APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1980, p. 1074-1075
0099-2240/80/05-1074/02$02.00/0

Vol. 39, No. 5

Isolation of *Methanobacterium bryantii* from a Deep Aquifer by Using a Novel Broth-Antibiotic Disk Method

E. M. GODSY

U.S. Geological Survey, Water Resources Division, Menlo Park, California 94025

Methanobacterium bryantii was isolated from a mixed-culture enrichment of a water sample from a deep aquifer by using a complex growth medium supplemented with antibiotic susceptibility disks to inhibit the growth of non-methanogenic bacteria.

During a recent reconnaissance for microbial activity in native unpolluted groundwater, samples from the Floridan Aquifer near West Palm Beach, Fla., consistently contained low numbers of methanogenic bacteria. Attempts to isolate the methanogens by using enrichment culture (13), roll tube (1, 6), and spread plate (3) techniques with formate, methanol, acetate, and H_2-CO_2 as substrates were not successful.

After failure with conventional techniques, a simple method for obtaining pure cultures of methanogenic bacteria was devised by taking advantage of the fact that methanogenic bacteria can grow in the presence of certain antibiotics that inhibit the growth of most other bacteria (7-9). This report describes a method for the isolation of a methanogenic bacterium from a mixed culture using a complex growth medium supplemented with antibiotic susceptibility disks (BBL Microbiology Systems, Cockeysville, Md.).

The broth medium was composed of the following (per liter): KH_2PO_4, 0.75 g; K_2HPO_4, 0.89 g; NH_4Cl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.36 g; trace metal solution (13), 9.0 ml; vitamin solution (12), 5.0 ml; yeast extract (BBL Microbiology Systems), 1.0 g; Trypticase (BBL Microbiology Systems), 1.0 g; sodium formate, 2.5 g; sodium acetate, 2.5 g; isovaleric acid, 0.5 ml; valeric acid, 0.5 ml; isobutyric acid, 0.5 ml; 2-methylbutyric acid, 0.5 ml; 2-mercaptoethanesulfonic acid (HS-CoM; Pierce Chemical Co., Rockford, Ill.), 0.1 mg; 0.2% resazurin, 1.0 ml. The pH was adjusted to 7.2, and the medium was boiled and cooled under a stream of oxygen-free nitrogen gas. Ten milliliters of a solution containing 25.0 g of $Na_2S \cdot 9H_2O$ and 25.0 g of cysteine-hydrochloride (pH 7.2) per liter was added to 1 liter of the cooled solution prepared above. The medium was dispensed by the method of Holdeman et al. (6) and sterilized at 120°C (1.05 kg/cm^2) for 15 min.

The broth-antibiotic disk medium was prepared by adding from one to five antibiotic susceptibility disks to tubes of sterile broth medium in serum tubes (no. 2048-00150; Bellco Glass Inc., Vineland, N.J.) capped with black rubber stoppers (no. 2048-11800; Bellco Glass Inc.) and crimped with aluminum seals (no. 224183; Wheaton Scientific Div., Wheaton Industries, Millville, N.J.). Antibiotics tested and expected concentrations of antibiotic (per milliliter) in broth per disk added (11) were as follows: penicillin G, 2 U; ampicillin, 2 μg; carbenicillin, 20 μg; cephalothin, 6 μg; vancomycin, 6 μg; tetracycline, 6 μg; clindamycin, 0.4 μg; chloramphenicol, 6 μg; erythromycin, 3 μg; and kanamycin, 6 μg.

Tubes of sterile broth medium were inoculated with water samples collected in sterile 100-ml serum bottles by previously described procedures (4). Water samples were obtained from wells that are located on the grounds of the West Palm Beach Regional Water Pollution Control Plant. The wells extended 657 to 673 m below the land surface and flowed freely under an artesian head.

After inoculation, the gas phase in each tube was exchanged for a pressurized atmosphere (2.1 kg/cm^2) of 80% H_2-20% CO_2. The tubes were incubated at 30°C for 4 weeks.

The presence of methane in the headspace was monitored periodically by gas chromatography with an Infotronics 15-C3 gas chromatograph fitted with thermal conductivity detectors and a Poropak Q column (2.4 m by 6 mm). The column was operated at 50°C with helium as the carrier gas. Methane was quantitated by a Spectra-Physics model 6300 digital integrator.

After 4 weeks, all methane-positive tubes were examined under phase contrast and epifluorescence with an American Optical Fluorstar microscope fitted with a 50-W mercury vapor lamp and Fluor Cluster no. 2073 (American Optical Co., Buffalo, N.Y.). Presumptive identification of methanogens was made on the basis of persistent fluorescence believed to be due to the presence of a fluorescence at 420 nm in the methanogens (2, 10). The fluorescing cells were

cylindrical rods with blunt rounded ends, 10 to 15 by 0.5 to 1.0 μm, often unevenly crooked and occurring both singly and in chains.

Samples of methane-positive tubes that contained fluorescing rods were inoculated into the broth-antibiotic disk medium, and the gas phase was exchanged for a pressurized atmosphere of 80% H_2–20% CO_2. The tubes were incubated at 30°C.

Total methane production in the headspace after 3 days averaged 1.2 μmol in all tubes except those containing chloramphenicol. No methane or cellular growth was observed in chloramphenicol-containing medium after 21 days. Total methane production in the headspace averaged 76 μmol in all growth-supporting media after 7 days of growth.

Before antibiotic treatment, only an occasional cell in the mixed cultures was observed to fluoresce. After antibiotic treatment, enrichment of fluorescent bacteria was noted in all media, but only in the presence of cephalothin (6 to 30 μg/ml) and clindamycin (1.6 and 2.0 μg/ml) was it noted that the cultures contained only fluorescing cells. The appearance of the cells in the cephalothin- and clindamycin-containing media was as described above.

Samples from the cephalothin- and clindamycin-containing media were transferred to fresh broth medium with a pressurized 80% H_2-20% CO_2 atmosphere, broth medium with a nitrogen atmosphere, peptone-yeast extract-glucose broth (6), supplemented brain heart infusion broth (6), and broth medium with a pressurized 80% H_2-20% CO_2 atmosphere containing 10^{-3} M bromoethanesulfonic acid, a potent inhibitor of methane production (5).

After 21 days of incubation at 30°C, methane and cellular growth were observed only in the broth medium with the H_2-CO_2 atmosphere.

The organism isolated was gram positive and did not require yeast extract, Trypticase, acetate, vitamins, volatile fatty acids, or HS-CoM for methane production and growth. The organism could use only H_2 as a growth substrate. Methane production or growth did not occur when formate, acetate, or methanol was used as growth substrate. The organism demonstrated a lack of susceptibility to all of the antibiotics tested except chloramphenicol. The organism was identified as *Methanobacterium bryantii* (1) according to the key of Balch et al. (1). A pure culture of *M. bryantii* (strain M.o.H.) exhibited similar biochemical characteristics and antibiotic susceptibilities.

The broth-antibiotic disk method offers a means by which methanogenic bacteria can be easily isolated from mixed cultures of bacteria.

I thank R. S. Oremland and G. G. Ehrlich for their helpful discussions.

The *M. bryantii* (strain M.o.H.) subculture was obtained from a culture donated to R. S. Oremland by J. G. Ferry.

ADDENDUM

After this paper was submitted, a publication appeared in which the authors used penicillin to purify mixed cultures containing a thermophilic strain of *Methanosarcina* (S. H. Zinder and R. A. Mah, Appl. Environ. Microbiol. **38**:996–1008, 1979). Also, a paper appeared in which the authors tested the resistance of strains of *Methanobacterium* to a number of antibiotics (W. P. Hammes, J. Winter, and O. Kandler, Arch. Microbiol. **123**:275–279, 1979).

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. **Doddema, H. J., and G. D. Vogels.** 1978. Improved identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **36**:752–754.
3. **Edwards, T., and B. C. McBride.** 1975. New method for the isolation and identification of methanogenic bacteria. Appl. Microbiol. **29**:540–545.
4. **Godsy, E. M., and G. G. Ehrlich.** 1978. Reconnaissance for microbial activity in the Magothy Aquifer, Bay Park, New York, four years after artificial recharge. J. Res. U.S. Geol. Surv. **6**:829–836.
5. **Gunsalus, R. P., J. A. Romesser, and R. S. Wolfe.** 1978. Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum*. Biochemistry **17**:2374–2377.
6. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobic laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg.
7. **Jones, J. B., B. Bowers, and T. C. Stadtman.** 1977. *Methanococcus vannielii*: ultrastructure and sensitivity to detergents and antibiotics. J. Bacteriol. **130**:1357–1363.
8. **Kandler, O., and H. Hippe.** 1977. Lack of peptidoglycan in the cell walls of *Methanosarcina barkeri*. Arch. Microbiol. **113**:57–60.
9. **Kandler, O., and H. König.** 1978. Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. Arch. Microbiol. **118**:141–152.
10. **Mink, R. W., and P. R. Dugan.** 1977. Tentative identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:713–717.
11. **Wilkins, T. D., and T. Thiel.** 1973. Modified broth-disk method for testing the antibiotic susceptibility of anaerobic bacteria. Antimicrob. Agents Chemother. **3**: 350–356.
12. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882–2886.
13. **Ziekus, J. C.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.