Methanogenesis in low sulfate hot spring algal-bacterial mats
by Kenneth Andrew Sandbeck

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
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Abstract:
Methanogenesis in algal-bacterial mats in the effluent channels of low sulfate hot springs (Yellowstone National Park) was studied. Methanogenesis was found to be greatest 13-23 C lower than the upper temperature limit for mat development which was about 73 C. Samples from various temperature regimes of the mat (44-60 C) all showed increased methane production upon incubation at elevated temperatures (65-70 C) indicating that the reason for maximal methanogenesis occurring below the upper temperature limit for mat development was not a lower upper temperature limit for methanogenic bacteria involved in anaerobic degradation. Methanogenic bacteria isolated from various temperature regimes of the mat also showed increased methane production and growth upon incubation at elevated temperatures. It appears that methanogenesis is not limited by temperature. Methane production and primary productivity exhibited similar tempera ture distributions indicating methanogenesis might be limited by the availability of methanogenic precursors, the amount of which is probably a direct function of the rate of formation of algal-bacterial organic matter. Experiments designed to determine the relative importance of labelled methane precursors indicated that acetate was not an important precursor of methane at either high or low acetate concentrations. At high acetate concentrations, acetate was apparently diverted photoheterotrophically into cellular material. Autoradiograms prepared from mat material incubated with 2-14C-acetate showed that acetate was. rapidly incorporated into very long filamentous bacteria. Dark incubation reduced photoheterotrophic incorporation of acetate. Experiments with NaH14CO3 showed that radioactive methane was produced rapidly from H14CO3 and that CO2 reduction accounted for at least 70-80% of the methane evolved from algal-bacterial mat samples. Apparently, CO2. is the main precursor of methane because competition for acetate by other inhabitants of this microbial community, possibly photoheterotrophic bacteria, may preclude acetate as a major methane precursor.

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ABSTRACT

Methanogenesis in algal-bacterial mats in the effluent channels of low sulfate hot springs (Yellowstone National Park) was studied. Methanogenesis was found to be greatest 13-23 C lower than the upper temperature limit for mat development which was about 73 C. Samples from various temperature regimes of the mat (44-60 C) all showed increased methane production upon incubation at elevated temperatures (65-70 C) indicating that the reason for maximal methanogenesis occurring below the upper temperature limit for mat development was not a lower upper temperature limit for methanogenic bacteria involved in anaerobic degradation. Methanogenic bacteria isolated from various temperature regimes of the mat also showed increased methane production and growth upon incubation at elevated temperatures. It appears that methanogenesis is not limited by temperature. Methane production and primary productivity exhibited similar temperature distributions indicating methanogenesis might be limited by the availability of methanogenic precursors, the amount of which is probably a direct function of the rate of formation of algal-bacterial organic matter. Experiments designed to determine the relative importance of ^{14}C labelled methane precursors indicated that acetate was not an important precursor of methane at either high or low acetate concentrations. At high acetate concentrations, acetate was apparently diverted photoheterotrophically into cellular material. Autoradiograms prepared from mat material incubated with 2-^{14}C-acetate showed that acetate was rapidly incorporated into very long filamentous bacteria. Dark incubation reduced photoheterotrophic incorporation of acetate. Experiments with $NaH^{14}CO_3$ showed that radioactive methane was produced rapidly from $H^{14}CO_3^-$ and that CO_2 reduction accounted for at least 70-80% of the methane evolved from algal-bacterial mat samples. Apparently, CO_2 is the main precursor of methane because competition for acetate by other inhabitants of this microbial community, possibly photoheterotrophic bacteria, may preclude acetate as a major methane precursor.

INTRODUCTION

The production of methane by microorganisms is a common occurrence in a wide variety of anaerobic environments where organic matter is available for decomposition (98,172,178). Methanogenesis (the biological production of methane), occurs in the rumen and intestinal tract of animals, anaerobic waste digestors, freshwater and marine sediments and the wetwood of living trees. Methane production by microorganisms has also been reported in hot springs (157,178) and lakes (52). Production of methane by microorganisms in sediments, marshes and bogs is continuous and this methane can ignite spontaneously. Colloquially, the transient, amorphous blue lights seen sometimes above these environments are referred to as "will-o-the-wisp" (175). In these anaerobic environments, methanogens (methanogens = microorganisms which produce methane), are the terminal organisms of the anaerobic microbial food chain. By consumption of the fermentation products of higher trophic levels, particularly H_2/CO_2 and acetate, they allow anaerobic decomposition to proceed (30). The methane production reaction is very important in the carbon and other cycles in nature because it results in the degradation of complex organic material to the gaseous products CO_2 and CH_4 with a relatively small growth yield of bacteria. In this way, a large amount of organic material is destroyed, but most of the substrate energy is retained in the methane (90%) (31).

Methane is produced by a small group of morphologically diverse bacteria. These bacteria are unified by their ability to produce methane as an end product during energy metabolism. Many detailed reviews discuss the taxonomy, physiology, biochemistry and activity of these bacteria (98,102,139,172,178).

Barker has reviewed the historical aspects of microbial methane production (7). The historical study of methane production is interesting and warrants a brief summary here. Volta is credited with the first observation of methane production in nature. In 1776, he reported that large quantities of a combustible gas were continuously being formed in the sediments of lakes and ponds in Italy. Volta also noticed that there seemed to be a direct correlation between the amount of plant material and the amount of gas produced, and from this concluded that the gas was formed from this plant material.

In 1806, William Henry found that this combustible gas was identical to a synthetic illuminating gas, methane. In 1868, Bêchamp, a student of Pasteur, provided evidence that methane production was a microbiological process. Tappeiner, in 1882, provided more adequate proof that methane production was a microbiological process.

Toward the latter part of the 19th century, cellulose was

thought to be a substrate for methane-producing bacteria. Later, though, it was believed that methanogenesis was a two stage process as cellulose could be decomposed without the production of methane, but the products of cellulose fermentations could be used by a methane-producing culture. However, around this time, Omelianski reported the isolation of Bacillus methanigenes which supposedly could form methane from cellulose.

During this century the study and knowledge of methanogenesis has grown a great deal. However, it has been only in the past 10 or 15 years that the true substrates (used by pure cultures) of methane-producing bacteria have been identified. Even at this point in time, much remains to be learned about methanogenic bacteria and their activities in nature or in manipulated environments such as anaerobic waste digestors. As an example, thermophilic production of methane has received attention as of late as a more efficient means of waste conversion (154).

Methanogenic Bacteria

Interest in methane-producing bacteria has increased lately and the main reason for this increased interest is due to the role methanogenic bacteria play in the anaerobic conversion of organic matter to methane (a fuel gas). Interest has also been stimulated by the idea (2,3,61,171) that methanogens comprise a unique type of

life distinctive from other procaryotic (including chloroplasts and mitochondria) and eucaryotic cell types. The basis for this distinction is not only unique ribosomal oligonucleotide patterns and sequences, but also the lack of muramic acid-containing peptidoglycan cell walls (61,81,84). Recently, Balch, et al. (2) proposed a new taxonomic scheme for the methanogens based on their phylogenetic relatedness as revealed by 16S rRNA comparisons. Additionally, methanogenic bacteria appear to possess unique ether-linked lipids (151). These findings further strengthen the distinction of methanogenic bacteria as the independent Family Methanobacteriaceae made earlier on the basis of common metabolic and physiological criteria (29).

The methanogenic bacteria as a group consist of very few recognized species belonging to five genera distinguished on the basis of cellular morphology (2,29,128). The fact that rod-, coccus-, irregular coccus-, spirillum- and pseudosarcina-shaped cells have been found to produce methane suggests diverse phylogenetic origins. As a group, methanogens have a severely restricted range of energy sources and most isolates use only H_2 or H_2 and formate (29). Previously, a metabolic property believed common to all methanogens, the ability to use H_2 as an electron donor in the reduction of CO_2 to methane, was touted as the one major unifying characteristic of

these bacteria (29,98,172,178). Recently, however, Zehnder, et al. (176) and Zinder and Mah (188) have isolated a rod and pseudosarcina, Methanobacterium soehngenii and Methanosarcina barkeri, respectively, which do not use H_2 and CO_2 as precursors of methane, but do actively use acetate as a methane precursor. Carbon monoxide is converted to CO_2 and CH_4 by Ms. barkeri and Methanobacterium formicicum (29). Ms. barkeri is the only isolate which may use either acetate or methanol as an energy source (30).

Only one true thermophilic methanogen has been isolated, Methanobacterium thermoautotrophicum (184), but Zinder and Mah (188) isolated a strain of Ms. barkeri which grew optimally at 50 C and did not grow at 60 C. M. thermoautotrophicum grows optimally in the temperature range 65-70 C.

Physiology, Biochemistry and Structure of Methanogenic Bacteria

The substrates for methanogenesis have aroused much controversy in the past. Early workers (7) reported that a wide variety of alcohols, fatty acids, cellulose and H_2/CO_2 could be used by methanogenic bacteria. However, much of this work was done with impure enrichment cultures and resulted in invalid results. With the advent of improved, specialized techniques for the isolation and cultivation of these strict anaerobes (27,33,70,73), many of the above mentioned substrates were shown to have been metabolized by contami-

nants in the enrichment culture. Previously, only H_2/CO_2, formate, CO, methanol and acetate had been shown to be substrates for axenic cultures of methanogenic bacteria (29). It appears now though (186, 187), that methyl mercaptan may serve as a methane precursor; however, this work was done with natural algal-bacterial mat or sediment material and not with pure cultures. Also, compounds such as methyl amines have been shown to contribute to methane formation (67,116, 162,188). Only recently has acetate conversion to methane been studied in pure cultures (97,159,176). Zeikus, et al. (182) reported that in a mineral salts acetate medium, acetate could serve as a methane precursor in Ms. barkeri and M. thermoautotrophicum only in the presence of added H_2. Mah, et al. (97) believe that Zeikus found it necessary to add H_2 for acetate conversion because of the selection and pregrowth conditions he employed. Mah, et al. (97) and Zehnder, et al. (176) have isolated pure cultures capable of growth and methane production on acetate as the sole energy source. The isolation of these bacteria in pure culture has done much to explain the frequently observed phenomenon that acetate is the main precursor of methane in most anaerobic systems (38,42,69,80, 88,98,107,136).

Despite the recent progress in obtaining methanogens in pure culture which can convert acetate to methane, a great deal remains

to be learned about organisms responsible for acetate conversion to methane. In addition, virtually nothing is known about the biochemistry of acetate conversion to methane or the means by which methanogens obtain energy from this reaction (98,172,175,178). However, a general scheme has been proposed for both CO_2 and acetate conversion to methane to account for results observed by researchers.

Basically, during the process of methane production, electrons generated in the oxidation of H_2 and formate are used in the reduction of CO_2 to methane; whereas electrons generated in the oxidation of acetate and methanol are used in the reduction of intact methyl groups of these substrates to methane (36,97,125,126,140,159). As electrons flow from donor to acceptor, ATP synthesis is presumed to occur via an electron transport mechanism (54). Stadtman and Barker (140) proposed a branching scheme for the reduction of CO_2 or methyl groups to methane to account for methane production from either CO_2 or methyl carbons. This presumably occurred through a common methylated intermediate, and this scheme replaced the earlier theory of Van Niel (see 6).

The belief of Stadtman and Barker that there was a common methylated intermediate involved in the reduction of CO_2 or methyl carbons was substantiated by the discovery of McBride and Wolfe (101) of coenzyme M (CoM), a terminal methyl carrier (mercaptoethane

sulfonic acid). This coenzyme has been studied by others (143, 144, 145) and appears to be active in the methyl reductase system of all methanogenic bacteria. CoM is found in all the methanogenic bacteria, except Methanobacterium ruminantium strain M1 where it is required as a growth factor (144) and this led to the use of this microorganism as a bioassay system for CoM (4). More recent evidence on the ability of CoM to transfer C_1 units of differing redox potential, led Gunsalus, et al. (64) to propose that CoM may be the common carrier of C_1-carbon from CO_2 or methyl groups.

Electrons removed by methanogens in the oxidation of H_2 or formate by M. ruminantium were shown to pass through a low potential electron carrier, called factor 420 or F_{420} (152), to NADP before ultimately reducing methyl CoM to methane. This factor was originally isolated from Methanobacterium MoH (46), but was found to be present in all methanogenic bacteria examined (61,64) and is also unique to methanogenic bacteria (61). The structure of F_{420} was recently presented as a riboflavin analog (59). F_{420} autofluoresces blue-green when excited by long wavelength ultraviolet light (46). In addition, some methanogenic bacteria have been shown to possess other chromophoric factors (65) of unknown function.

Although the role of CoM and F_{420} in acetate metabolism is unknown, a role is suggested by the inhibition of growth and methano-

genesis from acetate in a pure culture of Ms. barkeri by viologen dyes (97).

Even less is known about the pathway for fixation of CO_2 into cell carbon compared to known routes of CO_2 fixation (51,146,180). While many methanogens appear to be autotrophic (178), it appears that methanogenic bacteria lack a complete Calvin cycle, and they do not possess the enzymes necessary for CO_2 fixation in the serine or hexulose pathway (162). However, it is interesting that Weimer and Zeikus (163) have shown that M. thermoautotrophicum and Ms. barkeri are deficient in different tricarboxylic acid (TCA) cycle enzymes. This thought becomes more provocative in regard to the fact that some methanogenic bacteria have been shown to excrete organic compounds (178) so that one might infer that some cross-feeding of CO_2 fixation intermediates may take place in natural environments. Also, the contribution of acetate in the formation of major amounts of cell carbon in M. ruminantium (34) suggests that methanogenic bacteria may be mixotrophic or even heterotrophic. This makes sense in terms of anabolic metabolism as methanogens in nature are often found in a rich organic environment. The stimulation of methanogenic bacteria by growth factors apparently provided by nonmethanogenic associates in mixed cultures (111,127,132,147,159,185) suggests that methanogenic bacteria prefer symbiotic associations for optimal

nutritional conditions.

Not only are methanogens unique in regard to their physiology, they are also somewhat unique in regard to their structure. As mentioned previously, methanogenic bacteria lack a muramic acid-containing peptidoglycan cell wall and are thus resistant to antibiotics whose action is against cell wall synthesis such as penicillin, vancomycin and cycloserine (81). They also resist lysozyme and detergents such as lauryl sulfate (84). Electron microscopy showed an absence of an electron dense cell wall layer in Methanococcus vannielii (81) and a variety of other unusual cell wall and cytological structures in other methanogens (91,179). Evidently, the unusual ribosomal sequences of methanogenic bacteria (61) do not impart differences in ribosomal function or structure as chloramphenicol was found to inhibit M. vannielii (81).

Ecology of Methanogenic Bacteria

In nature organic matter is degraded anaerobically through several trophic levels (69,90,98,150). Organic polymers are degraded to a wide variety of sugars, fatty acids, alcohols, H_2 and CO_2. While other microorganisms further ferment the sugars and alcohols, a third group of organisms (30,31,175), the proton-reducing, acetogenic bacteria degrade higher fatty acids to acetate, H_2 and CO_2 which are subsequently used by the terminal organisms of the

anaerobic food chain, methane-producing bacteria. These methane-producing bacteria have been studied in a wide variety of environments.

Probably the most extensively studied methanogenic environments are the rumen and anaerobic sewage sludge digestors. Methanogenesis in these environments has been extensively reviewed (68,69,71,74,75, 87). Methanogenesis also occurs in sediments and soils and these environments are probably the most significant global source of methane (88). In these environments and others (gastro-intestinal tract, wetwood of trees and algal-bacterial mats), methanogenic bacteria are obligately linked to nonmethanogenic bacteria for the provision of substrates. This is true in all natural environments except in the case of anaerobic environments where methanogenic substrates are provided in emanating geothermal gases (53,178).

In natural environments methanogens readily form symbiotic associations with other anaerobic microorganisms. This accounts for the wide occurrence of impure cultures obtained in the past. This symbiotic association is well demonstrated by the "Omelianski symbiosis". Methanobacterium omelianski was thought to be a pure culture in the past, and, as such, was used for many biochemical and physiological studies. It apparently converted ethanol and CO_2 to acetate and methane. However, in 1967, it was shown by Bryant, et al.

(35) to be a symbiotic association between two microorganisms: a nonmethanogenic S-organism which oxidized ethanol to acetate and H_2 (eq. 1), and a methanogen, Methanobacterium strain MoH, which used the H_2 to reduce CO_2 to CH_4 (eq. 2). At pH 7.0:

S-organism $H_2O + CH_3CH_2OH \rightarrow CH_3COO^- + H^+ + 2H_2$ (eq. 1)

Methanobacterium strain MoH $H^+ + HCO_3^- + 4H_2 \rightarrow CH_4 + 3\ H_2O$ (eq. 2)

Growth of the S-organism on ethanol was greatly inhibited by the presence of H_2; however, when co-cultured with the methanogen, good growth occurred. The methanogen removed the "toxic" hydrogen and allowed the growth of the S-organism, while the S-organism produced H_2 and allowed the growth of the methanogen.

Since the resolution of the Omelianski symbiosis, much work has been done to elucidate the nature of the interaction between methanogens and other fermentative anaerobes. The work of Wolin and others (28,77,105,130,174) over the last decade has further clarified the concept of interspecies hydrogen transfer. Fermentative anaerobes generate reduced NAD (NADH) during the oxidation of organic substrates. In the absence of H_2-consuming species (methanogens), electrons from NADH are disposed of by the production of reduced end products such as ethanol, lactate, or propionate. When methanogens are present, carbohydrate-fermenting anaerobes produce increased

amounts of H_2 as a reduced end product. Acetate production increases and the production of other reduced end products such as ethanol or lactate is greatly reduced. The oxidation of NADH to produce H_2 is thermodynamically unfavorable if H_2 is present, but the reaction becomes increasingly favorable as the partial pressure of H_2 decreases (174). Methanogens consume H_2 as rapidly as it is produced and by keeping the partial pressure of H_2 very low, they make feasible the production of H_2 from NADH. If H_2 is the primary electron sink instead of reduced organic compounds, pyruvate (from glycolysis) is converted primarily to acetate (148) so that the nonmethanogen obtains more ATP when grown in the presence of a methanogen.

Weimer and Zeikus (161) reported a similar symbiotic interaction when Clostridium thermocellum was grown on cellulose in co-culture with M. thermoautotrophicum. Another interesting example of interspecies hydrogen transfer between sulfate-reducing bacteria and methanogens was described by Bryant, et al. (32). Desulfovibrio vulgaris and D. desulfuricans grew poorly on lactate in a low sulfate medium, but grew well when co-cultured with a methanogen. The sulfate reducers were able to grow in the absence of sulfate by using the methanogenic organism as an electron sink. Zeikus (178) has summarized that interspecies hydrogen transfer reactions result in the following changes in co-culture: i) increased substrate utiliza-

tion, ii) different proportions of reduced end products, iii) more ATP produced by the nonmethanogen, iv) increased growth of both symbionts and v) displacement of unfavorable equilibria.

Methanogens using methanol and acetate have also been shown to form symbiotic associations with other anaerobes. Zhilina and Zavarin (185) described several microorganisms which grew commensally with Methanosarcina in a methanol enrichment. The associates (nonmethanogens) were unable to grow in pure culture which indicated the existence of a symbiotic relationship between these microorganisms. Additionally, Mah and co-workers (98,159) have reported a stable interaction between Ms. barkeri and nonmethanogens in an acetate enrichment. The nonmethanogens could not use acetate as an energy source and it appeared that the nonmethanogens depended on the pseudosarcina for nutrient requirements.

It is generally assumed that in aquatic systems organic decomposition is limited by the rate of polymer degradation (164). Many workers (30,49,99,119) have concluded that organic biopolymer (e.g. cellulose) degradation limits the rate at which gas production occurs in anaerobic systems such as anaerobic waste digestors. As shown by Shea (131), in normal sludge digestion only about 3% of the total hydrogen-utilizing capacity of the hydrogen-oxidizing methanogens is utilized. He concluded therefore that methane production from CO_2

and H_2 can never be the rate limiting step in anaerobic digestion as has been claimed by others. McCarty (103) concluded that the decomposition of lipids and volatile acids appears to be the overall rate limiting step in gas production in sewage sludge digestors. Kaspar and Wuhrmann (85) state that in sewage sludge the limiting factor for complete anaerobic minerilization of biodegradable organic matter is found in the boundary conditions for the exergonic oxidation of propionate. Bryant (31) states the problem most clearly in that one can not separate any set of reactions from another in trying to determine the rate limiting step involved in gas production. He concludes that the rate limiting reactions in the methane fermentation (in sewage) often involve the degradation of fatty acids, but this depends on the efficiency of H_2 utilization. Indeed, it may be impossible to state conclusively that one reaction involved in organic decomposition limits the rate of gas production as all the reactions in complete anaerobic mineralization are intimately interconnected (31).

In other environments, the addition of several compounds has been shown to stimulate methanogenesis. Hydrogen was shown to stimulate methane production in marine sediments (113,115) and Winfrey, et al. (167) reported that in Lake Mendota sediments, methanogenesis was greatly increased by added H_2. Hungate (72) and Czerkawski, et al. (50) have shown that the amount of methane produced in the rumen is

proportional to the dissolved H_2 concentration. Acetate is not an important methane precursor in the rumen since it is drawn off for the energy needs of the animal (72). This was corroborated by the findings of Opperman, et al. (112) that only 2-2.5% of the rumen methane was derived from acetate. The methane precursors formate and acetate have been observed to stimulate methanogenesis and lactate also stimulated methanogenesis in Lake Vechten sediments (37).

In the past, much effort has been directed toward determining what the major precursors of methane are in various anoxic environments. Methanol is not considered to be a product of anaerobic decomposition and is not thought to be a methanogenic precursor in natural environments (80). Even though formate is a major product in anaerobic fermentations, it is not considered per se as an important methane precursor as it is readily cleaved to CO_2 and H_2 by a large number of anaerobic bacteria (178). In studies on the rumen, Hungate, et al. (76) concluded that formate was metabolized primarily by nonmethanogenic microorganisms.

It would appear then that H_2/CO_2 and acetate are the major in situ precursors of methane (31,98,172,175,178). Inhibition of methanogenesis with methane analogs (8,38,142,149), viologen dyes (173) or fluoroacetate (38) resulted in the accumulation of H_2. Other researchers have investigated the importance of acetate as a methane

precursor. Smith and Mah (136) determined that 73% of the methane produced in sludge was produced from acetate. Jeris and McCarty (80) reported that 70% of the methane produced in anaerobic sewage was derived from acetate. Cappenberg calculated that 75% of the methane evolved from Lake Vechten sediments was produced from acetate (38). Koyama (88) calculated that the methyl position of acetate accounted for 60% of the methane produced in rice paddy soils and that 20-30% of the methane produced was derived from CO_2. Belyaev and coworkers (13,78) have shown that in two Russian lakes most of the methane produced was derived from the methyl position of acetate. Winfrey and Zeikus (170) reported that CO_2 accounted for up to 41% of the methane formed in Lake Mendota sediments. It appears that the major precursor of methane (H_2/CO_2 or acetate) is determined by various conditions and relationships which vary from environment to environment. In this regard, some of the work in this study was directed toward determining the importance of acetate or CO_2 as methane precursors in algal-bacterial mats.

Cappenberg and others (40,41,42,169,170) have shown that acetate is also oxidized in sediment systems. He found that 2-^{14}C-acetate could be metabolized to $^{14}CO_2$ and that the amount of $^{14}CO_2$ evolved from 2-^{14}C-acetate increased with the addition of sulfate. This is an interesting observation because, in the past, sulfate-reducing

bacteria were not believed to use acetate as an electron donor (92, 148). Russian researchers (129,137) have shown that certain strains of Desulfovibrio can grow on acetate with added H_2 and CO_2. Sorokin (137) reported that acetate was used for biosynthetic purposes and not respired to CO_2. Badziong, et al. (1) observed the same phenomenon in that acetate was required only for cell carbon in strains of Desulfovibrio. Other recent work has shown how acetate might be respired to CO_2 in sediments. Pfennig and Biebl (120) isolated a new species of bacterium, Desulfuromonas acetoxidans, which could oxidize acetate to CO_2 using elemental sulfur as a terminal electron acceptor. Widdel and Pfennig (165) isolated a new species of Desulfotomaculum which coupled the oxidation of acetate to CO_2 with the reduction of sulfate to H_2S. CO_2 has also been shown to be produced from the methyl position of acetate by pure cultures of a methanogenic bacterium (182). Zehnder, et al. (176) isolated an acetate-decarboxylating, non-hydrogen-oxidizing methanogen and they speculated that some acetate may be completely oxidized to obtain the necessary reducing equivalents for cell biosynthesis.

Sulfate has been shown by several workers to inhibit methane production in sediments (37,93,100,169). It was demonstrated by Martens and Berner (100) that methanogenesis did not occur in marine sediments until sulfate was depleted. They speculated, as have

others, (48) that the inhibition may be due to competition for available hydrogen, or may be related to the relative free energy yield available from sulfate reduction versus carbonate reduction. Winfrey and Zeikus (169) showed that inhibition of methanogenesis in Lake Mendota sediment was due to competition for available substrates. Cappenberg (37) suggested that the inhibition of methanogenesis by sulfate may be due to the production of toxic levels of H_2S. His hypothesis was supported by the isolation of a sulfide sensitive methanogen from Lake Vechten (39). However, it has been reported that the accumulation of 6.25mM sulfide did not significantly affect methane production in digested sludge (see 98). Others (160,169) have also shown that sulfide accumulation did not affect methane production in algal-bacterial mats or lake sediments. It seems that sulfide may be inhibitory in some environments while having little effect in others. Interestingly, Oremland and Taylor (115) reported the concommitant activities of methane production and sulfate reduction in marine sediments, but the rate of methane production was considerably lower than in sulfate-free sediments. Since the work of Oremland and Taylor, other workers have also noted the concommitant activity of methane production and sulfate reduction (108,160,168). Ward (158) reported that methanogenesis occurred in the presence of about 20,000mg $SO_4^=$/liter in Great Salt Lake sediment.

Numerous other factors have been shown to influence methanogenesis in natural anoxic sediments. Temperature may often limit maximal rates of methane production. Zeikus and Winfrey (183) found that maximal methanogenesis occurred at 35-42 C, more than 10 C higher than the maximum in situ temperature. Cooney and Wise (49) found that there were two temperature optima for methanogenesis in thermophilic sewage sludge digestors, but that methane production was greatest at the higher temperature, 60 C. In the work of Cooney and Wise it appeared that there were two populations or strains of methanogens and the population that operated at 60 C was the more efficient.

Methanogenic bacteria are probably the strictest anaerobes known (98,172,178) and require low Eh values for growth to proceed. In rice paddies, methanogenesis was not observed until the Eh decreased to less than -250mV (89). Oremland and Tahlor (114) noticed diurnal fluctuations in methane levels in the rhizosphere of Thalassia testudinum and suggested the possibility of inhibition of methanogenic bacteria by oxidizing conditions. Nitrate has also been shown to inhibit methanogenesis in fresh water (47,93), marine sediments (5) and flooded soils (5,14). It was suggested by Bollag (14) that nitrate inhibition may be due to a rise in sediment Eh. Thus, the absence of reducing conditions might limit methanogenesis.

Systematic studies on the natural pH range for methanogens have not been reported. However, Van den Berg, et al. (153) reported a narrow pH range (pH 6-7.5) at which methanogenesis was maximal in waste digestors. Ward (157) reported that methanogenesis occurs in alkaline hot spring algal-bacterial mats at pH 8.5.

Enumeration of Methanogens

Many workers have estimated the numbers of methanogenic bacteria in anaerobic environments. In lake sediments workers found numbers of methanogenic bacteria that ranged from 10^2 to 10^7 cells per milliliter of sediment (11,12,13,37). These numbers are considerably lower than those reported for the rumen and digestor sludge. About 10^7 to 10^9 methanogens per milliliter have been reported in sludge (87,109,134) and in the rumen (75,117,134). Ward reported a maximum value of 10^8 methanogens per algal-bacterial mat subcore which roughly translates to 10^8 methanogens per cubic centimeter of a hot spring algal-bacterial mat (157).

Recently, techniques other than most probable number (MPN) estimates have been developed which may yield more accurate counts. Edwards and McBride (58) enumerated methanogens in sewage sludge using the UV fluorescence of F_{420}, a co-factor present in all known methanogenic bacteria. By counting fluorescent colonies, these workers obtained results comparable to those reported above. Mink

and Dugan (106) have similarly shown that methanogens can be tentatively enumerated in pure and mixed cultures based on their autofluorescing properties. Strayer and Tiedje (141) have used a fluorescent antibody specific for a strain of Methanobacterium to enumerate methanogens in lake sediments. They obtained counts at least an order of magnitude higher than with MPN techniques, but it must be remembered that the fluorescent antibody technique may count nonviable or moribund cells.

The Microbiology of Low Sulfate Hot Spring Algal-Bacterial Mats

The biology of natural hot springs has attracted the interest of scientists for many reasons over the years (21). Many surprising discoveries have been made over the past 15 years during a period Zeikus, et al. (181) referred to as the "golden era of thermophily".

One of the most interesting findings regarding microbial life at high temperatures was the report that the upper temperature limit for procaryotic microorganisms was not found in boiling springs (92 C) of Yellowstone National Park (15,16,25). Extreme thermophilic bacteria become macroscopically visible in the temperature range 88-75 C (17).

The upper temperature limit for photosynthetic life is reached as the effluent water from low sulfate, alkaline, siliceous hot springs cools to about 73 C (17,43,44). In the region below 74 C

to about 40 C, the growth of photosynthetic microorganisms results in the development of a thick (1-3cm) algal-bacterial mat. The mat is substantially reduced by metazoan grazing below about 40 C (166). In Octopus Spring, the photosynthetic components of the mat are a photosynthetic flexibacterium, Chloroflexus aurantiacus (123) and a cyanobacterium, Synechococcus lividus (104) which is embedded in the filament matrix of the flexibacterium. Another photosynthetic cyanobacterium, Mastigocladus laminosus can be found as a minor component in the algal-bacterial mat of some springs (Wiegert Channel in this study), and in some springs (43) it is the primary phototrophic component.

Chloroflexus is a good example of the unusual microflora unique to these hot springs. It is apparently related to the green sulfur bacteria by virtue of the presence of bacteriochorophyll c (122,123 124) and the presence of "chlorobium vesicles" (123). Also, Chloroflexus resembles the green sulfur bacteria as it has similar lipid chemistry (86), a similar G + C ratio (123) and has the ability to grow photoautotrophically using sulfide as an electron donor (45,94). On the other hand, Chloroflexus shares the property of anaerobic photoheterotrophic growth with the nonsulfur purple bacteria (96,124). Additionally, Chloroflexus resembles the cyanobacteria by virtue of similar carotenoid chemistry (66), gliding

motility and filamentous morphology (123).

Brock (20) has mentioned that these springs are good environments for ecological studies because they are essentially steady state systems with low species diversity. However, the recent isolation by Zeikus, et al. (181) of Thermoanaerobium brockii, the observation of heterotrophic bacteria, and the isolation of other novel bacteria from alkaline hot springs (26,79, unpublished results of this laboratory and personal communication - Steve Zinder) indicate that while species diversity may be low in terms of the major species which make up the mat, a variety of microorganisms reside within the algal-bacterial mat environment.

Most of the previous studies on the algal-bacterial mats of hot springs have been directed toward determining photosynthesis and production of the mat. The combined effect of sunlight and high temperature results in rates of photosynthesis as high as found anywhere in nature (20). In Octopus Spring, primary production of the mat is accomplished by both Synechococcus and Chloroflexus (57), but the provision of organic compounds for photoheterotrophic or heterotrophic growth of Chloroflexus by Synechococcus was suggested by Bauld and Brock (10). Indeed, in pure culture, Chloroflexus is a more vigorous photoorganotroph than photoautotroph (44). Photosynthesis in the mat is restricted by self-shading to the upper few

millimeters of the mat (9,19,57) and natural populations of the algal-bacterial mat were found to adapt to changes in light intensity (95).

Positive aerotaxis by the motile Chloroflexus in the dark was suggested by Doemel and Brock (57) as the mechanism for upward growth of the mat. Upward growth sometimes leads to the formation of raised mat structures which resemble precambrian algal or bacterial fossil stromatolites (55,155,156). In fact, Chloroflexus is a sediment trapping organism as are the stromatolite-forming blue-green bacteria (55). The laminations evident in a cored sample of the mat may be due to the differential migration of Chloroflexus in response to reduced light intensity or positive aerotaxis at night (55). Growth of the mat appears to be in balance with mat decomposition, but Doemel and Brock (57) speculated that there may be two rates of decomposition, occurring, that which is complete after 2-4 weeks and decomposition of more recalcitrant material which is complete after a year. Zinder, et al. (187) suggested that decomposition was most rapid in the top 3 millimeters of the mat, but was the main biological process below 3 millimeters. Rapid decomposition near the surface of the mat was suggested as a result of the lack of correlation between protein and thickness between inert carborundum layers after burial (57). In Octopus Spring, Ward (157) found maximal methano-

genesis near the mat surface. Additionally, Zinder, et al. (187) found maximal H_2S production near the mat surface. They also found that the production of volatile organic sulfur compounds in anaerobic decomposition was inhibited by light which was probably due to inhibition of anaerobic microorganisms by oxygen produced during cyanophyte photosynthesis. Ward (157) suggested that since organic matter accumulated below the upper layer of maximal mat decomposition, there might be limitations imposed on anaerobic decomposition at lower depths or a resistance of some organic components of the mat to anaerobic decomposition.

Brock and co-workers found that maximum primary production in the mat appeared between 48 and 59 C, but found a maximum standing crop between 55 and 60 C (18,23). Peary and Castenholz (118) found that "temperature strains" existed for Synechococcus and similarly, Bauld and Brock (9) found that photosynthesis by Chloroflexus in natural algal-bacterial mat samples was greatest at the environmental temperature where they were found. Brock and Brock (24) also showed that temperature strains exist for heterotrophic bacteria present in the mat. They found that for each temperature tested, the optimum for glucose incorporation was an experimental temperature similar to the environmental temperature of the sample.

Zeikus (178) isolated a H_2-using thermophilic methanogenic

bacterium similar to M. thermoautotrophicum from the algal-bacterial mat present in the effluent channel of Octopus Spring. The fact that M. thermoautotrophicum has an optimum for growth and methane production at 65-70 C (184) seems incongruous with the reported findings of Ward (157) that methanogenesis in these mats was maximal at 45-50 C. Accordingly, some of the experiments performed in this study addressed the problem of the temperature limitation of methanogenesis observed in these springs.

The purpose of this research was to examine in detail methane production in this environment because these algal-bacterial mat systems provided a natural high temperature environment in which anaerobic decomposition to methane could be observed. Specific objectives were i) to study the temperature relations and adaptation of the bacteria responsible for methane production and ii) to study carbon and electron flow to determine if substrate flow in these natural environments was similar to other anaerobic environments which have been studied extensively.

MATERIALS AND METHODS

Study Areas

The major research area used in this study was Octopus Spring, an alkaline hot spring (pH 8.0) located about 0.15 km SSE of Great Fountain Geyser in the White Creek drainage in Yellowstone National Park. At this spring, experiments were undertaken only in the southernmost effluent channel. Another research area used in this study was in a meadow, also in the Lower Geyser Basin of Yellowstone National Park, adjacent to Firehole Lake Drive. Springs in this meadow were collectively referred to as Serendipity Springs because of their chance discovery in early 1968 by M.L. and T.D. Brock (60). The study area in the Serendipity Springs group was a plywood channel (1.2 m wide x 24 m long) constructed by Dr. Richard Wiegert (Univ. of Georgia) by diverting the effluent of a spring so that it constantly flowed down the artificial channel. The pH of the piped in water ranged from 6.0 to 7.0 (60), depending on the concentration of the free CO_2. Three other springs in Yellowstone National Park were also investigated initially to study methane production versus temperature. Two of the springs, Twin Butte Vista and Mushroom Spring are located about 0.10 km SE and 0.13 km NNE of Great Fountain Geyser, respectively. The third spring is referred to as "West Thumb A". It can best be described as the first major spring located north of the West Thumb Geyser area that empties into Yellowstone Lake.

Only the northernmost effluent channel was sampled. All officially named springs in the Firehole Lake area are shown on a map in T.D. Brock's book, Thermophilic Microorganisms and Life at High Temperatures (22).

Sampling and Experimental Protocol

A) Sampling

Whole cores were removed from the algal-bacterial mat with a no. 4 brass cork borer (50.3 mm^2) and transferred directly to one dram glass vials (Kimble 14.5 x 45 mm) which were sealed anaerobically (73, except that no copper reducing column was used in the field) under a stream of 100% helium (Linde). Butyl rubber stoppers (A.H. Thomas, recessed butyl rubber stoppers, size 00) were used to effect a seal and keep the vials anaerobic during later manipulations. Anaerobically tubed samples were quickly placed in insulated coolers that contained water which was 5 C warmer than the in situ temperature. During transit to the laboratory (approximately 2 hours), samples cooled slightly but this procedure ensured that samples would be kept within 5 C of their indigenous temperatures. In the laboratory, samples were transferred to incubators that matched the in situ temperature (except in the case of temperature strain experiments where samples were incubated at several different temperatures). All additions were made from anoxic stock solutions at the time of sample

collection except in the case of some 2-^{14}C-acetate experiments where additions were made after returning to the laboratory.

B) Metabolism of Radioactively Labelled Compounds

The following processes were assayed as described below on anoxically tubed samples:

1. Primary Production

Replicate vials received 0.1 ml (2 µCi) of a 20 µCi/ml stock solution of $NaH^{14}CO_3$ (44.5 mCi/mmol, New England Nuclear) diluted in sterile anoxic distilled water (pH 8.0). The samples were incubated in the effluent channel for 1.5 hours and biological activity was terminated by the addition of 0.5 ml formalin. The addition of formalin was accompanied by extremely vigorous shaking to ensure that the formalin permeated the gelatinous sample. "Light" replicate vials had their stoppers taped only at the top so that upon incubation the core was exposed to sunlight. "Dark" replicates were taped lengthwise and wrapped in aluminum foil to exclude light during incubation. At the laboratory, a gas headspace subsample was removed for determination of the specific activity of CO_2. After acidification with 0.1 ml 50% sulfuric acid to ensure removal of ^{14}C-carbonate species, radioactivity was determined by homogenizing the core (teflon tissue homogenizer) and adding 0.1 ml of the homogenate to 10 ml Aquasol (New England Nuclear). A model LS 100-C liquid scintillation counter

(Beckman) was used to determine radioactivity. Radioactivity was measured on the $^{3}H + {}^{14}C$ window and the gain was set at 240. Correction for differences in counting efficiency were made by the automatic external standard method. The specific activity of CO_2 (dpm/nmole) was divided into the dpm in the cell fraction to convert results to moles of carbon fixed during the incubation. Duplicate vials were then averaged and the amount of CO_2 fixed in darkened vials was subtracted from the amount of CO_2 fixed in the light to give light stimulated primary productivity. The cores differed in length, and since activity is not proportional to length (157), all results were reported on a per core basis.

2. Metabolism of 2-^{14}C-Acetate

i) Replicate vials received 0.2 ml (0.2 µCi) of a 1 µCi/ml stock solution of 2-^{14}C-acetate (sodium salt, 44 mCi/mmol, New England Nuclear). Vials were taped lengthwise with masking tape to secure the stoppers. After two hours, biological activity was terminated as above. Analysis of gas headspace subsamples for $^{14}CO_2$, CO_2, $^{14}CH_4$ and CH_4 were made as described in the analytical methods section. Incorporated radioactivity was determined by filtering a 0.1 ml aliquot of a homogenized sample (teflon tissue homogenizer) diluted with 0.9 ml distilled water through a 0.45 µm membrane filter (Millipore). The filtrate of this mixture was retained in a

two dram glass vial (Kimble, 16 x 60 mm) and a 0.1 ml aliquot of the filtrate was used to determine unincorporated radioactivity. After the filtrate had been obtained, the filter was rinsed with 0.5 to 1.0 ml distilled water. When the filters had dried, they were exposed to concentrated HCl fumes overnight to remove any unincorporated radioactivity remaining on the filters. Headspace volume and liquid volume of each vial were determined by displacement with water so that results could be corrected to a per core basis. Additionally, pH was determined for correction of $^{14}CO_2$ (gas) to total $^{14}CO_2$ as described in the analytical methods section.

ii) Replicate vials received 0.1 ml (1 µCi) of a 10 µCi/ml stock solution of 2-^{14}C-acetate (sodium salt, 44.0 mCi/mmol, New England Nuclear). Vials were taped lengthwise as above. Light and dark replicates at 55 C were prepared as above. $^{14}CO_2$ and $^{14}CH_4$ were determined as described in the analytical methods section. Incorporated and unincorporated radioactivity were determined as above.

iii) Experimental vials were treated as above except replicate vials received 0.1 ml (1 µCi) of a 10 µCi/ml stock solution of 2-^{14}C-acetate (sodium salt, 44.0 mCi/mmol, New England Nuclear) after methanogenesis had been established (48 hours after coring). $^{14}CO_2$ and $^{14}CH_4$ were followed over time and determined as described in the analytical methods section. Incorporated and unincorporated radio-

activity were determined as above and measured 71 hours after the radiolabel had been added.

3. Metabolism of Tritiated Acetate

The study was performed in the same fashion as those experiments designed to determine the metabolism of 2-^{14}C-acetate, but only in Octopus Spring at 50 C. Replicate vials received 0.1 ml (2 µCi) of a 20 µCi/ml stock solution of ^{3}H-acetate (sodium salt, 2 Ci/mmol, New England Nuclear). Both short time incubations (3 hours) and long time course experiments (4 days) were performed. Gaseous subsamples were analyzed for $C^{3}H_4$ as described below. A parallel experiment was performed with material obtained from a dairy cow manure digestor (10 day turnover time, 8.8% solids, 86% volatile solids) to prepare $C^{3}H_4$ for use in ensuring its detection by the method described below. In other studies (unpublished results of this laboratory), it was determined that about 80% of the methane produced in this system came from the methyl group of acetate. By comparing the minimum and maximum peak responses to $C^{3}H_4$ (assuming a stoichiometric conversion of ^{3}H-acetate to $C^{3}H_4$ and that further incubation did not result in increased amounts of $C^{3}H_4$ -- this would maximize error), it was calculated that no more than 0.29% of the added 2 µCi of ^{3}H-acetate (in the algal-bacterial mat experiment) could have been converted to $C^{3}H_4$, or it would have been detected. Incorporated and unincorporated

radioactivity were determined as described above in the 2-^{14}C-acetate labelling experiments. Radioactivity was measured as above using only the ^{3}H window and gain of 270 to correspond to the gain setting used for quench curves of tritium.

4. Conversion of $NaH^{14}CO_3$ to $^{14}CH_4$

To determine the importance of CO_2 as a methane precursor, experiments were undertaken in which 0.1 ml (2 µCi) of a 20 µCi/ml stock solution of $NaH^{14}CO_3$ (44.5 mCi/mmol, New England Nuclear) was added to replicate vials. Radioactive CH_4 and CO_2 were followed over time as described below. Correction to nmoles CH_4 evolved from CO_2 was obtained by dividing total dpm $^{14}CH_4$ by the specific activity of CO_2.

The ratio, sp act CH_4/sp act CO_2 gives an indication of the importance of CO_2 as a methane precursor (80). It was found that over the initial incubation period, the ratio of the specific activities of $^{14}CH_4$ to $^{14}CO_2$ increased. To ensure that specific activity comparisons were made after the ratio appeared to level off (8 hours), vials were flushed (5 minutes) with 100% helium gas (Linde) that flowed through a heated copper column 24 hours after incubation. Subsequent readings were taken every few hours. To determine if the initial rise in the ratio of the specific activities of $^{14}CH_4$ to $^{14}CO_2$ was due to an increase in the importance of certain methane

formation reactions, an experiment was performed where 100% hydrogen (Linde) was added (0.1 ml) at the time of sampling and again at the time of flushing to see if this affected the ratio obtained. Also, acetate (0.1 ml to achieve a final concentration of 1mM in an estimated 2.5 ml sample) was added at sampling and flushing times to see if the ratio obtained was affected. To determine if the specific activity ratio obtained (which was always less than 1.0; 1.0 indicates 100% of the evolved methane was derived from CO_2 reduction) indicated error in measurements or isotopic preference for ^{12}C over ^{14}C, a parallel experiment was performed with pure cultures of methanogenic bacteria isolated as described below. Culture conditions were identical to those described later, but the headspace of culture tubes contained only H_2 and the only added methane precursor was CO_2 which was added in the form of $NaH^{14}CO_3$ (0.1 ml (2 μCi) of a 20 Ci/ml stock solution, 44.5 mCi/mmol, New England Nuclear).

C) Analytical Methods

1. Headspace Gases

Gas samples were removed from the headspace of vials using a helium flushed gas tight syringe. Hamilton syringes were used initially for methane production experiments. A glasspak syringe (Becton-Dickinson) attached to a mininert valve (Supelco) (to minimize loss of sample due to pressure differences) was used in later experiments.

Gas subsamples (0.2 ml) were analyzed by gas chromatography-gas proportion analysis. Concentrations were corrected to STP. For analysis of CH_4, $^{14}CH_4$, CO_2 and $^{14}CO_2$ a Carle model 8500 thermal conductivity gas chromatograph equipped with a 3.2 mm OD x 2.3 m (1/8 inch x 7.5 foot) stainless steel column packed with 80/100 mesh Poropak N (Supelco) was coupled to a Packard model 894 gas proportion analyzer. Helium make-up gas was added after combustion (at 750-800 C) in the gas proportion analyzer to increase total flow to 70 ml/minute so that the flow of propane quench gas through the gas proportion analyzer was an optimal percentage (10%) of the total flow. The gas chromatograph was operated isothermally at 50 C. This method of analysis for CH_4 and CO_2 and radioactivity in these gases was similar to the method reported by Nelson and Zeikus (110). Gas concentrations were calculated by comparison of peak area to that of standards using a Spectra-Physics model 4100 computing integrator. Radioactivity was calculated by comparison of peak area to the responses of standards to determine disintegrations per minute (based on standardization by liquid scintillation counting) using a Spectra-Physics Minigrator. C^3H_4 was detected in the same way, but with the gas proportional counter furnace turned off to prevent combustion of methane. The sensitivity to C^3H_4 was determined by preparing C^3H_4 from ^3H-acetate using contents of a dairy cow manure digestor as

described previously. The total amounts of CH_4 and $^{14}CH_4$ were calculated by comparison of subvolume to the gas headspace volume. Total amounts of CO_2 and $^{14}CO_2$ per vial were determined by correction for the difference between subsample and headspace volume, and also for gas solubility and dissociation equilibria according to Stainton (138).

More sensitive methane analysis was performed on a Varian 3700 series flame ionization gas chromatograph using a 3.2 mm x 1.83 m (1/8 inch x 6 foot) stainless steel column packed with 60/80 mesh Poropak Q (Supelco) with a helium carrier flow at 40 ml/minute and an isothermal oven temperature of 50 C. Injector and detector temperatures were 60 and 150 C, respectively. Gas concentrations were calculated by comparison of peak area to that of standards using a Spectra-Physics model 4100 computing integrator.

2. Autoradiograms

Autoradiograms of material incubated with 2-^{14}C-acetate were prepared after the method of Brock and Brock (16). A thin film of homogenate was smeared onto a precleaned glass slide (precleaned glass slides - VWR Scientific) and allowed to air dry. The slides were put through a series of five distilled water baths (one minute each) to remove any unincorporated radioactivity. Slides were then dipped for five seconds in photographic emulsion (Kodak NTB2) under a Kodak no. 2 safelight. Slides were exposed for about one month in total darkness

and then developed in total darkness with Kodak D-19 developer and fixed with Kodak fixer. Slides were examined using a Leitz Ortholux II microscope equipped for interference contrast optics. Photomicrographs were taken with a Nikon Microflex model EFM semi-automatic photomicrographic attachment at 500X using Kodak Panatomic-X film. Exposure time was about one second. Negatives were then enlarged to 5" x 7" prints on silver bromide print paper (Koda Bromide F-4) to achieve better contrast.

3. Other Methods

pH was taken in the field using colorpHast pH paper (MC/B Manufacturing Chemists, Inc.). pH was measured in the laboratory with a pH Master pH meter (VWR Scientific) and a glass combination electrode. Temperature was taken with a mercury thermometer.

D. Isolation and Culturing of Thermophilic Methanogenic Bacteria

1. Isolation

Enrichment, isolation and maintenance of a thermophilic methanogen was done in a medium (basal medium-BM) that contained the following per liter distilled water (all chemicals used were reagent grade): KH_2PO_4, 0.15g; Na_2HPO_4, 1.05g; NH_4Cl, 0.53g; $MgCl_2 \cdot 6H_2O$, 0.20g; cysteine-HCl, 0.5g; .0001g resazurin and 10 ml mineral elixir B. Mineral elixir B contained per liter distilled water: nitrilotriacetic acid, 1.5g; $FeCl_2 \cdot 4H_2O$, 0.3g; $MnCl_2 \cdot 4H_2O$, 0.1g; $CoCl_2 \cdot 6H_2O$,

0.17g; $ZnCl_2$, 0.1g; $CuCl_2$, 0.02g; H_3BO_3, 0.1g; Na molybdate, 0.01g. Mineral elixir B was prepared by adding the nitrilotriacetate to 200 ml distilled water and adjusting the pH to 6.5 with KOH. This solution was added to 600 ml distilled water at which time the remaining components of mineral elixir B were added in the order as they appear above. The volume was then brought to one liter with distilled water. The pH of the medium was adjusted to 9.2 so that the final pH was about 7.1 (±0.1) after all additions had been made. The medium was made anoxic by a modification of the Hungate Technique (73). The medium was boiled under 100% helium (Linde), but dispensed under 20% CO_2/80% H_2 (Linde) with a 5 ml repipet (L/I Repipet). Each roller culture tube (150 X 16 mm, Bellco) fitted with a butyl rubber stopper (A.H. Thomas, recessed butyl rubber stoppers, size 00) received 5 ml of medium. Roller culture tubes were autocalved in a tube press. $Na_2S \cdot 9H_2O$ (pH 13) was added after autoclaving to achieve a final concentration of 0.03%. All additions to the medium after autoclaving were made from sterile anoxic stock solutions. The basal medium (BM) was modified by adding 0.2% yeast extract and 0.2% trypticase for use in checking for heterotrophic contaminants (BM + TYE). A contaminant organism was isolated on BM + TYE (see Results) by dilution to extinction, and a portion of this "spent" medium that was rendered sterile by autoclaving and filtration through a 0.45

membrane filter (Millipore) was needed to supplement BM (BM + S) so that methanogenic bacteria could be isolated by dilution to extinction. Growth in the highest dilutions containing methane and blue-green autofluorescing cells was serially diluted repetitively until 1) all culture members exhibited similar morphology and fluorescence (the ability to autofluoresce was observed in a Leitz Ortholux II Microscope equipped with vertical illumination with ultraviolet light from an HBO-200W mercury lamp that passed through a Leitz B cube excitation and emission filter combination), and 2) inoculation into BM + TYE with a helium atmosphere yielded no growth. Since methanogenic bacteria have been shown to resist certain antibiotics (2), the isolation of the thermophilic methanogens by dilution to extinction was carried out with the sodium salt of either penicillin G (Eli Lilly), or ampicillin (Sigma) added to each dilution to reach a final concentration of 300 µg/ml of medium. Isolates were obtained at 50, 55, 60, and 65 C using Octopus Spring algal-bacterial mat obtained at each of those temperatures as inoculum. To determine if growth could occur on formate, acetate, or methanol, BM + S was used with a helium atmosphere. Sodium formate, sodium acetate or methanol (sterile anoxic stock solutions) were added to reach a final concentration of 1.0%.

2. Temperature Strain Experiments

Temperature strain experiments were performed with the

isolates using BM + S to parallel temperature strain experiments done with natural mat material. Each isolate was grown in duplicate at 50, 55, 60, 65, 70, 75 and 80 C and methane production was followed over time. Each experimental tube received a 0.1 ml inoculum from a turbid culture which had been pregrown in BM + S at the temperature at which it had been isolated. In addition, each isolate was grown in duplicate at the temperatures listed above and optical density was measured after 72 hours relative to an uninoculated blank on a Varian model 635 or a Gilford model 250 spectrophotometer at 660 nm (1 cm light path). Tubes analyzed for optical density were flushed daily with 20% CO_2/80% H_2 and given (via a 10 ml glasspak syringe fitted with a mininert valve) about 2.5 final atmospheres of the CO_2/H_2 gas mixture. Stoppers were held in place by tape in tubes used for optical density determinations. After 48 hours, tubes used for optical density determinations were supplemented again with 0.05 ml of a sterile anoxic 3% $Na_2S \cdot 9H_2O$ solution (because sulfide was presumably lost through daily gas headspace flushings).