New Method for the Isolation and Identification of Methanogenic Bacteria

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A new technique is reported for the rapid growth and detection of methanogenic bacteria by using petri plates. The method employs an anaerobic glove box containing an inner chamber with separate gas-flushing facilities. The numbers of methanogenic bacteria recovered from domestic sewage sludge are comparable to those recovered by other methods. The methanogenic organisms isolated from sludge include *Methanosarcina*, *Methanospirillum*, *Methanobacterium* strain M.o.H., and *Methanobacterium formicicum*. Identification of colonies containing methanogenic bacteria is facilitated by taking advantage of the unique fluorescence properties of these organisms. Colonies as small as 0.5 mm can be detected by exposing them to long-wave ultraviolet light.

Methanogenic bacteria have traditionally been isolated and cultured by the techniques described by Hungate (6). These methods provided the environment necessary to grow the extremely O_2-sensitive methanogenic bacteria.

Modifications of the original procedure have been described by Bryant and Robinson (3) and more recently by Holdeman and Moore (5). An excellent review of the methodology was written by Wolfe (8). Essentially, the technique consists of preparing and inoculating media in the absence of O_2. The medium is sealed from the aerobic environment with a butyl rubber stopper. Since petri plates do not lend themselves to this type of operation, "roll tubes" were prepared in which agar was spread evenly over the inner surface. Bacteria were imbedded in the agar or were streaked along the surface, making the roll tube analogous to a petri plate.

The drawbacks of the roll tube are: (i) the difficulty in observing and picking isolated colonies, particularly in low dilutions where colonies are crowded; and (ii) the inability to use roll tubes for routine genetic procedures such as replica plating.

Aranki and Freter (1) have described an anaerobic chamber that maintains O_2 levels of less than 10 μl/liter. The chamber was large and permitted the use of standard bacteriological techniques for the cultivation of very strict anaerobes. However, O_2 levels were still too high for the cultivation of methane bacteria. In this paper we describe an apparatus that maintains the low redox potential necessary for the growth of methane bacteria and permits the

use of routine bacterial culturing procedures. A technique for the rapid identification of colonies of methane bacteria will also be discussed.

MATERIALS AND METHODS

Growth medium. Methanogenic organisms were grown in a medium essentially as described by Bryant et al. (2): trypticase, 4 g; mineral no. 1 (3), 60 ml; mineral no. 2 (3), 60 ml; cysteine hydrochloride, 0.5 g; 0.1% resazurin, 1.5 ml; clarified rumen fluid, 300 ml; agar, 15 g; and distilled water to 1 liter. Cysteine hydrochloride was added to liquid media and the pH was adjusted to 7.5. After sterilization in the autoclave, the pH was 7.0 ± 0.1.

Bacteria. *Methanobacterium* strain M.o.H. was a gift of M. Bryant, University of Illinois, Urbana. All other methanogenic organisms were isolated from domestic sewage sludge obtained from an anaerobic sewage digester. They were identified on the basis of microscopic morphology, methane production, and the substrates utilized as precursors for methane biosynthesis.

Anaerobic chamber. The anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) was modified as shown in Fig. 1. A molded port (38-mm diameter) in the vinyl chamber bag was fitted with two rubber stoppers, one outside and one inside the chamber. The stoppers were fitted with two 300-mm lengths of 7-mm stainless-steel tubing, through which influent and effluent gases were passed. Chamber oxygen levels were measured with a trace oxygen analyzer (Lockwood and McLorie, Inc., Worsham, Pa.) modified for use in the presence of gases containing 5% CO_2. The chamber temperature was maintained at 37 C and at a relative humidity of 50%. Bacterial transfer loops were sterilized in an electrical incinerator. Standard aseptic procedures were followed.

Methane measurements. Methane was measured

in a Bendix model 2500 gas chromatograph using a silica gel column.

Chemicals. Palladium (0.5% wt/wt)-coated aluminum pellets were purchased from Engelhardt Industries, Toronto, Ontario, Canada. H_2-CO_2 (80:20, vol/vol) and N_2-H_2-CO_2 (85:10:5, vol/vol/vol) were purchased from Matheson of Canada Ltd., Whitby, Ontario.

Growth chamber. The methanogenic bacteria were grown in an anaerobic box illustrated in Fig. 2. The box, an ultra-low oxygen chamber (ULOC), used in our laboratory measured 45 by 20 by 23 cm. A box of this size holds 50 petri plates. The box was made of 6-mm acrylic plastic and cemented with no. 2 catalyzed cement, and edges were machined to insure leakproof construction. The lid (A) may be of 6-mm tempered glass if positive pressures are desired. The gasket was molded into place by pouring silicone rubber (Dow-Corning-B) into a machined channel (F) and then milling the top surface to expose 0.8 mm of the rubber (G). This provided a precision-mating elastomer surface resistant to moisture and chemical deterioration. The lid crossbars (E) were made of 6-mm hard anodized aluminum strips and were fastened to the lid with three stainless-steel screws. The screws were sealed with epoxy cement. The crossbars have a deep cut-out on the ends to accommodate the swing clamps (C). The swing clamps were custom machined of aluminum and were cemented in position on the sides of the box so that they swing through a 180° arc to fit into the crossbar cut-outs. The clamp-tightening nuts (D) on the swing clamps are of nylon-based material. The lid is fitted with two ball valves (B) (Whitey no. 4BF2-316) for gas exchange. A steel-mesh container of palladium catalyst pellets was placed in the box to remove traces of oxygen present in the incoming gas. Catalyst was inactivated by reaction with volatile sulfur compounds and was regenerated by heating the pellets to 160 C for 2 h. It is advisable to reactivate the catalyst every 2 weeks, or more often if large numbers of volatile sulfur-producing bacteria are cultivated. The effect of volatile sulfur compounds was minimized by trapping them in absorbent cotton soaked in 200 ml of 50% (vol/vol) glycerol-water, 0.5 g of $AgNO_3$, and 0.3 ml of 3 N H_2SO_4. Excessive moisture, which can cause serious contamination problems, was controlled with dried silica gel. A raised steel-mesh floor keeps plates above moisture that may collect in the bottom of the ULOC if the silica gel is not replaced at the appropriate time.

Brewer anaerobic jars fitted with vacuum and gassing ports can be used in place of the ULOC. They suffer from being small, and the metal jars must be opened to see the cultures.

Preparation of media. Media were prepared without anaerobic precautions. Plates of agar medium were poured in air, allowed to solidify, and then placed in the chamber. If the plates were placed in the chamber within 2 h of pouring, the resazurin in the medium was reduced in 4 h. If the plates were left in air overnight, they required 24 h to become colorless. Plates stored in the chamber for 4 months supported
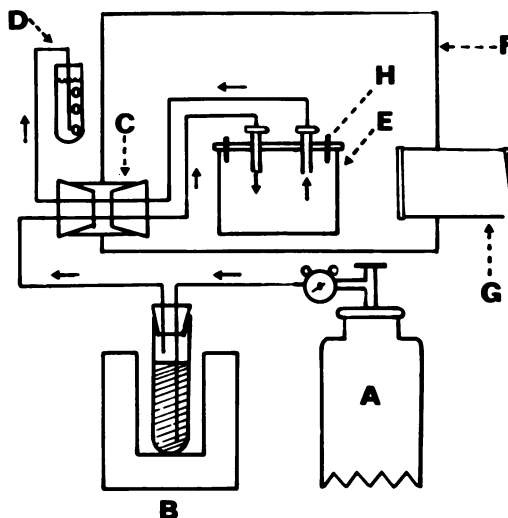

FIG. 1. *Modified anaerobe chamber containing inner ultra-low oxygen chamber (ULOC), with apparatus for supply of oxygen-free gas. (A) Gas mixture tank; (B) reduced copper-oxygen scrubber with heater; (C) stoppered portal for gas-flushing lines; (D) bubble flow meter to measure effluent gas; (E) ultra-low oxygen chamber (ULOC); (F) anaerobic chamber; (G) entry lock; and (H) corner snap clamps.*

the growth of methane bacteria. The Eh at which resazurin becomes colorless is -50 to -60 mV. This high value is unlikely to support the growth of methanogenic bacteria, and therefore, as a matter of routine, plates were allowed to reduce for 48 h before use. Glass rather than plastic petri plates are preferred, since it was observed that media in plastic dishes required several times longer to reduce. Plates were stored in plastic bags to prevent drying.

RESULTS

Growth of methanogenic bacteria. The cultures were plated in the outer chamber (F, Fig. 1) and immediately placed in the ULOC. The ULOC was then flushed with H_2-CO_2 (80:20). When the gas phase was used as a nutrient source, it was necessary to periodically flush the chamber with H_2-CO_2. The interval between flushing was a function of the number of organisms cultured. Methane analysis of effluent gases afforded a convenient indicator of growth which did not necessitate opening the ULOC. Methane analysis is also a good method for determining when flushing is complete. If the ULOC is filled with plates of methanogenic bacteria, it is advisable to continuously pass a slow stream of gas through the apparatus to ensure that there is a constant nutrient supply.

Colonies of methanogenic bacteria appeared

as early as 5 days and were always seen after 10 to 14 days of incubation. In 21 days, colonies of strain M.o.H. and *Methanospirillum* were 5 mm in diameter. Liquid cultures were prepared by subculturing from the petri plates to tubes. The tubes were stoppered and transferred out of the chamber, where they were gassed and incubated by standard techniques (8).

Oxygen concentration. Oxygen concentration was measured in the outer chamber and in the flushing gas. The outer chamber registered 2 to 5 μl of O_2 per liter. The ULOC flushing gas was always less than 1 μg/ml. Lower recovery rates were observed when organisms were plated in an atmosphere with greater than 10 μl of O_2 per liter. Therefore, the ULOC was never opened if the outer chamber contained more than 10 μl of O_2 per ml. Oxygen tensions in the outer chamber were measured daily. Flush-

ing gas was measured every 2 weeks. The ULOC was flushed before it was opened to prevent contaminating the catalyst in the outer chamber with volatile sulfur compounds.

Occasionally we had trouble with contamination of the plates. This problem was eliminated by keeping moisture in the ULOC to a minimum and by placing filters in the incoming gas lines.

Recovery. To be useful, the system described in this paper should be at least as effective as previously described procedures in terms of numbers of organisms and species isolated. Domestic sewage sludge and strain M.o.H. were chosen as test systems. Fourteen-day-old liquid cultures of strain M.o.H. having an optical density (660 nm) of 0.6 were serially diluted and plated in the outer chamber. Plating efficiency was as high as 30% of that expected from total
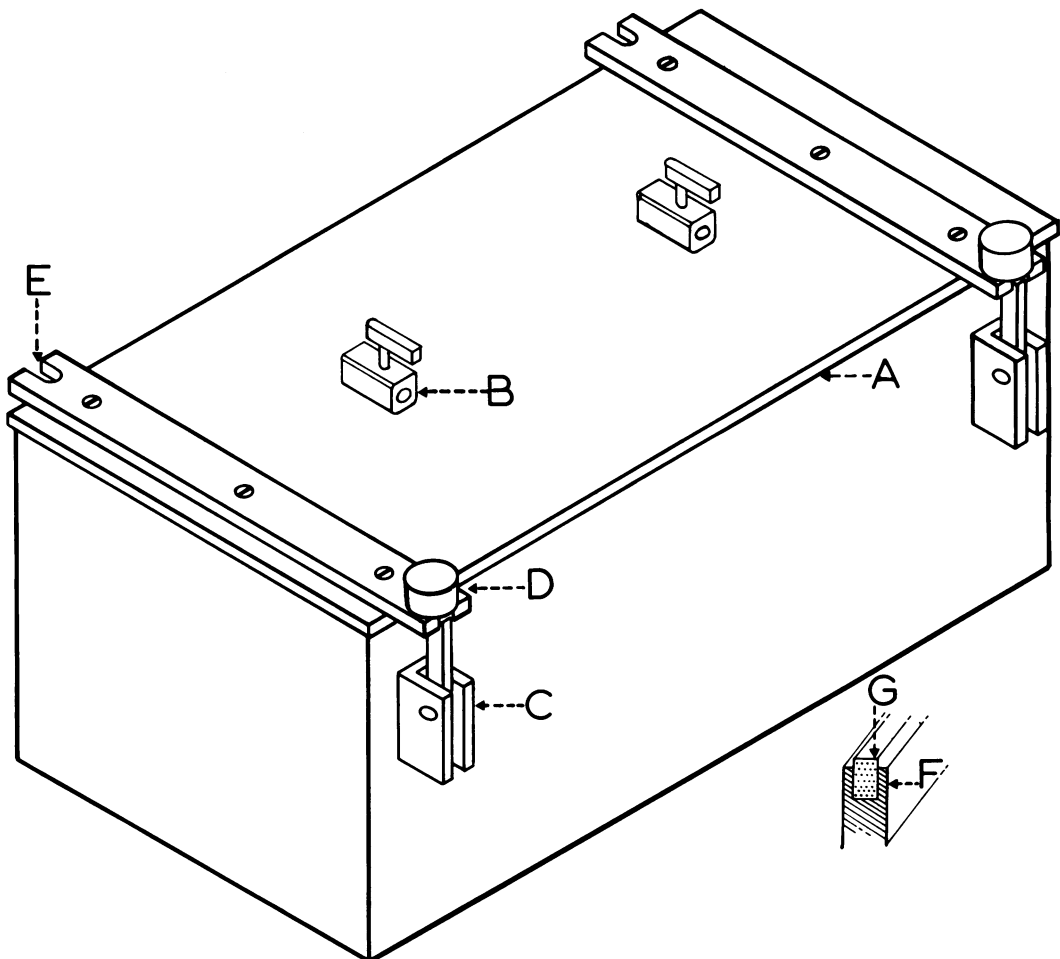

Fig. 2. *Ultra-low oxygen chamber. (A) Lid; (B) gassing valve; (C) swing clamp in the closed position; (D) clamp-tightening nut; (E) crossbar; (F) detail of wall showing machined channel; (G) molded silicone gasket.*

microscopic counts (Table 1). Sludge was obtained from an anaerobic sewage digester and immediately transported to the laboratory and placed in the anaerobic chamber. Samples were serially diluted, plated, and incubated in the ULOC. The plates were observed every 2 days and colonies were marked as they became visible. Twenty-five days after inoculation, every colony on one plate was subcultured into tubes of liquid medium, stoppered, and removed from the anaerobic chambers to a shaking water bath. After 14 days, the head space over the liquid was analyzed for methane. All methane-positive cultures were examined microscopically. Sludge samples contained 10^8 to 10^9 methanogenic bacteria per ml. This is comparable to the numbers obtained with the roll tube method (7). Gas analysis and microscopy showed that over 80% (25/30) of the colonies in a 10^7 dilution were methanogenic (Table 1). The species isolated from a single sewage sludge sample included *Methanosarcina barkeri*, *Methanobacterium* strain M.o.H., *Methanobacterium formicicum*, and *Methanospirillum*.

Identification of methanogenic bacteria. Colonies of methanogenic bacteria were identified on petri plates by taking advantage of a fluorescent pigment peculiar to this metabolic group of bacteria. Cheeseman et al. (4) reported that strain M.o.H. synthesizes a low-molecular-weight compound, F_{420}, which in the oxidized form fluoresces when excited by long-wave ultraviolet light. During active metabolism, 80% of F_{420} exists in the oxidized form (A. M. Roberton, personal communication). F_{420} is cell bound, and therefore its fluorescence is confined to the colony. The large amount of F_{420} in the cell and its high extinction coefficient prompted us to determine whether it could be visualized in colonies of strain M.o.H. There was ample F_{420} to permit direct visual observation of the methanogenic colonies when they were exposed to long-wave ultraviolet light (Fig. 3). Figure 3A is a photograph taken in visible light of a mixed culture containing strain M.o.H. and unidentified non-methanogenic organisms. Figure 3B is a photograph of the same plate taken in ultraviolet light. In the latter, the methanogenic colonies are bright white spots. Fluorescence due to F_{420} is typically blue-green and is readily distinguishable from the white-yellow fluorescence occasionally observed in non-methanogenic colonies. The practicability of ultraviolet fluorescence for screening mixed cultures was tested by using dilutions of sewage sludge. In all cases only colonies containing methanogenic bacteria fluoresced blue-green. This was proven by sub-

TABLE 1. Recovery of methanogenic organisms[a]

Source	Dilution	Colonies	CFU/ml[c]	% Methanogenic
Strain M.o.H.[b]	10^{-6}	35	3.5×10^7	100
Sewage sludge	10^{-7}	30	3×10^8	83

[a] This experiment was repeated three times, using different cultures of strain M.o.H. and different sludge samples. Similar results were obtained in each experiment.

[b] 1.1×10^9/ml as determined by microscopic enumeration in a Petroff-Hauser counting chamber.

[c] CFU, Colony-forming units.

culturing all fluorescent colonies into liquid media, incubating them anaerobically, and assaying for methane. Fluorescent colonies were not always pure, but they were all methanogenic. *Methanobacterium* strain M.o.H., *Methanospirillum*, *Methanobacterium formicicum*, and *Methanosarcina* have been identified by this procedure. It is possible to identify colonies of methanogenic bacteria when they are less than 0.5 mm in diameter with the use of a dissecting microscope or hand lens and an ultraviolet light. This identification technique offers a simple, rapid, and sensitive method for isolation and enumeration of methanogenic bacteria. Methanogenic colonies can be identified in roll tubes, but the fluorescence is not as intense as that observed on petri plates. This is probably due to light scattering at the agar-glass interface and absorption of light by the growth medium.

DISCUSSION

The system described offers a number of practical advantages in culturing methanogenic bacteria. Media are prepared by standard aerobic methodology, thus eliminating time-consuming anaerobic preparation methods. Special culture techniques are not required, and additional sensitivity and speed are gained by superior visibility and accessibility of colonies in high-density cultures. The use of ultraviolet light fluorescence selection also increases speed and sensitivity. The methanogenic organisms grow rapidly and are detectable sooner than by roll tube methods. Standard genetic procedures such as replica plating can be used to study these organisms.

The fact that the culture chamber is itself enclosed in an atmosphere of very low oxygen provides an additional safety margin against accidental exposure to high oxygen levels. Filtering of the ULOC and the outer gas chamber
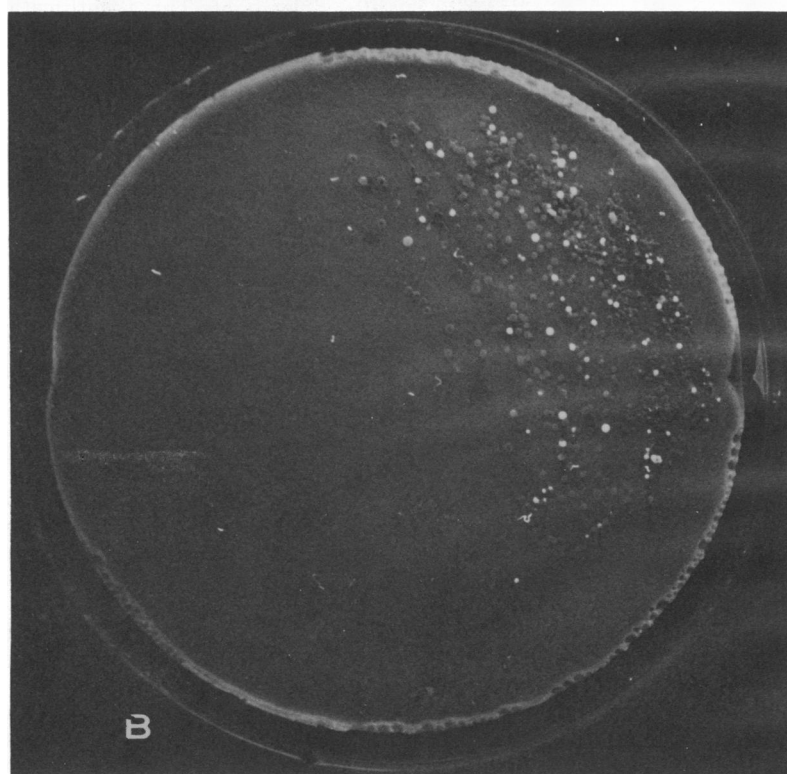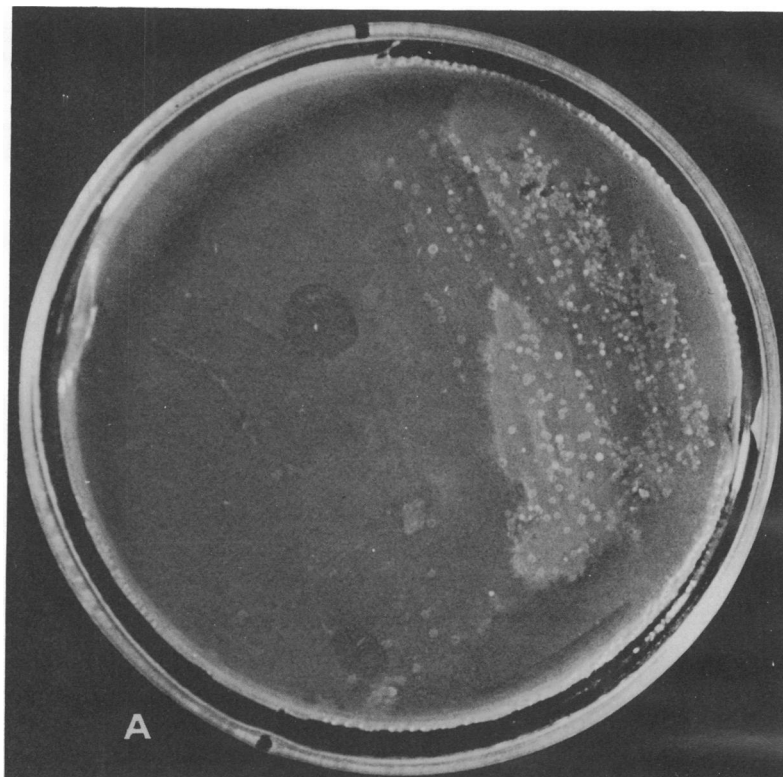
FIG. 3. *Ultraviolet identification of methanogenic colonies. (A) Photograph taken under visible light; (B) photograph showing fluorescent colonies under long-wave ultraviolet light.*

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provides an atmosphere very low in potential contaminants and eliminates many difficulties of that sort.

It has been our experience that all fluorescent colonies have been methanogenic and all non-fluorescent colonies have been non-methanogenic. However, the possibility exists that there are methanogenic bacteria that have no F_{420} or only low levels, or, conversely, that there are non-methanogenic bacteria that exhibit similar ultraviolet fluorescence. Fluorescence is presumptive evidence for methanogenic bacteria, but definitive proof requires further characterization.

We have shown that the ULOC technique is comparable to previously described anaerobic methods. Equivalent numbers and species of methanogenic bacteria can be isolated from a natural system. Furthermore, the combination of petri plate methodology and fluorescence observation permits detection of methanogenic colonies earlier than with conventional methods. This means that methanogenic organisms present in low numbers can be identified before they are overgrown by more numerous and rapidly growing organisms. Fluorescence also enables one to visualize a single methanogenic colony in the midst of large numbers of non-methanogenic colonies. This precludes the necessity of plating all colonies to assess the total numbers of methanogenic organisms.

LITERATURE CITED

1. Aranki, A., and R. Freter. 1972. Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. Am. J. Clin. Nutr. 25:1329–1334.
2. Bryant, M. P., B. C. McBride, and R. S. Wolfe. 1968. Hydrogen-oxidizing methane bacteria. I. Cultivation and methanogenesis. J. Bacteriol. 95:118–1123.
3. Bryant, M. P., and I. M. Robinson. 1961. An improved nonselective culture medium for rumen bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy. Sci. 44:1446–1456.
4. Cheeseman, P., A. Toms-Wood, and R. S. Wolfe. 1972. Isolation and properties of a fluorescent compound, factor$_{420}$, from *Methanobacterium* strain M.o.H. J. Bacteriol. 112:527–531.
5. Holdeman, L. V., and W. E. C. Moore (ed.). 1972. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg.
6. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14:1–49.
7. Smith, P. H. 1966. The microbial ecology of sludge methanogenesis. Dev. Ind. Microbiol. 7:156–161.
8. Wolfe, R. S. 1971. Microbial formation of methane. Adv. Microb. Physiol. 6:107–146.
9. Wolin, E. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. 238:2882–2886.