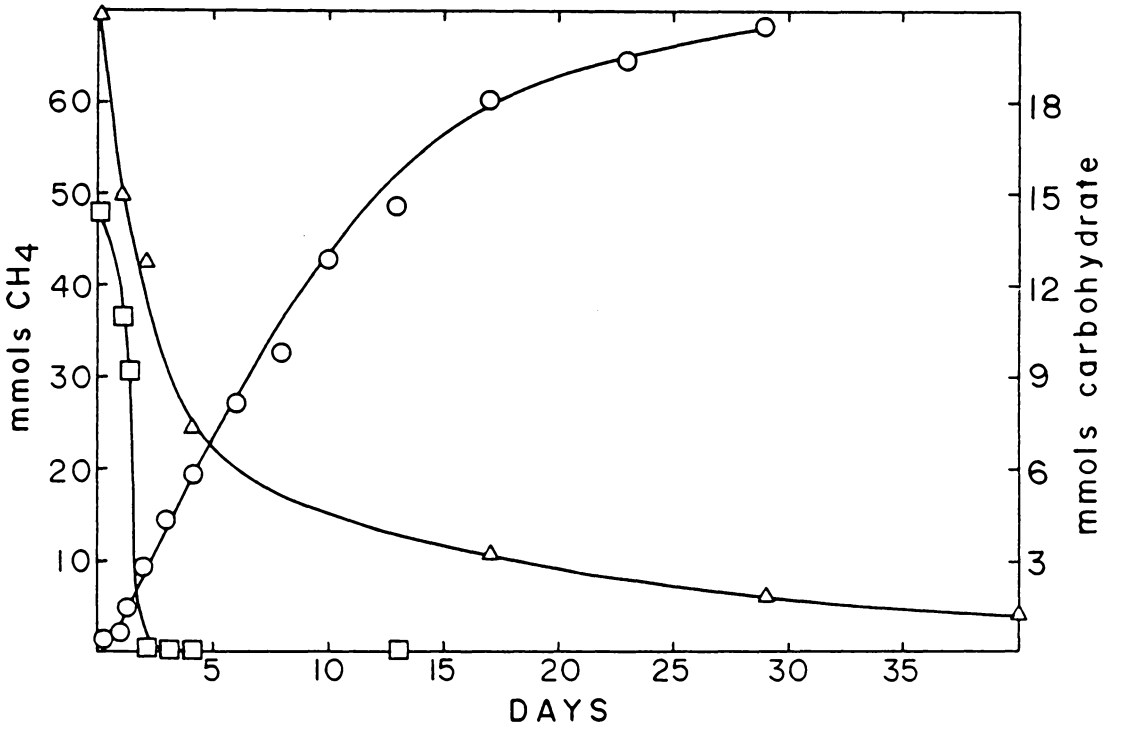

FIGURE 2. Time course study of kelp fermenters showing the decrease in mannitol (□) and alginate (△) and total production of CH_4 (○) after feeding with kelp.

tration of mannitol and alginate simultaneously decreased and formate, acetate, propionate, butyrate, succinate and ethanol were produced (Figure 3). The dissolved hydrogen concentration approached 13 µM (Figure 4). After 24 hours the hydrogen concentration rapidly declined to a steady state concentration of 60 nM. During this period CH_4 production increased and the only detectable intermediates were acetate, succinate and propionate. These results indicate that during the initial fermentation of the sugars the increased hydrogen concentrations resulted in a shift towards more reduced fermentation products such as ethanol. As methanogenesis increased the hydrogen concentration was lowered which allowed the fermentative bacteria to shift toward more oxidized products. The results suggest that interspecies hydrogen exchange has an important role in the marine methanogenic food chain. These results also suggest that a complete methanogenic food chain analogous to that described in fresh water environments is present in the marine environment. The high concentrations of succinate and propionate may be attributed to the degradation of alginate by species such as Cytophaga (A. F. Cappiapuoti and J. R. Forro, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, 076, 252). In addition 45% of the estimated sugar content was converted to CH_4 indicating that the kelp was an efficient substrate for methanogenic fermentation. Subsequent replenishments resulted in immediate CH_4 formation, less intermediate accumulation, and more rapid substrate utilization indicating that the population dynamics shifted towards more efficient degradation (data not shown).

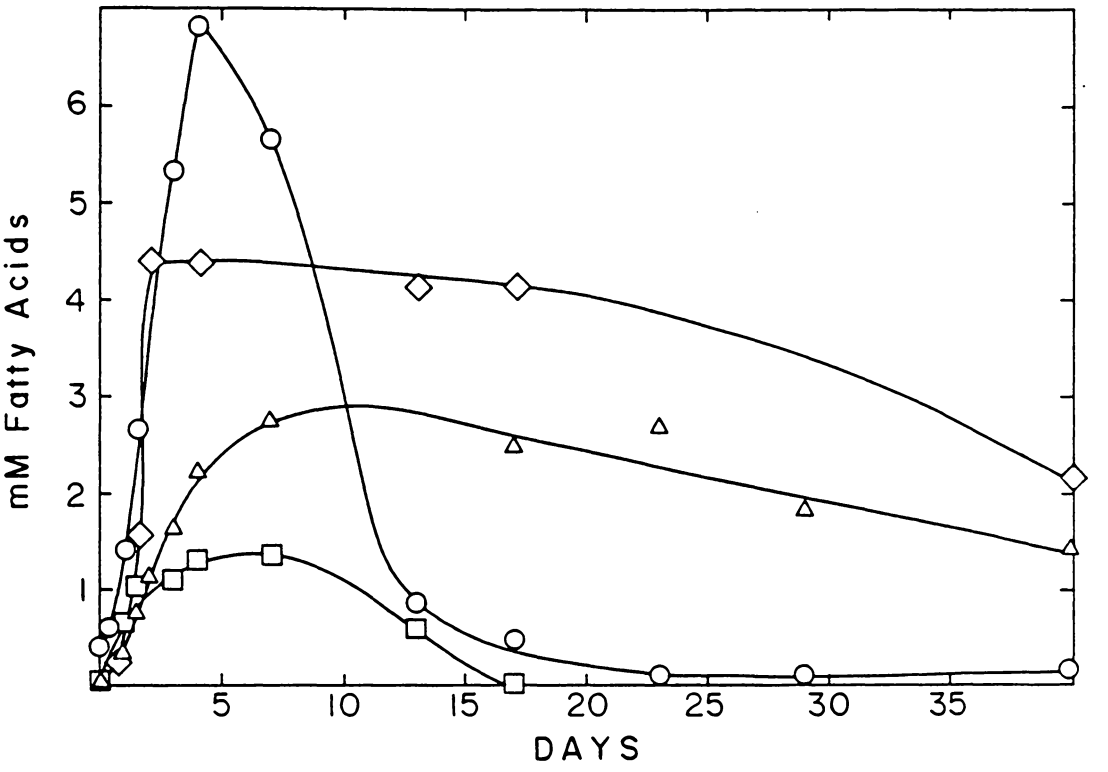

FIGURE 3. Time course study of kelp fermenters showing the concentrations of acetate (○), propionate (△), succinate (◇) and butyrate (□) after feeding with kelp.

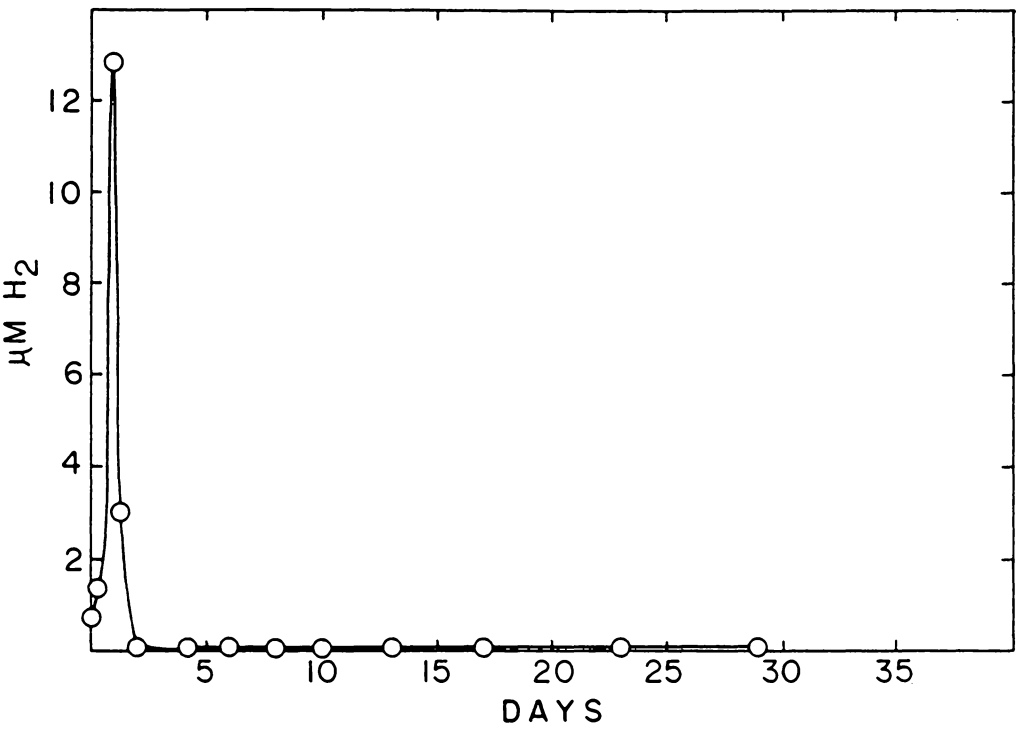

FIGURE 4. Time course study of kelp fermenters showing the dissolved H_2 concentration after feeding with kelp.

Organisms in the Consortium

Hydrogen and Formate-Oxidizing Methanogens. Three morphologically distinguishable isolates were obtained from H_2 roll tubes. Strain H2A is a nonmotile disc-shaped cell 2.0 ± 0.2 µm in diameter and 0.25 µm thick by phase contrast microscopy. Several fimbria-like structures and a subunit cell wall, characteristic of a protein cell wall, were visible by electron microscopy (Figure 5a). This isolate is similar to a previously described disc-shaped methanogen, Methanoplanus limicola (45). Strain H2E is a nonmotile flat rod 2.5 ± 0.9 µm in length by 0.8 µm in width and 0.25 µm thick. Fimbria-like structures, a protein-type subunit cell wall, and sharply angled terminal ends are visible by electron microscopy (Figure 5b). Strain H2S is a motile, helical rod 9.6 ± 1.3 by 0.4 µm in size, often occurring in filaments greater than 100 µm in length. Electron micrographs show a striated outer envelope and bundles of flagella at each end (Figure 5c). This isolate is similar to the freshwater species Methanospirillum hungatei (9) but has a much higher salt tolerance (32). All isolates obtained from formate roll tubes were similar to strain H2E.

Acetate-utilizing Methanogenic Bacteria. Seven isolates were obtained that each expressed three distinct morphovars at different growth phases: single irregular cocci, aggregates of cells, and communal cysts that contained an aggregation of single cells within a common envelope. One of these isolates, designated Methanosarcina acetivorans strain C2A, is described elsewhere as a new species and is the first marine acetotrophic methanogen (40) (Figure 5d). Large numbers of rod-shaped cells were observed in acetate enrichment cultures

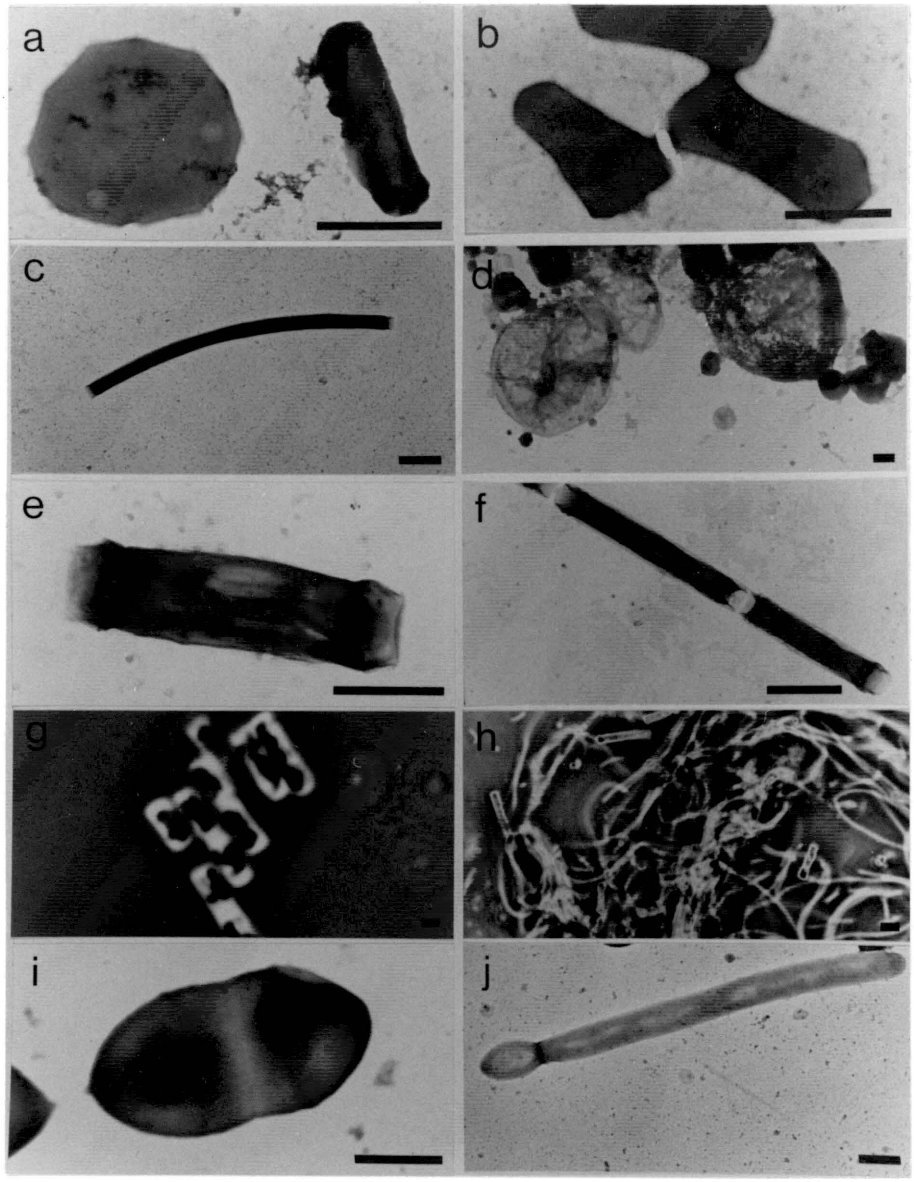

FIGURE 5. Electron (EM) and phase contrast photomicrographs of isolates and enrichments from kelp fermenters. Negatively stained electron photomicrographs of the H_2-utilizing strains H2A (a), H2E (b), and H2S (c). Negatively stained EM photomicrographs of the acetate-utilizing strain C2A (d) showing irregular cocci and communal cysts, and the acetate utilizing rods distinguishable by size (e,f). Phase contrast photomicrographs of the larger acetate-utilizing rod showing refractile inclusions (g) and a butyrate-degrading consortium showing long chains of rods with some acetate-utilizing type rods (h). EM photomicrographs showing the mannitol degrading coccus (i) and the sporeforming cytophaga-type isolate from alginate (j). All bars = 1 µm.

that would not colonize on agar. Two cells were distinguishable by size; 3.5 ± 0.5 by 1.0 µm and 2.3 ± 0.4 by 0.8 µm (Figures 5e,f,g). Both rods have flat ends, internal refractile bodies that do not appear in stationary phase cultures, and often form long chains. Electron micrographs show a striated cell wall similar to the protein cell wall of Methanothrix soehngenii (15). These rods become more predominant in older cultures which suggests that they have a slower growth rate than the irregular cocci.

Propionate- and Butyrate-Utilizing Acetogenic Bacteria. The acetogenic bacteria were not obtained in pure culture. The predominant cells of the enrichments were slightly curved rods 9.5 by 0.5 µm that formed long chains and rods 2.9 by 0.5 µm (Figure 5h). Both cell types were usually found in close association with cells similar to the hydrogen-utilizing methanogenic strains H2E and H2S, and with the acetate utilizing strain C2A and the flat-ended rods. Both cell types did not fluoresce under longwave ultraviolet light (29) and unlike methanogens they were sensitive to vancomycin (10).

Mannitol and Alginate Fermenting Bacteria. All colonies obtained on mannitol roll tubes consisted of cocci 2 µm in diameter that often occured in pairs and are similar in morphology to Leuconostoc species isolated previously from a kelp fermenter (A. F. Cacciapuoti and J. R. Forro, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, 976, p. 252) (Figure 5i). Colonies obtained from alginate roll tubes consisted of an elongated, sporeforming rod, 10 by 3 µm that produced a yellow pigment in the medium. These are characteristics of Cytophaga which has also been previously found in kelp fermenters by Cacciapuoti and Forro (Figure 5j).

The following conclusions are drawn from the results presented in this study. 1) A complete kelp degrading methanogenic consortium consisting of a close association of three physiologically distinct groups of bacteria was obtained from marine sediments. 2) This marine microbial food chain is not inhibited by high salt concentrations found in kelp (17) which causes inhibition in sewage digesters and methanogenic bacteria (26,32,43). 3) The food chain effectively degrades the two major components of kelp, alginate and mannitol, simultaneously and efficiently, with greater than 90% of the estimated content of alginate and mannitol being degraded to CH_4. 4) The population of methanogenic bacteria in the marine consortium is equally diverse in morphology and physiology to that found in a freshwater consortia. 5) The marine consortium has a high capacity for maintaining low H_2 partial pressure and our results suggest that interspecies H_2 exchange has an important regulatory role. 6) The high concentrations of acetate and propionate indicate that utilization of these substrates may be limiting in this system. Rapid degradation of these intermediates may be achieved as the population dynamics of the system is established in a continuous feed system. Subsequent transfers of the kelp enrichment cultures resulted in an increased rate of degradation and a more stable pH, factors which improve the rate and reliability of the system. Characterization of the described isolates is currently underway to in order to optimize methanogenesis from kelp in this system.

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SECTION IV. ISOLATION AND CHARACTERIZATION OF A METHYLOTROPHIC MARINE METHANOGEN, *METHANOCOCCOIDES METHYLUTENS* GEN. NOV., SP. NOV.

SUMMARY

A new genus of marine methanogenic bacteria is described that utilizes trimethylamine, dimethylamine, methylamine, and methanol as substrates for growth and methanogenesis. Methane was not produced from H_2-CO_2 (80:20), sodium formate, or sodium acetate. Growth on trimethylamine was stimulated by yeast extract, Trypticase, rumen fluid or B-vitamins. The optimal growth temperature was 30 to 35°C. The maximum growth rate was between pH 7.0 and 7.5. Na^+ (0.4 M) and $MgSO_4$ (0.05M) were required for maximum growth. Colonies of the type strain, TMA-10, were yellow, circular, and convex with entire edges. Cells were nonmotile, nonsporeforming, irregular cocci 1 μm in diameter which stained Gram negative and occurred singly or in pairs. Micrographs of thin sections revealed a monolayered cell wall approximately 10 nm thick which consisted of protein. Cells were lysed in 0.01% sodium dodecylsulfate or 0.001% Triton X-100. The deoxyribonucleic acid base composition was 42 mol% guanine plus cytosine. *Methanococcoides* is the proposed genus and *Methanococcoides methylutens* is the type species. TMA-10 is the type strain (ATCC 33938).

INTRODUCTION

In anaerobic marine sediments, methanogens are inhibited by the sulfate reducing bacteria which compete for the same substrates (27,31).

Methanogenesis is greater in sulfate depleted sediments that receive large amounts of organic matter (23) and in sediments that are not readily replenished with sulfate, such as marine trenches (6,25) and elevated portions of marine marshes (15). Methylated amines and methanol, not known to be utilized by sulfate reducing bacteria, are potential substrates for methanogens in marine sediments (24). Trimethylamine oxide, produced by marine animals, is reduced to trimethylamine (TMA) by organisms that utilize it as an electron acceptor for anaerobic respiration (33,38). TMA is present in marine algae (14) and also may be formed from microbial degradation of choline, a component of plants and animal tissues (7). Methanol is a product of pectin degradation (30). We describe here the isolation and characterization of a marine methylotrophic methanogen that utilizes TMA, methylamine, and methanol as substrates for growth and methanogenesis.

(This work was reported in part at the 82nd Annual Meeting of the American Society for Microbiology [K. R. Sowers and J. G. Ferry, Abst. Annu. Meet. Am. Soc. Microbiol. 1982, I 100, p. 111].)

MATERIALS AND METHODS

Source of inoculum. Sediment (0 to 60 cm) was obtained by a diver assisted with SCUBA at a depth of 19.8 m from the Sumner branch of Scripps canyon located near La Jolla, California. The sediment consisted of an interwoven mat of algae, sea grass debris, and sand deposited by longshore currents.

Media. Sterile media were prepared under a N_2-CO_2 (80:20) atmosphere by a modification of the Hungate technique (2). Enrichment

medium contained a mixture of 20% deionized water and 80% artificial sea water (18a) augmented with the following constituents at the indicated final percent compositions (wt/vol): NH_4Cl, 0.05; Na_2CO_3, 0.1; Na_2HPO_4, 0.035; NaH_2PO_4, 0.030; cysteine-HCl$\cdot H_2O$, 0.025; $Na_2S\cdot 9H_2O$, 0.025; resazurin, 0.0001; $FeSO_4$, 0.001; TMA$\cdot$HCl, 0.12. In addition, 1% (vol/vol) of vitamin solution and 1% (vol/vol) of trace element solution were added (37). The pH of the medium was adjusted to 7.5 by the addition of 6N HCl before autoclaving. Roll tubes were prepared by addition of 2% purified agar to the medium. Maintenance medium contained the following constituents at the indicated final percent compositions (wt/vol) in deionized water: NaCl, 2.34; $MgSO_4$, 0.63; Na_2CO_3, 0.50; yeast extract (Difco), 0.10; NH_4Cl, 0.5; KCl, 0.08; $CaCl_2\cdot 2H_2O$, 0.014; Na_2HPO_4, 0.06; resazurin, 0.0001; cysteine-HCl$\cdot H_2O$, 0.025; $Na_2S\cdot 9H_2O$, 0.025; TMA$\cdot$HCl, 0.30. In addition, 1% (vol/vol) of vitamin solution and 1% (vol/vol) of trace element solution were added to the medium. The pH was adjusted to 7.5 with 6 N HCl before autoclaving. Maintenance slants contained 1% purified agar (Difco). The final pH of the media was 7.2 after equilibration with 101 kPa of N_2-CO_2 (80:20).

Enrichment and isolation. A screwtop polyethylene container was filled with sediment and transported to the laboratory in an anaerobic jar (BBL Laboratory Systems). Approximately 5 g of sample was added to 45 ml of enrichment medium contained in a stoppered 160 ml serum vial. The culture was incubated under an atmosphere of N_2-CO_2 (80:20) at 25°C in the dark. After methane production subsided, 5 ml of the culture was inoculated into 45 ml of fresh sterile enrichment medium. Serial dilutions of the fifth subculture were inoculated into agar roll tubes

(25 x 150 mm) by the procedure described by Hungate (8) and modified by Bryant (5). Colonies were transferred to slants containing filter-sterilized vancomycin (100 mg/liter) that was added to molten autoclaved maintenance medium.

Analytical procedures. Growth experiments were performed in culture tubes (16 x 150 mm) that contained 10 ml of maintenance medium unless indicated otherwise. Tubes were sealed with a butyl rubber stopper that was secured with an aluminum crimp collar (2). Growth was monitored spectrophotometrically at 550 nm with a Bausch and Lomb Spectronic 20.

Methane was assayed with a gas chromatograph (Model 2440; Varian Instruments) equipped with a flame ionization detector. The column was 0.32 by 182.88 cm stainless steel which contained silica gel (80/100 mesh; Supelco). The column oven was operated at 100°C, and N_2 was the carrier gas. Purified methane (Airco) was used as a standard. Chromatographic data were integrated and concentrations calculated with a CDS-111 data system (Varian).

Crude cell-free extracts were prepared anaerobically by passing the cell slurry through a French pressure cell followed by centrifugation of the suspension (28). The assays for hydrogenase and formate dehydrogenase were performed by following hydrogen- or formate-dependent reduction of coenzyme F_{420} at 420 nm (ϵ_{420} = 42.5 $mM^{-1}cm^{-1}$) with a Perkin Elmer model 552 spectrophotometer (28). Coenzyme F_{420} was partially purified from the cell free extract by elution from a DEAE-cellulose column (0.9 by 6 cm) with 1 M KCl. The concentration of F_{420} was determined spectrophotometrically (28). Protein was determined by

the BioRad dye-binding assay, using bovine serum albumin as a standard (4).

Cell wall preparation. Cell paste (10 g wet weight) was suspended in 20 ml of salt buffer (pH 7.0) which contained trisodium citrate (15 mM), NaCl (400 mM), and $MgSO_4$ (50 mM). The cells were lysed as described above. Whole cells were removed by centrifugation at 3000 x g for 15 min. The cell wall fragments were removed from the supernatant solution by centrifugation at 48000 x g for 15 min and washed three times in salt buffer. The wall fragments were resuspended in salt buffer containing 2% Triton X-100 to remove cell membranes, then washed once in salt buffer and twice in buffer containing only trisodium citrate. The sample was then resuspended in buffer with RNase A (0.25 mg) and T_1 RNase (1,880 units) and incubated at 35°C for 30 min. DNase (2650 units) and $MgCl_2 \cdot 6H_2O$ (4.1 mg) were added, and the suspension was incubated for an additional 30 min. The sample was washed twice with deionized water and dialyzed overnight in 6 liters of 10 mM EDTA at 1°C. The sample was dialyzed twice in 6 liter deionized water for 3 hours and then freeze-dried.

The freeze-dried cell walls were acid hydrolyzed for 24, 48, and 72 hours and the hydrolysates was analyzed with a Beckman 121 amino acid analyzer. Cysteine and methionine were determined after performic acid oxidation. The organic content was determined by measuring the difference in mass after heating at 425°C overnight.

Moles percent of guanine plus cytosine (G+C). Cells were lysed with sodium dodecyl sulfate, and DNA was obtained from the lysate by the procedure of Marmur (21). Moles percent G+C was determined by the

thermal denaturation method with a Gilford model 2400 Spectrophotometer (11). Bacteroides fragilis (VPI 2553) and Escherichia coli B DNA were used as standards.

Microscopy. Phase-contrast micrographs were made with a Leitz Dialux microscope. Cells were fixed for electron microscopy by adding 1 ml of an 8% aqueous solution of glutaraldehyde to a 10 ml broth culture. The culture was gently inverted several times and allowed to stand for 30 min. A drop of the cell suspension was transferred to a collodion-coated grid for 1 min, then washed twice with double distilled water. Cells were negatively stained for 2 min with a filtered 0.5% aqueous solution of uranyl acetate. Cells for thinsection electron microscopy were fixed as described above, washed twice with double distilled water, and then resuspended in a 2% aqueous solution of osmium tetroxide for 1.5 h. The cells were then washed twice and embedded in 2% Noble agar (Difco). The agar blocks were suspended in a 0.5% aqueous uranyl acetate solution for 16 h before dehydration with a graded series of aqueous-ethanol mixture. The ethanol was replaced with acetone before embedding in Spurr's medium (32). Thin sections were made with a Sorvall model MT2b ultramicrotome and poststained with lead citrate and uranyl acetate (35). Electron micrographs were taken with a JEOL JEM 100B transmission electron microscope.

Chemicals. Purified agar and yeast extract were from Difco Laboratories (Detroit, Mich.). Trypticase was from BBL Microbiology Systems (Cockeysville, Md.). Trimethylamine, dimethylamine, and methylamine were from Aldrich Chemical Co. (Milwaukee, Wis.). Bovine serum albumin RNase A, T_1 RNase, Triton X-100, SDS, and vitamins were obtained from

Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were reagent grade.

RESULTS

Enrichment cultures became turbid 6 days after inoculation with sediment. The predominant organisms in the enrichments were small irregular cocci that fluoresced blue-green when examined with a UV-fluorescence microscope by the method of Mink and Dugan (22). Surface colonies of these organisms were yellow, circular, and convex with entire edges. The colonies were 0.5 mm in diameter 5 days after inoculation and 1.5 mm after 14 days. Microscopically pure colonies were reisolated in roll tubes that contained enrichment medium and vancomycin (100 mg/liter). Strain TMA-10 was used throughout this study to represent organisms with these characteristics.

Cells of strain TMA-10 were irregular cocci with an average diameter of 1.0 μm (Fig. 1A). Larger cells, 2 to 3 μm in diameter, were occasionally present and were more numerous in older cultures. Cells became spherical and then lysed when either NaCl or $MgSO_4$ was omitted from the medium. Although strain TMA-10 stained Gram negative, thin-sections revealed the absence of an outer membrane typical of a gram-negative cell wall (Fig. 1C and D). Cell walls were not typically Gram-positive, as they consisted of a very thin monolayer approximately 10 nm thick. Cells were lysed by the addition of sodium dodecyl sulfate (final concentration, 0.01%) or Triton X-100 (final concentration, 0.001%) to the medium. All of these characteristics are very similar to those of the marine genera Methanococcus and Methanogenium which

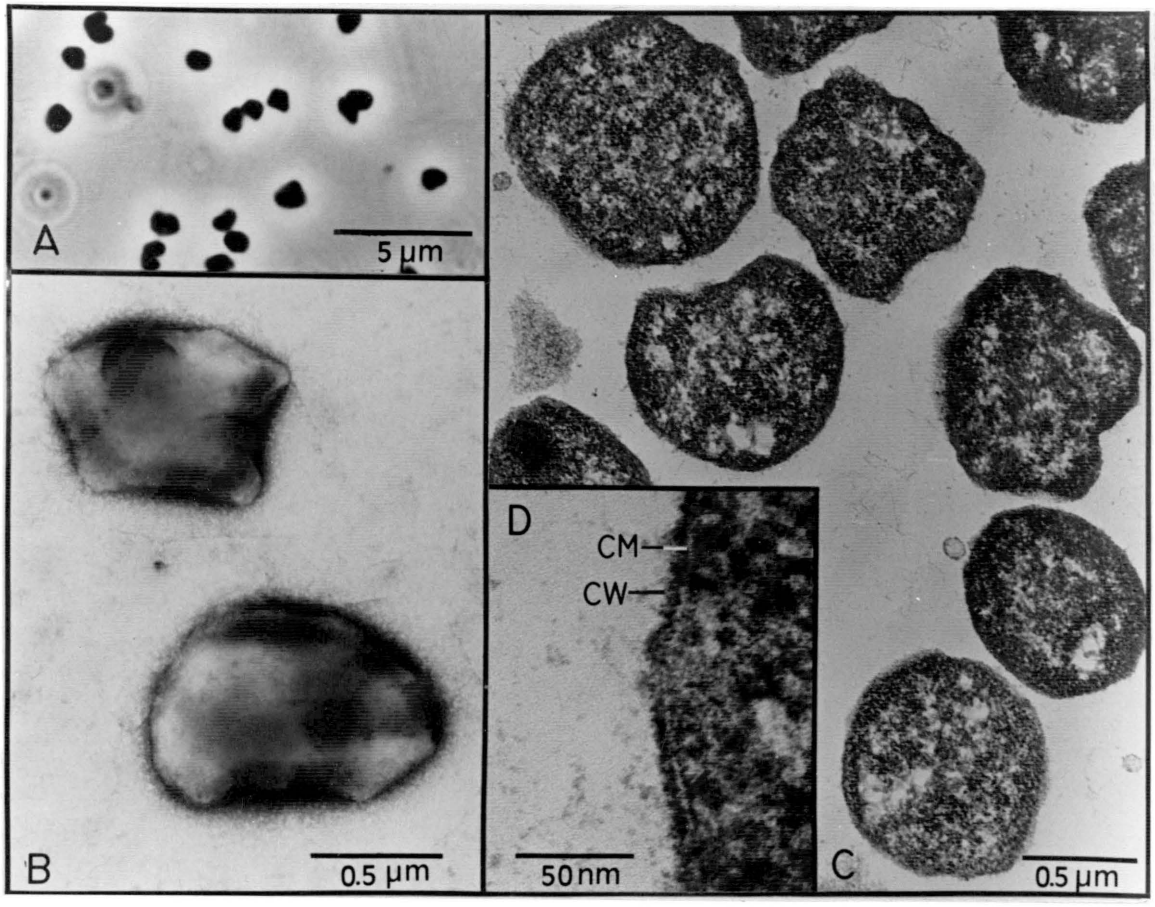

Fig. 1 A-D. Electron and phase-contrast photomicrographs of strain TMA-10. (B) Negative stain of whole cells illustrating their irregular shape. (C) Thin section of whole cells. (D) Thin section showing the cell wall (CW) and cytoplasmic membrane (CM).

contain protein cell walls (12,13,26). Analysis of the acid hydrolyzed cell wall preparation of strain TMA-10 revealed a wide distribution of amino acids but no amino sugars (Table 1). Furthermore, 93.2% of the organic material was recovered as amino acids, indicating that the cell walls consisted of protein. Motility in strain TMA-10 was not observed, and electron micrographs of negatively stained cells revealed the absence of flagella and pili (Fig. 1B).

Growth and methanogenesis were supported by the methylotrophic substrates TMA, dimethylamine-hydrochloride (1.62%), methylamine, or methanol. Strain TMA-10 previously grown with TMA did not grow on or produce methane from H_2-CO_2 (80:20), sodium formate (0.5%), sodium acetate (0.5%), or calcium acetate (0.5%) within 90 days after inoculation. The minimum doubling time obtained with maintenance medium that contained TMA was 5.2 h. Yeast extract (0.1% [wt/vol]), Trypticase (0.1% [wt/vol]), rumen fluid (10% [wt/vol]), or B vitamin solution (1% [vol/vol]) (37) stimulated growth four-fold compared with growth in maintenance medium containing only formate (0.1% [wt/vol]) and acetate (0.1% [wt/vol]) or without additions. Growth temperature was optimal at 30 to 35°C and no growth occurred at 40°C (Fig. 2). The effect of Na^+ was determined by adjusting the NaCl concentration in maintenance medium. The maximum growth rate occurred between 0.24 and 0.64 M Na^+ (Fig. 3). No growth occurred at 0.043 or 1.2 M Na^+. KCl did not substitute for NaCl. Greater than 10 mM $MgSO_4$ was required for growth, and the optimal concentration was 50 mM, which is also the concentration present in seawater (Fig. 4). $MgCl_2$ could be substituted for $MgSO_4$, but $MnSO_4$, $CoSO_4$, $NiSO_4$ or $FeSO_4$ did not substitute for $MgSO_4$ indicating

TABLE 1. Amino acid content of the hydrolyzed cell wall preparation from strain TMA-10.

Amino acid	µmol/mg dry weight	molar ratio (His = 1.0)
Asp	0.187	4.14
Glu	0.177	3.92
Gly	0.152	3.39
Ala	0.147	3.24
Leu	0.134	2.97
Val	0.123	2.72
Lys	0.121	2.67
Ile	0.113	2.50
Ser	0.100	2.26
Arg	0.091	2.01
Thr	0.079	1.72
Pro	0.070	1.56
Phe	0.057	1.27
Met	0.052	1.15
Tyr	0.052	1.15
His	0.045	1.00
Cys	0.025	0.55
Trp	ND	ND

ND = not determined.

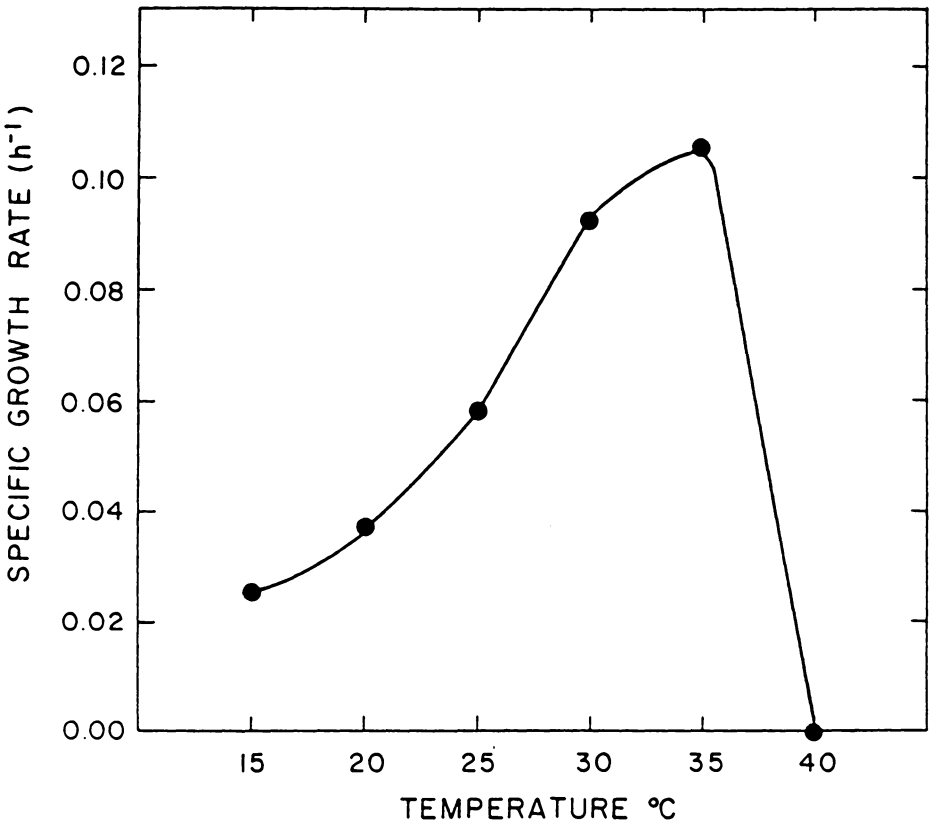

Fig. 2. Effect of temperature on the growth rate of strain TMA-10.

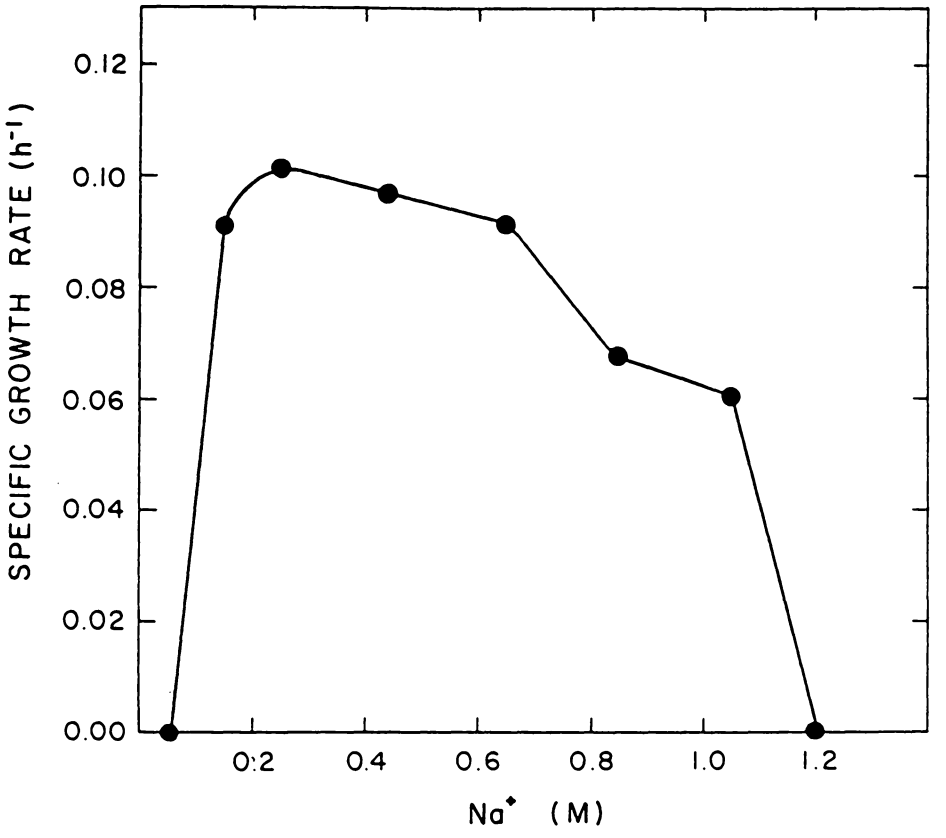

Fig. 3. Effect of Na^+ concentration on the growth rate of strain TMA-10. The final Na^+ concentration was corrected for Na_2CO_3, $NaHPO_4$ and Na_2S present in the medium.

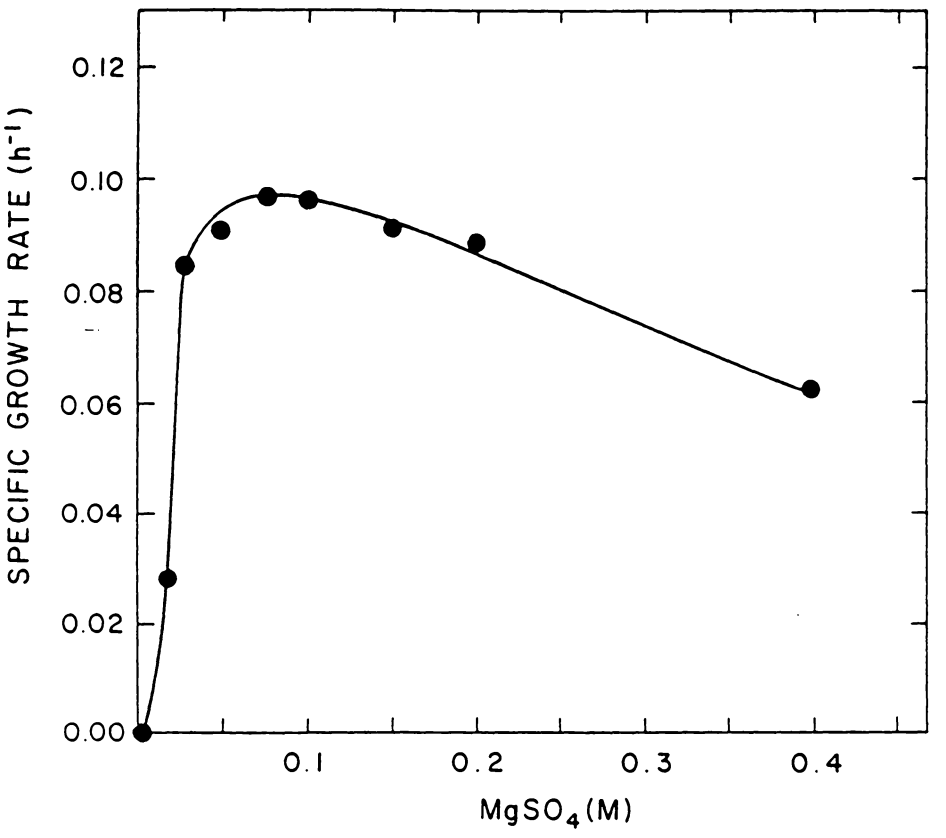

Fig. 4. Effect of $MgSO_4$ concentration on the growth rate of strain TMA-10.

that the divalent cation Mg^{++} was specifically required. Growth and methanogenesis occurred in maintenance medium between pH 6.0 and 8.0, and maximum growth rates occurred between pH 7.0 and 7.5.

Cell-free extracts from TMA-grown cells of strain TMA-10 contained 176 ng of coenzyme F_{420} per mg of protein, which was 11 to 25-fold greater than that reported for Methanosarcina barkeri strain 227 (3). Hydrogen- or formate-dependent reduction of coenzyme F_{420} by cell-free extract from TMA-grown cells was not detected under the specified conditions. Strain TMA-10 contained coenzyme M (R. White, personal communication).

The polar lipid fraction of strain TMA-10 consisted of 2,3-diphytanyl glycerol diethers in addition to an unidentified glycerol ether component not previously detected in methanogens (T. Langworthy, personal communication). Dibiphytanyl diglycerol tetraethers were not apparent. The DNA base composition was 42 ± mol% G+C.

DISCUSSION

Described methylotrophic methanogens include the genus Methanosarcina, and the species Methanococcus mazei and Methanothrix soehngenii. These all utilize acetate for growth and methanogenesis (9,20). The inability of strain TMA-10 to utilize acetate as a sole energy source is unique among described methylotrophic methanogens. However, acetate-utilizing methanogens have been isolated which cannot grow on acetate subsequent to growth on methanol (R. Mah, personal communication). It therefore cannot be ruled out that unknown factors would enable strain TMA-10 to grow on acetate. Attempts to isolate

this species from the same inoculum with acetate as the sole energy source were unsuccessful.

Approximately 70% of the methane produced in freshwater sediments and sewage is derived from the methyl group of acetate (19). However, sulfate-reducing bacteria are the predominant acetate-utilizing organisms in sulfate-rich marine sediments (17,31,36). The sulfate reducing population may also inhibit methane production from hydrogen in the presence of marine sulfate concentrations by lowering the hydrogen partial pressure below that necessary for utilization by methanogens (16,18). The production of methylated amines and methanol may account for some of the methanogenic activity observed in sulfate-rich marine sediments. Desulfovibrio vulgaris degrades choline to TMA, acetate, and ethanol, but when D. vulgaris is cocultured with Methanosarcina barkeri, TMA is further metabolized to methane (34). Oremland et al. (24) reported the accumulation of TMA and methanol in sulfate-rich marine sediments when methanogenesis was selectively inhibited by the addition of bromoethanesulfonic acid. The apparent inability to use H_2-CO_2 and acetate, and the good growth on TMA and methanol indicates that strain TMA-10 is particularly adapted for growth in the sulfate-rich sediments from which it was isolated.

An interesting property of this isolate is the apparent absence of coenzyme F_{420}-dependent hydrogenase, a constitutive enzyme of Methanosarcina barkeri (3). Coenzyme F_{420} is an electron carrier closely linked to hydrogenase and formate dehydrogenase of the methanogens (10, 29). Although these results are preliminary, high concentrations of coenzyme F_{420} and the apparent absence of coenzyme F_{420}-dependent

hydrogenase and formate dehydrogenase in extracts of strain TMA-10 suggest additional functions for this electron carrier.

Direct immunofluorescence (S-probe) serotyping revealed no detectable relationship between strain TMA-10 and the families Methanobacteriaceae, Methanomicrobiaceae and Methanococcaceae. Cross-reactions with members of the Methanosarcinaceae indicate that strain TMA-10 is more related to the Methanosarcinaceae family than to any other family, but did not reveal a reaction pattern that identified strain TMA-10 as any known member of the genus Methanosarcina (E. Conway de Macario, personal communication).

We propose that strain TMA-10 be placed in the order Methanomicrobiales as described by Balch et al. (1) based on direct immunofluorescence (S-probe) serotyping, absence of dibiphytanyl diglycerol tetraethers in the lipid fraction, and the ability to use methylotrophic substrates. Although the mol percent G+C is comparable to that observed for the genus Methanosarcina, several features clearly distinguish strain TMA-10 from members of the genus Methanosarcina. These include a negative gram reaction, a thin protein cell wall, sodium dodecyl sulfate sensitivity, the inability to use acetate, a high coenzyme F_{420} concentration, and a requirement for Na^{+} and Mg^{++}. We therefore propose that this methylotrophic methanogen be placed in a newly described genus of methane-producing bacteria: Methanococcoides. The type species is Methanococcoides methylutens, and TMA-10 is the type strain.

The following genus description is suggested: Methanococcoides (Me.tha'no.coc.coi'des) gen. nov., Sowers and Ferry. Methanococcus

established genus plus -ides, Gr. adj. suffix similar to; Methanococcoides M.L. neut. n. organisms similar to methanococcus. Members of this genus are nonsporeforming, nonmotile, highly irregular cocci 1 to 3 μm in diameter which stain gram negative and occur singly or in pairs. Surface colonies are yellow, circular, and convex with entire edges and fluoresce blue-green under longwave UV light. Cells are lysed by sodium dodecyl sulfate and possess a thin protein cell wall (10 nm). Growth and methanogenesis occur with the methylotrophic substrates TMA, dimethylamine, methylamine, and methanol. DNA base composition of the only described strain is 42 mol%. Sodium and magnesium are required for growth. Organisms are found in anaerobic marine sediments.

The formal species description is Methanococcoides methylutens (methy.lu'tens) sp. nov., Sowers and Ferry. Methyl mod. chem. word, utens L. part. adj. using; methylutens using methyl.

Morphology and colony characteristics are the same as those described for the genus: temperature optimum, 30 to 35°C; pH optimum 7.0 to 7.5; source, anaerobic sediments from the Sumner branch of Scripps Canyon located near La Jolla, California; physiology, fastidious anaerobe - TMA, dimethylamine, methylamine and methanol serve as substrates for growth and methanogenesis; H_2-CO_2, formate, and acetate do not; nutrition, optimal growth at 0.44 M NaCl and 25 mM or greater $MgSO_4$; yeast extract, Trypticase, rumen fluid, or B vitamins stimulate growth; type strain, TMA-10. This strain has been deposited in the American Type Culture Collection, Rockville, Md. as number 33938.

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SECTION V. TRACE METAL AND VITAMIN REQUIREMENTS OF *METHANOCOCCOIDES METHYLUTENS* GROWN WITH TRIMETHYLAMINE

SUMMARY

Trace organic nutrient and metal requirements for the growth of *Methanococcoides methylutens* strain TMA-10 were determined in defined medium that contained trimethylamine as the substrate. Biotin was the only organic supplement required in place of yeast extract, Trypticase or a mixture of 8 B-vitamins. Fe and Ni were required for growth and low concentrations of Fe^{++} (< 5 µM) and Ni^{++} (< 0.25 µM) provided limited growth. In the absence of added Co the growth rate was reduced by 94% and growth was limiting at concentrations below 0.1 µM. Stimulation of growth by Se, Mo, B, Al, Zn, Mn or Cu could not be demonstrated.

Key Words: Cobalt, Iron, Nickel, Biotin, *Methanococcoides methylutens*, Trace elements, Vitamins, Methanogens

INTRODUCTION

Comprehensive studies on the trace metal and vitamin requirements of methanogenic bacteria have been reported for *Methanobacterium thermoautotrophicum* (Schonheit et al., 1979; Taylor and Pirt, 1977), *Methanococcus voltae* (Whitman et al., 1982) and *Methanosarcina barkeri* (Scherer and Sahm, 1981). The vitamin content of *M. thermoautotrophicum* and *M. voltae* have been reported (Leigh, 1983), and the elemental compositions of 10 methanogenic species was determined by inductively-coupled plasma

emission spectrometry (Scherer, Lippert and Wolff, 1983). M. barkeri is the only species in these studies that uses acetate, methanol and methylamines as substrates for growth. Recently, methanogenic bacteria have been described that use only methanol or methylated amines for growth (Sowers and Ferry, 1983; König and Stetter, 1982; Zhilina, 1984). One of these species, Methanococcoides methylutens, was isolated from a marine canyon and some nutritional requirements have been reported (Sowers and Ferry, 1983). We describe here the effect of trace metals and organic supplements on the growth of M. methylutens when grown in defined medium with trimethylamine.

MATERIALS AND METHODS

Culture conditions. Methanococcoides methylutens strain TMA-10 (= DSM 2657, = ATCC 33938) was maintained on agar slants as previously described (Sowers and Ferry, 1983). Sterile media, described below, were prepared under an oxygen-free N_2-CO_2 (80:20) atmosphere by a modification of the Hungate technique (Balch and Wolfe, 1976). Growth experiments were done with culture tubes (16 by 150 mm; Bellco Glass Inc., Vineland, NJ) that contained 10 ml of medium and were sealed with butyl rubber septa. Cultures were incubated at 30°C and growth was measured by determining absorbance at 550 nm with a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, NY). All results are reported as the mean value of triplicate cultures.

Vitamin-free medium. The procedure for the organic growth factor experiments has been described (Sowers et al., 1984). All glassware was washed with concentrated sulfuric acid, rinsed with charcoal-treated

water and heated at 450°C overnight. The medium contained the following constituents in demineralized water that was treated with activated charcoal (percent weight/vol): NaCl, 2.34; $MgSO_4$, 0.63; Na_2CO_3, 0.5; NH_4Cl, 0.5; trimethylamine-HCl, 0.3; KCl, 0.08; Na_2HPO_4, 0.06; $CaCl_2 \cdot 2H_2O$, 0.014; $Na_2S \cdot 9H_2O$, 0.025; cysteine-HCL$\cdot H_2O$, 0.025; and resazurin, 0.0001. In addition 1% (vol/vol) of trace metal solution (Wolin et al., 1963) was added and the pH of the medium was adjusted to 7.2 with 6 N HCl before dispensing. Cysteine was deleted from the medium when testing for stimulation by Casamino acids. The growth factors were added to each tube of medium (0.1 ml) from 100-fold stock solutions before autoclaving. The final concentrations of growth factors tested were 0.1% (wt/vol) yeast extract, Trypticase, Casamino acids, 1% (vol/vol) vitamin mixture (Wolin et al., 1963) and short chain fatty acid mixture that included 0.5 mls/l of each of the following components: formic acid, acetic acid, valeric acid, 2-methylbutyric acid, isobutyric acid, isovaleric acid.

Metal-free medium. Trace metal requirements were determined using the procedure of Whitman et al. (1982) with modifications described below. All glassware was acid washed and butyl rubber stoppers and polypropylene pipet tips were treated with $NaHCO_3$/EDTA solution as previously described (Whitman et al., 1982). Gas cannula needles were covered with Teflon tubing to prevent metal contamination. A Chelex-100 column (1.5 x 18 cm, Bio-Rad Laboratories, Richmond, CA) was pretreated and the sodium form generated as previously described (Davey et al., 1970). The flow rate was adjusted to 5 ml/min and the column was equilibrated with sodium acetate (0.5 M, pH 5.1), then rinsed with 5

bed volumns of demineralized H_2O. This pH was chosen in order to adsorb Mo in addition to Co, Cu, Fe, Ni and Zn (Riley and Taylor, 1968). 6-liters of demineralized H_2O were passed through the column and collected for rinsing and medium dilution. The medium was prepared in two parts. Part I contained the following components in demineralized H_2O (percent wt/vol): NaCl, 4.68; $MgSO_4$, 1.26; NH_4Cl, 1.0; trimethylamine-HCl, 0.6; KCl, 0.6; Na_2HPO_4, 0.12; $CaCl_2 \cdot 2H_2O$, 0.028; cysteine-HCl, H_2O, 0.05; biotin, 0.004. The pH was adjusted to 5.1 with 1 N HCl before passing through the Chelex-100 column. The first 100 ml were discarded and the remaining medium was collected in a flask continuously purged with N_2-CO_2. Part II contained the following components in demineralized H_2O (percent wt/vol): Na_2CO_3, 2.5; $Na_2S \cdot 9H_2O$, 0.125. The pH was adjusted to 8.0 with 1N HCl, passed through a Chelex-100 column equilibrated with Na_2HPO_4 buffer (0.5 M; pH 8.0) and collected as described above. Part I (500 ml), part II (200 ml) and boiled Chelex-treated water (300 ml) were anaerobically mixed together in a 2 l flask and dispensed into culture tubes that contained the metal salts to be tested. Cells were harvested by centrifugation and washed three times in Chelex-treated medium. Cells were then suspended in a volume of Chelex-treated medium required to obtain an absorbance of 0.01 with a 1% inoculum.

Chemical sources. Yeast extract was from Difco Laboratories (Detroit, MI). Trypticase was from BBL Microbiology Systems (Cockeysville, MD). Vitamin-free, salt-free Casamino Acids were from ICN Nutritional Biochemicals (Cleveland, OH). All vitamins were from Sigma Chemical Co. (St. Louis, MO). Trimethylamine-HCl and the ultra-pure

(99.999%) metal salts $CoSO_4 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, and $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ were from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade.

RESULTS

No significant growth occurred in vitamin-free mineral medium unless a B-vitamin mixture or biotin alone was added (Figure 1). The addition of biotin with or without the complete vitamin mixture resulted in the same amount of growth and the individual deletion of any one vitamin, except biotin, had no significant influence on growth. Equivalent growth also occurred when yeast extract or Trypticase replaced biotin (data not shown). However, no growth occurred when either vitamin-free Casamino acids or a volatile fatty acid mixture was added to vitamin-free mineral medium and these supplements were not stimulatory when added to medium that also contained biotin. The results indicate that biotin is the only organic growth supplement required by this strain of M. methylutens.

The trace metal requirements of M. methylutens were determined in medium that was treated with Chelex-100 ion-exchange resin to remove divalent metal cations. Each metal was added at various concentrations in the presence of fixed amounts of the other two metals as indicated in Figure 2. Final culture densities were limited by concentrations below 5 µM Fe, 0.25 µM Ni and 0.10 µM Co. No growth occurred in medium without added Fe. A final absorbance of 0.3 was consistently obtained throughout serial transfers in medium without added Ni. In medium without added Co, the specific growth rate decreased from 0.19 h^{-1}, the

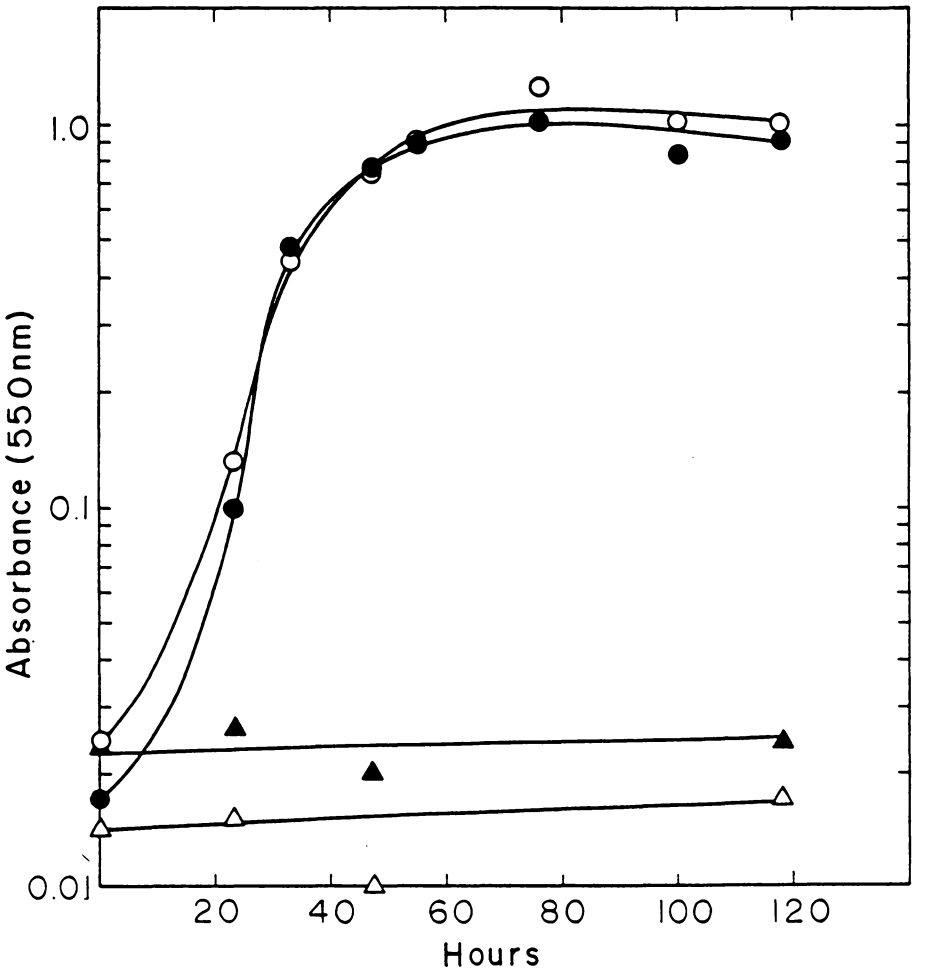

Fig. 1. Effect of biotin and seven other vitamins on growth of Methanococcoides methylutens with trimethylamine. Additions include complete vitamin mixture with (●) and without (▲) biotin, biotin only (○), and no vitamins (△). The final concentration of biotin was 0.02 mg/l.

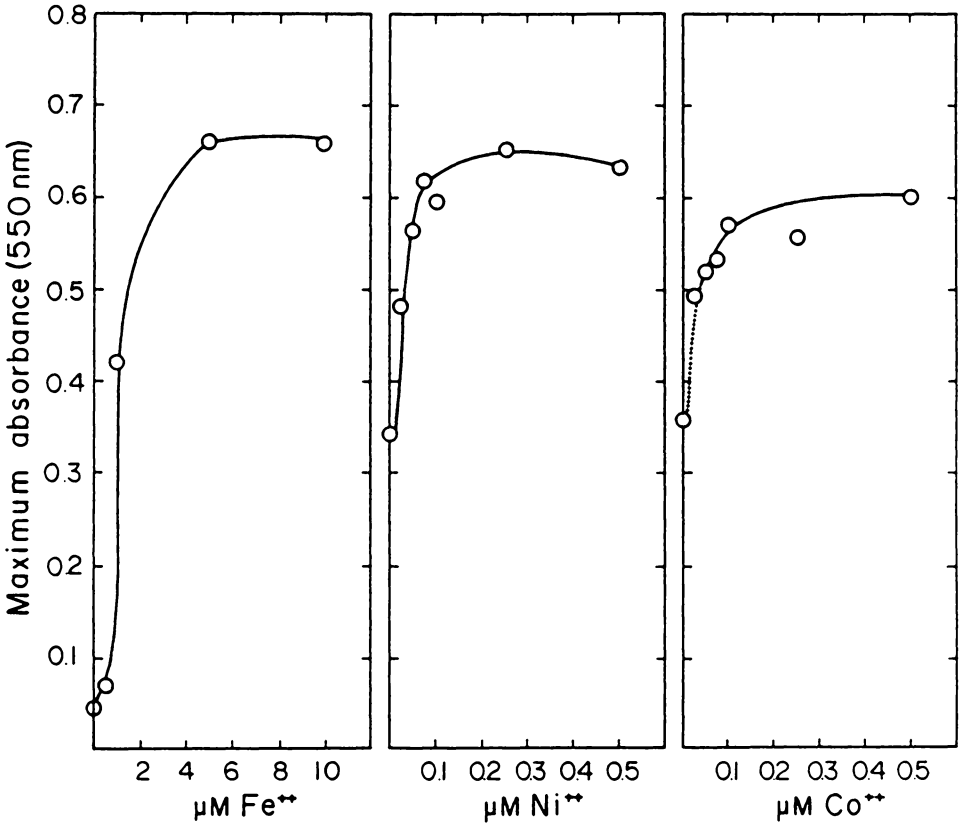

Fig. 2. Effect of Ni, Fe and Co on the growth of Methanococcoides methylutens with trimethylamine. Each metal salt was added at the indicated concentrations with optimum concentrations of the other two metals. The optimum concentrations in μmols/l are: Fe, 4.0; Co, 0.5; and Ni, 0.5. The values represent maximum absorbance at the end of growth with the concentrations of metals indicated. The values on the dotted line of the Co curve were obtained after 1 week rather than 2 to 3 days because of a slower growth rate.

growth rate at optimum Co concentations, to 0.011 h^{-1}. This lower growth rate was reproducible in subsequent transfers. Other metal salts were tested for their ability to stimulate growth in medium that contained optimum concentrations of Fe, Ni and Co. The following metal salts failed to stimulate growth when individually added at the indicated final concentrations in µmols/l: $MnSO_4$, 30; Na_2MoO_4, 10; Na_2O_3Se, 10; $Zn(C_2H_3O_2)_2$, 6; H_3BO_3, 2; $CuSO_4$, 0.4; $AlKO_8S_2$, 0.2. Tungsten, an antagonist of molybdoenzymes, was added to medium to further test for an Mo requirement, but there was no inhibition at concentrations as high as 1 mM Na_2WO_4.

DISCUSSION

The methanogenic archaeobacteria synthesize several unique cofactors including coenzymes M, F_{420} and F_{430} (Eirich et al., 1978; Keltjens et al., 1983; McBride and Wolfe, 1971), but FAD (Lancaster, 1981) and vitamins (Leigh, 1983) ubiquitous among the eubacteria have also been reported in methanogenic bacteria. The addition of biotin to mineral medium is stimulatory to the growth of Methanobacterium bryantii (Bryant et al., 1967), and is required by Methanothrix soehngenii (S. Zinder, personal communication). Leigh (1983) has reported that Methanobacterium thermoautotrophicum contains biotin although the concentration is approximately one order of magnitude below that found in eubacteria. Among the Methanosarcina only Methanosarcina barkeri strain Fusaro requires riboflavin (Scherer and Sahm, 1981); Methanosarcina sp. strain TM-1 requires p-aminobenzoic acid (S. H. Zinder, personal communication) and Methanosarcina acetivorans strain C2A has

no vitamin requirements (Sowers et al., 1984). Methanolobus tindarius, which is phenotypically similar to M. methylutens is reported to have no vitamin requirements although B-vitamins are stimulatory (Konig and Stetter, 1982). Since the vitamin requirements among methanogenic species are diverse (Smith and Mah, 1981) and the requirements of several strains in each species have not been determined, it is possible that the vitamin deficiencies may be auxotrophic characteristics at the strain level. Such characteristics, if at the strain level, could be useful markers for genetic analyses.

The metal requirements of M. methylutens were similar to those reported for other methanogenic bacteria. Previous reports indicate that Fe is required for growth of several genera (Schönheit et al., 1979; Whitman et al., 1982; Patel et al., 1978; Patel et al., 1976) and stimulates methane production from acetate in fermenters (Murray and van den Berg, 1981; van den Berg et al., 1980; Hoban and van den Berg, 1979). The growth-limiting concentration of Fe (< 5 mM) for M. methylutens was within the range reported for Methanobacterium thermoautotrophicum (Schönheit et al., 1979) and Methanococcus voltae (Whitman et al., 1982). Fe may be required for the synthesis of cytochromes which have been reported in all acetotrophic and methylotrophic species tested (Kühn et al., 1983; Kühn and Gottschalk, 1983) including M. methylutens (Jüssofie, 1984; Ferry, unpublished results). Iron-containing superoxide dismutase (Kirby et al., 1981), ferredoxin (Hatchikian et al., 1982) and non-heme iron-sulfur proteins (Scherer and Sauer, 1982; Lancaster, 1980) have also been reported in methylotrophic species. HiPiP iron-sulfur signals appear in electron paramag-

netic resonance spectra of M. methylutens whole cells (M. Barber, personal communication).

Growth could not be completely inhibited when Ni was not added to Chelex-treated medium. However, growth stopped at an absorbance of 0.3 when Ni was not added indicating that the low concentration of Ni remaining in the medium was rapidly depleted. Based on a ratio of final absorbances at various concentrations it was estimated that there was less than 0.025 μM Ni remaining in the Chelex-treated medium. Other investigators have reported low levels of growth in medium without added Ni (Schönheit et al., 1979; Whitman et al., 1982). The growth-limiting concentration of Ni for M. methylutens was in the same range reported for other methanogenic bacteria (Schönheit et al., 1979; Whitman et al., 1982). Ni is a component of hydrogenase (Graf and Thauer, 1981) and of Factor F_{430} (Diekert et al., 1981). Ni is also a component of the carbon monoxide dehydrogenase in M. barkeri (Krzycki and Zeikus, 1984).

The concentration of Co that limited growth of M. methylutens was 10-fold higher than the limiting concentration reported for M. thermoautotrophicum (Schönheit et al., 1979). Although the absence of Co did not completely inhibit M. methylutens, the growth rate was reduced 94% compared with a reduction of 50% for Methanococcus voltae (Whitman et al., 1982). Co may be required for corrinoids which have been found in all methanogenic bacteria tested (Krzycki and Zeikus, 1980) and have been identified in the methanol methyltransferase of M. barkeri (van der Meijden et al., 1983).

Mo is required for growth of M. thermoautotrophicum (Schönheit et al., 1979) and stimulates growth of M. barkeri (Scherer and Sahm, 1981). Scherer et al. (1983) have shown that Mo accumulates in M. barkeri which suggests that it is an essential element. However, a requirement for Mo by M. methylutens could not be demonstrated. The addition of Mo to medium did not stimulate the growth of M. methylutens and the addition of W, an antagonist of molybdoenzymes, did not affect growth. Although Se stimulates growth of other methanogenic bacteria (Jones and Stadtman, 1977; Scherer and Sahm, 1981; Jones et al., 1983; Jones et al., 1983; Whitman et al., 1979) it did not stimulate growth of M. methylutens under the conditions described. Other metals have been shown to accumulate in the cells of methanogenic species (Whitman et al., 1982; Scherer et al., 1983) including Zn and Cu but as in reports on other species a physiological requirement for Zn and Cu could not be demonstrated for M. methylutens.

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SECTION VI. DEGRADATION OF METHYLATED AMINES IN A MARINE SEDIMENT BY METHANOGENIC ARCHAEOBACTERIA

SUMMARY

The distribution of methanogens in sediments from a marine canyon was determined by the most probable numbers technique. Distribution patterns were determined for the methanogenic substrates hydrogen-carbon dioxide, acetate, and trimethylamine. The populations of hydrogen- and acetate-utilizing methanogens range from 4×10^4 to 9×10^5 and 1×10^4 to 4×10^5 per 100 ml, respectively, and population density exhibited little variation with depth. In contrast, the trimethylamine-utilizing population ranged from 4×10^5 to 1×10^9 with the highest population density occurring at 2 cm and decreasing with depth. Results indicate that methanogens were able to utilize trimethylamine in the presence of high sulfate concentrations associated with marine surface sediments. Data presented also indicate that non-acetate-utilizing methylotrophic methanogens may exist in the marine environment which are uniquely adapted for utilization of methylated amines as substrate.

INTRODUCTION

In anaerobic marine sediments, methanogens are inhibited by the sulfate reducing bacteria which are more efficient competitors for the same substrates (9,10,20). Low rates of methanogenesis coexist with sulfate reduction but the mechanism is unknown (21). It was recently observed that the methanogenic substrates trimethylamine (TMA) and

methanol accumulated in a sulfate-rich marine sediment in which methanogenesis was inhibited (15). TMA and methanol but not H_2, acetate or formate stimulated methanogenesis in an alkaline and moderately hypersaline lake that contained 15 mM sulfate (14). TMA and methanol are not known to be utilized by sulfate reducers and they are produced in the marine environment (4,6,19,24). Recently isolated marine methanogens utilize these compounds as substrates but are unable to utilize acetate and hydrogen (8,23). Data is presented which suggests that these methylotrophic methanogens co-exist with sulfate-reducing bacteria in the sulfate-rich upper marine sediment layers.

MATERIALS AND METHODS

Sediment was obtained from the Sumner Branch of Scripps canyon at a depth of 15-23 meters near LaJolla, California. The sediment consisted of an interwoven mat of algae, sea grass debris and sand. Core samples were obtained by a diver assisted with SCUBA. The sampling device was a 61 cm length of 7.6 cm (O.D.) stainless steel tubing with 1.2 cm holes drilled 2 cm apart. Strips of electrical tape were used to cover the holes prior to sampling. The leading edge of the device was sharpened to facilitate cutting through plant material with minimal disruption of the sediment profile. The diver inserted the entire length of the device into the sediment and the top was capped with a rubber bung. The sampler was removed from the sediment and the bottom was capped with a rubber bung. The entire unit was transported to the lab and subsampled within 3 hours. Subsamples (10 ml) were obtained with a 10 ml plastic syringe with the end removed. Subsamples were

aseptically transferred to 160 ml serum vials that contained 90 ml of artificial medium without substrate. Sterile media were prepared under a N_2-CO_2 (80:20) atmosphere by a modification of the Hungate technique, (2). The artificial medium contained a mixture of 20% deionized water and 80% artificial sea water (9a) augmented with the following constituents at the indicated final percent compositions (w/v): NH_4Cl, 0.1; Na_2CO_3, 0.25; Na_2HPO_4, 0.06; cysteine-HCl$\cdot H_2O$, 0.025, $Na_2S\cdot 9H_2O$, 0.025; resazurin, 0.001. In addition 1% v/v each of vitamin and trace element solutions (25) and 10% (v/v) clarified rumen fluid were added. Substrates were added in the following amounts: sodium acetate, 0.16% (w/v); trimethylamine$\cdot$HCl, 0.15% (w/v). Medium for hydrogen utilizers was made by displacing the N_2-CO_2 gas phase with 101 kPa of H_2-CO_2 (80:20). The pH was adjusted to 7.5 prior to autoclaving. Anaerobiosis was maintained by a stream of O_2-free N_2:CO_2 (80:20). The vial was sealed with a butyl rubber septum and serial dilutions were prepared from 10^{-2} to 10^{-7} by the sequential transfer of 0.5 ml to 8 ml stoppered serum vials that contained 4.5 ml of artificial medium with substrate. Controls without substrate were prepared at 10^{-2} dilution for each depth. Each dilution was prepared in triplicate and was incubated at 20°C in the dark. After 8 weeks incubation each dilution vial was examined for total methane production by first measuring the total volume of head space gas with a water lubricated 10 ml glass syringe followed by analysis of methane by gas chromatography. The chromatograph (model 2440, Varian Instruments) was equipped with a flame ionization detector and a 0.32 x 182.88 cm stainless steel column packed with silica gel (80/100 mesh). The oven was operated at 100°C and nitrogen

was the carrier gas. The distribution of methane positive cultures was used to calculate the most probable numbers (1).

RESULTS AND DISCUSSION

Results in Figure 1 indicate that TMA-utilizing methanogenic organisms were most numerous in sediment immediately below the surface and numbers decreased with depth. The distribution of these organisms was unlike those of the acetate- and hydrogen-utilizers which occurred in lower numbers and with less variation. These data suggest that TMA metabolism by methanogenic organisms was not influenced by the presence of sulfate.

Senior et al. (21) observed that methanogenesis occurred in the presence of active sulfate reduction in marine sediments, although at lower rates. More methane evolved from the sediment surface than could be accounted for by concentrations of acetate and carbon dioxide present and they concluded that this excess methane may be the result of alternative methanogenic substrates. The results of Oremland (15) suggest coexistence of sulfate reduction and methanogenesis from methylated amines and methanol and results of the present study would indicate that these compounds may be the alternative substrates suggested by Senior et al. If methylated amines are important substrates for methanogenesis then methane would be expected to accumulate in the upper layers independent of the sulfate concentration. Profiles of dissolved methane in marine sediments have indicated that methane and sulfate are mutually exclusive (5). This observation has promoted the conclusion that methanogenesis does not occur in the upper sulfate layers. This

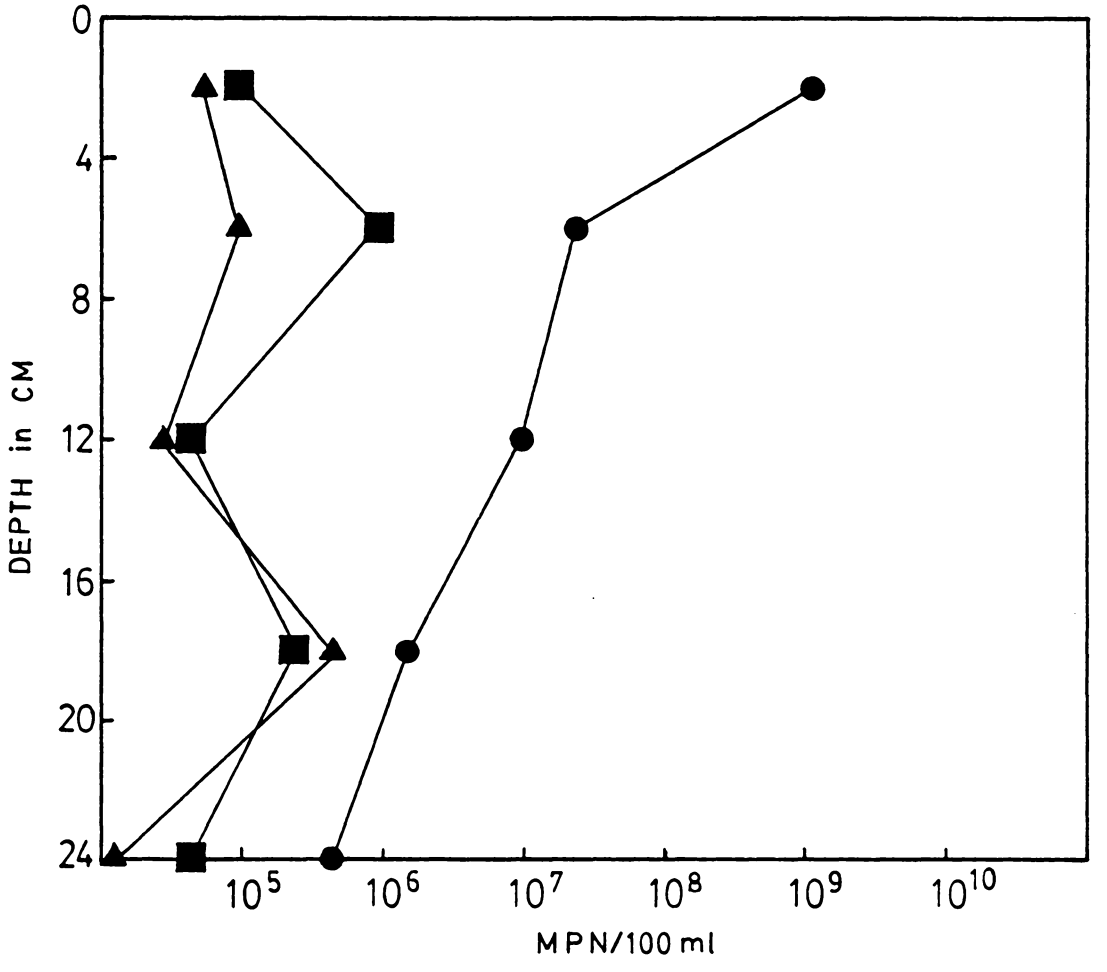

Fig. 1. Methanogenic populations (MPNs) that use trimethylamine TMA (●), H_2-CO_2 (80:20) (■), and acetate (▲). Serial dilutions were prepared from 10^{-1} to 10^{-7} for each substrate in triplicate at the indicated depths.

effect could be alternatively explained by sulfate-dependent anaerobic methane oxidation (7,17,18). It has been shown that anaerobic methane oxidation occurs throughout the sulfate-reducing zone in marine sediments, with maximum rates occurring at the base of the sulfate reducing zone. This could explain the absence of expected methane accumulation in the upper sediments due to utilization of amines or methanol. An alternative fate of the methylated amines may be oxidation by methylotrophic anaerobic respiration in the presence of nitrate (22).

The results in Figure 1 show that amine-utilizers occur in numbers 10^4 greater than acetate- and hydrogen-utilizers. This suggests the presence of methanogenic organisms that are unable to utilize hydrogen and acetate. Methanococcoides methylutens isolated from these sediments may represent this group. Further support for this is the inability to isolate strains of organisms similar to M. methylutens in sodium acetate enrichments (Sowers and Ferry, in press).

Methanosarcina barkeri is the predominant methylotrophic methanogen observed in the rumen (13,16). M. barkeri is well adapted for growth in this environment because hydrogen is the major methanogenic substrate in the rumen (3) and this species can use both hydrogen and methylated amines (26). Furthermore, hydrogen and the methylotrophic substrate methanol are used simultaneously by M. barkeri (11). The marine methylotroph M. methylutens appears to be best adapted to sulfate-rich marine sediments with its inability to use hydrogen and acetate which are utilized more efficiently by the sulfate reducers. The results support an important function for methanogens in the degradation of TMA in anaerobic marine sediments.

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SECTION VII. *METHANOSARCINA ACETIVORANS* SP. NOV., AN ACETOTROPHIC METHANE-PRODUCING BACTERIUM ISOLATED FROM MARINE SEDIMENTS

SUMMARY

A new acetotrophic marine methane-producing bacterium that was isolated from the methane-evolving sediments of a marine canyon is described. Exponential phase cultures grown with sodium acetate contained irregularly shaped cocci that aggregated in the early stationary phase and finally differentiated into communal cysts that released individual cocci when ruptured or transferred to fresh medium. The irregularly shaped cocci (1.9 ± 0.2 mm in diameter) were gram negative and occurred singly or in pairs. Cells were nonmotile, but possessed a single fimbria-like structure. Micrographs of thin sections showed a monolayered cell wall approximately 10 nm thick that consisted of protein subunits. The cells in aggregates were separated by visible septation. The communal cysts contained several single cocci encased in a common envelope. An amorphous form of the communal cyst that had incomplete septation and internal membrane-like vesicles was also present in late exponential phase cultures. Sodium acetate, methanol, methylamine, dimethylamine, and trimethylamine were substrates for growth and methanogenesis; H_2-CO_2 (80:20) and sodium formate were not. The optimal growth temperature was 35 to 40°C. The optimal pH range was 6.5 to 7.0. Both NaCl and Mg^{2+} were required for growth, with maximum growth rates at 0.2 M NaCl and 0.05 M $MgSO_4$. The DNA base composition was 41 ± 1% guanine plus cytosine. *Methanosarcina aceti-*

vorans is the proposed species. C2A is the type strain (DSM 2834, ATCC 35395).

INTRODUCTION

Low rates of methanogenesis occur in sulfate-rich marine sediments where sulfate reduction is the predominant terminal process in the anaerobic degradation of organic matter (34,35). Methanogenesis predominates in marine environments where sulfate is readily depleted, which include the lower depths of marine sediments (24), sediments that receive large amounts of organic matter (26), and the elevated portions of marine marshes (14). Although acetate is the major precursor for methanogenesis in these sulfate-depleted sediments (31), marine acetotrophic methane-producing bacteria have not been isolated.

Several methanogenic bacteria that utilize H_2 or formate for growth have been isolated from marine sediments (7,9,11,28-30,39,40). Recently, two marine methylotrophic species, "Methanococcoides methylutens" (36) and Methanolobus tindarius (15), were described, but neither utilizes acetate for growth. We describe here the first marine methanogenic bacterium reported that utilizes acetate in addition to methanol and methylated amines as substrates for growth and methanogenesis.

MATERIALS AND METHODS

Source of inoculum. Marine sediment was obtained as previously described (36) from the Sumner branch of Scripps Canyon located near La Jolla, Calif.

Media. Sterile media were prepared under a N_2-CO_2 (80:20) atmosphere by a modification of the Hungate technique (2). All gases were passed through a column of reduced copper filings at 350°C to remove traces of O_2. Enrichment medium was as previously described (36) with 0.02 M sodium acetate as the substrate. The enrichment medium contained artificial seawater supplemented with the following constituents at the indicated final percent compositions (weight/volume): NH_4Cl, 0.05; Na_2CO_3, 0.1; Na_2HPO_4, 0.035; NaH_2PO_4, 0.030; cysteine-HCl•H_2O, 0.025; Na_2S•$9H_2O$, 0.025; $FeSO_4$, 0.001; resazurin, 0.0001. In addition 1% (vol/vol) of vitamin solution and 1% (vol/vol) of trace element solution were added (43). The final pH of the medium was 7.2. Roll tubes contained enrichment medium with the addition of 2% purified agar. Maintenance medium (36) contained the following constituents at the indicated final percent compositions (weight/volume) in demineralized water: NaCl, 2.34; $MgSO_4$, 0.63; Na_2CO_3, 0.5; yeast extract, 0.1; NH_4Cl, 0.05; KCl, 0.08; $CaCl_2$•$2H_2O$, 0.014; Na_2HPO_4, 0.06; cysteine-HCl•H_2O, 0.025; Na_2S•$9H_2O$, 0.025; resazurin, 0.0001. In addition, 1% (vol/vol) each of vitamin and trace element solutions (43) and 0.05 M of the indicated substrate were added to the medium. The final pH of the medium was 7.2. Strains were maintained on agar slants of maintenance medium with sodium acetate contained in 16 by 150 mm anaerobe tubes (Bellco Glass, Inc., Vineland, N.J.) that were sealed with butyl rubber stoppers.

Enrichments and isolation. Enrichments were started by adding sediment (5 ml) to 160 ml serum vials (Wheaton Scientific, Millville, N.J.) that contained 45 ml of enrichment medium. The vials were purged

with N_2-CO_2 (80:20) and sealed with butyl rubber septa secured by aluminum crimp collars (2). The cultures were incubated in the dark at 25°C. After methane production subsided, 5 ml of the culture was anaerobically transferred into a new vial of sterile enrichment medium. This procedure was repeated for 5 successive transfers, and then 1 ml of the culture was transferred to an agar slant of maintenance medium that contained 0.05 M sodium acetate and vancomycin (100 mg/liter). The use of agar slants enhanced growth, and the vancomycin inhibited the growth of nonmethanogenic organisms. When the liquid of syneresis became turbid, serial dilutions of the culture were inoculated into tubes (25 by 150 mm) of molten agar maintenance medium containing sodium acetate and then rolled (4). Colonies were picked with a bent, sterile Pasteur pipette. The plug of agar containing a colony was transferred to the liquid of syneresis in a sterile agar slant of maintenance medium. All strains were maintained by monthly transfer on agar slants incubated at 20°C.

Large scale production of cell material. Cells for biochemical characterization and determination of the DNA base composition were grown in a 12-liter fermenter (model MP-114, New Brunswick Scientific Co., Inc., Edison, N.J.) that contained 10 liters of maintenance medium. The fermentor was operated as a pH-stat (32) with acetic acid as the growth substrate.

Growth methods. The most probable number of acetotrophic methane-producing bacteria in sediments was determined as previously described (10) by using enrichment medium.

Growth experiments were performed in culture tubes (16 by 150 mm, Bellco Glass, Inc.) that contained 10 ml of maintenance medium. Trimethylamine (0.05 M) was used as the substrate to avoid the formation of aggregates. The tubes were sealed with butyl rubber septa secured with aluminum crimp collars. Cultures were anaerobically transferred with a syringe (2). Cultures were incubated at 40°C. Growth was followed at 550 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb Inc., Rochester, N.Y.). The results are reported as the mean of values from triplicate cultures.

Glassware for growth factor experiments was cleaned in concentrated sulfuric acid, rinsed three times with tap water and six times with deionized water, and then heated at 425°C overnight. Media were prepared with demineralized water that was passed at 5 ml/min through a 1.5 by 18 cm column that contained activated charcoal (Bio-Rad Laboratories, Richmond, Calif.). The culture tubes were sealed with new butyl rubber septa that had been rinsed with charcoal-treated water.

Growth at each indicated pH was determined by substituting for sodium carbonate the following buffers at a final concentration of 0.1 M: pH 5.5 and 6.0, 2(N-morpholino)ethanesulfonic acid (pKa, 6.1); pH 6.5 and 7.0, piperazine N-N'-bis(2-ethanesulfonic acid) monosodium monohydrate (pKa, 6.8); pH 7.5, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pKa, 7.5); pH 8.0 and 8.5, N,N-bis(2 hydroxyethyl)glycine (pKa, 8.3). All cultures were incubated at 35°C.

Molar growth yields were determined during exponential growth in a pH-stat. The dry weight was determined by fixing 10 ml of culture material with 2% glutaraldehyde for 15 min and then filtering the cells

on a predried filter (0.2 μm pore size, Millipore Corp., Bedford, Mass.). The cells and filter were washed with deionized water, dried overnight at 90°C, and weighed. One optical density unit corresponded to a cell mass of 0.323 g (dry weight) per liter.

Analytical Methods. Coenzyme F_{420} was partially purified from cell extract by DEAE-cellulose column chromatography, and its concentration was determined spectrophotometrically (32). Protein was determined by the Bradford assay with bovine serum albumen as a standard (3). Cell walls were isolated, and the amino acids and amino sugars analyzed as previously described (36). Methane was assayed with a gas chromatograph equipped with a flame ionization detector (36).

Mole percent of guanine plus cytosine. Cells were lysed with sodium dodecyl sulfate (SDS). DNA was isolated and purified by the procedure of Marmur (23). The moles percent G+C was determined by thermal denaturation with a spectrophotometer and thermal programmer (model 2400, Gilford Instrument Laboratories, Inc., Oberlin, Ohio) (8). DNA from Bacteroides fragilis (VPI 2553) and Escherichia coli b were used as standards.

Microscopy. Phase contrast micrographs were made with a Leitz Dialux microscope. Negative stain and thin section electron micrographs were prepared as previously described (36). The procedure for UV fluorescence microscopy was as previously described (25) with a Leitz Orthoplan fluorescence microscope.

Chemicals. Purified agar, and yeast extract were from Difco Laboratories (Detroit, Mich.). Trypticase was from BBL (Cockeysville, Md.). Activated charcoal was from Bio-Rad. Trimethylamine, dimethyl-

amine, and methylamine hydrochloride were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Bovine serum albumin, vancomycin, RNase A and T_1, DNase I, Triton x-100, SDS, and all organic buffers and vitamins were obtained from Sigma Chemical Co. (St. Louis, M.O.). Vitamin-free, salt-free Casamino Acids were from ICN Nutritional Biochemicals (Cleveland, Ohio). All other chemicals were reagent grade.

RESULTS

Enrichment and isolation. A survey of sediment samples by a most probable number dilution series indicated that the acetotrophic methane-producing population ranged from 530 to 4,300/cm^3 in the first 20 cm of a depth profile. Strain C2A was isolated from an enrichment culture inoculated with the upper 10 cm of this sediment. The predominant organisms in this enrichment were irregularly shaped cocci that occurred singly or in pairs and fluoresced blue-green when examined by UV fluorescence microscopy. Colonies in roll tubes were pale yellow and were 0.5 mm in diameter after 14 days of incubation. Surface colonies were smooth, circular, and convex with entire edges. Several colonies of irregular cocci were isolated, and strain C2A was selected for further study.

Cell morphology. Acetate-grown cultures of strain C2A in the exponential phase contained irregular cocci with an average diameter of 1.9 ± 0.2 μm (Fig. 1a). A single fimbria-like appendage was visible in electron micrographs of negatively stained cells (Fig. 2a). These structures did not have the helical appearance of flagella, and motility was not observed in wet mounts. Electron-dense granules were seen in

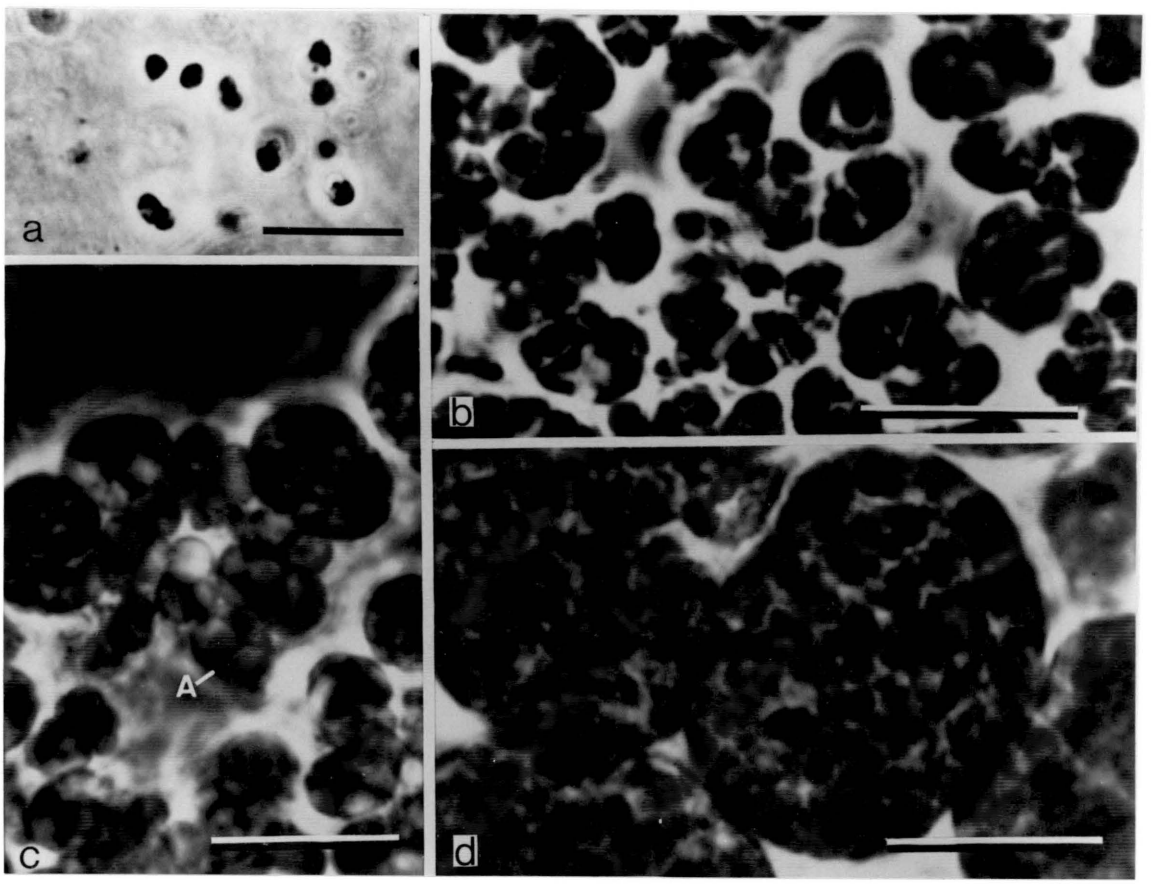

Fig. 1 a-d. Phase-contrast micrographs of strain C2A grown on acetate.
a. Single cells from an exponential phase culture (bar = 5 µm).
b. Cell aggregates from a late exponential phase culture (bar = 10 µm).
c. Aggregates of large cells (A) (bar = 10 µm).
d. Communal cysts from a late exponential phase culture (bar = 10 µm).

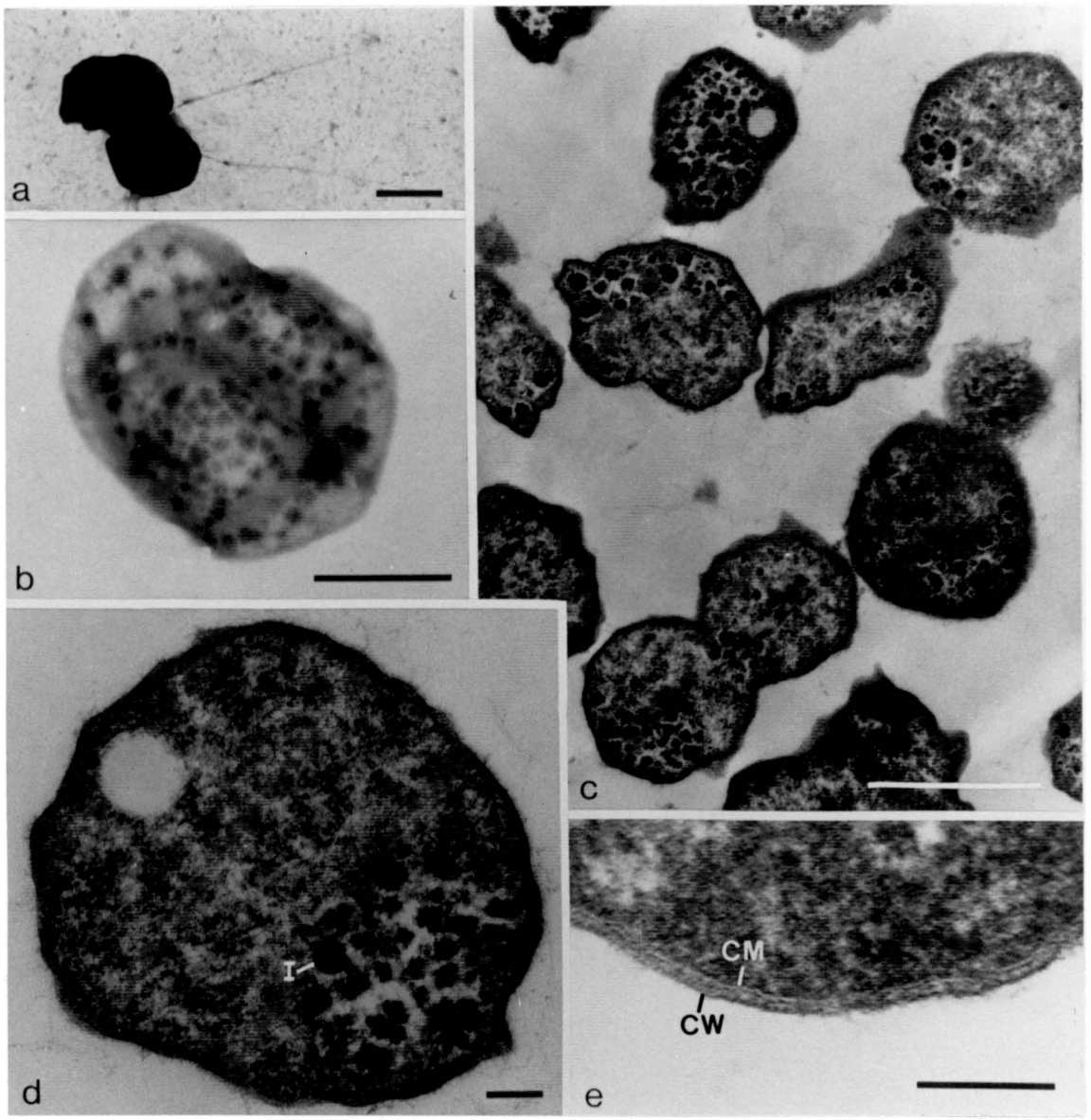

Fig. 2 a-e. Electron micrographs of strain C2A single cells grown on acetate.

a. A negative stain of whole cells with fimbria-like structures (bar = 1 µm).

b. A negative stain of a whole cell showing irregular shape and inclusions (bar = 0.5 µm).

c-d. Thin sections showing inclusions (I) (c: bar, 1 µm; d: bar, 0.2 µm).

e. A thin section showing the cell wall (CW) and cytoplasmic membrane (CM) (bar = 100 nm).

electron micrographs of negatively stained whole cells (Fig. 2b) and thin sections (Fig. 2c and d). Although cells stained gram negative, thin sections revealed a monolayer cell wall 10 nm thick that is characteristic of described marine methanogenic bacteria with a protein cell wall (7,9,11,15,28-30,36,39,40) (Fig. 2e). Pelleted cells were lysed immediately when suspended in maintenance medium that contained SDS (0.005%, wt/vol) or Triton x-100 (0.01%, vol/vol). Lysis also occurred when cells were suspended in maintenance medium from which either NaCl or $MgSO_4$ was omitted or when $MnSO_4$ was substituted for $MgSO_4$. Lysis did not occur when KCl was substituted for NaCl. Acid-hydrolyzed cell wall preparations contained a wide distribution of amino acids, which accounted for 98% of the dry weight (Table 1). No amino sugars were detected. These results show that strain C2A contains an osmotically fragile protein cell wall.

As acetate-grown cultures approached the stationary phase, aggregates of 2 to 12 cells predominated (Fig. 1b). Loosely associated aggregates of larger cells (3 to 4 μm in diameter) were also present (Fig. 1c). The predominant form in stationary phase cultures was a communal cyst comprised of many single cocci within a common envelope (Fig. 1d). When these communal cysts were ruptured by applying pressure to the cover slip, single, irregular cocci were released that resembled cells from exponential phase cultures. These cysts ruptured and released single cocci several days after transfer to fresh medium that contained acetate. The release of individual cocci occurred sooner when the cysts were transferred to medium that contained methanol or

Table 1. Amino acid content of the hydrolyzed cell wall preparation from strain C2A

Amino Acid	µmol/mg (dry weight)	Molar ratio (His = 1.0)
Glu	0.326	5.66
Ala	0.319	5.53
Gly	0.307	5.32
Asp	0.292	5.07
Leu	0.242	4.20
Lys	0.196	3.40
Ile	0.172	2.98
Val	0.157	2.72
Pro	0.145	2.52
Thr	0.145	2.51
Ser	0.136	2.37
Arg	0.131	2.27
Met	0.102	1.76
Phe	0.096	1.67
Tyr	0.074	1.28
His	0.062	1.00
Cys	0.029	0.51
Trp	ND[a]	ND[a]

[a]ND, Not determined.

methylated amines. Aggregates or communal cysts were not present in cultures grown with methanol or methylated amines.

Thin-section electron micrographs of late exponential phase, acetate-grown cultures showed an amorphous form of the communal cyst. This form contained incomplete septation, electron dense granules and internal structures that resembled membrane vesicles (Fig. 3a and b). All of these components were enclosed within a common envelope approximately 6 nm thick. The internal membranes were approximately 10 nm thick and appeared to have subunit structure (Fig. 3c). These membranes appeared as concentric rings in cross section which indicated that they may be closed vesicles. Some cysts contained both single cells and components of the amorphous form within a common envelope (Fig. 3d). Communal cysts contained fully developed cells all surrounded by a thin (6 nm) envelope (Fig. 3e).

Physiology. Sodium acetate, methanol, methylamine, dimethylamine, and trimethylamine supported growth with doubling times of 24.1, 5.2, 6.7, 7.8, and 7.3 h, respectively. Acetate-grown cells did not grow or produce methane when transferred to medium that contained H_2-CO_2 (80:20) or sodium formate as the sole substrate. The molar growth yield of strain C2A was 2.4 g (dry weight) per mol of acetate consumed.

Strain C2A did not require exogenous growth factors. Trypticase or vitamins (42) did not stimulate growth, but yeast extract or vitamin-free Casamino Acids were slightly stimulatory (data not shown). The optimal NaCl range for growth was between 0.1 and 0.6 M (Fig. 4). No growth occurred without NaCl. Neither Na_2SO_4 nor KCl could be substituted for NaCl, which indicated that both Na^+ and Cl^- were required.

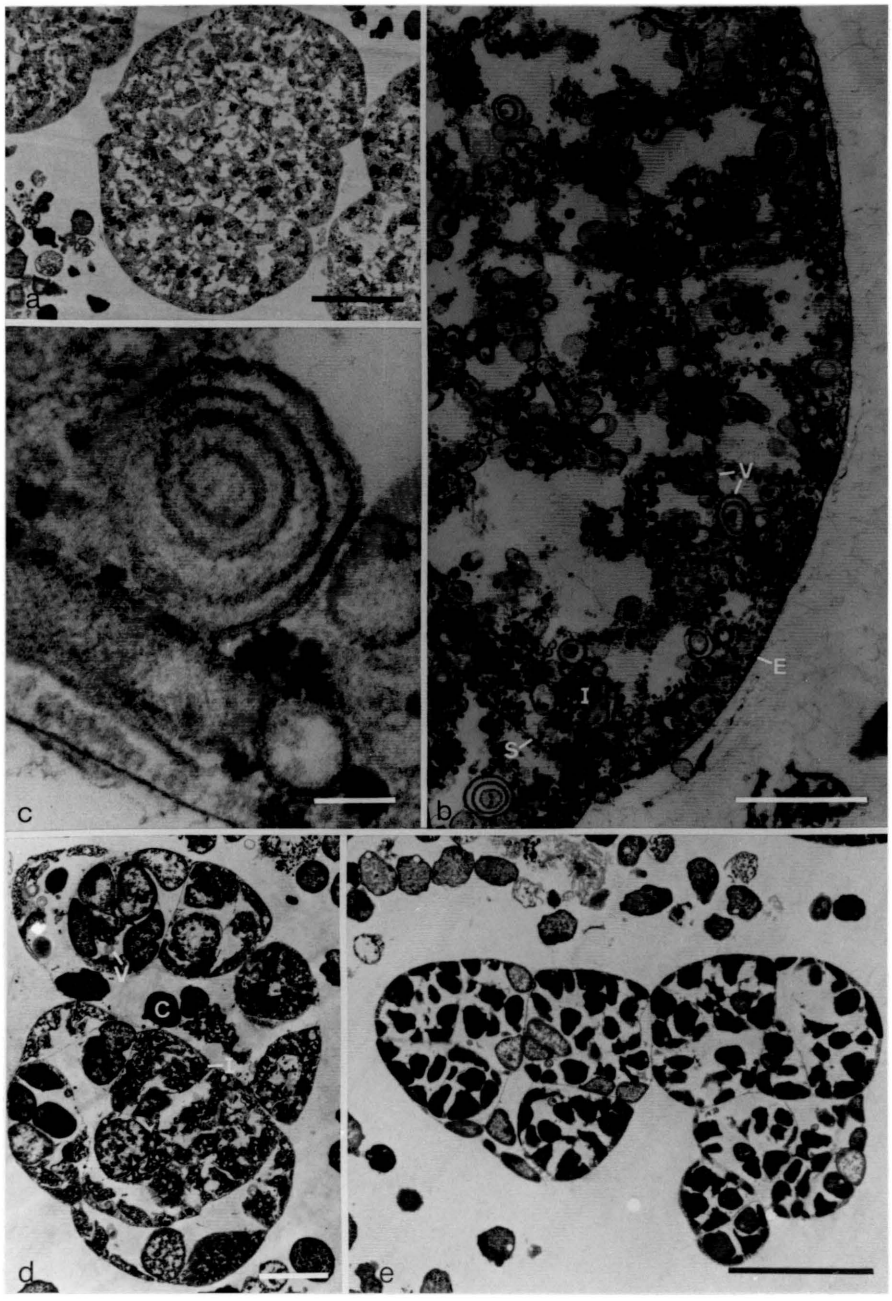

Fig. 3 a-e. Electron micrographs of strain C2A cysts.
a,b. Thin sections of the amorphous form showing cytoplasmic inclusions (I), internal vesicles (V), incomplete septation (S) and the external envelope (E) (a: bar = 5 µm; b: bar = 1 µm).
c. A thin section of the amorphous form showing internal vesicles with apparent subunit structure (bar = 100 nm).
d. A thin section showing an incompletely formed communal cyst containing individual cells (C), cytoplasmic inclusions (I), and internal vesicles (V) (bar = 2 µm).
e. A thin section of a communal cyst containing only whole cells enclosed by a common envelope (bar = 5 µm).

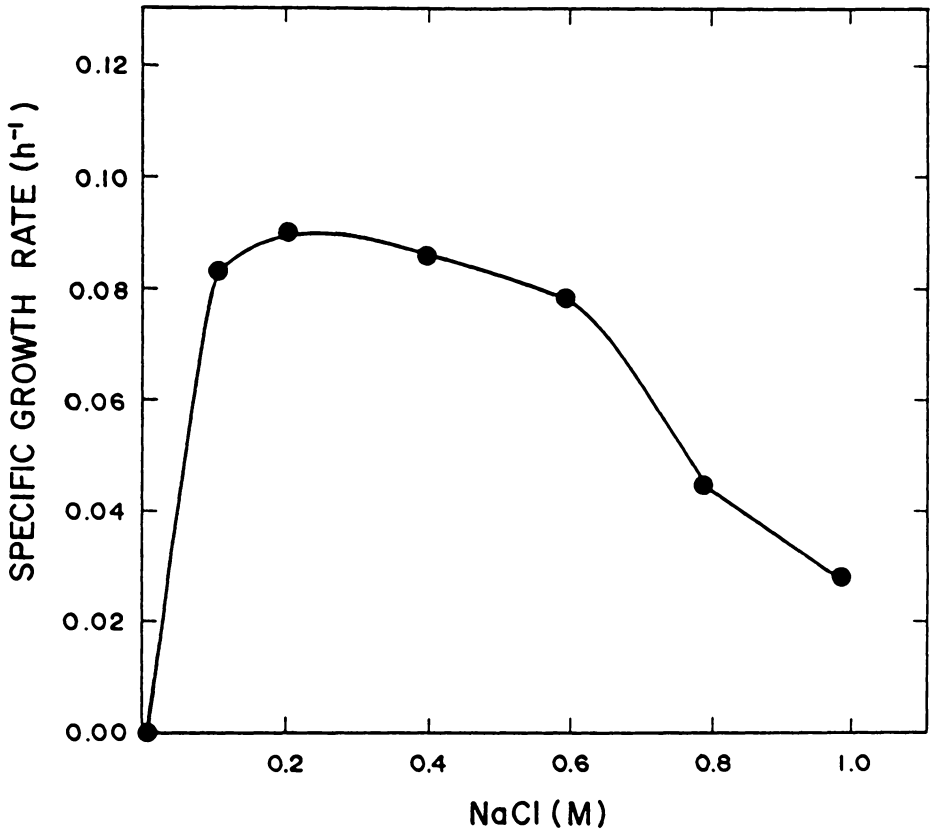

Fig. 4. The effect of NaCl concentration on the growth rate of strain C2A with trimethylamine. K_2CO_3 and $KHPO_4$ were substituted for Na_2CO_3 and $NaHPO_4$. The final Na^+ concentration included Na_2S in the medium.

Maximum growth rates occurred with $MgSO_4$ concentrations between 0.05 and 0.1 M (Fig. 5). No growth occurred in the absence of $MgSO_4$. $MgCl_2$ substituted for $MgSO_4$, which indicated that only Mg^{++} was required. Growth occurred over a pH range of 5.5 to 8.0, and the maximum rate of growth was near pH 6.5 (Fig. 6). No significant inhibition was observed with any of the buffers tested, and the pH of the cultures did not change significantly during growth. The maximum rate of growth was at 40°C, and no growth was detected below 10°C or above 50°C (Fig. 7).

Cell extracts of acetate-grown cells contained 46 ng of coenzyme F_{420} per mg of protein. The DNA base composition was 41 ± 1 mol% G+C.

DISCUSSION

Methanogenic isolates that utilize H_2 or formate have been described from a diversity of marine habitats (7,9,11,28-30,39,40). Recently, two methylotrophic methane-producing marine isolates were described that utilize methylated amines and methanol, but not acetate (15,36). Strain C2A is the first marine methanogenic bacterium described that utilizes acetate as a substrate.

Methanogenesis has been reported in a variety of marine sediments. Low rates of methane production occur in sulfate-rich marine sediments (34,35) where sulfate-reducing bacteria outcompete methanogenic organisms for H_2 and acetate (16,17,19,21,33). The methane produced in these sulfate-rich marine sediments may also be derived from methylated amines, which are not utilized by the sulfate-reducing bacteria (13,27, 41). Methane production predominates in sulfate-depleted marine sediments that receive high organic loading (26) and in the tidal zones of

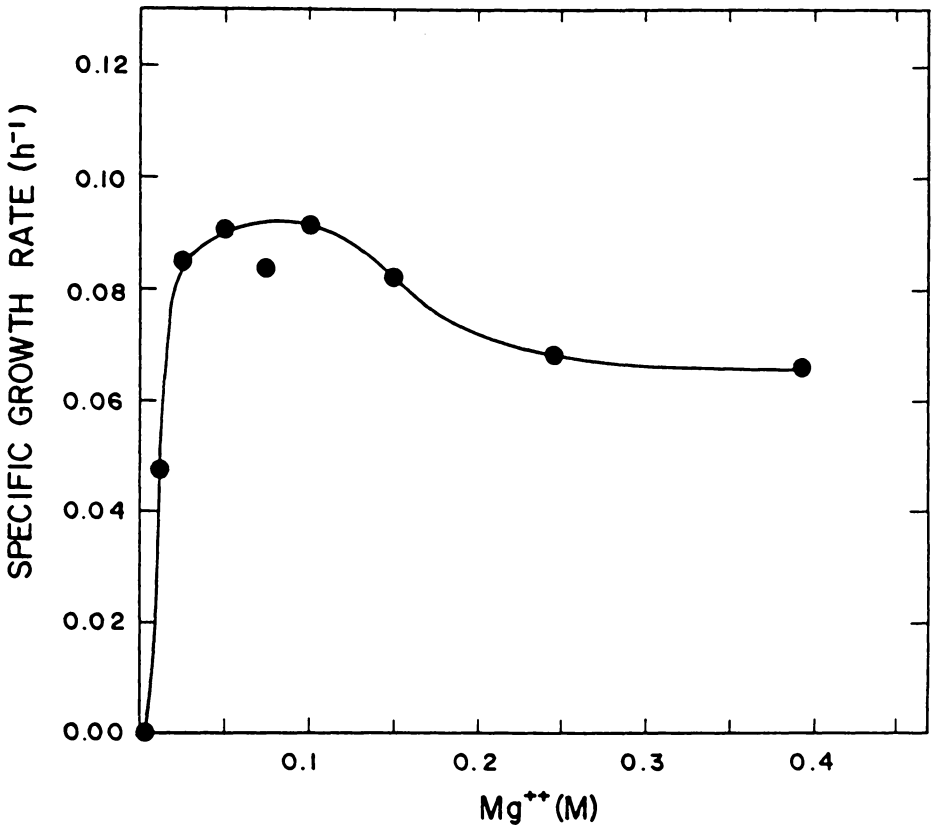

Fig. 5. The effect of Mg^{++} on the growth rate of strain C2A with trimethylamine.

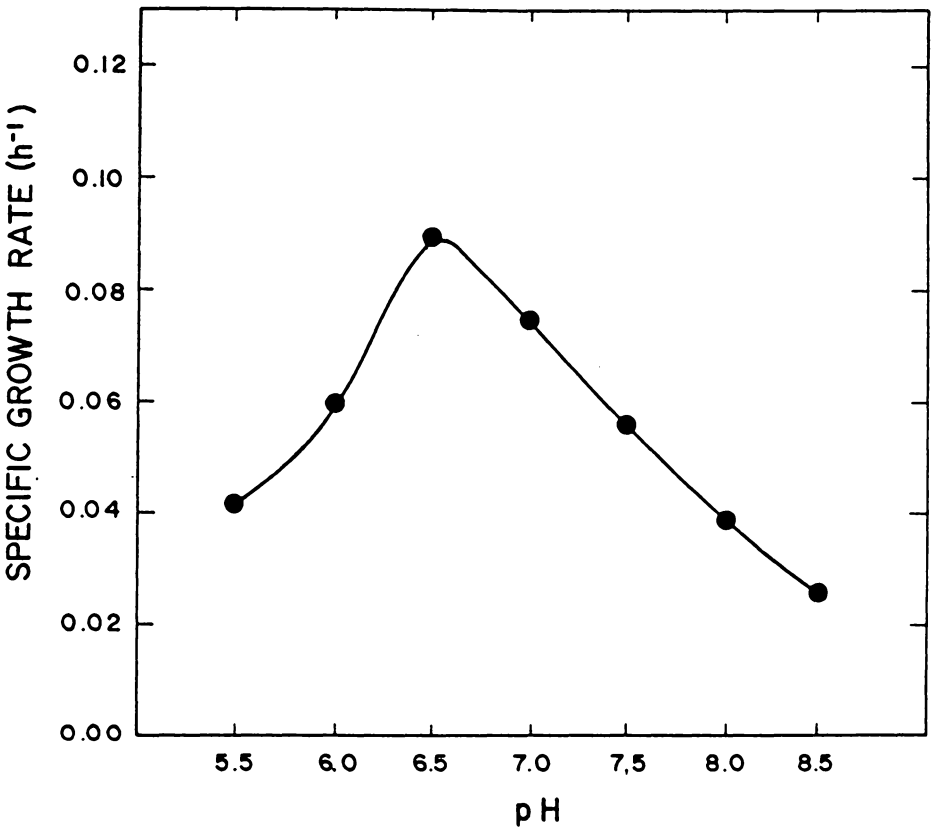

Fig. 6. The effect of pH on the growth rate of strain C2A with trimethylamine. See materials and methods for the buffer used at each pH.

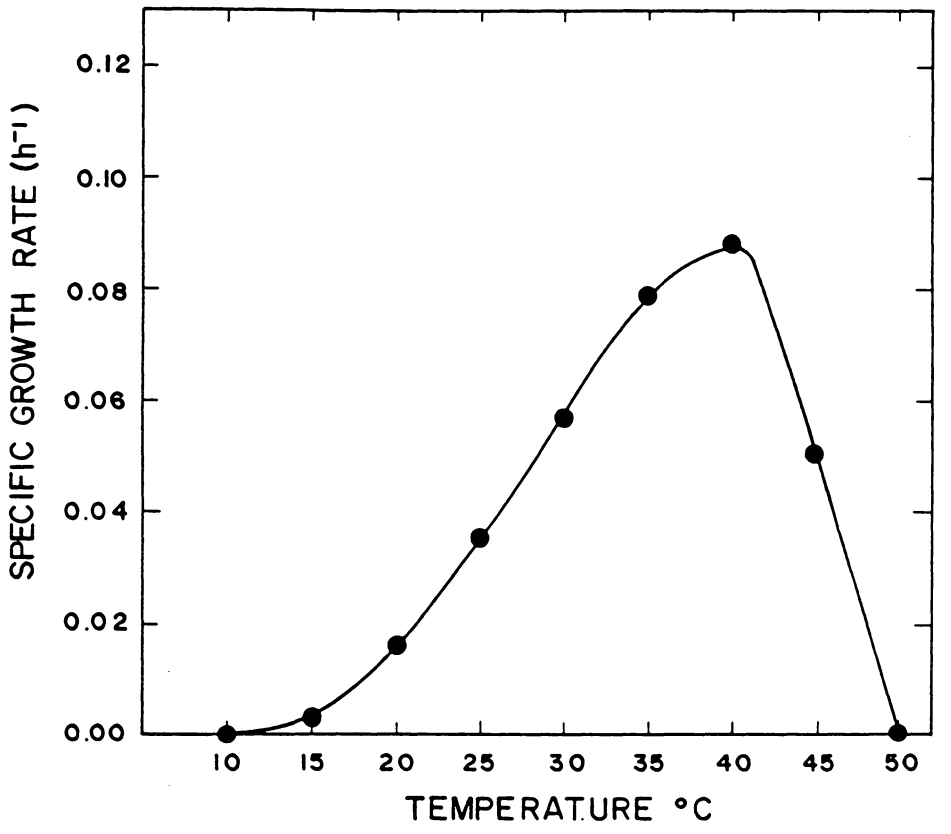

Fig. 7. The effect of temperature on the growth rate of strain C2A with trimethylamine.

marshes (14). Acetate is a major substrate for methanogenesis in nonmarine sediments (5,20) and may also be a major substrate for methane production in sulfate-depleted marine sediments (31). Strain C2A was isolated from a marine canyon with large deposits of kelp and sea grass where acetotrophic methane-producing bacteria like strain C2A may be important in the degradation of organic matter.

The morphology of strain C2A changed during growth on acetate and depended upon the phase of growth. Only single cocci were present in exponential phase cultures. As cultures approached late exponential phase, methanosarcina-like cell aggregates appeared among the single cocci. These aggregates developed into communal cysts comprised of a loose arrangement of single cocci enclosed within a common envelope. These cysts were often present in old cultures and may provide a means of survival. Late exponential phase cultures also contained a morphological form that resembled a communal cyst, but had incomplete septation and internal membrane-like structures. This amorphous form may be an intermediate stage between the aggregate and communal cyst.

Although the various cell morphologies of strain C2A were similar to the acetotrophic isolates Methanosarcina mazei (22) and Methanosarcina sp. of morphovar 3 (43), there were significant differences. The cell wall of strain C2A was thinner than that of Methanosarcina sp. of morphovar 3 and consisted of protein rather than the SDS-insensitive heteropolysaccharide present in M. mazei cell walls (22, S. F. Hurst, R. W. Robinson, and A. S. Bleiweis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, J10, p. 92). "Microcysts" and "pseudococci" reported in cultures of Methanosarcina sp. of morphovar 3, were not present in

cultures of strain C2A. Communal cysts of strain C2A were unlike the macrocyst described for Methanosarcina sp. of morphovar 3, which contained tightly packed "pseudococci" and "polygonal" cells (43).

Like all other described marine methanogenic bacteria (1,7,9,11,15, 28-30,36,39,40), strain C2A is gram-negative and SDS sensitive and has a protein cell wall. Protein cell walls are found only in certain members of the archaeobacteria, which include methanogenic bacteria, extreme halophiles, and thermophilic sulfur oxidizing bacteria (12,37, 38). The cell wall of strain C2A contained a high proportion of the negatively charged amino acids aspartate and glutamate, a feature found in all described archaeobacterial protein cell walls (12,36-38). The ability to lyse strain C2A by the substitution of $MnSO_4$ for $MgSO_4$ indicates that Mg^2 is required to maintain the integrity of the cell wall. Perhaps Mg^2 maintains the integrity of the cell wall by binding loosely with cell wall proteins to reduce the electrostatic repulsions between the negatively charged subunits (18,37). The inability to substitute another divalent cation for Mg^2 may result from differences in their binding properties. Lysis in the absence of NaCl appears to be an osmotic effect since KCl could be substituted for NaCl. The ability to gently lyse strain C2A by elimination of NaCl or $MgSO_4$ or by treatment with SDS is advantageous for biochemical and genetic studies of methylotrophic and acetotrophic metabolism.

Immunological fingerprinting (6) by indirect immunofluorescence (S-probe) showed that strain C2A cross-reacted with members of the Methanosarcinaceae, but did not cross-react completely with presently described strains (E. Conway de Macario, personal communication). rRNA

hybridization studies showed that strain C2A had greater than 85% homology with described species of Methanosarcina (K. R. Sowers, J. L. Johnson and J. G. Ferry, submitted for publication).

We propose that strain C2A be placed in the family Methanosarcinaceae as described by Balch et al. (1), based on DNA-RNA homologies, immunological fingerprinting and the ability to use acetate and other methylotrophic substrates. Phenotypic characteristics that distinguish this strain from members of the Methanosarcina are the presence of a gram-negative protein cell wall, SDS-sensitivity, and a requirement for NaCl and Mg^{2}. Characteristics that distinguish it from "M. methylutens" and M. tindarius are the ability to use acetate as a sole substrate and the formation of methanosarcina-like aggregates and communal cysts. We therefore propose that this strain be placed in a newly described species of methanogenic bacteria, Methanosarcina acetivorans. C2A is the type strain.

The following species description is proposed: Methanosarcina acetivorans sp. nov. (a.ce.ti´vo.rans). L.n. acetum, vinegar; L. part. adj. vorans, consuming; N.L. mas. adj. acetivorans, consuming acetic acid.

Morphology: Morphovars of this species, when grown on acetate, occur as irregular cocci, septated cell aggregates, or communal cysts that contain several individual cocci within a common envelope. Individual cocci are 1.9 µm in diameter and nonmotile. Surface colonies are pale yellow, circular, and convex with entire edges. Cells are lysed by SDS and possess a thin (10 nm) gram-negative cell wall. Nutrition: Acetate, methanol, methylamine, dimethylamine, and

trimethylamine are growth substrates; H_2-CO_2 (80:20) and sodium formate are not; NaCl (0.2 M optimum) and Mg^{2+} (0.05 to 1.0 M optimum) are required for growth; no exogenous growth factors are required; the optimum temperature is 35 to 40°C; the optimum pH is 6.5 to 7.0; it is a strict anaerobe. The source is anaerobic sediments from the Sumner branch of Scripps Canyon located near La Jolla, California. The type strain is C2A. This strain has been deposited in the German Collection of Microorganisms, Göttingen, Germany, as number DSM 2834 and in the American Type Culture Collection, Rockville, MD as number ATCC 35395.

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SECTION VIII. GROWTH OF ACETOTROPHIC, METHANE-PRODUCING BACTERIA IN A pH AUXOSTAT

SUMMARY

Three acetotrophic Methanosarcina species, which included marine, nonmarine, and thermophilic strains, were grown on acetate in a 10-l pH auxostat. Specific growth rates and molar growth yields were constant throughout growth. Cell yields were up to 18-fold greater than previously reported. These properties of the pH auxostat indicate that it is a preferred culture method for the biochemical study of methanogenesis from acetate.

INTRODUCTION

The biochemical study of methanogenesis from acetate requires suitable methods for the large-scale culture of methanogenic bacteria on acetate. Batch culture methods presently used have several disadvantages: (i) the pH and acetate concentration fluctuate widely because large amounts of substrate are consumed [5,8]; (ii) several weeks (3-4) are required to obtain maximum cell yields [1,4,6,7,9,20]; (iii) final cell yields are low [1,4,6,9,20]; and (iv) enzyme activities of cell extracts among cultures are variable [11]. The present study describes a modification of a continously-fed batch culture, the pH auxostat [11], that produces high yields of acetate-grown methanogenic bacteria in minimum time.

MATERIALS AND METHODS

Strain sources for Methanosarcina barkeri, "Methanosarcina thermophila" (Methanosarcina sp. strain TM-1) and Methanosarcina acetivorans are described [16,17]. Media were prepared anaerobically under N_2 by a modification of the Hungate technique [3]. Methanosarcina barkeri and "Methanosarcina thermophila" were grown in DPB medium that contained the following constituents in demineralized water at the indicated final percent concentrations (wt/vol): NH_4Cl, 0.14; K_2HPO_4, 0.13; KH_2PO_4, 0.13; NaCl, 0.05; $MgSO_4$, 0.05; $Na_2S \cdot 9H_2O$, 0.027; $CaCl_2 \cdot 2H_2O$, 0.006; $Fe(NH_4)_2(SO_4)_2$, 0.001; cysteine-HCl$\cdot H_2O$, 0.027; yeast extract (Difco, Detroit, MI), 0.01; Trypticase (BBL, Cockeysville, MD), 0.01; sodium acetate, 0.41. In addition, 1% (vol/vol) each of vitamin and trace element solutions [21] and Antifoam-C (Sigma, St. Louis, MO) were added. Methanosarcina acetivorans was grown on M-C2 medium as previously described [16], except that Na_2CO_3 was omitted. Growth studies were conducted in 14-l fermenters (model MP-114, New Brunswick Scientific) that contained 10 l of medium. The media were prepared without the addition of $Na_2S \cdot 9H_2O$ and the pH was adjusted to 6.8 before autoclaving at 121°C for 50 min. After cooling the media to the appropriate growth temperatures under N_2, 5 ml of a sterile $Na_2S \cdot 9H_2O$ solution (50% wt/vol) was added and the same amount added every 48 h thereafter during growth. $Na_2S \cdot 9H_2O$ was sterilized by autoclaving in a serum vial containing N_2. "M. thermophila" was grown at 50°C; all other species were grown at 35°C. The medium was sparged with N_2 at 600 ml/min and stirred at 400 rpm. The fermenters were inoculated with 1-l batch cultures or by transferring 1-l of culture from another fermenter. The

fermenter was modified to operate as a pH auxostat by installing autoclavable reference and pH electrodes connected to a pH controller as previously described for the growth of formate-utilizing methanogenic bacteria [12]. Acetic acid (10 N) was delivered from a calibrated reservoir through Teflon tubing (I.D. 1.5 mm, Bethesda Research Laboratories, Bethesda, MD). As acetate was used by the culture, the pH controller compensated for the increase in pH by adding acetic acid, thereby maintaining a constant pH and substrate concentration. Late exponential phase cultures were harvested anaerobically with a continuous flow centrifuge [12] and stored in liquid N_2. Acetate [8] and CH_4 [16] were assayed with a gas chromatograph as described.

RESULTS AND DISCUSSION

The growth characteristics of each strain are shown in Figure 1. After an initial lag, exponential growth coincided with exponential acetate consumption and CH_4 formation which indicates that catabolism and anabolism were coupled uniformly. The ratios of CH_4 produced and acetate consumed (Fig. 1) approached the expected ratio of 1:1 [5,13,14, 22]. The slightly higher ratio of CH_4 to acetate towards the end of the growth curves may be attributed to errors in the determination of CH_4. Each culture was maintained at pH 6.8 ± 0.2. Acetate concentration in each culture was maintained at 50 ± 5 mM which does not limit the growth rates of M. barkeri and "M. thermophila" [14,20,22]. Furthermore, acetate was maintained below inhibiting concentrations for M. barkeri [14].

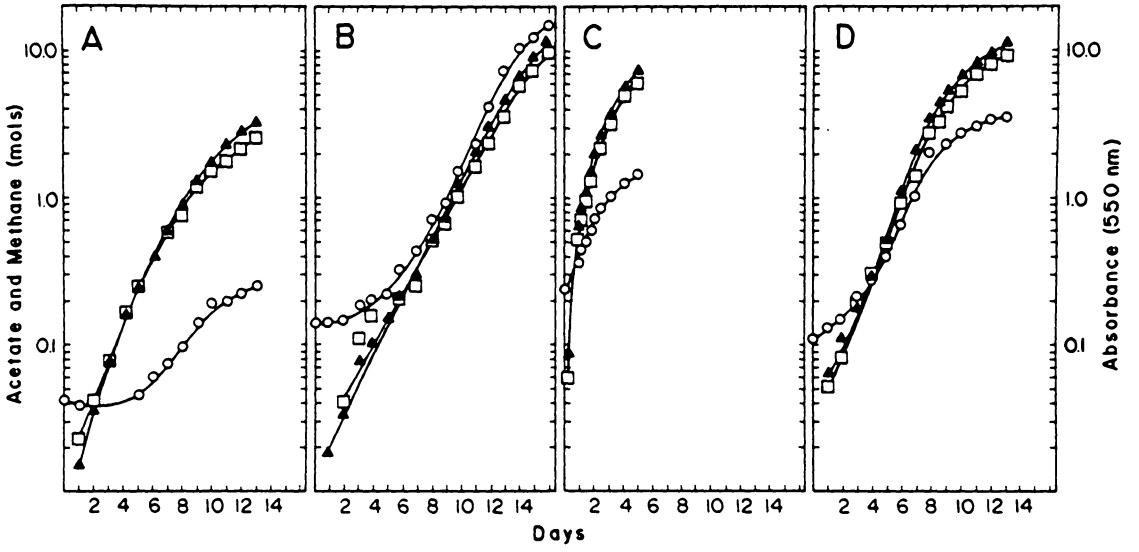

Figure 1. Growth of M. barkeri strain MS (A), M. barkeri strain 227 (B), "M. thermophila" (C), and M. acetivorans (D) in a pH auxostat. Absorbance (○) was determined at 550 nm in a 1 cm light path with a Hitachi spectrophotometer (model 100-60). Total acetate utilized (□) was determined from depletion of 10 N acetic acid in the reservoir. Total methane produced (▲) was determined by the equation $Y_{t2}-Y_{t1}/2.303\ m$, where Y is μmol CH_4/min at the time points t_1 and t_2, and m is the slope [12].

The pH auxostat has several advantages compared to previous methods for large-scale cultivation of acetate-grown methanogenic bacteria [11]. Optimal pH and acetate concentration were maintained throughout growth which resulted in maximum growth rates over a longer time. The molar growth yields from the pH auxostat (Table 1) were equivalent to maximum values reported from batch cultures [14,15,20,22], but the total cell yields were higher. The final dry and wet weight yields of cells (Table 1) were from 3.6 to 17-fold greater than yields reported for M. barkeri strain MS [20] and strain 227 [9,20]. Wet weight yields of 0.21 g/day/liter and 1.0 g/day/l for M. barkeri strains MS and 227, respectively, were obtained in the pH auxostat compared with reported values of 0.05 g/day/liter [103] and 0.04-0.05 g/ day/ liter [6] for the same strains in noncontinuously fed batch cultures. Since only a small amount of free energy is available from the conversion of acetate to methane ($\Delta G^{o'}$ = -31.0 kJ/mol of CH_4 produced) [18], a large amount of substrate is required. The pH auxostat provides a constant concentration of acetate, even at high cell density, which results in a high yield of metabolically active cells. Cell extracts of M. barkeri strain 227 grown in the pH auxostat contained methyl coenzyme M methylreductase activity three-fold greater (unpublished results) than activities reported from extracts of cells grown in non-continuously fed batch cultures [1]. Alternative substrates, such as H_2-CO_2, methanol and methylated amines, provide high yields of acetotrophic methanogenic bacteria [1,5,19]; however, acetate metabolism by these cells appears to be repressed [2,10,14,20, S. H. Zinder, Abstr. Annu. Meet. Am. Soc. Microbiol., 1982, I 88, p. 109]. The pH auxostat is an ideal source of

Table 1. Growth of acetotrophic methane-producing bacteria on acetate with a pH auxostat.

Species	Specific growth rate (hr^{-1})[a]	Final yield (g wet wt/10 liters)	Final yield (g dry wt/10 liters)[b]	Molar growth yield (g dry wt/mol acetate)[c]
M. barkeri strain MS	0.028	27	3	1.2
M. barkeri strain 227	0.029	163	20	2.7
"*M. thermophila*"	0.059	59	10	1.7
M. acetivorans	0.033	35	12	2.4

[a]Growth rates are mean values calculated from the exponential growth phase (Fig. 1).

[b]10 ml of culture from a pH auxostat was passed through a pre-dried filter (0.2 μm pore size, Millipore Corp., Bedford, MA). The cells and filter were washed in demineralized water, then dried overnight at 90°C and reweighed. The dry weight of osmotically sensitive *M. acetivorans* was determined as previously described [16].

[c]The molar growth yields were determined from absorbance and acetate consumption in exponential growth (Fig. 1). One optical density unit corresponded to the following dry weights (g/liter): *M. barkeri* strain MS, 0.79; *M. barkeri* strain 227, 0.13; "*M. thermophila*", 0.54; *M. acetivorans*, 0.32.

cells for the biochemical study of methanogenesis from acetate because of the simplicity of this culture method and the high yields of acetate-grown cells provided in a minimum time.

ACKNOWLEDGEMENTS

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SECTION IX. PHYLOGENIC RELATIONSHIPS AMONG THE METHYLOTROPHIC METHANE-PRODUCING BACTERIA AND EMENDATION OF THE FAMILY METHANOSARCINACEAE

SUMMARY

The phylogenetic relationships among 7 strains of methylotrophic methane-producing bacteria were determined by ribosomal ribonucleic acid rRNA hybridization and deoxyribonucleic acid DNA homology techniques. The strains tested had deoxyribonucleic acid guanine-plus-cytosine DNA contents of 39 to 43 mol% and represented a diversity of phenotypic characteristics. Our results indicate that the strains should be divided into 6 species within the genera Methanosarcina, Methanolobus, and Methanococcoides. The genus Methanosarcina includes Methanosarcina barkeri strains MS and 227, Methanosarcina sp. strain TM-1, Methanosarcina acetivorans strain C2A, and Methanosarcina mazei strain S-6. The genus Methanolobus is represented by Methanolobus tindarius strain Tindari 3, and the genus Methanococcoides is represented by Methanococcoides methylutens strain TMA-10. Despite phenotypic similarities between Methanolobus tindarius and Methanococcoides methylutens, we propose that these species remain in separate genera based on differences in ribosomal ribonucleic acid rRNA homology and fractional $\Delta T_{m(e)}$ values. The divisions indicated by DNA deoxyribonucleic acid homology experiments complemented the ribosomal ribonucleic acid hybridization results. Phenotypic characteristics were consistent with these phylogenetic divisions; an apparent exception was

cell wall composition, which is a conserved trait. Methanosarcina acetivorans had only a thin protein cell wall, but all other strains of Methanosarcina previously studied have been reported to have heteropolysaccharide cell walls. We present evidence which indicates that a protein component may be associated with the heteropolysaccharide cell wall of Methanosarcina barkeri strain 227.

INTRODUCTION

The methane-producing bacteria belong to the archaeobacteria, which, based on 16S oligonucleotide cataloging (6), are phylogenetically distinct from the eubacteria and eucaryotes. The most comprehensive phylogenetic study of the methanogenic bacteria was done by Balch et al. (1), who proposed the presently accepted divisions among this group. In that study, the Methanosarcinaceae, the only methanogenic family with methylotrophic species, was represented by three strains of one species. Since then several methylotrophic methane-producing species have been described. These include Methanosarcina mazei (17), Methanothrix soehngenii (8), Methanolobus tindarius (15), Methanococcoides methylutens (26) and Methanosarcina acetivorans (25a). The taxonomic positions of only Methanothrix soehngenii (28) and Methanosarcina mazei (17) are phylogenetically classified by 16S ribosomal ribonucleic acid (rRNA) oligonucleotide cataloging; the other taxa are based on phenotypic characteristics and mol% guanine-plus-cytosine (G+C) contents of deoxyribonucleic acid (DNA). Another isolate, Methanosarcina sp. strain TM-1, also has been described (35) but a specific epithet has not been proposed. The electrophoretic patterns

of ribosomal proteins suggest that there are important phylogenetic differences between phenotypically similar strains of Methanosarcina that may not be detected by rRNA oligonucleotide cataloging (5). Although the methanogenic bacteria have been previously studied by rRNA hybridization techniques, only one methylotrophic strain was included in that study (29). In this study the phylogenetic relationships of 7 strains of methylotrophic methane-producing bacteria were determined by rRNA hybridization and DNA reassociation techniques, and taxonomic divisions are proposed.

MATERIALS AND METHODS

Bacterial strains. Methanosarcina barkeri strain MS (= DSM 800) was obtained from M. P. Bryant. Methanosarcina barkeri strain 227 (= DSM 1538), Methanosarcina sp. strain TM-1 (= DSM 1825), and Methanosarcina mazei strain S-6 (= DSM 2053) were obtained from R. A. Mah. Methanolobus tindarius strain Tindari 3 (= DSM 2278) was obtained from K. O. Stetter. Methanococcoides methylutens strain TMA-10 (= DSM 2657) and Methanosarcina acetivorans strain C2A (= DSM 2834) have been described previously (26,27).

Media and growth conditions. Sterile media were prepared anaerobically in an atmosphere containing N_2 and CO_2 (80:20) (7). Gases were passed through a column of reduced copper filings at 350°C to remove traces of O_2. Nonmarine strains were maintained on agar slants of MPB medium that contained the following constituents in demineralized water (in final concentrations, wt/vol) : Na_2CO_3, 0.3%; NH_4Cl, 0.14%; K_2HPO_4, 0.13%; KH_2PO_4, 0.13%; NaCl, 0.05%; $MgSO_4$, 0.05%; cysteine-$HCl \cdot H_2O$,

0.027%; $Na_2S \cdot 9H_2O$, 0.027%; $CaCl_2 \cdot 2H_2O$, 0.006%; $Fe(NH_4)_2(SO_4)_2$, 0.001%; yeast extract, 0.1%; Trypticase, 0.1%; sodium acetate, 0.41%. In addition 1% (vol/vol) vitamin solution and 1% (vol/vol) trace element solution were added to the medium (31). The pH of the medium was adjusted to 7.0 with HCl before autoclaving. The agar slants used for maintenance of Methanosarcina acetivorans, Methanococcoides methylutens and Methanolobus tindarius have been described (25a,26). Cultures were stored at 20°C and transferred monthly.

Large scale production of cell material. The cells used for extraction of nucleic acids and for cell wall isolation were grown in a 14-liter fermentor modified to operate as a pH auxostat (22) with acetic acid as the substrate. Strains that were unable to use acetate were grown on trimethylamine hydrochloride. The trimethylamine hydrochloride concentration was monitored with a gas chromatograph that contained a column (0.64 by 183 cm) packed with Carbopak B-4% Carbowax 20M-0.8% K_3OH_4 (Supelco, Inc., Bellafonte, PA). Trimethylamine hydrochloride was added to the culture to replace the depleted substrate. Late exponential phase cultures were harvested with a continuous flow centrifuge as described previously (22) and were stored in liquid nitrogen.

Cell wall preparation. Cell paste was suspended in (15 mM) trisodium citrate buffer at pH 7.0 at a ratio of 1:2 (vol/vol) and was passed twice through a French pressure cell at 137 MPa. Cell walls were isolated and purified as described previously (26) with the following modification: whole cells were separated from cell wall fragments by centrifugation in a sucrose gradient. The lysate was layered over a

linear (25 to 40%) sucrose gradient in citrate buffer (40 ml) in a tube (25 by 9.0 cm) and centrifuged at 3,000 x g for 15 min. The cell wall fragments that banded near the surface of the gradient were removed with a Pasteur pipette. These cell wall preparations were free of whole cells, as determined by phase-contrast microscopy. Analyses for amino acids and amino sugars were performed as described previously (26).

DNA isolation. The frozen cells were suspended in a 0.15 M NaCl-0.01 M ethylenediaminetetraacetic acid salt (NaCl/EDTA) solution (pH 8.0) supplemented with 0.2% (vol/vol) ribonuclease T_1 RNase (2,500 U/ml) (10). The DNA was then isolated by one of two methods. Strains that had protein cell walls (strains C2A, TMA-10, and Tindari 3) were lysed by adding 20% sodium dodecyl sulfate to a final concentration of 1%. The DNA was then isolated by the ethanol precipitation method of Marmur (19). Strains with heteropolysaccharide cell walls (strains MS, 227, TM-1, and S-6) were lysed by one passage through a French pressure cell at 137 MPa into a flask that contained 20% sodium dodecyl sulfate (final concentration, 1%). Then 50 μg of proteinase K per ml was added to the lysate, and the preparation was incubated at 50°C for 1 h. The DNA was then isolated by the hydroxylapatite procedure (10), with the following modification: after the phenol extraction, the preparations were dialyzed in NaCl/ethylenediaminetetraacetic acid buffer to remove the phenol and then retreated with ribonuclease before hydroxylapatite extraction. The DNA preparations were stored in 15 mM NaCl-1.5 mM sodium citrate (pH 7.0) at -20°C.

RNA isolation. rRNA was isolated as described previously (10). The procedure was modified by suspending the ribonucleic acid (RNA) pellet in 3 M sodium acetate (pH 6.0) after the first ethanol precipitation (25). The RNA was centrifuged and resuspended in 150 mM NaCl-15 mM sodium citrate (1X SSC) at pH 7.0 before the second ethanol precipitation. RNA preparations were stored in 1X SSC supplemented with 0.5% sodium dodecyl sulfate at -20°C.

Competitor rRNA preparations were adjusted to a concentration of 2 mg/ml. The 23S and 16S rRNA components were isolated by sucrose gradient centrifugation (25) and combined for labeling (11). The purities of the preparations were determined by polyacrylamide gel electrophoresis (2). The preparations were labeled with ^{125}I as described previously (25).

(G+C) content of DNA. The G+C contents of the DNA preparations were determined by thermal denaturation with a spectrophotometer and thermal programmer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) (10). DNA from Escherichia coli b was isolated by both of the methods described above and was used as a standard.

DNA homologies. The S1 nuclease procedure (10) was used to determine DNA homologies. The reassociation reaction mixtures contained 10 μl (0.025 μg) of labeled DNA, 50 μl (20 μg) of unlabeled DNA and 50 μl of a buffer that contained 880 mM NaCl and 1 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) at pH 7.0. Labeled DNA was denatured by heating in boiling water for 5 min. The reaction mixtures were incubated at 62°C for 24 h. S1 nuclease-resistant fragments were precipitated with HCl (21) and collected on type GF/F glass fiber

filters (Whatman Ltd., Kent, England). The filters were dried and counted with a gamma scintillation counter (Beckman Instruments Inc., Irvine, Calif.).

rRNA hybridizations. The competition rRNA hybridization procedure which we used has been described previously (11). The hybridization reaction mixtures contained 10 µl (0.3 µg) of labeled rRNA, 25 µl (50 µg) of competitor rRNA, and approximately 4 µg of DNA immobilized on a nitrocellulose filter (3 by 9 mm), all contained in 600 mM NaCl-60 mM ethylenediaminetetraacetic acid (4X SSC)-1 mM HEPES buffer (pH 7.0) supplemented with 45% formamide. The hybridization reaction mixtures were incubated at 50°C for 16 h.

rRNA-hybrid thermal stabilities. The procedure used to determine the thermal stability profiles of rRNA hybrids with DNA immobilized on nitrocellulose filters has been described previously (10,11). The elution buffer was 4X SSC-1 mM HEPES (pH 7.0) containing 50% formamide. The temperatures used to obtain the dissociation profiles were 35° through 85°C at 5°C increments.

Chemicals. Purified agar and yeast extract were obtained from Difco Laboratories, Detroit, Mich. Trypticase was from BBL Microbiology Systems, Cockeysville, Md. Trimethylamine hydrochloride was from Aldrich Chemical Co., Inc., Milwaukee, Wis. Ribonuclease A, ribonuclease T_1, deoxyribonuclease I, sodium dodecyl sulfate, cysteine hydrochloride, and all organic buffers and vitamins were from Sigma Chemical Co., St. Louis, MO. Acrylamide, bisacrylamide, and DNA grade hydroxylapatite were from Bio-Rad Laboratories, Richmond, Calif.). ^{125}INa was

from New England Nuclear Corp., Boston, Mass. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

The G+C contents of 7 strains of methylotrophic methane-producing bacteria (Table 1) were determined with standards which were isolated by using the same technique that was used for the DNA preparations assayed. The values for Methanosarcina barkeri strains MS and 227, Methanosarcina sp. strain TM-1, and Methanosarcina mazei were consistently 1 mol% lower than the values obtained by the buoyant density technique (R. Mah, personal communication). The G+C content of Methanolobus tindarius was 6 mol% lower than value 45.9% previously reported (15), but the reason for this discrepancy is not known.

Levels of DNA homology (Table 1) were determined with ^{125}I-labeled DNAs from five reference strains. A dendrogram (Fig. 1) prepared from the DNA homology values by average linkage clustering showed two distinct groups with only 2% DNA homology. Methanosarcina sp. strain TM-1, Methanosarcina acetivorans, and Methanosarcina mazei each had less than 30% homology with all of the other strains tested. Methanococcoides methylutens and Methanolobus tindarius had no significant DNA homology either with one another or with the other strains tested. These results indicated that the seven strains tested could be separated into six species (9). Inclusion of strain 227 in the species Methanosarcina barkeri based on a DNA homology value of 96% is consistent with the conclusion of Balch et al. (1) who used rRNA oligonucleotide cataloging. All of the other strains showed levels of DNA homology with

Table 1. Levels of DNA homologies and G + C contents[a]

Strain	G+C content (mol%)	% DNA homology with reference DNA from strain:				
		MS	227	TM-1	C2A	T3
MS	39	100[b]	98	34	27	
227	39	93	100	33	25	
TM-1	42	24	23	100	18	
C2A	42	12	11	14	100	
S-6	42	19	16	24	28	
TMA-10	42	1	1	3	2	4
Tindari 3	40	2	2	2	2	100

[a] *Methanosarcina barkeri* strains MS and 227, *Methanosarcina* sp. strain TM-1, and *Methanosarcina acetivorans* strain C2A, *Methanosarcina mazei* strain S-6, *Methanococcoides methylutens* strain TMA-10, and *Methanolobus tindarius* strain Tindari 3.

[b] Homologous values are reported as 100%.

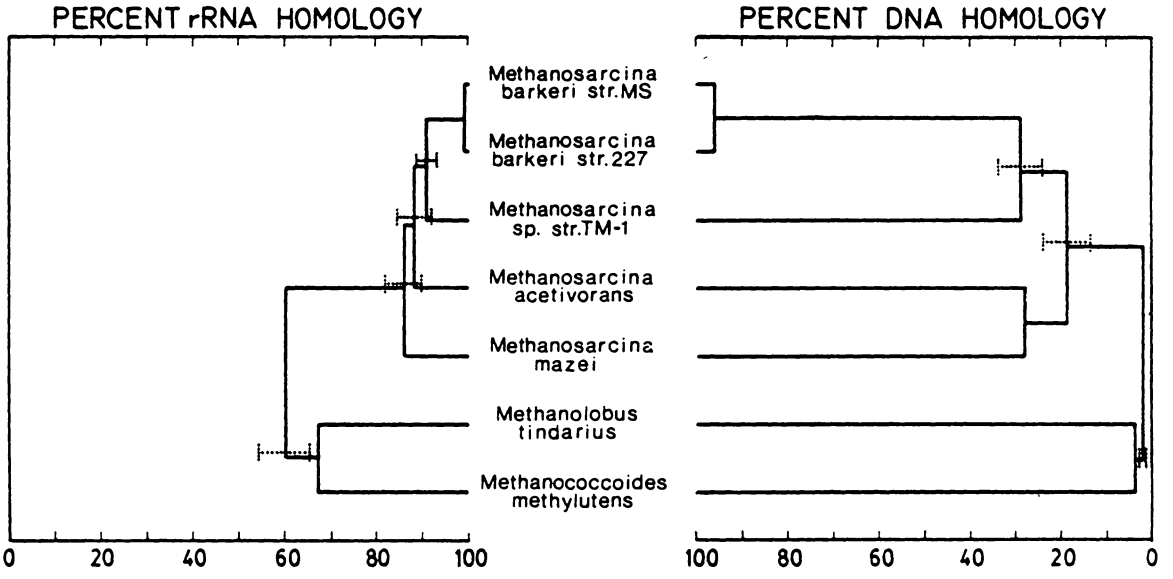

Fig. 1. Dendrograms of the phylogenetic relationships among the methylotrophic methane-producing bacteria. The dendrograms are based on average linkage clustering of the rRNA and DNA percent homology values from Tables 1 and 2. The standard deviations from the means are indicated by dotted lines.

each other that were well below 60%, which is the accepted value for division at the genospecies level (9).

Cross hybridization values of rRNAs obtained by the competition method from the seven strains were determined with ^{125}I-labeled rRNAs from six reference strains (Table 2). The values for Bacteroides fragilis VPI 2553 (parent strain of strain ATCC 25285) and Bacteroides uniformis VPI 0061 (parent strain of strain ATCC 8492) rRNAs, which were included as heterologous controls, were below 12%. Thermal stability results are reported as midpoint temperatures and differences in midpoint temperatures ($\Delta T_{m(e)}$) (Table 3). The rRNA homology values obtained by the competition technique were compared with the $\Delta T_{m(e)}$ values (correlation coefficient, 0.89) (Fig. 2). A similar correlation coefficient was observed for the two methods with the eubacteria (11). The oligonucleotide sequence similarity values (S_{AB}) of Methanosarcina barkeri strain MS relative to Methanosarcina mazei (S_{AB}, 0.8) (17) and Methanococcoides methylutens (S_{AB}, 0.52) (C. Woese, personal communication) are in agreement with the rRNA homology values (Table 2).

A dendrogram of the rRNA homology values from Table 2 is shown in Fig. 1. The results of rRNA hybridization experiments (Fig. 1) indicated that there are the following two distinct groups above the species level: group I which includes Methanosarcina barkeri, Methanosarcina sp. strain TM-1, Methanosarcina acetivorans, and Methanosarcina mazei, and group II, which includes Methanolobus tindarius and Methanococcoides methylutens. Phenotypic characteristics of the strains are compared in Table 4. Although group I varied with respect to the ability to use H_2-CO_2, all species in this group use acetate as a

Table 2. Levels of RNA homology

Strain	% RNA homology with reference RNA from strain:					
	MS	227	TM-1	C2A	TMA-10	T3
MS	100[a]	98	88	83	60	50
227	100	100	89	84	61	55
TM-1	94	93	100	84	58	54
C2A	93	91	91	100	69	55
S-6	92	88	86	79	61	54
TMA-10	68	65	60	61	100	63
Tindari 3	71	67	62	61	71	100
2553	8	8	5	10	9	12
0061	10	6	3	11	6	

[a]Homologous values are reported as 100%.

Table 3. $\Delta T_{m(e)}$) values of DNA-rRNA thermal stability profiles

Strain	$\Delta T_{m(e)}$ (°C) with reference RNA from strain:					
	MS	227	TM-1	C2A	TMA-10	T3
MS	68.0[a]	0	5.3	3.8	12.9	8.0
227	0	68.0	5.0	3.8	12.7	9.0
TM-1	3.9	4.0	70.5	4.5	12.4	8.5
C2A	2.4	2.1	4.2	68.6	12.3	8.1
S-6	2.9	2.9	4.2	3.4	12.2	8.2
TMA-10	9.9	10.8	9.7	12.3	70.6	6.0
Tindari 3	9.7	10.0	9.7	10.9	7.2	64.6

[a]Homologous values are reported as midpoint temperatures.

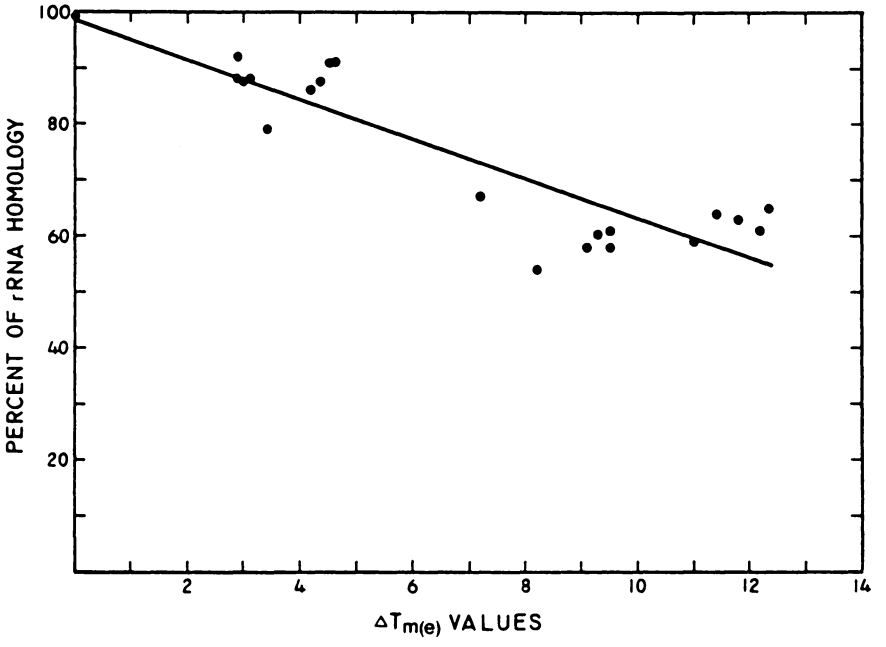

Fig. 2. Relationship of rRNA homology values to $\Delta T_{m(e)}$ values. Data from Tables 2 and 3.

Table 4. Phenotypic characteristics of the methyltrophic methane-producing bacteria[a]

Strain	Growth with:[b]		Optimum growth temp (°C)	Cell wall composition	C_{20} phytanyl glycerol ethers	Flagella or fimbriae
	H_2-CO_2	Acetate				
MS	+	+	35	H[d]	+	-
227	+	+	35	H	ND[e]	-
TM-1	+/-	+	50	H	ND	-
C2A	-	+	40	P	ND	+
S-6	+/-	+	40	H	ND	-
TMA-10	-	-	35	P	+	+
Tindari 3	-	-	25	P	ND	+

[a]Data obtained from references 1,15,16,18,25,26,35 and S. H. Zinder, personal communication.

[b]In addition to methanol and methylated amines.

[c]+, positive; -, negative; +/-, H_2-CO_2, used slowly.

[d]H, Heteropolysaccharide; P, protein.

[e]ND, Not determined.

substrate. The inability of group II species to use acetate appeared to be the only phenotypic characteristic that divides the two groups. The apparent morphologies do not support the division since morphovar III was expressed by both groups. In addition, a methylotrophic isolate that is unable to utilize acetate and expresses morphovars I and III and communal cysts has been described (34). Other phenotypic characteristics cannot be used to divide the two groups (Table 4).

An apparent anomaly is the presence of species with different cell wall types within the genus Methanosarcina (Table 4). All of the freshwater strains of Methanosarcina studied have rigid, thick cell walls composed of galactosamine, glucose, mannose, and glucuronic acid or galacturonic acid in varying proportions (12). The marine species, Methanosarcina acetivorans, has thin cell walls (10 nm) composed of protein subunits (25a). Cell wall structure is a conserved characteristic which has coincided with phylogenetic divisions at the genus level in previous studies of the archaeobacteria (1,12). Despite the gross morphological and chemical differences between heteropolysaccharide and protein cell walls, the results of immunological fingerprinting (3,4) indicated that there are common antigenic determinants between Methanosarcina acetivorans and species with heteropolysaccharide cell walls (E. Conway de Macario, personal communication). Likewise, Methanolobus tindarius and Methanococcoides methylutens, which also have protein cell walls, reacted as strongly or more strongly with heteropolysaccharide cell wall species than with each other. One possible explanation for these results is that all members of group I possess a protein cell wall with a heteropolysaccharide outer layer. Zeikus and Bowen

(32) described the cell wall of Methanosarcina barkeri strain MS in electron micrographs as having laminated "amorphous" outer layers which were attached to an electron dense layer adjacent to the cell membrane. A 10 nm electron dense inner layer, which is the thickness of a protein cell wall (15,25a,26) was also reported by Zhilina (33) in thin-section electron micrographs of Methanosarcina sp. Although previous investigators observed no or only trace amounts of amino acids in heteropolysaccharide cell wall preparations (12,13, S. F. Hurst, R. W. Robinson and A. S. Bleiweis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, J10, p. 92), treatment of the cell wall preparations included incubation with trypsin or sodium dodecyl sulfate, which would have completely solubilized a protein cell wall layer (14). Acid-hydrolyzed cell wall preparations contained a significant proportion of amino acids (17.32% of the total volatile mass) and an amino acid distribution (Table 5) similar to that in protein cell walls (14,20,25a,26,28,30). Cysteine, aspartic acid, and glutamic acid were predominant. The ash content of the preparations was 14.4% of the total dry weight; 17.0% of the volatile fraction was recovered as galactosamine, and 0.54% was recovered as glucosamine. The high proportion of cysteine in the cell wall preparation of Methanosarcina barkeri (Table 5) has not been reported previously in protein cell walls. The volatile mass not accounted for (65%) may contain uronic acids and neutral sugars (S. F. Hurst and A. S. Bleiweiss, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984. I81, p. 135), as well as residual water. These results suggest that methanogenic bacteria with a heteropolysaccharide cell walls also have an inner protein cell wall layer. This hypothesis is supported by

Table 5. Amino acid and amino sugar content of the hydrolyzed cell wall preparation of Methanosarcina barkeri strain 227

Compound	Concn (μmol mg. dry wt)	Molar ratio[a]
Amino acids		
Cys	181.2	3.08
Asp	124.3	2.11
Glu	101.2	1.72
Thr	100.8	1.71
Ser	86.7	1.47
Gly	77.8	1.32
Ala	76.8	1.30
Leu	59.3	1.01
His	58.9	1.00
Val	52.3	0.89
Lys	45.7	0.77
Ile	41.4	0.71
Pro	39.4	0.67
Met	21.3	0.36
Tyr	19.1	0.32
Phe	18.9	0.32
Arg	17.0	0.29
Trp	ND[b]	ND
Amino sugars		
Galactosamine	409.8	ND
Glucosamine	25.9	ND

[a]Based on a value of 1.00 for histidine.

[b]ND, Not determined.

the fact that all other known species in the order Methanomicrobiales have a protein cell wall (12). Since no single step mutations are known which lead to an altered cell wall type (24), Methanosarcina acetivorans may have lost the ability to produce the heteropolysaccharide outer layer of its cell wall. Alternatively, non-marine species may have developed the heteropolysaccharide for osmotic protection.

As a result of these observations we propose that the family Methanosarcinaceae be emended to include the species Methanosarcina acetivorans, Methanolobus tindarius and Methanococcoides methylutens, as described below.

Family II, Methanosarcinaceae. Methanosarcinaceae Balch and Wolfe 1979 (emend.) (Me.tha.no.sar.cin.a´ce.ae. M.L. fem. n. Methanosarcina type genus of the family; -aceae ending to denote family; M.L. fem. pl. n. Methanosarcinaceae the Methanosarcina family.) Spherical or pleomorphic cells 1.0 to 2.5 µm in diameter. Members of this family express one or more of the following three cellular arrangements: morphovar I is an aggregation of cocci, up to 100 µm in diameter; morphovar II is a smaller aggregation of cells, up to 30 µM in diameter; and morphovar III cells are arranged singly. Some species also form communal cysts of several single cells within a common envelope. Cell division planes are irregular. A species may have protein cell wall 10 nm thick which has a negative Gram reaction or a cell wall layer composed of heteropolysaccharide that stains gram positive or gram variable. Cells are nonmotile mesophiles to thermophiles. Energy for growth comes from the oxidation of H_2 with the reduction of CO_2 to methane or from metabolism of methanol, methylated amines (methylamine, dimethylamine, trimethyl-

amine or ethyldimethylamine), or acetate with formation of methane and CO_2 as end products; ammonia is produced from amine degradation. Gas vacuolated forms have been reported.

We propose that rRNA homology groups I and II be divided at the genus level based on a rRNA homology level of 60% (Table 1) and an S_{AB} value of 0.52 between Methanosarcina barkeri strain MS and Methanococcoides methylutens (Woese, personal communication). Although these values are higher than those most used to divide genera of eubacteria (6,24), they fall within the genus-species division value for the methanogenic bacteria proposed by Balch et al. (1). This division is further supported by the DNA-rRNA hybridization data of Tu et al. (29). The midpoint temperatures of the thermal melting curves for DNA-rRNA hybrids from that study were normalized by using a value of 20°C as the maximum heterologous $\Delta T_{m(e)}$ value (11). The phylogenetic relationships are expressed as the fractional $\Delta T_{m(e)}$ values between heterologous (0.0) and the homologous (1.0) strains in Figure 3. The fractional $\Delta T_{m(e)}$ value between group I and group II was lower than most values between other genera of methane producing bacteria. In addition, species in group I could use acetate but those in group II could not. All species in group I, which includes Methanosarcina barkeri (18), Methanosarcina acetivorans (25a), Methanosarcina mazei (16), and Methanosarcina sp. strain TM-1 (35), are in the genus Methanosarcina as described by Balch et al. (1).

The Group II strains showed virtually no DNA homology (4%), which indicates that they are separate species. The different G+C values (Table 5) and phenotypic characteristics (Table 6) support this conclu-

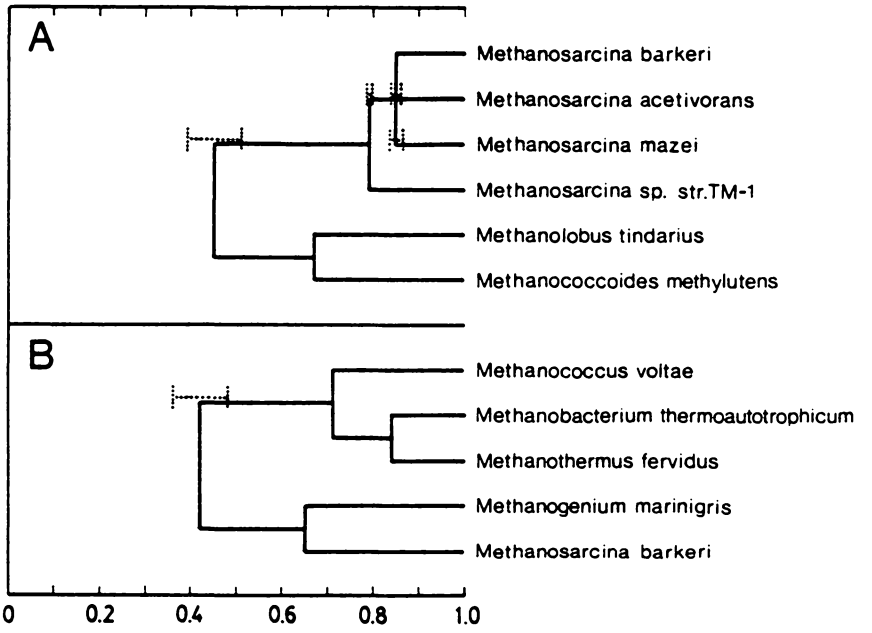

Fig. 3. Dendrograms of the relationship among the methanogenic bacteria based on $\Delta T_{m(e)}$ values from this study (A) and from the study of Tu et al. (29) (B). Data from both studies were normalized by using a value of 20°C for the maximum heterologous $\Delta T_{m(e)}$ value. The phylogenetic relationships are expressed as fractional $\Delta T_{m(e)}$ values ranging from 0.0 to 1.0. Fractional $\Delta T_{m(e)} = 20 - \Delta T_{m(e)}/20$. The standard deviations from the means are indicated by dotted lines.

Table 6. Phenotypic differences between *Methanolobus tindarius* strain Tindari 3 and *Methanococcoides methylutens* strain TMA-10[a]

Species	Source	Flagellum or fimbriae	Internal membranes	Uniform inclusions	Vitamins required	Maximum growth temp (°C)	Optimum growth temp (°C)
Methanolobus tindarius	Marsh pond	+	+	+	-	45	25
Methanococcoides methylutens	Marine canyon	+	-	-	+	35	35

[a]Data obtained from references 15 and 26.

sion. The level of rRNA homology between Methanolobus tindarius and Methanococcoides methylutens was only 7% greater than the levels of rRNA homology values between these two species and Methanosarcina (Fig. 1). In addition, when fractional $\Delta T_{m(e)}$ values obtained in this study are compared with values obtained by Tu et al. (29), the value between Methanococcoides methylutens and Methanolobus tindarius is lower than the values between other genera of the methanogenic bacteria (Fig. 3). Although Methanolobus tindarius (15) and Methanococcoides methylutens (26) are phenotypically similar (Table 6), results of this study suggest that they are phylogenetically distant. As more methylotrophic strains become available and their phylogenies are determined, the current division may become less distinct, and the merging of these genera may be warranted. The maintenance of separate genera may also be impractical for identification purposes if more distinguishing phenotypic characteristics are not found in other strains. The genera Methanolobus and Methanococcoides have been described elsewhere (15,26).

Despite the morphological diversity among the methylotrophic methane-producing bacteria, these organisms appear to be closely related at the level of rRNA homology. As the phylogenetic relationships of more strains are determined, the generic division observed in this study may become more or less pronounced, warranting changes in the proposed divisions. The phylogenetic divisions obtained with DNA-rRNA and DNA-DNA hybridization techniques showed good agreement with phenotypic characteristics and complemented the oligonucleotide sequencing data of Balch et al. (1) for the methanogenic bacteria.

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SUMMARY AND DISCUSSION

This investigation is the first reported description of a complete polymer-degrading methanogenic consortium from marine sediments. Labelling studies by several investigators (135,190,198) indicate that sulfate reduction is the predominant terminal process of polymer-degradation consortia in marine sediments because the sulfate reducing bacteria have a greater affinity (lower K_s) for hydrogen and acetate than do the methanogenic bacteria (113,123,126,188). Since sulfate is available from seawater (128) marine sediments are readily replenished before depletion of sulfate by the sulfate reducing bacteria. However, in intertidal sediments during low tide (108) and in sediments that receive high organic loading such as eutrophic estuaries (24) and marine trenches (68,167), sulfate is depleted and methanogenesis is the predominant terminal process of the polymer-degrading anaerobic consortia. An analogous situation has been reported in freshwater sediments: in oligotrophic lake sediments sulfate reducing bacteria can out-compete the methanogenic bacteria for substrate (126) but in eutrophic lake sediments where sulfate is rapidly depleted methanogenesis is the predominant terminal process (148,195,210,236).

Both methanogenic and sulfate-reducing bacteria are essential for the degradation and subsequent recycling of organic compounds in the environment. In the absence of respiratory electron acceptors such as oxygen or nitrate, organic compounds are degraded by fermentative bacteria because these organisms can dispose of electrons from energy-yielding oxidations by reducing their products (20). The methanogenic and sulfate-reducing bacteria facilitate complete oxidation of organic

matter by diverting electrons away from product reduction. Some products of the fermentative bacteria are further oxidized by the hydrogen-producing acetogenic bacteria. The oxidized products of the fermentative and acetogenic bacteria and the reducing equivalents in the form of hydrogen are substrates for the methanogenic and sulfate-reducing bacteria which produce carbon dioxide and methane, and carbon dioxide and hydrogen sulfide, respectively. Methane is eventually oxidized to carbon dioxide (41,68,168,227) thus completing the carbon cycle.

Methanogenesis compliments sulfate reduction by degrading organic matter in marine sediments with low sulfate concentrations. In oligotrophic marine sediments where sulfate reduction is predominant methanogenesis occurs concurrently although at low rates (190,198). If sulfate is depleted the methanogenic bacteria assume the terminal role of the consortium. The methanogenic bacteria may also have a unique niche in the degradation of methylated amines which are readily available in the marine environment (160,161).

Despite the important role of methanogenic bacteria in marine degradation only one marine species had been described before this project was begun (206). During the course of this research many marine species have been isolated and described by several laboratories (74,87,78,170,171,173,233,234). However, all of these species were isolated with hydrogen as a substrate. Acetotrophic methanogenic bacteria are essential in freshwater methanogenic consortia (125) but no marine species had been reported before Methanosarcina acetivorans (Section VII). A strictly methylotrophic methanogen, Methanococcoides methylutens, was described (Section II) and reported concurrently with a strain isolated by K. O. Stetter (112).

Kelp Degradation by a Marine Methanogenic Consortium

Kelp degradation studies have been reported by several investigators but the source of inoculum was usually sewage sludge. Methanogenesis from this source was often inhibited by the high salt concentrations in kelp (140). In this study (Section III) the source of inoculum was a marine canyon that contained an indigenous methanogenic, kelp-degrading consortium. The intermediate products of kelp degradation reported in this study were similar to kelp degradation products reported by others (A. F. Cappiapuoti and J. R. Furro, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, 076, p. 252). Acetate and propionate were the predominant intermediates in kelp degradation as reported in sludge digestion studies. Although these results would suggest that acetate and propionate are rate limiting steps as in freshwater methanogenic consortia (97), kinetic studies would be necessary to show this. The morphology of the mannitol- and alginate-fermenting bacteria were similar to those previously reported (A. F. Cappiaputoi and J. F. Furro, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, 076, p. 252). The appearance and depletion of propionate and butyrate suggests that hydrogen-producing acetogenic bacteria were present (16,142) or that sulfate reducing acetogenic bacteria were functioning as proton reducers (21,220). Enrichment cultures yielded rod-shaped bacteria in close association with methanogenic bacteria. This is the first report that demonstrates the presence of hydrogen-producing acetogenic bacteria in the marine methanogenic consortium.

Hydrogen-utilizing Methanogenic Bacteria

The hydrogen-utilizing bacteria isolated from the kelp enrichment were particularly interesting because almost all of the isolates were unlike methanogens from freshwater methanogenic consortia (Section III). They had an irregular shape, an osmotically sensitive cell wall, and grew at marine salt concentrations like marine methanogens reported by others (74,87,89,170,171,173,233,234). The only isolate that resembled a freshwater species was a hydrogen-utilizing spirillum, strain H_2S, but this is likely a new species because unlike Methanospirillum hungatei (162) it can tolerate marine salt concentrations. All previously reported marine strains are divided from freshwater strains at the species level (3). All the other strains isolated in this study are morphologically similar to previously described strains and require physiological and phylogenetic characterization before they can be identified.

Acetate-utilizing Methanogenic Bacteria

The methyl group of acetate accounts for 60% of the total methane in freshwater sediments. Acetate is also a major methane precursor in methanogenic marine sediments (175) and a major intermediate in the kelp fermenters (Section III, Figure 3), yet no marine acetotrophs have been previously reported. Three different methanogenic bacteria were observed in enrichment cultures that contained acetate and mineral medium (Section III). Two chain-forming rods with square ends that could be differentiated by size were predominant in cultures with low acetate concentrations (0.2 and 2 mM) or in stationary phase cultures

that contained 20 mM acetate when inoculated. The proliferation at low substrate concentration and morphology are characteristics of the freshwater species Methanothrix soehngenii (79), except that the marine bacteria contained refractile bodies. This observation is particularly interesting because rod-shaped acetate degraders are the predominant acetotrophic methanogens in freshwater consortia (52,79,153,252).

The other acetotrophic methanogen was isolated and further characterized (Section VII). This isolate was designated Methanosarcina acetivorans and is the first report of an acetotrophic methanogen indigenous to marine sediments. M. acetivorans expresses three distinct morphovars which are similar to those expressed by the freshwater species Methanosarcina mazei (131) and the moderately halophilic methylotroph Methanococcus halophila (257). However, M. acetivorans is irregularly shaped and has an osmotically sensitive cell wall which are characteristics typical of all other reported marine methaogenic bacteria. In addition this species requires sodium chloride and magnesium at concentrations contained in seawater.

In order to study the physiology of M. acetivorans and produce enough genetic material for phylogenetic studies a mass culturing technique, the pH auxostat, was developed for this and other species of acetotrophic methanogens (Section VIII). The technique was successful for growing marine or non-marine, and mesophilic or thermophilic strains yielding up to 16-fold the cells grown by conventional batch culture techniques. This technique was used to grow cells for preliminary studies on acetate catabolism (M. J. Nelson, K. R. Sowers and J. G. Ferry. Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I25, p. 125).

Since these organisms are difficult to grow and grow slowly on acetate, the pH auxostat will greatly facilitate studies on acetotrophic physiology.

Methylotrophic Methanogenic Bacteria

Trimethylamine oxide is available for the marine environment as an excretory and degradation product of most marine fauna (62,98). This compound may be reduced to trimethylamine by respiratory bacteria as a terminal electron acceptor in anaerobic sediments (211,247). Trimethylamine oxide may also be converted to dimethylamine by a dismutation reaction and subsequently oxidized by methylotrophic bacteria in the presence of nitrate or oxygen (199). In the absence of alternative electron acceptors methylated amines may be degraded by methylotrophic methane-producing bacteria (160,151,235). Oremland (160,161) demonstrated that methanogenesis from trimethylamine was not effected by the addition of sulfate to sediments in enrichment cultures. However, King et al. (106) reported that methanogenesis from trimethylamine at in situ concentrations only accounts for 35 to 61% of the total methane production in intertidal sediments. These results suggest that although trimethylamine is not an exclusive substrate for methanogenesis, the methanogenic bacteria may be able to compete for this substrate more successfully than for other substrates. Population studies reported in this investigation (Sectin VI) indicate that trimethylamine-utilizing methanogens are most numerous in the upper sediment layers where the sulfate concentration is highest, which supports an important role for methanogenic bacteria in the degradation of trimethylamine. The isola-

tion of an exclusively methylotrophic methanogen, Methanococcoides methylutens further supports a unique role for methanogenic bacteria in amine degradation (Section IV). Although it is often difficult to grow acetotrophic strains in acetate when they are transfered from alternative substrates (13) all attempts to grow M. methylutens on acetate or hydrogen were unsuccessful. The recent reports of other exclusively methylotrophic methanogens, Methanolobus tindarius (112) and Methanococcus halophilus (257) and the phylogenetic study (Section IX) further support the unique metabolism of M. methylutens. Attempts to determine the contribution of trimethylamine to methanogenesis in Scripps Canyon sediments were unsuccessful because winter storms disrupted the sediment profile.

M. methylutens is irregularly shaped, has an osmotically sensitive cell wall, and requires sodium chloride and magnesium for growth like other methanogenic marine species. Trace metal requirements are similar to those of freshwater species except that stimulation by molybdenum and selenium could not be shown (Section V) (183,187,233).

Phylogeny of the Acetotrophic and Methylotrophic Methanogens

The phylogenetic positions of only three methylotrophic and acetotrophic methanogens had been determined by 16S rRNA oligonucleotide cataloging relative to the type strain Methanosarcina barkeri strain MS before this study (3,52,202). In order to determine the phylogenetic position of M. acetivorans and M. methylutens it was necessary to compare all known species of acetotrophic and methylotrophic methanogenic bacteria. Since other techniques (38) had reported differences

between strains that were not detectable by 16S rRNA oligonucleotide cataloging (3), DNA/DNA homologies and RNA/DNA hybridizations were used in this study. This procedure would detect differences between strains as well as genera. Results of this study indicated that M. acetivorans and M. methylutens were new species of methanogenic bacteria (Section IX). An apparent anomaly is the inclusion of M. acetivorans, that has a protein cell wall, with other members of the Methanosarcina that have a heteropolysaccharide cell wall. Cell wall structure is a conserved trait and methanogenic species with different cell wall types are phylogenetically divided at least at the genus level (3,93). Based on evidence presented in this study (Section IX) and by other investigators it is hypothesized that species with a heteropolysaccharide cell wall have a protein inner layer. Lack of a heteropolysaccharide cell wall layer in M. acetivorans may be the result of a single-step mutation that would not be reflected in the phylogenetic analysis.

Description of Methanococcoides methylutens and Methanolobus tindarius were published simultaneously and because of phenotypic differences from species of Methanosarcina a new genus was proposed for each. DNA homologies indicated that they are separate species and although the species have similar phenotypic characteristics RNA homology values did not conclusively warrant merging the species into one genus. More strains need to be isolated and their phylogenetic relationships determined before a final decision can be made on the taxonomy.

Methanosarcina sp. strain TM-1 is an acetotrophic thermophile that had been previously described but a specific epithet had not been

proposed (262). As a result of this study it was determined that this strain is a new species and a specific epithet is being proposed in conjunction with S. Zinder.

The physiological and phylogenetic results from this study indicate that the populations of methanogenic bacteria from marine sediments are both unique and as diverse as methanogenic bacteria described from non-marine environments.

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Physiological and Phylogenetic Characterizations of Marine Methanogenic Bacteria from a Kelp-degrading Consortium

by

Kevin R. Sowers

Committee Chairman: James G. Ferry

Anaerobic Microbiology

(Abstract)

Methanogenesis is the predominant terminal process of polymer degradation in anaerobic marine sediments depleted of sulfate; however, characterization of a marine consortium has not been previously reported. A marine methanogenic consortium consisting of fermentative, hydrogen-producing acetogenic and methanogenic bacteria is described. An acetotrophic methane-producing strain of bacteria was isolated. This isolate expresses three distinct morphovars: individual cocci, cell aggregates and communal cysts. Individual cocci are 1.9 μm in diameter and are nonmotile, but have fimbria-like structures. Cells have a thin protein cell wall. Acetate, methanol, methylamine, dimethylamine and trimethylamine are substrates for growth; formate and hydrogen are not. Sodium chloride and magnesium concentrations found in seawater are required for optimum growth. RNA homology values indicated that this isolate is a new species. Methanosarcina acetivorans is the proposed specific epithet. This is the first report of an acetotrophic methane-producing species indigenous to marine sediments. A method is described for mass culturing this and other acetotrophic methanogens using a pH auxostat.

A strictly methylotrophic methane-producing strain was also isolated and is described. Only trimethylamine, dimethylamine, methylamine and methanol were substrates for growth. Cells were nonmotile, irregular cocci 1 μm in diameter and had a thin protein cell wall. Sodium chloride and magnesium concentrations found in seawater were required for optimum growth. Biotin was the only organic supplement required for growth in mineral medium. Fe was required for growth; Ni and Co were stimulatory. This isolate is a new genus based on RNA homology. Methanococcoides is the proposed genus and Methanococcoides methylutens is the specific epithet. Population studies suggest that this species is uniquely adapted for methylated amine degradation in marine sediments.

The phylogenetic relationships of M. acetivorans, M. methylutens and other acetotrophic and methylotrophic species were determined by RNA and DNA homology techniques. Phylogenetic and physiological results in this study indicated that the population of methanogenic bacteria in marine sediments is both unique and equally diverse as the population of freshwater methanogenic species.