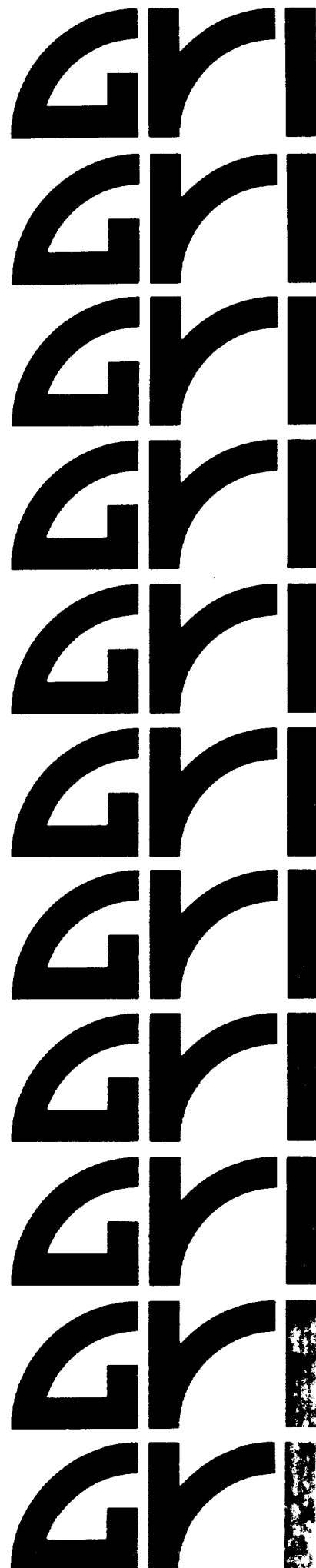


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MICROBIOLOGICAL STUDIES TOWARDS OPTIMIZATION OF
METHANE FROM MARINE PLANT BIOMASS

FINAL REPORT
(JULY 1979-JUNE 1982)

**Gas Research Institute
8600 West Bryn Mawr Avenue
Chicago, Illinois 60631**



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METHANE FROM MARINE PLANT BIOMASS

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(JULY 1979-JUNE 1982)

Prepared by
J. G. Ferry and J. S. Chen

Virginia Polytechnic Institute and State University
Blacksburg, Virginia 24061

For
GAS RESEARCH INSTITUTE
Contract No. 5014-363-0178

GRI Project Manager
Kathleen Locke
Basic Research Division

July, 1982

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RESEARCH SUMMARY

Title Microbiological Studies Towards Optimization of Methane from Marine Plant Biomass

Contractor Virginia Polytechnic Institute and State University

GRI Contract Number: 5014-363-0178

Principal Investigator J. G. Ferry

Report Period July 1979-June 1982
Final Report

Objective To reach a better understanding of the basic microbiology and biochemistry of the conversion of marine biomass to methane by anaerobic microorganisms.

Technical Perspective Greater than 90 percent of the energy in degradable biomass is recovered in the methane produced by bioconversion. Development of methods to improve the rate and reliability of the process depend on a better knowledge of the microorganisms and their biochemical pathways. This project has characterized anaerobic bacteria that convert marine biomass to methane and advanced our understanding of enzymes that catalyze these conversions. As California giant kelp was proposed as a potential energy crop, this study focused on its conversion to methane.

Results Laboratory-scale fermentors that converted kelp to methane were studied. The two major complex kelp constituents, alginate and mannitol, were degraded concurrently by microorganisms present in the fermentors. The kelp constituents were degraded to the simple compounds acetate, formate and hydrogen that were further converted to methane by the methane-producing bacteria.

Bacteria responsible for degradation of kelp constituents were isolated and studied in pure culture. The conditions for growth and optimum product formation were determined. Several isolates produced acetate as the main product of alginate degradation. Some of the isolates produced propionate and succinate from mannitol and alginate. These products are further metabolized to acetate and hydrogen gas by a separate type of anaerobic bacteria. Several methane-producing bacteria that utilize acetate were isolated. The conditions for growth and optimum substrate utilization were determined. One of the isolates is a novel marine organism Methanococcoides methylutens.

The conversion of formate to methane by methanogenic bacteria was studied. Formate dehydrogenase, an enzyme that catalyzes the utilization of formate, was isolated from the methane-producer Methanobacterium formicicum and studied in detail. The enzyme contains molybdenum in the active site. The molybdenum is contained in a small molecule not previously known to occur in methane-producing bacteria.

Technical
Approach

Stabilized laboratory-scale fermentors that converted kelp to methane were established with seed organisms from a marine sediment situated below a kelp bed. Quantification of compounds produced from the degradation of kelp was facilitated by development of a procedure for their detection in sea water medium. The fermentors were used as a source for the isolation of organisms specific for kelp conversion. Other kelp-degrading organisms were selected from the Anaerobe Laboratory culture collection. The use of sodium azide in buffer systems decreased the oxygen sensitivity of the formate dehydrogenase and allowed purification by conventional anaerobic column chromatography.

Project
Implications

As part of GRI's Basic Research program in Biomass, this grant addresses the conversion of biomass to methane by methanogens, a special class of microorganisms that occur in nature in highly complex communities. GRI is interested in biological conversion of biomass because the process produces only methane and carbon dioxide, is not adversely affected by high water content in the biomass, and can take place at ambient temperatures. At present, however, the physiology and biochemistry of methanogens are poorly understood.

This grant advanced our understanding of the biochemistry and nutritional requirements of the hydrogen and formate utilizing microorganisms. These organisms are important because they may be involved in the hydrogen buildup which frequently precedes digester failure.

A better method to analyze the intermediate products of the biological conversion process is a significant accomplishment. This method could be used to correlate substrate variations with methane yield and digester efficiency. This would lead to understanding the rate-limiting steps which would help in the development of more effective and reliable digestors.

GRI intends to continue support of this important area. Further studies will address the biochemistry of the acetate-utilizing methanogens.

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I. Introduction

A. Overall Project Objective. Conversion of biomass to methane is accomplished by a microbial food chain comprised of at least three metabolic groups of anaerobic microorganisms. The first two metabolic groups degrade biomass to hydrogen, formate and acetate which are the only substrates utilized by the third metabolic group, the methanogens. The overall objective of this research is a better understanding of the basic microbiology and biochemistry with the view to improve the rate and reliability of this process.

This objective can be further divided into three specific areas: (1) the isolation and characterization of methylotrophic (primarily acetate-degrading) methanogens, (2) a better understanding of the biochemistry of hydrogen and formate metabolism in methanogens, and (3) the identification of intermediates in the conversion of marine plant biomass to methane and the isolation and study of organisms responsible for their production.

B. Brief Description of the Project.

Laboratory-scale fermentors that converted kelp to methane were studied. The two major complex kelp constituents, alginate and mannitol, were degraded concurrently by microorganisms present in the fermentors. The kelp constituents were degraded to the simple compounds, acetate, formate and hydrogen that were further converted to methane by the methane-producing bacteria.

Fermentative bacteria that metabolize mannitol to produce H_2 , CO_2 , formate and acetate were obtained from the VPI Anaerobe Laboratory culture collection. These organisms were examined for their ability to ferment alginate; however, none of them were good alginate-utilizers. The product pattern of these organisms was evaluated with mannitol as the substrate, and the tests were performed under different growth conditions including a variation in hydrogen partial pressure.

Major efforts were then devoted to the isolation of alginate-fermenting bacteria from nature. We have obtained both facultative and obligate anaerobes that produce methanogenic substrates as their main products from alginate. All of them have been identified to the genus level, and several have been identified to the species level. Selected strains of alginate-fermenting bacteria were studied under different growth conditions for an evaluation of their productivity of useful compounds. Most isolates were found to produce acetate as the main product from alginate. Some alginate-fermenting bacteria are able to ferment mannitol, too, and paired cultures were tested for the simultaneous utilization of alginate and mannitol.

A low potential electron carrier was purified to near homogeneity from an alginate-fermenting organism, Bacteroides ovatus. It is a ferredoxin-type molecule even when the organism was grown in media low in iron. The molecular properties of the purified electron carrier have been studied.

Methane-producing bacteria that utilize acetate were isolated. The conditions for growth and optimum substrate utilization were determined. One of the isolates is a novel marine organism Methanococcoides methylutens.

The conversion of formate to methane by methanogenic bacteria was studied. Formate dehydrogenase, an enzyme that catalyzes the utilization of

formate, was isolated from the methane-producer Methanobacterium formicicum and studied in detail. The enzyme contains molybdenum in the active site. The molybdenum is contained in a small molecule not previously known to occur in methane-producing bacteria.

C. Rationale. Acetate is the major methanogenic substrate in the conversion of biomass to methane and its metabolism is a rate limiting step. However, relatively few of the methylotrophic methanogens have been isolated which has made this area of research difficult. We chose to isolate and characterize the marine methylotrophic methanogens to bolster the methylotrophic methanogen culture collection and identify superior strains to be used in future microbiological and biochemical studies. We chose the marine environment because this is unexplored as a source of methylotrophic methanogens and the project emphasized marine biomass.

The conversion of hydrogen and formate by methanogens is important because removal of these fermentation products facilitates the initial decomposition of biomass. We focused on formate because less was known than for the metabolism of hydrogen by methanogens. The biochemistry (specifically the purification) of formate dehydrogenase was chosen for study to: (1) increase our understanding of cofactor requirements in methanogens, (2) begin studies on the electron transport and energy conserving pathways, and (3) lay groundwork for future genetic studies of methanogens.

Because marine plant biomass, specifically the California giant kelp, was proposed as a potential energy crop for methane production, it was apparent that organisms which could convert kelp-ingredients into methanogenic substrates would be needed for a study on the microbiology of methane production from marine plant biomass. As carbohydrates constitute 85% of total organic matter of giant kelp (Macrocystis pyrifera) while alginate (42%) and mannitol (34%) together constitute 75% of total carbohydrates, we chose to study the alginate- and mannitol-fermenting organisms that produce hydrogen and acetate as their main products. Because there were no readily available cultures of alginate-fermenting bacteria for this study, we undertook the task of isolating such organisms from nature and characterized the relevant properties of these organisms.

D. Projected Benefits to the Gas Consumer.

Studies on methylotrophic methanogens will lead to faster rates of acetate conversion to methane. A better understanding of the microbiology of hydrogen and formate metabolism will make the conversion of biomass to methane more reliable. Increased rates and reliability will decrease capital costs of methane from biomass and ensure low priced continuous supplies of methane from marine and other biomass. The biochemistry of formate dehydrogenase will lay groundwork for a "second generation" of research efforts which will include genetic studies to further improve the process above that achieved by microbiological and biochemical manipulations.

The isolation and study of organisms that ferment alginate and mannitol is necessary if a defined bacterial population will be used to convert kelp into simpler chemicals to be used by the methanogenic bacteria. If the process should be developed for commercial production of methane, the gas consumers would benefit from the reliability of the process to which this study has contributed.

II. Technical.

A. Work Plan. The microbiological part of this proposal emphasized the conversion of marine biomass to methane; therefore, we investigated anaerobic marine methanogenic habitats. Also, the marine environment was chosen because this is unexplored as a source of methylotrophic methanogens. Inocula was obtained from marine canyons filled with decaying kelp and eel grass. The samples were obtained by a diver in cooperation with the Scripps Institute of Oceanography. Methanogenic kelp enrichment cultures were maintained in the laboratory and this was the source of all isolates and microbiological studies on mixed cultures. Our first task was to measure intermediates produced by fermentatives that are further converted to methane. Existing methods for the measurement of volatile fatty acids in sea-water medium were unacceptable; therefore, an improved method was developed.

Our approach to the isolation of methylotrophic methanogens was the use of the strictly anaerobic roll tube method using the substrates methanol, methylated amines and acetate. Methanol and methylamines were chosen because methylotrophic methanogens grow best on these substrates.

We planned to follow the ability of FDH to reduce the natural electron acceptor F_{420} during purification to determine the electron carriers and cofactors required for F_{420} reduction. In this way, we anticipated to delineate that portion of the electron transport chain closely linked to FDH. We discovered early on that it was necessary to develop new methods for the isolation of formate dehydrogenase (FDH) from M. formicicum because the FDH was extremely oxygen sensitive. Later this problem was overcome by the use of sodium azide in the buffers used for column chromatography.

Initially, the H_2 - and acetate-producing bacteria were to be obtained from the culture collection of the VPI Anaerobe Laboratory. The selected organisms would be tested for their efficiency to ferment alginate and/or mannitol under conditions relevant to the objective of the project, i.e., to produce methane from marine plant biomass. Therefore, salt tolerant anaerobes are desirable, and those organisms that can grow in a simple medium should also be useful. Because a quantitative approach was adopted for the evaluation of fermentation efficiency, appropriate methods had to be developed for the measurement of substrate utilization and product formation.

Information in the literature indicated that the availability of iron and the hydrogen partial pressure were two factors most likely to affect the production of hydrogen gas and acetate by anaerobic bacteria, we therefore studied the effect of these factors on selected organisms. When the availability of iron affects the fermentative pattern, such as a shift from acetate production to lactate production in low-iron medium, the kind of electron carrier found in the cell is expected to change, too. For example, the ferredoxin-type low potential electron carrier may disappear as it does not have a role in lactate production. Our plan was to examine the low-potential electron carrier and the fermentation pattern of selected alginate- and mannitol-fermenting organisms grown in low-iron medium. The effect of hydrogen partial pressure was tested by periodic, continuous, or no removal of the gas phase, which contained hydrogen gas produced by the culture. The growth rate and the product pattern were compared.

As it is desirable to have simultaneous utilization of alginate and mannitol in the fermentation, mixed populations were tested for their performance in media containing both alginate and mannitol.

B. Work Performed and Results.

1. Characterization of kelp-degrading methanogenic enrichment cultures.

A sensitive and reliable method for measurement of volatile fatty acids in sea water medium resulted in the identification of the intermediates and their pool sizes at various times after feeding kelp. The results are tabulated below:

Intermediate	Pool size (mM)		
	1 day	6 days	20 days
formate	0.1	0.05	0.06
acetate	1.3	6	0.1
propionate	0.4	2.5	2.2
butyrate	0.7	1.3	0.1
ethanol	0.2	-	-
succinate	4.5	4.5	4.2
dissolved H ₂	1.2 x 10 ⁻³	5 x 10 ⁻⁸	5 x 10 ⁻⁸

The times indicated are days after addition of frozen chopped kelp to the methanogenic kelp-degrading cultures. Succinate appeared in the highest concentration and was last to subside as the conversion of kelp to methane progressed. Ethanol production peaked two days after the addition of kelp and decreased rapidly to below detectable levels. The appearance and disappearance of ethanol correlated with dissolved hydrogen concentrations which ranged from 50 nM to 1.2 μM. The relationship between ethanol and hydrogen suggests that interspecies hydrogen transfer functions to regulate the proportion of reduced fermentation products. The volatile fatty acids which appeared in the highest concentrations were acetate and propionate.

2. Isolation of hydrogen-and formate-utilizing marine methanogens.

Sea-water media that contained either hydrogen or formate were inoculated with kelp-degrading enrichment cultures. Several strains of hydrogen- and, or, formate-utilizing methanogens were isolated by the use of anaerobic roll tubes. Morphologically, most of the strains resemble Methanospirillum hungatei or Methanobacterium formicicum. In addition, a new morphotype was isolated which may also represent a new genus. Each isolate is salt tolerant and will be useful in reconstitution of defined food chains.

3. Isolation and characterization of methylotrophic methanogens.

Sea-water media that contained methylotrophic substrates were inoculated with the kelp-degrading enrichments. Several morphological types were enriched with the various substrates. The acetate-degrading enrichments revealed morphotypes resembling Methanosarcina mazei and Methanobacterium soehngenii. Strains of M. mazei were obtained in pure culture and shown to be salt tolerant. Cultures of M. soehngenii contained minor contaminants but culture conditions were determined for optimum growth and maintenance of the cultures. All of the acetate-degrading methanogens are maintained in our culture collection and are available for future research.

A new genus of methylotrophic methanogen was isolated from the methylamine enrichments Methanococoides strain TMA-10 was studied in detail. Cells of strain TMA-10 were irregular cocci with an average diameter of 1.0 μm . Larger cells, 2-3 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 TMA-10 stained Gram negative, thin-sections revealed the absence of an outer membrane typical of a Gram-negative cell wall. 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 dodecylsulfate (final concentration, 0.01%) or Triton X-100 (final concentration, 0.001%) to the medium. These characteristics are similar to those of the marine genera Methanococcus and Methanogenium which contain protein cell walls. Motility in strain TMA-10 was not observed and electron micrographs of negatively stained cells revealed the absence of flagella and pili.

Growth experiments were performed in 16 x 150 mm culture tubes sealed with butyl rubber stoppers and secured with an aluminum crimp collar. Growth was followed spectrophotometrically at 550 nm with a Bausch and Lomb Spectronic 20. Growth and methanogenesis was supported by the methylotrophic substrates trimethylamine, dimethylamine, methylamine or methanol. Strain TMA-10, previously grown with trimethylamine, did not grow on or produce methane from $\text{H}_2:\text{CO}_2$ (80:20), sodium formate (0.5%), sodium acetate (0.5%), or calcium acetate (0.5%) 90 days after inoculation. The maximum doubling time obtained with medium that contained trimethylamine was 5.2 h. 0.1% (w/v) yeast extract, 0.1% (w/v) Trypticase, 10% (v/v) rumen fluid, or 1% (v/v) B-vitamin solution stimulated growth of strain TMA-10. The optimal growth temperature was 30-35°C and no growth occurred at 40°C. The effect of NaCl was determined by adjusting the NaCl concentration of the medium. The optimal growth rate was between 0.2 M and 0.6 M NaCl. No growth occurred in the absence of NaCl or at 1.2 M NaCl. 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 sea water. MgCl_2 could be substituted for MgSO_4 but MnSO_4 , CoSO_4 , NiSO_4 or FeSO_4 did not substitute for MgSO_4 .

Cell-free extracts 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 in Methanosarcina barkeri strain 227. Hydrogen- or formate-dependent reduction of coenzyme F_{420} by cell-free extract was not detected. Strain TMA-10 also contained coenzyme M (R. White, personal communication).

The polar lipid fraction of M. methylutens consisted of 2,3-diphytanyl glycerol diethers in addition to an unknown glycerol ether component (T. Langworthy, personal communication). Dibiphytanyl diglycerol tetraethers were not apparent. The mol percent G+C was 42%.

4. Microbiology of formate utilization by methanogens. The kinetics of formate metabolism in Methanobacterium formicicum and Methanospirillum hungatei were studied with log phase formate-grown cultures. The progress of formate degradation was followed by the formyltetrahydrofolate synthetase assay for formate and fitted to the integrated form of the Michaelis-Menten equation. The K_m and V_{max} for Methanobacterium formicicum were 0.58 mM formate and 0.037 mol formate $\text{h}^{-1} \text{g}^{-1}$ (dry weight). The lowest concentration of formate metabolized by Methanobacterium formicicum was 26 μM . The K_m and V_{max} for Methanospirillum hungatei were 0.22 mM and 0.044 mol formate $\text{h}^{-1} \text{g}^{-1}$ (dry weight). The lowest concentration of formate metabolized by Methanospirillum hungatei was 15 μM .

The apparent K_m for formate by formate dehydrogenase in cell-free extracts of Methanospirillum hungatei was 0.11 mM. The steady-state of formate concentrations in sewage sludge (0.017 to 0.13 mM) and kelp-degrading methanogenic enrichments (0.029 to 0.19 mM) suggests that formate could be directly metabolized by methanogens in these habitats. Also, degradation of unusual substrates or increased substrate loading may produce formate concentrations much greater than are reported for the steady-state. The K_m values for formate uptake by formate-grown isolates from each habitat are needed^m to draw conclusions.

The K_m and H_2 uptake by cultures of Methanobacterium formicicum was 6 μ M dissolved H_2^m . Formate and H_2 were equivalent electron donors for methanogenesis when both substrates were above saturation; however, H_2 uptake was severely depressed when formate was above saturation and the dissolved H_2 was below 6 μ M as shown below.

H_2 (μ M)	Total CH_4 production (mmol/h per g [dry wt])	H_2 uptake (mmol/h per g [dry wt])	CH_4 production ^b supported by H_2	
			(mmol/h per g [dry wt])	(% of total)
106	16	37	9.3	58
15.5	17	32	8.0	47
7.2	20	29	7.3	37
1.7	17	13	3.3	19
0.9	15	6	1.5	10
0.5	12	4	1	8

^aThe formate concentration was 48 mM.

^bValues were calculated with the assumption that 4 H_2 are consumed per CH_4 produced. The balance of the total CH_4 is assumed to be supported by formate.

It is interesting to postulate that nonformate-utilizing methanogens are the primary organisms which maintain low dissolved H_2 concentrations in anaerobic habitats when the formate concentration is greater than the K_m for formate uptake of formate-utilizing methanogens. The depression of H_2 -supported methanogenesis by saturating formate is also interesting because it implies a form of substrate regulation of metabolism.

5. Biochemistry of methanogenesis. Soluble formate dehydrogenase from Methanobacterium formicicum was purified 71-fold with a yield of 35%. Purification was performed anaerobically in the presence of 10 mM sodium azide which stabilized the enzyme. The purified enzyme reduced, with formate, 50 μ mol of methyl viologen per min per mg of protein and 8.2 μ mol of coenzyme F_{420} per min per mg of protein. The apparent K_m for 7,8-didemethyl-8-hydroxy-5-deazariboflavin, a hydrolytic derivative of coenzyme F_{420} , was 10-fold greater (63 μ M) than for coenzyme F_{420} (6 μ M). The purified enzyme also reduced flavin mononucleotide ($K_m = 13 \mu$ M) and flavin adenine dinucleotide ($K_m = 25 \mu$ M) with formate, but did not reduce NAD^+ or $NADP^+$. The reduction of $NADP^+$ with formate required formate dehydrogenase, coenzyme F_{420} , and coenzyme F_{420} : $NADP^+$ oxidoreductase.

The formate dehydrogenase had an optimal pH of 7.9 when assayed with the physiological electron acceptor coenzyme F₄₂₀. The optimal reaction rate occurred at 55°C. The molecular weight was 288,000 as determined by gel filtration. The purified formate dehydrogenase was strongly inhibited by cyanide ($K_1 = 6 \mu\text{M}$), azide ($K_1 = 39 \mu\text{M}$), α, α -dipyridyl, and 1,10-phenanthroline. Denaturation of the purified formate dehydrogenase with sodium dodecyl sulfate under aerobic conditions revealed a fluorescent compound. Maximal excitation occurred at 385 nm, with minor peaks at 277 and 302 nm. Maximal fluorescence emission occurred at 455 nm. The excitation and emission spectra of this cofactor are distinct from the spectra of all fluorescent cofactors previously reported in methanogens.

Purified formate dehydrogenase from *M. formicicum* was also examined by EPR spectroscopy. The air oxidized enzyme exhibited no EPR signals. $\text{S}_2\text{O}_4^{2-}$ reduction produced several paramagnetic species. The 10K spectrum showed a major species ($g_{\text{av}} = 1.971$) typical of Fe/S cluster(s). At 193K, a rhombic spectrum was observed ($g_{\text{av}} = 2.005$) which showed hyperfine coupling to at least one ^1H and a broad hyperfine pattern at high field. Simulations yielded g-values of 2.018, 1.003 and 1.994 with coupling constants to 2 equivalent ^1H of $A_1 = 4.5\text{G}$, $A_2 = 6\text{G}$ and $A_3 = 5.5\text{G}$. The 193K signal was found in formate-reduced whole cells using manual mixing or freeze-quench methods. Reduction in D_2O showed the ^1H were exchangeable. CN^- or N_3^- addition resulted in changes in g-value ($G_{\text{av}} = 1.988$). $^{13}\text{CN}^-$ or $^{15}\text{C}^{15}\text{N}^-$ gave spectra identical to $^{12}\text{C}^{14}\text{N}^-$. Cells grown in the presence of $^{95}\text{MoO}_4^{2-}$ produced additional splittings and increased amplitude of the high field hyperfine pattern. The signal and FDH activity was absent in whole cells grown in the presence of WO_4^{2-} . These data allow assignment of the 193K species to Mo^{V} . The anomalous g-values indicate a significant difference between the Mo center in this enzyme and that of other Mo-containing proteins.

6. Selection and characterization of alginate- and mannitol-fermenting bacteria for the production of methanogenic substrates.

a. Mannitol-fermenting bacteria. Among the mannitol-fermenting bacteria, strains of *Clostridium sphenoides* and *Clostridium sartagoformum* produce hydrogen gas and acetate as the main products. Some strains also produce formate and butyrate as additional products. The levels of acids produced are below 20 mM when the organisms were grown in a peptone-yeast extract-based medium containing 1% (w/v) mannitol. When mannitol was replaced by alginate (1%, w/v), some growth could be observed while the peptone-yeast extract basal medium supported no significant growth. The amount of acids produced in the alginate-containing medium was not significantly higher than what was obtained in the basal medium containing no additional carbohydrates. It was decided that efficient alginate-fermenting bacteria would be more readily obtained by isolation with the enrichment culture technique.

b. Isolation and identification of alginate-fermenting bacteria. An anaerobic liquid medium containing alginate as the sole organic substrate was used in setting up the enrichment cultures, and a total of six enrichment cultures was established. The cultures were maintained at room temperature (ca. 20°C) and were transferred into fresh liquid medium [16% (v/v) inoculum] on the sixth day following original enrichment. Periodic samples showed that acetate was the early major product in all cultures, with formate increasing in older cultures. Propionate and butyrate were also produced in some cultures. Standard microbiological methods were then applied under anaerobic conditions for the isolation of organisms from isolated colonies formed on agar medium. The isolates were further grown up in liquid medium and re-isolated from agar medium to establish the purity of the isolates.

Fourteen pure cultures representing different colony types were obtained from this experiment. Among these 14 alginate-fermenting bacterial strains, eight are anaerobes and six are facultative anaerobes. Detailed biochemical characterization was carried out with all strains for their identification.

The facultative anaerobes were found to be members of Klebsiella and Citrobacter. With the collaboration of Drs. W. E. C. Moore and L. V. Holdeman of this department, the anaerobes were found to be Bacteroides ovatus, Clostridium clostridiiforme and a previously undescribed species of Cytophaga. All strains produced acetate and CO₂ as the main products from alginate. Some strains also produced H₂ as a main product. Other minor products include lactate, propionate and succinate. The facultative strains could also ferment mannitol and one of them produced formate, too.

One anaerobe (Bacteroides ovatus strain 1S2a2) and one facultative anaerobe (Citrobacter sp. strain 5E2) were selected for additional studies on the "kinetics" of alginate utilization and product formation. The results are presented in Tables 1 and 2. The common features of these two organisms are their ability to produce acetate to a level of 70-80 mM when over 85% of the added alginate is fermented. Lactate and/or succinate are produced to a much lower level (less than 10 mM).

Table 1. Fermentation of alginate by an anaerobe (Bacteroides ovatus) [pH 6.8, 34°C].

Time of Incubation (hours)	Alginate (mM)	Acetate (mM)	Succinate (mM)
0	63	0	0
25	57	20	3.5
30	44	30	6.0
40	14	53	7.0
50	8	72	9.0

The medium also contained (per liter): peptone, 0.5 g; pepticase, 0.5 g; and yeast extract, 1 g.

Table 2. Fermentation of alginate by a facultative anaerobe (Citrobacter sp.) [pH 6.8, 34°C].

Time of Incubation (hours)	Alginate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
0	68	0	0	0
20	37	33	2	2
30	15	70	8	3
40	10	80	10	3

The medium also contained (per liter): peptone, 0.5 g; pepticase, 0.5 g; and yeast extract, 1 g.

c. Growth of Bacteroides ovatus strain 1S2a2 under different conditions.

Growth in defined medium. A defined medium was developed which included the basal medium of Varel and Bryant [Appl. Microbiol. 28:251-257 (1974)] with the addition of methionine (6.4 mg/l) and potassium phosphate (16 mM final concentration, pH 7.1). Either glucose or alginate was the carbon source. B. ovatus 1S2a2 showed consistent growth in these two media after five serial transfers. The results indicate that B. ovatus 1S2a2 does not have a complex nutritional requirement. However, the growth rate was significantly improved by the addition of pepticase or yeast extract.

Effect of hydrogen partial pressure. B. ovatus produces hydrogen gas as a major product. B. ovatus was grown in either sealed culture tubes or in flasks that were continuously sparged with N₂ to remove gas products. The fermentation pattern of the two types of cultures was compared and no difference was observed. Similar results were also observed with Clostridium pasteurianum, a hydrogen-producing anaerobe. The mannitol-fermenting organisms, C. sphenoides and C. sartagoformum, were tested by a different method: the cultures were grown in sealed tubes but the excess gaseous products were released daily up to six days in one set of tubes while the other set of tubes was kept sealed throughout the experiment. Again, the fermentation pattern was the same for the two sets of cultures. The results suggest that metabolism of these fermentative organisms is not very sensitive to variations in hydrogen partial pressure. A recent report [Lobos, Lamed and Su. Abstr. Annu. Meeting Am. Soc. Microbiol. 018. 1982] suggests that in static cultures of H₂-producing organisms, H₂ may become supersaturated in the culture medium and a shift in fermentation pattern can then be observed. It was also reported that a hydrogen partial pressure of 2 atm was required to show an effect in a stirred culture. Therefore, in a stirred fermentor with a mixed population of bacteria, the hydrogen partial pressure may not be a significant factor pertaining to the fermentation pattern of the H₂-producing bacteria.

Effect of heme. Dr. Wilkins and his coworkers of this department [Sperry, Appleman and Wilkins. Appl. Environ. Microbiol. 34:386-390. 1977] reported that Bacteroides ovatus had an absolute requirement for heme for growth in defined media containing glucose as the carbon source. Heme is apparently needed for the synthesis of a cytochrome(s) which is involved in the formation of succinic acid (an undesirable product for this study). We have examined the fermentation pattern of B. ovatus 1S2a2 in an alginate medium containing 5, 1, 0.1 and 0.01 µg/ml of heme. The cultures were passed through each test medium three times before the product pattern in that medium was scored. This study has shown that in 24-hr cultures the level of heme in the medium affects the fermentation pattern significantly. The molar ratio of acetate/succinate showed a five-fold increase when the level of heme in the medium was decreased from 5 to 0.01 µg/ml. However, the production of acetic acid is maximal with 1 µg/ml added heme under the test conditions; the concentrations of acetic acid obtained were 36, 42, 22 and 25 mM, respectively, when the corresponding heme concentrations were 5, 1, 0.1 and 0.01 µg/ml. It appears that the optimal level of heme for an alginate medium is between one and five µg/ml so that the yield of acetic acid is maximized.

Effect of carbon source. The fermentation pattern of B. ovatus has been examined in media containing glucose, maltose, mannitol, cellobiose or starch in the place of alginate; the heme concentration was kept at 5

µg/ml throughout the experiment. The cultures were previously passed through the test media before the product pattern in these media was compared. While B. ovatus achieved only moderate growth with mannitol as the carbon source, it showed good growth with any of the other four carbohydrates as the carbon source. However, the fermentation pattern found in these media is very different from what is observed with alginate as the carbon source, i.e., succinate (13-21 mM) is produced to a higher level while acetate (8-13 mM) is significantly lower. The results suggest that the strain of B. ovatus that we isolated is somewhat specialized in converting alginate to acetic acid.

Coculture of alginate-fermenting and mannitol-fermenting bacteria. Cocultures of an alginate-fermenting bacterium (B. ovatus) and a mannitol-fermenting bacterium (C. sphenoides) were grown in a medium containing both alginate and mannitol. The utilization of alginate and mannitol in the coculture was monitored during the course of growth. The fermentation pattern was also examined. Two types of inoculum were used in the experiment: one consisted of a mixed culture of the two organisms and the other consisted of two individual cultures which were separately inoculated into the experimental cultures. In both cases, alginate-utilization was faster than mannitol-utilization. It appears that more elaborate studies are needed in order to select the proper pair of organisms and growth conditions to achieve concerted utilization of alginate and mannitol. The situation can be much more complicated when a defined, mixed population of organisms is required to metabolize an array of substrates in a concerted manner.

d. Hydrogenase and low-redox potential electron carrier of B. ovatus 1S2a2. Hydrogenase is the enzyme which is responsible for the production of hydrogen gas by B. ovatus. The enzyme was separated from the low-potential electron carrier when cell-free extracts of B. ovatus 1S2a2 were chromatographed on a DEAE-cellulose column. Only one type of low-potential electron carrier was found in this organism, and it is a ferredoxin with a molecular weight above 10,000. The ferredoxin was produced in media containing only 8 µM of added iron, which is a low level that usually triggers the synthesis of flavodoxin to replace ferredoxin in other organisms. The level of the low-potential electron carrier is about two times higher in alginate-grown cells than in glucose-grown cells, and the main fermentation product is acetate only in the alginate medium. It is not clear why acetate is the main product only from alginate, but it is reasonable that the level of the low-potential electron carrier is higher when acetate is the main product.

C. Findings.

Acetate, propionate and succinate are the major products produced by the fermentation of marine biomass (primarily kelp and eel grass) by enrichment cultures of marine origin. These cultures contain a large number of salt tolerant methanogens including a new genus that utilized methylotrophic substrates. An examination of formate utilization by pure cultures suggests that formate is an important substrate for methanogens in nature. This study also indicates that formate influences the utilization of hydrogen, which implies a form of regulation of substrate utilization in species that use both substrates. Purification of formate dehydrogenase has revealed a new cofactor not previously reported in methanogens. EPR studies on this enzyme has shown that methanogens synthesize iron-sulfur centers of the Fe_4S_4 type, and the environment of the molybdenum centers are unlike any known molybdoenzymes studied to date.

Clostridium sphenoides and C. sartagoformum are mannitol-fermenting bacteria that produce H_2 and acetate as the main products. Bacteroides ovatus, Cytophaga sp., Citrobacter spp. and Klebsiella spp. are alginate-fermenting bacteria that produce acetate as the main product and some also produce H_2 . H_2 partial pressure has no detectable effect on the fermentation pattern of C. sphenoides, C. sartagoformum and B. ovatus under normal growth conditions. The low-redox potential electron carrier of B. ovatus is a ferredoxin.

III. Major Achievements of the Project.

A. Research findings.

The following list the major research accomplishments.

1. Development of an improved technique for measurement of fermentation products and methanogenic precursors in sea water media. The technique is more sensitive and less variable than previously reported techniques.
2. Identification of intermediates and their pool sizes in stabilized methanogenic kelp-degrading enrichment cultures.
3. Development of culture conditions for and establishment of highly enriched methanogenic cultures that utilize hydrogen, formate, acetate and propionate.
4. Isolation and characterization of a new methylotrophic methanogen from the marine habitat.
5. Isolation of new strains of hydrogen- and formate-utilizing methanogens from the marine environment.
6. Development of a method for purification of the oxygen sensitive formate dehydrogenase from Methanobacterium formicicum.
7. Discovery of a new methanogen cofactor and characterization of the iron-sulfur and molybdenum centers in M. formicicum formate dehydrogenase.
8. An increased understanding of formate metabolism in anaerobic microbial food chains.
9. Development of the groundwork for future genetic studies on formate and hydrogen metabolism in methanogens.
10. Fermentation of alginate is an area where literature information is scarce. We have identified several strict and facultative anaerobes that ferment alginate to produce acetate as the main product. Bacteroides ovatus was found to be an efficient alginate-fermenting organism, and a ferredoxin was purified from this organism. Low-potential electron carriers have not been previously isolated by other investigators from the genus Bacteroides.

B. Graduate students.

The following graduate students were supported in part by this grant:

1. Kevin R. Sowers. Candidate for the Doctor of Philosophy degree.
2. Harold D. May. Candidate for the Doctor of Philosophy degree.

C. Abstracts.

The following abstracts resulted from research supported by this grant:

1. Schauer, N. L., and J. G. Ferry. 1980. Characterization of formate dehydrogenase in Methanobacterium strain JF-1. Abstr. Annu. Meet., Am. Soc. Microbiol. K 85, p. 140.
2. Schauer, N. L., and J. G. Ferry. 1981. Isolation and characterization of formate dehydrogenase from Methanobacterium formicicum. Fed. Proc. 40:1666.
3. Sowers, K. R., and J. G. Ferry. 1982. Characterization of methanosarcina strain TMA-10, a new marine methanogen. Abstr. Annu. Meet., Am. Soc. Microbiol. I 100, p. 111.
4. Barber, M. J., L. M. Siegel, N. L. Schauer, H. D. May, and J. G. Ferry. 1982. Methanogenesis: a novel molybdenum center in Methanobacterium formicicum. Fed. Proc. 41:891.

D. Publications.

The following publications resulted from research supported by this grant:

1. Schauer, N. L., and J. G. Ferry. 1980. Metabolism of formate in Methanobacterium formicicum strain JF-1. J. Bacteriol. 142:800-807.
2. Schauer, N. L., and J. G. Ferry. 1982. Properties of formate dehydrogenase in Methanobacterium formicicum. J. Bacteriol. 150:1-7.
3. Schauer, N. L., D. P. Brown, and J. G. Ferry. 1982. Kinetics of formate metabolism in Methanobacterium formicicum and Methanospirillum hungatii. Appl. Environ. Microbiol. 44:549-554.
4. Sowers, K. R., and J. G. Ferry. 1982. Isolation and characterization of a methylotrophic marine methanogen, Methanococoides methylutens gen. nov., sp. nov. Appl. Environ. Microbiol. (in press).

IV. Major Technical Problem Areas Encountered.

In the study on the fermentative organisms, we could only carry out very limited tests on paired cultures for the simultaneous utilization of alginate and mannitol. As expected, there seem to be rather stringent requirements for a condition that will allow a paired culture to accomplish the task. We isolated and characterized several alginate-fermenting bacteria, but it turned out that much more physiological information about the alginate- and mannitol-fermenting bacteria was needed before a properly designed experiment on paired cultures could be performed. The practical limits of the project did not permit a more involved undertaking in that direction.

V. Conclusions and Recommendations.

A. Characterization of a naturally occurring marine microbial food chain has provided a starting point from which the process can be further understood and improved. It is recommended that these cultures be used as inocula in the large scale fermentation studies funded by GRI.

B. Organisms have been isolated from a stabilized kelp-degrading methanogenic enrichment culture of marine origin. The isolates are representative of two metabolic groups that comprise the microbial food chain. The isolation of acetate-degrading methanogens is most significant. This research has established a basis for study of the individual components of the food chain and interactions between individual components. Microbiological and biochemical studies on acetate-degrading methanogens is recommended.

C. The isolation of formate dehydrogenase, the enzyme that initiates formate conversion to methane, has provided techniques applicable to the study of methanogens on a molecular basis and revealed information on a general class of enzymes which function in methanogenesis. Further work is needed on the regulation of substrate utilization and other aspects of metabolism to take advantage of the groundwork produced by this study.

D. Bacteroides ovatus is an efficient alginate-fermenting organism for the production of acetate and hydrogen gas. Cytophaga, Citrobacter and Klebsiella also contain active alginate-fermenting species. Clostridium sphenoides and C. sartagoformum are mannitol-fermenting organisms that produce acetate and hydrogen gas as the main products. In order to achieve simultaneous utilization of alginate and mannitol by a paired culture, growth parameters must be carefully controlled. This study again raises the question whether it is practical to use a defined but mixed population of bacteria to carry out a concerted fermentation of a complex substrate such as kelp. Obviously, more study in this area may bring us closer to finding a definite answer, whether positive or negative, to the question.