APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1983, p. 691-697 Vol. 45, No. 2
0099-2240/83/020691-07$02.00/0
Copyright © 1983, American Society for Microbiology

Methanogenic Bacteria from the Bondyuzhskoe Oil Field: General Characterization and Analysis of Stable-Carbon Isotopic Fractionation

S. S. BELYAEV,[1]† R. WOLKIN,[1] W. R. KENEALY,[1] M. J. DENIRO,[2,3]‡ S. EPSTEIN,[2] AND J. G. ZEIKUS[1]*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706[1]; Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, California, 91125[2] and Department of Earth and Space Sciences, University of California, Los Angeles, California 90024[3]

Received 20 June 1982/Accepted 21 October 1982

Selective enrichment culture techniques were employed to obtain mixed cultures of methanogenic rods and sarcina from surface flooding waters and deep subsurface (~1650 m) oil-bearing sedimentary rocks and formation waters sampled from an old oil field in the U.S.S.R. previously reported to display active biological methanogenesis. The methanogens were selectively isolated as colonies on agar petri dishes that were incubated in a novel container. The general cellular and growth features of three *Methanobacterium* isolates were determined. These strains grew optimally at 37 to 45°C in anaerobic pressure tube cultures with a doubling time of 16 to 18 h on H_2-CO_2 and proliferated as autotrophs. Acetate addition significantly enhanced the final cell yield. Growth of these strains was completely inhibited by either 0.6 g of sodium sulfide per liter or 31.0 of sodium chloride per liter, but growth was not inhibited by either 0.3 g of sodium sulfide per liter or 1.0 g of sodium sulfate per liter. One novel isolate, *Methanobacterium* sp. strain ivanov, was grown on H_2-CO_2, and the stable-carbon isotopic fractionations that occurred during synthesis of methane, cell carbon, and lipids were determined. The results of this study were used to examine the anomalous relationship between the isotopic and chemical compositions of natural gas occurring in the deep subsurface environment of the oil field.

Bacterial methanogenesis appears as a ubiquitous process in anaerobic ecosystems in which organic matter is being vigorously decomposed in the absence of excess exogenous electron acceptors (e.g., O_2, NO_3^{2-}, SO_4^{2-}). Methanogenic bacteria have been isolated from a variety of environments that include freshwater and marine surface sediments, gastrointestinal tracts of animals, thermal springs, sewage sludge and other anaerobic digestors, and wetwoods of living trees (29). Radioactive tracer and enrichment culture studies have demonstrated microbial methanogenesis associated with deep subsurface natural gas deposits in oil-bearing strata and formation waters (3, 4, 22). Recently, *Methanobacterium bryantii* was isolated from a deep unpolluted aquifer (18), and *Methanobacterium thermoautotrophicum* was isolated from deep waters of a thermal spring (31).

The purpose of this study was to isolate and characterize methanogenic bacteria from an old oil field in the Middle Volga region of the U.S.S.R., where previous studies established that microbial methanogenesis was actively occurring in the deep subsurface environment (S. S. Belyaev and M. V. Ivanov, *Proceedings of the Fifth International Symposium on Environmental Biogeochemistry, Stockholm, 1982*, in press). These investigators reported the following environmental microbial methanogenesis measurements: $\delta^{13}C$ methane values of −52 to −69; 2,500 to 6,000 total methanogens × L^{-1}; a methanogenesis rate of 195 to 227 ml of CH_4 per liter × 10^{-6}/day; and as high as 69% of the methane originating from [2-^{14}C]acetate. The general physiological properties of the isolated hydrogen-oxidizing methanogens described here and in vitro studies of the carbon isotopic fractionation occurring during methane formation by

† Present address: Institute of Biochemistry and Physiology and Microorganisms, Academy of Sciences-U.S.S.R., Pushchino-na-Oke, U.S.S.R.

‡ Present address: Department of Earth and Space Sciences, University of California, Los Angeles, Los Angeles, CA 90024.

them indicate that microbial methanogenesis is of modern origin in this oil field.

MATERIALS AND METHODS

Environmental characteristics and samples. The Bondyuzhskoe oil field is located on the right bank of the Kama River in the northeastern part of the Tartar Republic, U.S.S.R. Detailed environmental characterization of the oil field was reported by Belyaev and Ivanov (in press). The oil-bearing rocks are sandstones and siltstones of the Upper Devonium that have in situ temperature of 30 to 50°C and a 20 to 30% liquid content. The deep subsurface waters vary in salinity from 1 to 33% and in pH from 7.8 to 5.7, depending on the amount of fresh river water that penetrates the hypersaline sediments during field flooding on secondary oil recovery. The oil from this field has a paraffinic content of 4%, a density of 0.84, and an elemental composition (in percent) of: S, 2.2; C, 85; H, 12.4; N, 0.2; O, 0.2. $\delta^{13}C$ values for methane dissolved in deep subsurface groundwaters range between −38 and −52 per mil at salinities greater than 14%, whereas values range from −52 to −69 per mil at salinities less than 14%. Gas samples have C_2^+ (i.e., ethane, propane, and higher hydrocarbon gas) concentrations of 35.2 to 41.7% (Belyaev and Ivanov, in press).

Anaerobic groundwaters were collected from the operational oil wells 47 and 295 of the Bondyuzhskoe oil field from a depth of 1,650 to 1,690 m. Anaerobic oil-bearing rock cores with an approximate diameter of 75 mm were collected from a depth of 1,673 to 1,676 m during the drilling of a new oil well. In addition, samples of the anaerobic injection water (a mixture of ground water and surface water) were taken. Anaerobic samples were taken as described by Belyaev et al. (4) and were processed immediately for enrichments.

Chemicals. All chemicals were reagent grade. N_2-CO_2 (95:5, vol/vol) and H_2-CO_2 (80:20) gas mixtures were obtained from Matheson Scientific, Inc. (Joliet, Ill.) and were passed over heated (370°C) copper filings to remove traces of O_2. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Media. Liquid media and solutions were prepared and sterilized under a strictly anaerobic N_2, N_2-CO_2 (95:5), or H_2-CO_2 (80:20) atmosphere by methods used previously (31). Enrichment medium contained (in grams per liter of distilled water): NH_4Cl, 0.75; $NaHCO_3$, 2.0; $MgCl_2 \cdot 6\ H_2O$, 0.2; K_2HPO_4, 1.0; KH_2PO_4, 1.0; CH_3COONa, 1.5; HCOONa, 1.5; $Na_2S \cdot 9\ H_2O$, 0.5; cysteine hydrochloride, 0.5; yeast extract (Difco Laboratories, Detroit, Mich.), 2.0; resazurin, 0.001; and 2 ml of trace mineral solution. Trace mineral solution contained (in grams per milliliter of distilled water): $MnCl \cdot 4\ H_2O$, 0.1; $CoCl_2 \cdot 6\ H_2O$, 0.17; $CaCl_2 \cdot 2\ H_2O$, 0.02; $FeCl_2$, 0.4; H_2BO_3, 0.019; $ZnCl_2$, 0.1; and $NaMoO_4 \cdot 2\ H_2O$, 0.1. The final pH of the enrichment medium was 6.8 to 7.2.

Maintenance medium contained the following nutrients (in grams per liter of distilled water): NaCl, 0.9; $MgCl \cdot 6\ H_2O$, 0.12; $CaCl_2 \cdot 2\ H_2O$, 0.1; NH_4Cl, 1.0; and (in milliliters per liter) trace mineral solution, 10; and vitamin solution (28), 5. Sterilized anoxic sulfide (0.25 g/liter) and KPO_4 buffer (40 mM) were added separately to medium by hypodermic syringe. The final pH of maintenance medium was 7.0.

Isolation medium contained (in grams per liter of distilled water): KH_2PO_4, 0.3; $Na_2HPO_4 \cdot 7\ H_2O$, 2.1; NH_4Cl, 1; $MgCl_2 \cdot 6\ H_2O$, 0.2; purified agar (Difco), 20; and (in milliliters per liter): trace mineral solution, 10; $FeSO_4$ (2.5%), 25×10^{-3}; resazurin (0.2%), 1; and vitamin solution, 5. After sterilization, the molten medium was transferred into an anaerobic chamber bag (Coy Laboratory Products, Ann Arbor, Mich.), and samples of approximately 20 ml were poured into sterile plastic petri plates. A few hours before plating, 0.10 ml of a cysteine-sulfide solution (1.25 g of each per liter) was spread on the surface of the agar. Cultivation techniques employed procedures designed for growth of fastidious anaerobes in sealed culture tubes or in culture chambers (2, 6, 13).

Isolation and growth. A filter-sterilized penicillin G solution (6 g/100 ml of distilled water) and a streptomycin solution (6 g/100 ml of distilled water) were also spread (0.1 ml of each) on the surface of the agar medium to enhance the isolation of pure cultures. After inoculation of the isolation medium inside the anaerobic glove bag, the petri plate cultures were transferred to a pressure steel paint pot (W. R. Brown Corp., Chicago, Ill.) modified for the anaerobic culture of up to 52 plates. The paint pot was then removed from the anaerobic chamber, pressurized to approximately 30 lb/in^2 with H_2-CO_2 (80:20), and placed inside a 30°C incubator. Growth of methanogens inside the paint pot was monitored by quantification of methane. Methane was measured with a Varian aerograph model 600-D gas chromatograph as described previously (21).

Crimp top sealed pressure tubes (Bellco Glass, Inc., Vineland, N.J.) that contained 2 to 3 atm (200 to 300 kPa) of H_2-CO_2 were used to grow methanogens in liquid medium. Growth was determined in these anaerobic culture tubes by measuring optical density at 660 nm. Absorbance was quantified directly by insertion of the anaerobic culture tube into a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.).

Cellular characterization. Bacteria were disrupted by passage through a French pressure cell at 400 lb/in^2. DNA was isolated and purified from the cell homogenate by the method of Marmur (19). DNA base compositions were calculated by the method of De Ley (10) from thermal denaturation in 0.015 M NaCl and 0.0015 M trisodium citrate as determined in a Gilford model 250 spectrophotometer equipped with a model 2527 thermoprogrammer. *Escherichia coli* DNA VIII, lot D-2001 from Sigma Chemical Co., served as a standard. Data reported represent the mean of three separate determinations. A Carl Zeiss photomicroscope was used as previously described (31) for phase-contrast observations, including determination of cell size and the epifluorescence of methanogen deazoflavin under UV light (20).

$^{12}C/^{13}C$ fractionation analysis. *Methanobacterium* sp. strain ivanov was grown on maintenance medium in a 12-liter Virtis fermentor gassed at 1.0 to 1.5 liter/min with an 80:20 mixture of H_2-CO_2. Reducing conditions were maintained at these high gassing rates by adding 1.5 g of sodium sulfide daily. Gas flowing into and out of the fermentor was sampled when the cultures were inoculated, approximately midway through the culture period, and when the cells were harvested. Carbon dioxide and methane were isolated from the gas samples, and the methane was combusted to CO_2 as described previously (11). Cells were har-

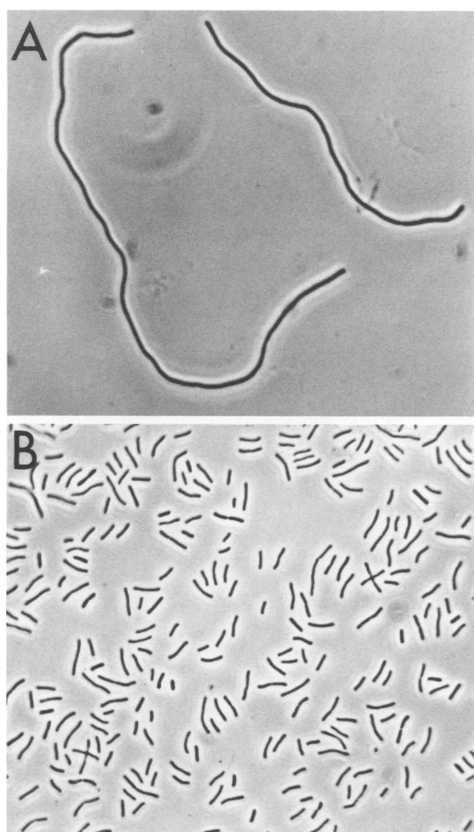

FIG. 1. Phase-contrast photomicrographs of oil field methanogens. (A) *Methanobacterium* sp. strain kuzentosov ×1,400. (B) *Methanobacterium* sp. strain ivanov ×500.

vested by centrifugation when the absorbance of the cultures at 660 nm approached 0.2. The lipids were isolated from samples of lyophilized cells by a modification of the procedure of Bligh and Dyer as described elsewhere (5, 12). Samples of the lipid fractions and total cells were converted to CO_2 for isotopic analysis by the combustion procedure of R. K. Stump and J. W. Frazer (Nucl. Sci. Abstr. **28**:7848, 1973). Carbon isotopic composition of CO_2 samples were determined by mass spectrometry. The results are reported relative to the Peedee Belemnite (PDB) carbonate standard, in which $\delta^{13}C_{PDB}$(per mil) = $\{[(^{13}C/^{12}C\ \text{sample})/(^{13}C/^{12}C\ \text{PDB})] - 1\} \times 10^3$. The uncertainty of the $\delta^{13}C$ values owing to sample preparation and measurement was ± 0.2 per mil for CO_2, cell carbon, and lipid samples and ± 1.0 per mil for CH_4 samples.

RESULTS

Enrichment, isolation and maintenance. Approximately 1 ml of sample water or 1 g of sample rock were added into N_2-gassed anaerobic culture tubes that contained 10 ml of enrichment medium. Oil-bearing sandstone samples were taken from the centers of the drilling cores. After inoculation, the culture tubes were gassed with H_2-CO_2 and sealed with a rubber bung. Enrichment cultures were incubated at 30 to 37°C and were periodically gassed with H_2-CO_2 and were transferred upon the detection of >10% methane in the headspace. Microscopic observation of methanogenic enrichment cultures inoculated with injection water, groundwater, or oil-bearing sandstone revealed a heterogeneous population of microorganisms that included irregular spherical shapes, spores, and a variety of rod types. A methanogenic enrichment culture from an oil-bearing sandstone inoculum contained both *Methanobacterium* spp. and *Methanosarcina* spp. as indicated by the presence of long filamentous rods and irregular spherical packets that fluoresced yellow-green under UV light in the microscope. Three distinct methanogenic strains were isolated from the Bondyuzhskoe oil field. Strain ivanov was isolated from oil-bearing sandstone. Strains kuznetsov and omeliansky were isolated from groundwater, and strain omeliansky was isolated from an injection water enrichment. Successful isolation of these strains was obtained by serial dilutions of enrichment cultures onto maintenance medium followed by plating of end dilutions on isolation medium. After 16 days of incubation on H_2-CO_2 at 37°C, the maximum colony diameter was 3 to 6 mm for strain ivanov, 2 to 3 mm for strain omeliansky, and ≤1 mm for strain kuznetsov. All colonies observed were smooth, uniformly round, mucoid, and greyish white.

Cellular features. All three strains appeared as irregular curved rods and existed singly, in chains, or as long filaments (Fig. 1). The average size of these organisms was 0.5 to 0.8 μm in width; length varied from 1.2 μm (strain ivanov) to 11 to 18 μm (strain kuznetsov). No spores were observed, and these cultures did not exhibit motility in wet mounts. Strain kuznetsov stained gram negative, whereas both strains ivanov and omeliansky stained gram positive.

All three strains fluoresced yellow-green when exposed to UV light, thus indicating the presence of the deazoflavin (13, 27). DNA isolated from strains omeliansky, ivanov, and kuznetsov had base compositions of 37.3, 36.6, and 48.8 (± 1) mol% guanine plus cytosine, respectively.

Growth and nutritional properties. The relationship between the growth rate of these methanogenic bacteria and temperature is shown in Fig. 2. These strains did not grow at 55 or at 10°C. The optimum temperature for growth of strains ivanov and omeliansky was 45°C. The optimum temperature for growth of strain kuznetsov was 37°C. The generation time for all

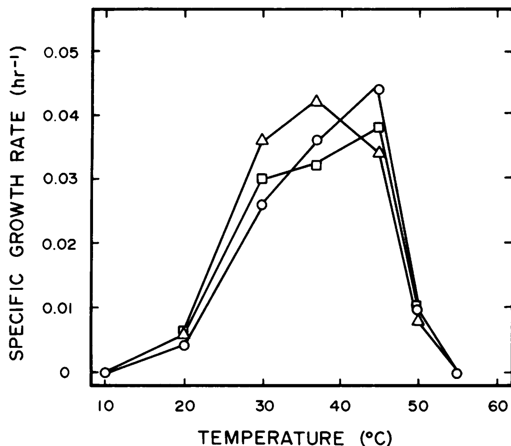

FIG. 2. Relationship between growth rate and incubation temperature for oil field *Methanobacterium* sp. Symbols: ○, strain ivanov; △, strain kuznetsov; □, strain omeliansky.

three strains at their respective growth temperature optima was 16 to 18 h.

The pH optimum for growth was between 6.5 to 7.2 for strain omeliansky and 7.0 to 7.4 for the other strains. Strain omeliansky grew within the pH range of 6.0 to 7.4, whereas strains ivanov and kuznetsov grew within the pH range of 6.5 to 8.2.

Nutritional studies were performed in maintenance medium at the optimum temperature for growth of each strain. All three strains were capable of growth on H_2-CO_2. Only strain kuznetsov grew on formate as the sole carbon and energy source. Methanol, methylamine, glucose, and acetate could not serve as the sole energy source for the growth of any strains.

All strains isolated were capable of autotrophic growth on H_2-CO_2 without vitamin supplementation. The addition of acetate to mineral salts medium enhanced the growth of all strains, whereas neither cysteine or more complex organic compounds in yeast extract or Trypticase (BBL) had a significant effect on growth.

All three strains were unable to grow in the presence of trace quantities of air, which turned the resazurin in maintenance medium light pink. Sodium sulfate at 1.0 g/liter did not influence growth, whereas 0.6 g of sodium sulfide per liter inhibited strains ivanov and kuznetsov by 31 and 100%. Growth of all strains was significantly inhibited by 11 g of NaCl per liter and was not detectable at 31 g of NaCl per liter.

Carbon isotopic fractionation. *Methanobacterium* sp. strain ivanov was grown in a mineral medium continually gassed with H_2 and CO_2, which served as the only energy and carbon source. Table 1 compares the $^{13}C/^{12}C$ ratios of the CO_2 sources and the CH_4 and also of the total cells and lipids synthesized by *Methanobacterium* sp. strain ivanov during different fermentor growth experiments at 37 and 46°C. The $\delta^{13}C$ values of the CO_2 remained unchanged throughout the growth periods because the cultures were gassed at relatively high flow rates and were harvested at relatively low cell densities. Under these conditions, the CO_2 reservoir is effectively infinite, ensuring that valid carbon isotopic fractionations between CO_2 and components synthesized by the cultures are measured. Carbon isotopes were more highly fractionated in methane than in total cell or lipid components at both growth temperatures. The total cell and lipid components had nearly equivalent $\delta^{13}C$ values at 37°C, whereas the lipid fraction of cells grown at 46°C was enriched in ^{13}C relative to total cell carbon.

DISCUSSION

The general cellular, growth, and nutritional properties of *Methanobacterium* sp. strains ivanov and omeliansky are similar to those reported for *M. bryantii*, whereas strain kuznetsov appears similar to *Methanobacterium formicicum* (1, 7). However, it is not possible at this time to assign the strains characterized here to these species or to new ones because more detailed comparative taxonomic studies, including DNA-DNA hybridization, DNA-RNA hybridization, and two-dimensional analysis of total cellular proteins, are required to establish species identity with absolute certainty. Notably, several properties of *Methanobacterium* sp. strain ivanov appear to differ from those reported for *M. bryantii* strain MOH (1, 8, 9, 13, 33), including DNA guanine plus cytosine content, lack of growth stimulation by yeast extract, formation of large colonies on agar plates, and

TABLE 1. $\delta^{13}C_{PDB}$ values (per mil) of the source CO_2 and CH_4, total cells, and lipids synthesized by *Methanobacterium* sp. strain ivanov

Carbon source	δ^{13}_{PDB} (per mil)			
	37°C in culture:		46°C in culture:	
	I	II	I	II
CO_2, inlet at inoculation	−9.4	−10.1	−28.1	−28.4
CO_2, inlet at midpoint	−9.4	−10.0	−28.4	−28.5
CO_2, inlet at harvest	−9.4	−10.1	−28.1	−28.4
CO_2, outlet at inoculation	−9.2	−10.1	−28.0	−28.2
CO_2, outlet at midpoint	−9.3	−10.0	−28.7	−28.6
CO_2, outlet at harvest	−9.2	−10.0	−28.7	−28.6
CH_4, outlet at harvest	−44.1	−43.4	−60.1	−61.9
Cells	−33.6	−32.4	−46.3	−51.6
Lipids	−34.0	−33.4	−40.3	−49.2

facile lysis by mild osmotic shock. These last features are significant for future use of this strain in initiating genetic studies of methanogens.

The isotopic fractionations observed between source CO_2 and CH_4 formed by *Methanobacterium* sp. strain ivanov at 37°C were similar to those reported for *M. bryantii* by Games et al. (17). The $\delta^{13}C$ values for methane and cell carbon formed by *Methanobacterium* sp. strain ivanov during growth on H_2-CO_2 were significantly different, an observation which Fuchs et al. (15) reported for *M. thermoautotrophicum*. The results of isotopic discrimination studies cannot be used to support or disprove the hypothesis that initial one-carbon transformation reactions in methanogen metabolism share common intermediates (24, 30), as Fuchs et al. (15) have proposed, because methane formation involves only one-carbon conversions (27, 30), whereas significant amounts of methanogen cell carbon are derived from other reactions (e.g., carboxylations of acetyl coenzyme A, phosphoenolpyruvate, succinyl coenzyme A) during which carbon isotopic fractionation might occur.

Methanobacterium sp. strain ivanov grown at 46°C differs from all other organisms studied to date, including *M. thermoautotrophicum* and *Methanosarcina barkerii* (Kenealy, DeNiro, Epstein, and Zeikus, unpublished observations), in that its lipid fraction is enriched in ^{13}C relative to the total cell carbon. DeNiro and Epstein (11) proposed that the ^{13}C depletion of the lipid fraction relative to cell carbon observed in nonmethanogens results from kinetic isotope effects which occur during the synthesis of acetyl coenzyme A from pyruvate by the pyruvate dehydrogenase complex, with the ^{13}C-depleted acetyl groups synthesized during this reaction then being polymerized to form the fatty acids that comprise the bulk of the lipid fraction. The observation of a reversed isotopic relationship between the cells and lipids of strain ivanov grown at 46°C is thus consistent with suggestions that the pyruvate dehydrogenase reaction does not contribute significantly to the formation of acetyl coenzyme A in methanogens (14, 24, 30). The temperature sensitivity of the isotopic relationship between lipids and cell carbon in *Methanobacterium* sp. strain ivanov should prove useful for further studies on the mechanism of carbon isotope fractionation associated with lipid synthesis in methanogens.

The relationship between the $\delta^{13}C$ values of CH_4 in the natural gases from the Bondyuzhskoe field and the chemical composition of the gases deviates markedly from those observed in other occurrences of natural gas. Gas samples from the Bondyuzhskoe field had C_2^+ concentrations that ranged from 35.2 to 41.7%. Hydrocarbons in gas samples with C_2^+ concentrations in this range are generally considered to be formed by thermal cracking of organic matter and usually have CH_4 with $\delta^{13}C$ values less negative than −50 per mil (16, 23). By contrast, natural gases produced by bacterial action in nature usually have low concentrations of C_2^+ (<0.1%) and CH_4 with $\delta^{13}C$ values more negative than −50 per mil, with values as low as −90 per mil reported (23). Thus, the high C_2^+ values and low $\delta^{13}C$ methane values (i.e., −69 per mil) in the Bondyuzhskoe are anomalies. The association in some of the gas samples of CH_4 of such low $\delta^{13}C$ values with large amounts of C_2^+ components can be explained by the mixing of CH_4 generated by methanogens in the oil-bearing strata with hydrocarbons derived from thermal cracking of petroleum. We have shown here that methanogens isolated from the deep subsurface in laboratory culture can grow under the general in situ environmental conditions (i.e., temperature, pH, salinity, reducing conditions) under which active biological methanogenesis occurs and under which anomalously low $\delta^{13}C$ methane values are found in the presence of high C_2^+.

The results reported here indicate that biological methane formation in the Bondyuzhskoe oil field is of modern origin and is caused by contamination with surface methanogens since the methanogens isolated were similar in physiological properties to other isolates from surface aquatic sediments (32) and the same methanogens were isolated from the flooding-injection waters and the deep subsurface rock or groundwater samples. In view of the biological activity measurements for methanogenesis reported (Belyaev and Ivanov, in press), the methanogens in this environment appear to have resulted from the injection of freshwater, since none of the isolated methanogens grow at the hypersalinities or low pH values which characterize uncontaminated groundwaters in the Bondyuzhskoe field (i.e., waters not displaying biological methanogenesis and having more positive $\delta^{13}C$-methane values). The phenomenon of modern microbial methanogenesis may occur in other oil fields where water flooding is used to enhance oil recovery.

Our in vitro studies of carbon isotope fractionation accompanying methane synthesis during growth on H_2-CO_2 suggest that methane formation from the reduction of CO_2 is not significant in the Bondyuzhskoe field. If methanogens fractionate carbon isotopes under the conditions that exist in the field to the same extent that we observed in the laboratory, the methane would be about 35 per mil more negative than the CO_2 source. This fractionation is not large enough to account for the difference of 40 to 55 per mil that was observed between CH_4 and coexisting CO_2

in Bondyuzhskoe field waters with salinities less than 3%, the limit of which our isolates did not grow. It is possible that methanogens fractionate carbon isotopes during CH_4 synthesis to a greater extent in the field than we observed in the laboratory. However, it is likely that methane generation from some other carbon source, namely acetate, also accounts for the low $\delta^{13}C$ values of methane in the Bondyuzhskoe field. Studies with [^{14}C]acetate indicate that methane generation from acetate is significant (up to 69% of the methane) in parts of the subsurface environment of this oil field (Belyaev and Ivanov, in press). There is no reason to believe that methane formation was due to the utilization of petroleum hydrocarbons because methanogens are limited to the metabolism of H_2-CO_2, acetate, and one-carbon substrates (e.g., CH_3OH) as energy sources (29).

Finally, as noted above, all the oil field methanogens, including *Methanobacterium* sp. strain ivanov, failed to grow at 3.0% NaCl. Thus, other strains must account for any methanogenic activity at the high salinity values that exist in parts of the oil field. In this regard we have isolated *Methanobacterium* strains from formation water injection filters on offshore oil platforms in the Gulf of Mexico that grow in 10% NaCl (Zeikus and co-workers, unpublished findings). The absence of growth inhibition for these isolates by high sulfate and moderate (i.e., 0.3 g/liter) sulfide concentrations supports the hypothesis that methanogenic bacteria are not inhibited by these sulfur species in freshwater or marine environments (25, 26). Their temperature and pH ranges for growth and their salinity, S^- and SO_4^{2-} tolerances suggest that these *Methanobacterium* strains and also other methanogens (e.g., acetate-degrading *Methanosarcina* strains) are active in the parts of the Bondyuzhskoe field with groundwater salinities less than 3% where methane of anomalously low $\delta^{13}C$ value is found associated with high C_2^+ concentrations.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and National Science Foundation EAR 79-26239. We gratefully acknowledge the research fellowship awarded to S.S.B. from the cooperative agreement between the U.S.S.R. and U.S.A. National Academies of Sciences. R.W. and W.R.K. were supported by predoctoral traineeships from the National Institutes of Health and by Cellular and Molecular Biology training grant 53 GM07215.

We thank A. N. Fuex of Shell Development Corp., Houston, Tex. for helpful discussions.

LITERATURE CITED

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
2. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32**:781–791.
3. **Belyaev, S. S.** 1979. Microbiological methane formation in various ecosystems, p. 205–219. *In* Role of microorganisms in natural turnover of gases. Edition Nann, Moscow. (In Russian.)
4. **Belyaev, S. S., U. S. Laurinavichus, and V. I. Gaitan.** 1977. Contemporary methane formation in quarternary and pliocene sediments of the Caspian Depression. Geokhimya **8**:1251–1256. (In Russian.)
5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
6. **Bryant, M. P.** 1972. Commentary of the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
7. **Bryant, M. P.** 1974. Methane producing bacteria, p. 472–477. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
8. **Bryant, M. P., B. C. McBride, and R. S. Wolfe.** 1968. Hydrogen-oxidizing methane bacteria. I. Cultivation and methanogenesis. J. Bacteriol. **95**:1118–1123.
9. **Bryant, M. P., S. F. Tzeng, I. M. Robinson, and A. E. Joyner.** 1971. Nutrient requirements of methanogenic bacteria. Adv. Chem. Ser. **105**:23–40.
10. **De Ley, J.** 1970. Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. J. Bacteriol. **101**:738–754.
11. **DeNiro, M. J., and S. Epstein.** 1977. Mechanism of carbon isotope fractionation associated with lipid synthesis. Science **197**:261–263.
12. **DeNiro, M. J., and S. Epstein.** 1978. Influence of diet on the distribution of carbon isotopes in animals. Geochim. Cosmochim. Acta **42**:495–506.
13. **Edwards, T., and B. C. McBride.** 1975. New method for the isolation and identification of methanogenic bacteria. Appl. Microbiol. **29**:540–545.
14. **Fuchs, G., E. Stupperich, and R. K. Thauer.** 1978. Acetate assimilation and the synthesis of alanine, aspartate, and glutamate by *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **117**:61–66.
15. **Fuchs, G., R. Thauer, H. Ziegler, and N. Stickler.** 1979. Carbon isotope fractionation in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **120**:135–137.
16. **Fuex, A. N.** 1977. The use of stable carbon isotopes in hydrocarbon exploration. J. Geophys. Explor. **7**:155–188.
17. **Games, L. M., J. M. Hayes, and R. P. Gunsalus.** 1978. Methane-producing bacteria: natural fractionations of the stable isotopes. Geochim. Cosmochim. Acta **42**:1295–1297.
18. **Godsy, E. M.** 1980. Isolation of *Methanobacterium bryantii* from a deep acquifer by using a novel broth-antibiotic disk method. Appl. Environ. Microbiol. **39**:1074–1075.
19. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
20. **Mink, R., and P. R. Dugan.** 1977. Tentative identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:713–717.
21. **Nelson, D. R., and J. G. Zeikus.** 1974. Rapid method for the radioisotopic analysis of gaseous end products of anaerobic metabolism. Appl. Microbiol. **28**:258–261.
22. **Rosanovo, E. P., and S. I. Kuznetsov.** 1975. Microflora of oil deposits, p. 196. Edition Nanna, Moscow. (In Russian)
23. **Schoell, M.** 1980. The hydrogen and carbon isotopic composition of methane from natural gases of various origin. Geochim. Cosmochim. Acta **44**:649–661.
24. **Weimer, P. J., and J. G. Zeikus.** 1979. Acetate assimilation pathway of *Methanosarcina barkeri*. J. Bacteriol. **137**:332–339.

25. **Winfrey, M. R., and J. G. Zeikus.** 1977. Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. Appl. Environ. Microbiol. **33**:275–281.
26. **Winfrey, M. R., and J. G. Zeikus.** 1979. Anaerobic metabolism of immediate methane precursors in Lake Mendota. Appl. Environ. Microbiol. **37**:244–253.
27. **Wolfe, R. S., and I. J. Higgins.** 1979. Methanogenesis, p. 267–370. *In* T. R. Quayle (ed.), Microbial biochemistry, vol. 21. University Park Press, Baltimore.
28. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882–2886.
29. **Zeikus, J. G.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.
30. **Zeikus, J. G.** 1980. Chemical and fuel production by anaerobis. Ann. Rev. Microbiol. **34**:423–464.
31. **Zeikus, J. G., A. Ben-Bassat, and P. W. Hegge.** 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. **143**:432–440.
32. **Zeikus, J. G., and M. R. Winfrey.** 1976. Temperature limitation of methanogenesis in aquatic sediments. Appl. Environ. Microbiol. **31**:99–107.
33. **Zeikus, J. G., and R. S. Wolfe.** 1972. *Methanobacterium thermoautotrophicum* sp. n., an anaerobic, autotrophic, extreme thermophile. J. Bacteriol. **109**:707–713.