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Application of the Fluorescent-Antibody Technique to the Study of a Methanogenic Bacterium in Lake Sediments†

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Fluorescent antibody (FA) was prepared for a methanogenic bacterium isolated from Wintergreen Lake pelagic sediment. The isolate resembles *Methanobacterium formicicum*. The FA did not cross-react with 9 other methanogens, including *M. formicicum* strains, or 24 heterotrophs, 18 of which had been isolated from Wintergreen Lake sediment. FA-reacting methanogens were detected in heat-fixed smears of several different lake sediments and anaerobic sewage sludge. Pretreatment of all samples with either rhodamine-conjugated gelatin or bovine serum albumin adequately controlled nonspecific absorption of the FA. Autofluorescent particles were observed in the sediment samples but, with experience, they could easily be distinguished from FA-reacting bacteria. FA direct counts of the specific methanogen in Wintergreen Lake sediments were made on four different sampling dates and compared with five-tube most-probable-number estimates of the total methanogenic population that was present in the same samples. The FA counts ranged from 3.1×10^6 to 1.4×10^7/g of dry sediment. The highest most-probable-number estimates were at least an order of magnitude lower.

Methods for enumerating methane-producing bacteria in anaerobic habitats require anaerobic culture techniques, prolonged incubation times, detection of methane (19, 26) and, in the case of roll tube methods, some way of determining which colonies are responsible for methane production (8, 19). While these methods represent the state-of-the-art for estimating the total methanogenic population, autoecological studies of specific species of methanogens are either extremely tedious or cannot be done at all.

The fluorescent-antibody (FA) technique, which is one of the most useful methods available for studying microbial autecology (11, 12, 21), has not been investigated with methane bacteria and has not been used in the anaerobic lake sediment habitat. One purpose of this paper is to report our evaluation of these applications of the FA technique. The second purpose is to compare two methods of enumeration: an FA direct count of a specific hydrogen-consuming methanogen and a most-probable-number (MPN) estimate of total methanogens in lake sediment. This comparison was made to determine whether hydrogen-consuming methanogenic bacteria were among the predominant methane formers in this habitat.

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MATERIALS AND METHODS

Media. The enrichment-purification medium for the methanogen contained the following (per liter of distilled water): K_2HPO_4, 0.225 g; KH_2PO_4, 0.225 g; NH_4Cl, 0.185 g; NaCl, 0.45 g; $MgCl_2 \cdot 6H_2O$, 0.076 g; $CaCl_2 \cdot 2H_2O$, 0.060 g; trace elements solution (24), 10 ml; vitamin solution (24), 10 ml; 0.1% (wt/vol) resazurin solution, 1.0 ml; CO_2-equilibrated 8% (wt/vol) Na_2CO_3, 25 ml; cysteine hydrochloride, 0.5 g; $Na_2S \cdot 9H_2O$, 0.5 g. The gas atmosphere of all anaerobically prepared culture tubes was oxygen-free H_2-CO_2 (50:50, vol/vol). An incubation temperature of 28°C was used for enrichment and isolation.

Counts of FA-reacting methanogens in the sediment were compared with estimates of the total methanogenic population made by the five-tube MPN technique (1). Three different MPN media were employed. All had a mineral composition that was identical to that of the enrichment-purification medium. In addition, the following organic substrates were added (per liter of medium): Trypticase, 2.0 g; yeast extract, 2.0 g; sodium formate, 2.0 g; sodium acetate, 2.0 g. The three media differed in the reducing agent and/or growth factors that were added (per liter of medium): (i) cysteine hydrochloride, 1.0 g; (ii) a 24-h culture of *Escherichia coli* grown aerobically at 37°C in tryptic soy broth (Difco), 40 ml; or (iii) $Na_2S \cdot 9H_2O$, 0.1 g; Ti(III) citrate (25), Ti(III), 0.6 mM; 2-methyl butyric

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acid, isovaleric acid, and isobutyric acid, 0.1 mM each. The reductants were chosen to avoid possible sulfide toxicity, which has been noted by Cappenberg (8).

All reducing agents were added 24 h before inoculation with sediment. Preliminary experiments showed that the added *E. coli* would reduce methyl viologen within this time. The method of preparing Ti(III) citrate (25) was modified such that all solutions were boiled under O_2-free nitrogen before mixing, and all manipulations were done anaerobically.

Anaerobic methodology. The anaerobic culture and isolation techniques used were those described by Hungate (15) with the modifications of either Bryant and Robinson (7) or Macy et al. (17). For some purification attempts, we used an anaerobic glove chamber (2) (Coy Manufacturing Co., Ann Arbor, Mich.) with a gas atmosphere of H_2-N_2 (10:90, vol/vol). Petri plate agar (enrichment-purification medium plus 1.5% [wt/vol] agar) streaks were incubated in anaerobic containers within the glove chamber. These containers were similar to those described by Edwards and McBride (10) and had a gas atmosphere of H_2-CO_2 (50:50, vol/vol). Exposure of the plates to the glove box atmosphere occurred only during inspection for growth and culture transfer to fresh medium.

Antigen preparation. The methanogenic culture that we used as the antigen in FA preparation was isolated from a dilution series of enrichment-purification medium that had been inoculated with lake sediment. The sediment was collected by a gravity corer from the 6-m depth of Wintergreen Lake (Kalamazoo County, Mich., R 9W, T 2N, Sec 8) in July. The medium was inoculated from a 10-fold dilution series of a homogeneous mixture of the upper 5 cm of the sediment core.

All attempts to obtain an axenic methanogenic culture were unsuccessful. However, contaminant levels could be kept at a minimum (less than 1% of the presumptive methanogen) by keeping the headspace H_2 concentrations high and by transferring the culture to fresh medium (10-fold dilution) every 2 days. Contaminant levels were monitored by microscopic observation of wet mounts of the culture. Cells to be used as antigen were grown in 200 ml of enrichment-purification medium, harvested by centrifugation, and killed by suspension in 4% (vol/vol) Formalin in 0.85% (wt/vol) sterile saline for 72 h.

Antibody preparation. The antigen suspension was injected into young adult male rabbits according to the schedule described by Schmidt et al. (22). Blood was collected every 2 weeks from a puncture of the marginal vein of the ear. At each bleeding, 30 ml of blood was collected in an equal volume of anticoagulent solution. Purification of the serum antibody and conjugation with fluorescein isothiocyanate followed the procedure of Schmidt et al. (22). The fluorescein isothiocyanate-to-protein ratio was 1:50 (wt/wt), and protein was determined by the method of Lowry et al. (16), with bovine serum albumin as the standard. Excess fluorescein isothiocyanate was removed from conjugated FA by passage through a Sephadex G-25 column that had been equilibrated with 0.12 M phosphate-buffered saline, pH 7.2. The FA was collected in 5-ml portions, preserved with 0.01% (wt/vol) Thimerosal (Sigma Chemical Co.), and frozen until needed.

FA stain of cultures and natural samples. The microscope slide staining procedure of Schmidt et al. (22) was used to stain heat-fixed cultures or natural samples. Nonspecific absorption of the FA was controlled by the prior addition of either rhodamine-conjugated gelatin (5) or 2% bovine serum albumin. The FA-stained smears were observed under a Leitz Ortholux microscope with incident ultraviolet light illumination. A 150-W Xenon lamp was used as the ultraviolet light source. The excitation filters were two KP490's. The dichroic beam-splitting mirror was a TK510, and the barrier filters were a K515 and a K510. Two thicknesses of Kodak Wratten gelatin filter no. 12 were added to the barrier filter assembly when photomicrographs were taken.

A modification of the soil procedure of Bohlool and Schmidt (6) was used to obtain direct counts of FA-reacting bacteria in lake sediments. Ten milliliters of sediment, 90 ml of filter-sterilized distilled water, and 0.1 ml of Tween 80 were mixed for 5 min in a blender. A flocculant [0.5 g of $Ca(OH)_2$ and 1.25 g of $MgCO_3$; dried overnight at 90°C] was added to this dispersed sediment, which was then vigorously shaken by hand for 2 additional min. Two drops of Antifoam B emulsion (Sigma Chemical Co.) were added during the last 10 s of shaking. The floc was allowed to settle, undisturbed, for 15 min. The supernatant liquid was then carefully removed by aspiration, and a sample was immediately filtered through a black Sartorius 25-mm diameter membrane filter (0.45-μm pore diameter). The procedure of Bohlool and Schmidt (6) as modified by Schmidt (21) was used to stain the bacteria on the filters with FA, except that 2% bovine serum albumin was used to control nonspecific absorption.

Stained bacteria were observed under a Leitz microscope with incident ultraviolet illumination as described above. Since the number of FA-reacting bacteria per microscope field followed a Poisson distribution, these data were normalized with a square-root transformation so that 95% confidence limits could be calculated. The bacteria on a minimum of three membrane filters were stained with FA on each sampling date, and 100 microscope fields per filter were counted. The number of FA-reacting organisms per gram (dry weight) of sediment was calculated by using the equation of Bohlool and Schmidt (6).

Since microorganisms are removed by flocculation (21), we attempted to correct for this by determining the percentage of total sediment bacteria lost during this step. Total bacteria present before and after flocculation were determined by the acridine orange-epifluorescence, direct counting method of Daley and Hobbie (9). Only green-fluorescing bacteria were counted.

For comparison with the FA direct counts, five-tube MPN estimates of the sediment methanogenic bacterial population were made by using the three MPN media described above. Tenfold serial dilutions of the sediment were made in enrichment-purification medium, with 1 min of Vortex mixing between each dilution. MPN tubes were inoculated from this dilution series. The tubes were incubated for 4 weeks at 37°C. At the end of this time, each tube was analyzed for methane production as determined by flame ionization gas chromatography. Other studies had shown

that the results did not change over a 2- to 6-week incubation period.

RESULTS

Description of methanogenic organism purified from the sediment. Figure 1 is a phase-contrast photomicrograph of single cells and a filament of the methanogen. The organism is gram positive and produces methane from hydrogen and carbon dioxide and from formate. It does not produce methane from acetate or methanol. The optimum growth temperature is 37°C. Chains and filaments are common, often exceeding 60 μm in length. Cellular dimensions are 0.5 μm in diameter by 3 to 6 μm. Surface colonies are pale yellow to tan and flat. These colonies fluoresce blue-green when exposed to long-wavelength ultraviolet light. This fluorescence is characteristic of methanogens and is due to oxidized factor 420, a compound that has only been found in methanogens (10). Individual cells of an actively growing culture exhibited a faint autofluorescence when observed microscopically under incident ultraviolet illumination. This allowed us to be certain that the morphotype reacting with the FA was a methanogen. This autofluorescence is too faint to distinguish in the sediment matrix and thus cannot be used to directly enumerate methanogens.

Tests on antibody prepared with the methanogen. Agglutination titers of serum collected from injected rabbits never exceeded 800. Although this value is low, FA prepared from this serum adequately stained the antigen (Fig. 2). An FA stain of a culture in which the contaminants were deliberately allowed to proliferate showed that the contaminants did not react with the FA.

Table 1 lists the control tests that Schmidt (20) states are necessary for the application of FA. Our results from these tests are listed in the right-hand column. Several tests were run to satisfy the requirement that the FA should stain antigen cells grown under various condi-

TABLE 1. *Control tests needed for the application of FA (from Schmidt [20])*

Component checked	Expected result	Actual result
Antigen		
Stain with "normal" serum FA	–	–
Stain with unconjugated homologous serum followed by homologous FA	–	–
Stain with homologous FA that has been absorbed with antigen cells	–	–
Stain of antigen cells grown under various conditions	?	+
Antibody		
FA stain of related organisms (see Table 2)	–	–
FA stain of unrelated organisms (see Table 2)	–	–

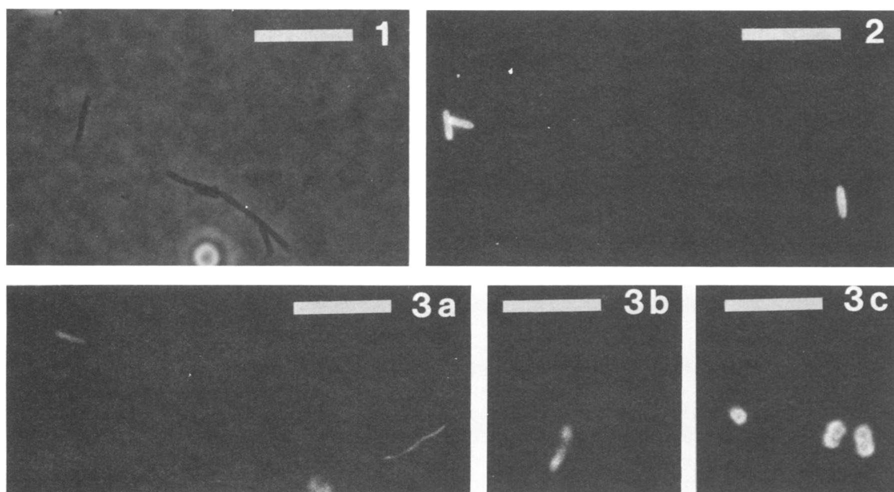

FIG. 1. *Phase-contrast photomicrograph of a short filament and single cells of a methanogenic bacterium isolated from Wintergreen Lake sediment and used to prepare the FA. Bar represents 10 μm.*

FIG. 2. *Photomicrograph of an FA-stained smear of the methanogenic isolate. Bar represents 10 μm.*

FIG. 3. *Photomicrographs of immunofluorescing cells in FA-stained smears of various habitats. (a) Wintergreen Lake pelagic sediment. (b) Sludge from an anaerobic sewage digestor. (c) Bovine rumen fluid. Bars represent 10 μm.*

tions. The FA reactions of the methanogenic culture at different stages of the growth cycle and after different periods of starvation were all +4. An FA reaction of +4 was arbitrarily defined as the FA reaction of the original antigen preparation. A freshly stained smear of this antigen was used as a reference for each stain. The FA reaction of the methanogen culture grown at different temperatures ranged from +2 for cells exposed to 4°C to +4 for cells grown at 28 and 37°C. The morphology of the methanogenic bacteria changed at the different growth temperatures, with single cells being the predominant morphotype at 4°C and filaments being the predominant morphotype at the higher temperatures.

Tests on the antibody (Table 1) included staining related and unrelated organisms to test the specificity of the FA preparation. The organisms tested are listed in Table 2. None were stained by the FA. Of particular importance to this study was the finding that no other organism isolated from Wintergreen Lake sediment reacted with the FA.

Qualitative survey of various habitats for FA-reacting bacteria. Heat-fixed smears of samples from several anaerobic methanogenic habitats were stained with FA to determine the distribution of this methanogenic strain. Samples stained included Burke Lake pelagic sediment, Wintergreen Lake pelagic and littoral sediments, anaerobic sewage sludge, and bovine rumen fluid. Photomicrographs of several of these FA-stained samples are presented in Fig. 3. In all sediment and in the anaerobic sewage sludge samples, the morphological shape shown in Fig. 3a and b was the only one that was stained with FA. The only FA-reacting organism in rumen fluid is shown in Fig. 3c. The differences in stain localization and morphology of the rumen organism compared with those of the sludge and sediment organisms was far more apparent to the eye than could be recorded by photography. Also, phase-microscopic examination of the stained rumen cells clearly showed that they were cocci.

Qualitative estimates of the FA-staining reaction of cells in these habitats ranged from +1 in Burke Lake sediment to +3 in all Wintergreen Lake sediments, +3 to +4 in anaerobic sewage sludge, and +2 in rumen fluid. Nonspecific absorption of FA by all samples was minimal when they were pretreated with either rhodamine-conjugated gelatin or BSA. Autofluorescent particles were not a problem in anaerobic sewage sludge or rumen samples, but were present in most of the sediment samples. These particles appeared to be partially degraded zooplankton and could easily be distinguished from FA-reacting bacteria.

Direct count FA and MPN estimates of the methanogenic bacteria in lake sediment. Estimates of the methanogenic population in the upper 5 cm of sediment cores collected from Wintergreen Lake are presented in Table 3. Cores were collected at times of active methanogenesis. The October and March samples were taken just before lake turnover and

TABLE 2. *Microorganisms tested for cross-reactivity with FA preparation; no cross-reaction observed*

Organism	Source of isolate	Contributor
Related organisms		
Methanobacterium strain M.o.H.	San Francisco Bay mud	M. P. Bryant
M. ruminantium PS	Anaerobic sewage sludge	M. P. Bryant
M. ruminantium M1	Rumen	M. P. Bryant
M. formicicum JF	Anaerobic sewage sludge, benzoate enrichment	J. G. Ferry
M. arbophilicum	Wetwood of trees	J. G. Zeikus
Methanobacterium sp.	Salt marsh sediment	M. J. B. Paynter
Methanosarcina MS	Anaerobic sewage sludge	M. P. Bryant
Methanosarcina U.B.S.	Lake Mendota sediment	J. G. Zeikus
Methanospirillum hungatii JF	Anaerobic sewage sludge, benzoate enrichment	M. P. Bryant
Unrelated organisms		
Four clostridial strains	Wintergreen Lake sediment	M. J. Klug
One other obligate anaerobe	Wintergreen Lake sediment	M. J. Klug
Six facultative anaerobes	Wintergreen Lake sediment	M. J. Klug
Four denitrifying strains	Wintergreen Lake sediment	Authors' laboratory
Three anaerobes isolated from a methanogenic enrichment	Wintergreen Lake sediment	Authors' laboratory
Bacterial smear of a sulfate-reducing enrichment	Wintergreen Lake sediment	Authors' laboratory
Six *Bacteroides* species	Various intestinal habitats	C. A. Reddy

TABLE 3. *Number of methane-producing bacteria in Wintergreen Lake sediment on different sampling dates as estimated by FA direct counts and by a five-tube MPN technique using three different media*[a]

Sampling date	No. of methanogens ($\times10^{-4}$) per g of dry sediment as determined by:			
	FA direct count	MPN with cysteine reductant	MPN with *E. coli* reductant	MPN with Ti(III) reductant
25 June 1976	1,400 (1,300–1,500)[b]	10 (3.5–27)	ND[c]	ND
3 September 1976	320 (300–330)	24 (6.4–59)	ND	ND
16 October 1976	630 (590–680)	6.6 (2.3–17)	66 (23–170)	ND
19 March 1977	310 (280–340)	2.6 (0.8–6.4)	11 (3.7–31)	16 (5.7–44)

[a] MPN tubes were scored as positive if methane was produced within 4 weeks.
[b] Numbers in parentheses indicate 95% confidence intervals.
[c] ND, Not determined.

after long periods of anoxia. After dispersion-flocculation, up to 10 ml of the supernatant could be passed through the membrane filters before they became clogged. For all FA membrane filter counts, the number of bacteria per field followed a Poisson distribution (G-test for goodness of fit [23]). Additional MPN media were used after the June sampling date in an attempt to improve the MPN estimates over that obtained with the cysteine-reduced medium.

Direct counts of acridine orange-stained bacteria before and after the flocculation of dispersed sediment indicated that 15% of the bacteria were lost during flocculation. This loss was estimated with the sediment collected in March 1977. The FA counts presented in Table 3 have been corrected for this flocculation loss.

To determine whether FA-reacting bacteria were present in MPN tubes, the contents of high-dilution, methane-positive tubes inoculated with the 16 October 1976 sediment sample were stained with FA. FA-reacting bacteria were present in all tubes tested.

DISCUSSION

As expected, an FA could be made that would react with the methane bacterium isolated (Fig. 2). The specificity of the FA was judged adequate, since no other methanogen nor a variety of nonmethanogens showed any cross-reaction with it (Table 2). FA-reacting bacteria in sediment and anaerobic sewage sludge had the same morphology as the methanogen (Fig. 1, 2, 3a and b). The FA-reacting organism in rumen fluid (Fig. 3c) did not; this coccoid cell was never observed in any stained preparations of our culture, lake sediments, or anaerobic sludge samples. When Hobson et al. (14) stained sheep rumen contents with FA prepared for *Selenomonas ruminantium,* they observed that, in addition to stained selenomonads, small cocci and occasionally large cocci also reacted. Their FA preparations cross-reacted with *Veillonella alcalescens* (*gazogenes*) and, when rumen contents were stained with FA that had been absorbed with *V. alcalescens,* no reaction was observed other than with selenomonad-like organisms. In a similar manner, it is likely that antibodies to microorganisms related to *V. alcalescens* were responsible for the FA staining of the rumen organisms we observed. It is possible that these cross-reacting cocci are normal inhabitants of the rabbit intestinal tract.

The value of the FA technique for certain autecological studies may be limited by a high degree of strain specificity of the FA if there is a high degree of antigen diversity among the organisms responsible for the activity under study. Strain-specific FA is not uncommon and has been used by Schmidt et al. in studying *Rhizobium* (22) or noted by other investigators attempting to use FA in autecological studies of *Thermoplasma* (3), *Sulfolobus* (4), *Bacillus* sp. (13), and *Butyrivibrio* (18). There apparently is some strain or species specificity shown by the FA used in this study, since it does not cross-react with *Methanobacterium* strain M.o.H. and *M. formicicum,* which are morphologically similar to our isolate. However, our finding that FA-stained cells of the correct morphology were present in all of the sediments and activated sludge that we examined suggests that the FA we prepared is not too strain specific and is thus of use in studying natural samples. Furthermore, the population of FA-stained cells was at least as great, if not greater, than the population estimates obtained with the best MPN media (Table 3). Though there could be more numerous methanogens than either of these approaches detect, present methodology suggests that the FA we prepared is adequate for detecting at least a significant fraction of the sediment methanogenic flora.

The intensity of staining that we observed varied. The weak staining of the methanogenic culture grown at 4°C and the observation of weakly FA-stained bacteria in winter samples

of Burke Lake pelagic sediment indicate that our FA may react poorly with cold-adapted or extensively aged cells. Our isolate apparently does not exhibit much antigenic variability, since the FA readily stained sediment methanogens 1.5 to 2.5 years after their original isolation, and it stained cells of laboratory cultures prepared under a variety of growth and starvation conditions.

No major problems were encountered with applying FA staining techniques to anaerobic lake sediments. Autofluorescent particles were initially a problem, but with experience it became easy to distinguish FA-reacting bacteria. In March, after anoxic conditions had caused a winter kill, autofluorescent particles were absent.

Comparison of the two methods that we used for enumerating sediment methanogens (Table 3) shows that the membrane filter FA direct counts were generally an order of magnitude greater than MPN estimates. Several explanations could account for this difference. The most likely is that all viable methanogenic cells do not grow in MPN media. The differences in estimates among media indicate the important influence of media on results. Other explanations are that the FA may be detecting a substantial number of dead cells and that a slightly more vigorous method of release and dispersion of cells was employed with the FA method. An error that could increase the FA/MPN recovery ratio is underestimation of flocculation losses. The value of 15%, which was determined for the predominant short rods in this sediment, may be too low for the more filamentous methanogenic cells.

Our enumeration results, both by FA and MPN (Table 3), compare favorably with the results reported by others for different lakes. Zeikus and Winfrey (26) have estimated the number of methanogens in Lake Mendota sediments and found as few as 10^2/g in shallow, winter sediments and as many as 10^6/g in sediments underlying deeper waters in the summer. Although Cappenberg (8) determined the number of methanogenic bacteria with sediment depth in Lake Vechten, comparisons with our results are difficult to make, since his estimates are reported on the basis of the number of organisms per liter of wet mud. Nevertheless, at a 5-cm sediment depth, the number of methane-producing bacteria per milliliter of wet Lake Vechten mud ranged from 2×10^3 to 8×10^5.

Although the time required to isolate the microorganism of interest and to prepare and test the resulting FA is long, we believe that this autecological technique has proved useful for detection and enumeration of the methanogen. Recently, Ward and Frea (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I89, p. 169) have used FA techniques to detect other methanogenic species in Lake Erie sediments. Thus, the FA techniques may be generally useful for autecological studies of methanogens.

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LITERATURE CITED

1. **American Public Health Association, American Water Works Association, and Water Pollution Control Federation.** 1971. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, Washington, D.C.
2. **Aranki, A., S. A. Seyd, E. B. Kenney, and R. Freter.** 1969. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. Appl. Microbiol. **17**:568-576.
3. **Bohlool, B. B., and T. D. Brock.** 1974. Immunofluorescence approach to the study of the ecology of *Thermoplasma acidophilum* in coal refuse material. Appl. Microbiol. **28**:11-16.
4. **Bohlool, B. B., and T. D. Brock.** 1974. Population ecology of *Sulfolubus acidocaldarius*. II. Immunoecological studies. Arch. Microbiol. **97**:181-194.
5. **Bohlool, B. B., and E. L. Schmidt.** 1968. Nonspecific staining: its control in immunofluorescence examinations of soil. Science **162**:1012-1014.
6. **Bohlool, B. B., and E. L. Schmidt.** 1973. A fluorescent antibody technique for determination of growth rates of bacteria in soil. *In* T. Rosswall (ed.), Modern methods in the study of microbial ecology. Bull. Ecol. Res. Commun. (Stockholm) **17**:336-338.
7. **Bryant, M. P., and I. M. Robinson.** 1961. An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy Sci. **44**:1446-1456.
8. **Cappenberg, T. E.** 1974. Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh water lake. I. Field observations. Antonie van Leeuwenhoek J. Microbiol. Serol. **40**:285-295.
9. **Daley, R. J., and J. E. Hobbie.** 1975. Direct counts of aquatic bacteria by a modified epifluorescent technique. Limnol. Oceanogr. **20**:875-882.
10. **Edwards, T., and B. C. McBride.** 1975. New method for the isolation and identification of methanogenic bacteria. Appl. Microbiol. **29**:540-545.
11. **Fliermans, C. B., B. B. Bohlool, and E. L. Schmidt.** 1974. Autecological study of the chemoautotroph *Nitrobacter* by immunofluorescence. Appl. Microbiol. **27**:124-129.
12. **Fliermans, C. B., and E. L. Schmidt.** 1975. Autoradiography and immunofluorescence combined for autecological study of single cell activity with *Nitrobacter* as a model system. Appl. Microbiol. **30**:676-684.
13. **Hill, I. R., and T. R. G. Gray.** 1967. Application of the fluorescent-antibody technique to an ecological study of bacteria in soil. J. Bacteriol. **93**:1888-1896.
14. **Hobson, P. N., S. O. Mann, and W. Smith.** 1962. Sero-

logical tests of a relationship between rumen selenomonads *in vitro* and *in vivo*. J. Gen. Microbiol. **29**:265–270.

15. **Hungate, R. E.** 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1–49.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **Macy, J. M., J. E. Snellen, and R. E. Hungate.** 1972. Use of syringe methods for anaerobiosis. Am. J. Clin. Nutr. **25**:1318–1323.
18. **Margherita, S. S., R. E. Hungate, and H. Storz.** 1964. Variation in rumen *Butyrivibrio* strains. J. Bacteriol. **87**:1304–1308.
19. **Paynter, M. J. B., and R. E. Hungate.** 1968. Characterization of *Methanobacterium mobilis,* sp. n., isolated from the bovine rumen. J. Bacteriol. **95**:1943–1951.
20. **Schmidt, E. L.** 1973. Fluorescent antibody technique for the study of microbial ecology. *In* T. Rosswald (ed.), Modern methods in the study of microbial ecology. Bull. Ecol. Res. Commun. (Stockholm) **17**:67–76.
21. **Schmidt, E. L.** 1974. Quantitative antecological study of microorganisms in soil by immunofluorescence. Soil Sci. **118**:141–149.
22. **Schmidt, E. L., R. O. Bankole, and B. B. Bohlool.** 1968. Fluorescent-antibody approach to study of rhizobia in soil. J. Bacteriol. **95**:1987–1992.
23. **Sokal, R. R., and F. J. Rohlf.** 1969. Biometry. W. H. Freeman and Co., San Francisco.
24. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882–2886.
25. **Zehnder, A. J. B., and K. Wuhrmann.** 1976. Titanium-(III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. Science **194**:1165–1166.
26. **Zeikus, J. G., and M. R. Winfrey.** 1976. Temperature limitation of methanogenesis in aquatic sediments. Appl. Environ. Microbiol. **31**:99–107.