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Shifts in Methanogenic Subpopulations Measured with Antibody Probes in a Fixed-Bed Loop Anaerobic Bioreactor Treating Sulfite Evaporator Condensate

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A fixed-bed loop, high-rate anaerobic bioreactor treating sulfite evaporator condensate was sampled when it reached steady state and afterwards following perturbations during a 14-month period. By using immunotechnology, it was observed that shifts in methanogenic subpopulations occurred in association with perturbations, such as restarting and relocating the biomass into a different tank. Methanogens related to *Methanobacterium bryantii* MoHG and *Methanobrevibacter smithii* ALI were numerous throughout the observation period, while *Methanosarcina mazei* S6 and *Methanosarcina thermophila* TM1 were found in the early and late samples, respectively. Also, *Methanobacterium formicicum* was more numerous at the top portion of the bioreactor, while *Methanobrevibacter arboriphilus* AZ and DC were at the bottom. Sample formalinization required for prolonged storage proved suitable for antigen preservation.

Interest in anaerobic bioreactors for wastewater treatment is currently on the rise, although their chemistry and engineering (1, 5, 7, 9, 17, 18, 20), as well as their microbiology (3, 6, 16, 20, 21–23), have been studied for many years. Antibody probes and immunotechnology have been developed which allow identification of methanogens and other bacteria associated with them by direct examination of samples, i.e., avoiding culture isolation procedures (10, 12). This immunologic approach has proven useful for examining axenic and enrichment cultures and for elucidating complex microbial communities (8, 12, 13, 15, 19). A survey of bioreactors in different laboratories and localities demonstrated a considerable diversity of methanogenic subpopulations (15). Another survey, involving a set of bioreactors operated in the same laboratory under standardized conditions, revealed distinctive patterns of methanogenic flora depending on bioreactor type, feedstock, and temperature (A. J. L. Macario, J. F. K. Earle, D. P. Chynoweth, and E. Conway de Macario, Syst. Appl. Microbiol., in press). In the course of these studies, antibody probes and immunotechnology have been standardized for measuring the sizes of methanogenic subpopulations and for quantitatively comparing samples. It was necessary, however, to determine whether this immunotechnology would be suitable for detecting time course shifts in methanogenic subpopulations and thus provide a means for monitoring bioreactors. To answer this question, a convenient experimental protocol requires examination of successive samples collected at intervals over several months from a single bioreactor.

In this report, data are described for the first time concerning the qualitative and quantitative changes of methanogenic subpopulations measured with a panel of antibody probes over a period of 14 months in a high-rate anaerobic bioreactor. This was operated at the same overall conditions but was subjected to temporary changes, such as withdrawing biomass, restarting, or totally relocating the methanogenic biomass into a different vessel. Samples had to be stored for testing, not only as they were collected but also at the end of the observation period, so that they could be analyzed simultaneously under the same conditions. A comparison was therefore carried out between aliquots stored after formalinization and those stored without this treatment.

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MATERIALS AND METHODS

Antibody probes. Antibody S and D probes were prepared as described previously (13, 15) from antisera against the following reference methanogens (2): 1, *Methanobrevibacter smithii* PS; 2, *Methanobacterium formicicum* MF; 3, *Methanosarcina barkeri* MS; 4, *Methanobacterium bryantii* MoH; 5, *Methanobacterium bryantii* MoHG; 6, *Methanosarcina barkeri* R1M3; 7, *Methanospirillum hungatei* (*Methanospirillum hungatii*) JF1; 8, *Methanobrevibacter ruminantium* M1; 9, *Methanobrevibacter arboriphilus* (*Methanobrevibacter arboriphilicus*) DH1; 10, *Methanobrevibacter smithii* ALI; 11, *Methanobacterium thermoautotrophicum* GC1; 12, *Methanobacterium thermoautotrophicum* ΔH; 13, *Methanococcus vannielii* SB; 14, *Methanococcus voltae* PS; 15, *Methanogenium marisnigri* JR1; 16, *Methanosarcina barkeri* 277; 17, *Methanogenium cariaci* JR1; 18, *Methanosarcina mazei* S6; 19, *Methanosarcina barkeri* W; 20, *Methanosarcina thermophila* TM1; 21, *Methanobrevibacter arboriphilus* (*Methanobrevibacter arboriphilicus*) AZ; 22, *Methanobrevibacter arboriphilus* (*Methanobrevibacter arboriphilicus*) DC; 23, *Methanomicrobium mobile* BP; 24, *Methanothermus fervidus* V24S; 25, *Methanolobus tindarius* Tindari; 26, *Methanococcus maripaludis* JJ; 27, *Methanosphaera stadtmanae* MCB3; 28, *Methanoplanus limicola* M3; 29, *Methanococcus thermolithotrophicus* SN1; 30, *Methanothrix soehngenii* Opfikon; and 31, *Methanothrix* sp. strain CALS-1.

Reference antigens. A panel of reference antigens composed of the methanogens listed above and others in our repository, as well as samples also in our repository from

bioreactors already studied, was used as standards for calibrating the probes and in the assays, for comparative analyses of experimental samples, and as positive controls in each assay as described earlier (12, 13).

Immunologic assays. Before being tested, samples were fractionated into fluid and solid phases by centrifugation (15,000 × *g*, 20 min, 4°C). The pellet (solid phase) volume was measured, and the pellet was washed three times with five times its volume of phosphate-buffered saline, pH 7.2. The pellet obtained after the last washing was measured and then resuspended in the volume of phosphate-buffered saline required to obtain a suspension containing enough cells to cover ~80% of the surface of a circle of a slide immunoenzymatic assay (SIA) under standardized conditions (12, 13).

SIA-constellation (12) was used to study all samples. Methanogens were identified by morphotype (unstained, gram-stained, and immunofluorescence preparations were examined by using phase-contrast, bright-field, and epifluorescence optics, and the samples were compared with standard reference antigens) and by antigenic fingerprint as described previously (11–13, 15). The antigenic fingerprint was determined by two independent assays, indirect immunofluorescence (IIF) and quantitative SIA, by using a panel of 31 calibrated S and D probes, respectively. Antigenic fingerprinting results were compared with information in a data bank pertaining to the 31 reference methanogens and to information about 200 other characterized methanogens. Identified morphotypes were counted after IIF was performed. Bacterial concentrations, or packet concentrations in the case of sarcinas, were determined per unit volume (1 ml) of sample pellet. When SIA was used, the results were expressed in A_{450} at peak readings per ml of sample pellet. The absorbance values represent the quantitative signal given by the immunoenzymatic reaction, which is proportional to the number of positive cells.

Bioreactor samples. A 20-liter fixed-bed loop bioreactor, run in pH-auxostat mode (U. Ney et al., manuscript in preparation), filled with microporous reticulated sintered glass (SIRANTM; Schott, Mainz, Federal Republic of Germany) (1), working at 39°C, and fed sulfite evaporator condensate (chemical oxygen demand [COD] of 43,000 mg/liter) was sampled three successive times over a 14-month period. The first sampling (samples A and D) was done when the bioreactor reached stable conditions, i.e., constant flow of substrate and biogas. After 7 months, modifications were introduced, i.e., pH 6.8 and a substrate COD of 45,000 mg/liter, and samples E and F were collected. Then the configuration was changed, and 7 months later samples G through J were collected (Table 1). The material thus obtained was fractionated into liquid and solid phases by centrifugation. The pellet was divided into two aliquots; one was suspended in Formalin fixative as described earlier (13), and the other was suspended in phosphate-buffered saline with antibiotics (vancomycin, penicillin G, and tetracycline, 10 μg of each per ml). The two aliquots were stored at 4°C.

The microorganism content was determined by using a roll tube method with serial dilutions up to 10^{-9} to get single colonies. The cell numbers were calculated by counting colonies in the highest dilutions showing growth (Ney et al., in preparation). The percentage of acetate and H_2-CO_2 utilizer methanogens as well as furfural-degrading organisms varied depending on the working conditions of the bioreactor.

TABLE 1. Bioreactor samples[a]

Sample	Formalinization	Time[b]	Substrate (mg COD/liter)	Loading rate (kg COD/m^3 per day)	Efficiency of COD reduction (%)
A	+	1	43,000	73	86
D	+	1	43,000	73	86
E	−	2	53,000	78	83
F	+	2	53,000	78	83
G	−	3	45,000	68	81
H	+	3	45,000	68	81
I	−	3	45,000	68	81
J	+	3	45,000	68	81

[a] Bioreactor working volume, 20 liters; temperature, 39°C; pH, 6.7 at time 1 and 6.8 at times 2 and 3; substrate, sulfite evaporator condensate consisting of aqueous acetic acid, furfural, and sulfite sulfate. The bioreactor was purged from free biomass and restarted after each sampling at times 1, 2, and 3 by backflushing. Samples I and J were taken from the bottom of the fixed bed, whereas the others were taken from the upper level.

[b] 1, When the bioreactor reached stable conditions; 2, 7 months later (after these samples were taken, the whole fixed bed, including the organisms, was transferred into another bioreactor of the same working volume but of different configuration); 3, 7 months after time 2.

RESULTS

Methanogenic subpopulations before and after the bioreactor stress. The concentration of methanogens antigenically related to *Methanobacterium formicicum* MF was comparatively low (10^5 cells per ml in early samples), but it increased in excess of fourfold in late sample H (Fig. 1a). A similar trend was observed for the methanogens antigenically related to *Methanobrevibacter arboriphilus* AZ and DC, but they reached peak concentrations in sample J (Fig. 1b and c). A methanogen antigenically related to *Methanobrevibacter smithii* ALI was in the 10^7/ml range throughout the observation period, reaching the highest levels in samples F and H (Fig. 1d). A methanogen more closely related to *Methanobrevibacter smithii* PS than to strain ALI was 10-fold less concentrated and also reached its highest values in sample F (Fig. 1e). A rod recognized by the probe for *Methanosarcina thermophila* TM1 increased in concentration from the initial samples, reaching the highest levels in samples H and J (Fig. 1f and 2). Methanogenic subpopulations antigenically related to *Methanosarcina barkeri* W and *Methanosarcina mazei* S6 (both acetoclastic) tended to decrease with time (Fig. 2g and h). Packets and single cocci were discriminated. A *Methanothrix* morphotype was found in all samples which reacted very weakly with the probe for Opfikon (not shown).

Quantitative SIA was applied to compare samples when the concentration of the methanogen under scrutiny was too high for IIF counting. This was the case for all samples of a methanogen antigenically related to *Methanobacterium bryantii* MoHG (Fig. 3a) and for the rod recognized by the probe for *Methanosarcina thermophila* TM1 in samples H and J (Fig. 3b). The methanogen related to strain MoHG was in the range of 10^3 to 10^4 A_{450}/ml (i.e., above 10^7 cells per ml) throughout the observation period. Under IIF, this methanogen appeared as long filaments (30 to 40 μm) in early samples (Fig. 4a and b) and as shorter rods in late ones (2 to 5 μm).

Comparison of samples taken from different levels of the bioreactor. A comparison of samples taken from the bottom of the fixed bed, such as sample J, with samples taken from upper levels, such as sample H, showed some stratification. For instance, in sample H, the methanogens related to *Methanobacterium formicum* MF, *Methanobrevibacter*

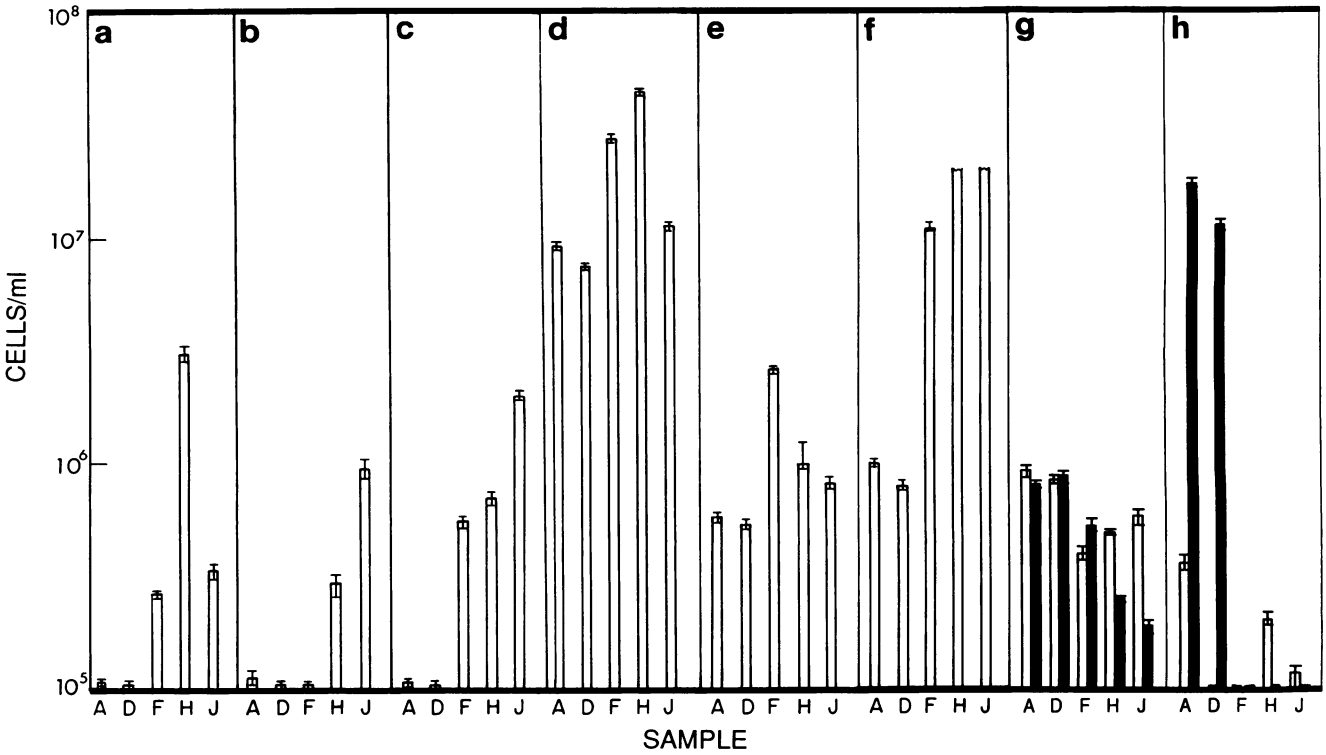

FIG. 1. Quantification of methanogens in samples A, D, F, H, and J antigenically related to *Methanobacterium formicicum* MF (probe no. 3) (a), *Methanobrevibacter arboriphilus* AZ (probe no. 21) (b) and DC (probe no. 22) (c), and *Methanobrevibacter smithii* ALI (probe no. 10) (d) and PS (probe no. 1) (e) and rod reactive with probe for *Methanosarcina thermophila* TM1 (probe no. 20) (f), *Methanosarcina barkeri* W (probe no. 19) (g), and *Methanosarcina mazei* S6 (probe no. 18) (h). Bars represent the numbers of reactive morphotype per milliliter of sample pellet. For g and h, open and closed bars represent packets and single cocci, respectively. Each bar represents the arithmetic mean ± the range (n = 2). The wavy lines at the top of the bars for samples H and J in panel f indicate abundance beyond that quantifiable by the method used.

smithii ALI, and *Methanosarcina thermophila* TM1 (rods) tended to be more abundant than in sample J. On the other hand, in the latter sample, the methanogens related to *Methanobrevibacter arboriphilus* AZ and DC tended to be more numerous than in sample H.

Comparison of formalinized with nonformalinized samples. A comparison of samples which had been formalinized at the time of sampling with identical aliquots that had not been fixed was carried out. Thirty such pairs of aliquots were examined at the end of the 14-month observation period. No significant differences were detected in 17 pairs. However, in the nonformalinized component of 13 pairs, reactive methanogens were fewer than in the formalinized aliquot (Fig. 5; data obtained with the probe for *Methanobacterium bryantii* MoHG are not shown in Fig. 5 because IIF counts were not done, as mentioned above [Fig. 3a]). Testing a sample immediately after collection and after storage, at the end of the experiment, produced the same results (data not shown).

DISCUSSION

Data regarding the invariability of the methanogenic population in a bioreactor at steady conditions have been demonstrated microbiologically by others (J. T. C. Grotenhuis, personal communication, and E. Koornneef, manuscript in preparation) and confirmed immunologically by us (unpublished data). Therefore, the data presented in this report about the time shifts in a methanogenic population can be attributed to the modifications introduced in the system. For example, the single-coccus form of a methanogen closely related to *Methanosarcina mazei* S6 was present at concentrations over 10^7/ml initially, but it decreased markedly in late samples, after the methanogenic biomass had been stressed by relocating it into a different tank. The packet form of this methanogen also decreased with time but not as dramatically as the single cocci. These downward trends coincided with an increase in methanogenic rods, such as those antigenically related to *Methanobacterium formicicum* MF and *Methanobrevibacter arboriphilus* AZ and that recognized by the probe for *Methanosarcina thermophila* TM1, presumably a *Methanothrix* immunotype (see below). These trends suggest that some methanogenic rods established their subpopulations more rapidly than sarcina after experiencing the condition changes brought about by restarting the bioreactor.

Interestingly, two immunotypes of *Methanobrevibacter smithii* were found to coexist in the bioreactor (Fig. 1d and e). One, reactive with the probe for *Methanobrevibacter smithii* ALI, was round and appeared mostly as single cells; the other, reactive with the probe for *Methanobrevibacter smithii* PS, was oval and appeared in pairs or in short chains. Their respective concentrations varied independently of each other during the 14-month observation period, although not as much as those of other methanogens. These data suggest that these two methanogens are indeed different immunotypes and confirm earlier observations concerning

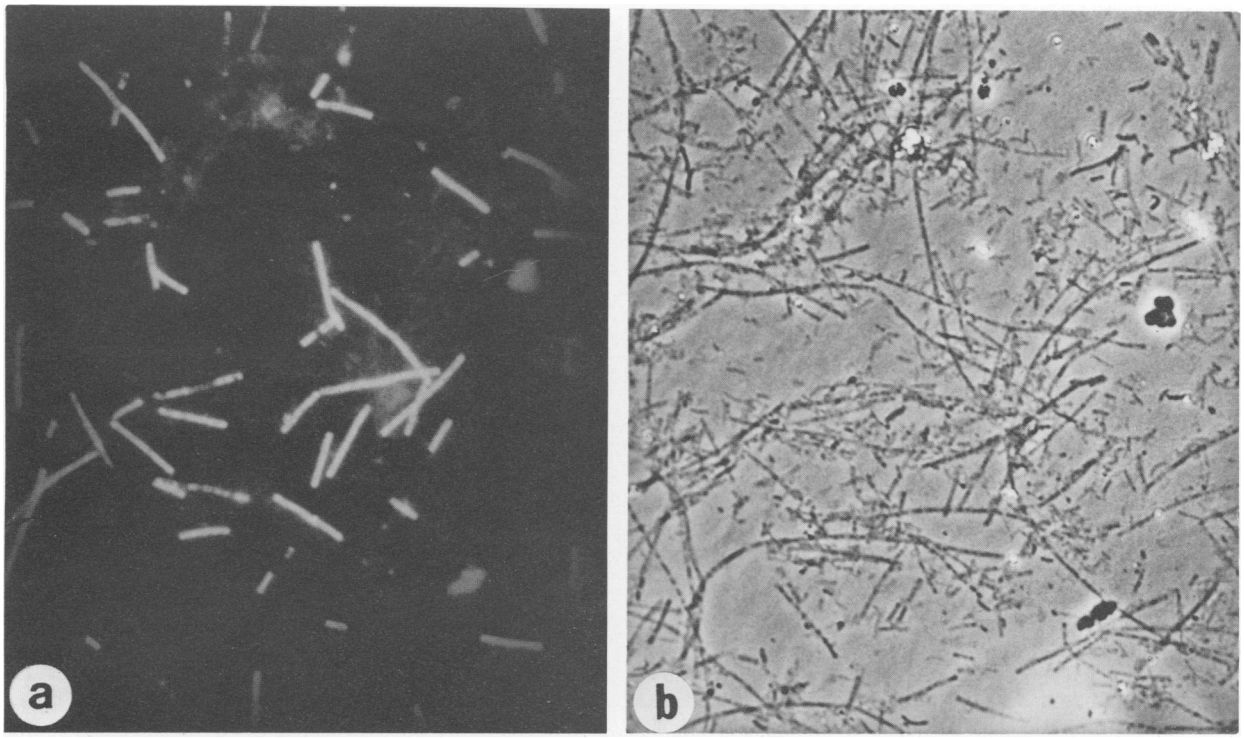

FIG. 2. Rods recognized by the probe for *Methanosarcina thermophila* TM1 (probe no. 20) in sample H. a, IIF; b, phase contrast of the same microscopic field. Magnification, ×4,000.

the immunologic diversity of *Methanobrevibacter smithii* (4).

At steady state, the bioreactor contained a methanogen antigenically related to *Methanobacterium bryantii* MoHG which was abundant in all samples (over 10^3 A_{450}/ml) and also showed variations with time. It appeared at the beginning, mostly as long filaments with 50% antigenic similarity to the reference methanogen. After the configuration of the bioreactor was changed (samples H and J), shorter rods predominated that were antigenically 100% similar to the reference immunotype. Phenotypic shifts from short to long rods have been observed also during batch growth of pure cultures of *Methanobacterium bryantii* after the exponential phase, when nutrients become limited as a result of prolonged cultivation (S. M. Schoberth, unpublished results). Whether the reverse shift observed in the bioreactor during this investigation is due to improved growth conditions needs further study.

Considering all samples as a group, it was observed that while the methanogens related to *Methanobacterium formicicum* MF and *Methanobrevibacter arboriphilus* AZ did occur at various times, they were not predominant, as in other bioreactors studied before (15). In the bioreactor described here, the predominant subpopulations were a sarcina antigenically related to *Methanosarcina mazei* S6 at the beginning and a rod related to *Methanosarcina thermophila* TM1 in late samples. In addition, the methanogens related to *Methanobacterium bryantii* MoHG and to *Methanobrevibacter smithii* ALI were comparatively abundant throughout the observation period.

A comparison of samples that had been taken from two different levels of the bioreactor did show some quantitative differences, but the same spectrum of methanogens was found in both.

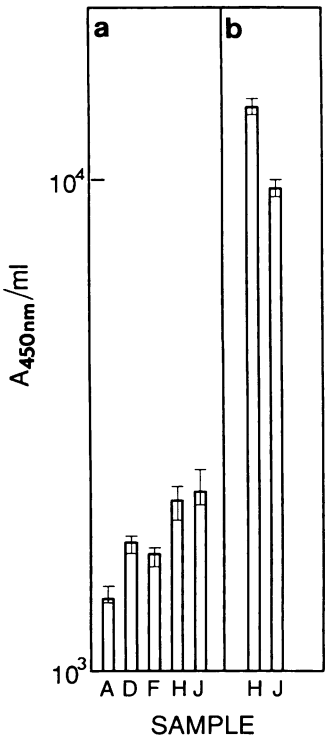

FIG. 3. Quantitative measurement of the load of the methanogens antigenically related to *Methanobacterium bryantii* MoHG (probe no. 5) (a) and rod reactive with the probe for *Methanosarcina thermophila* TM1 (probe no. 20) (b) using SIA. Bars represent A_{450} units per milliliter of sample pellet. Each bar represents the arithmetic mean ± the range (n = 2).

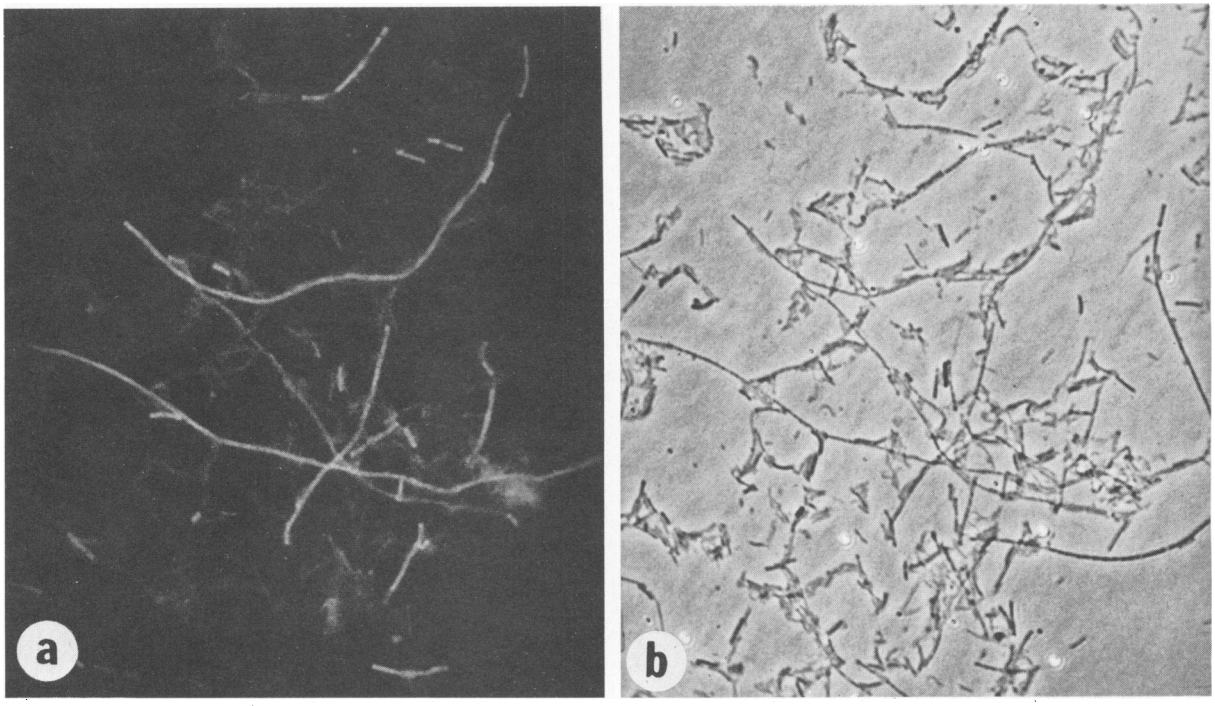

FIG. 4. Methanogen antigenically related to *Methanobacterium bryantii* MoHG (probe no. 5) in sample D. IIF (a) and phase contrast (b) of the same microscopic field are shown. Magnification, ×4,000.

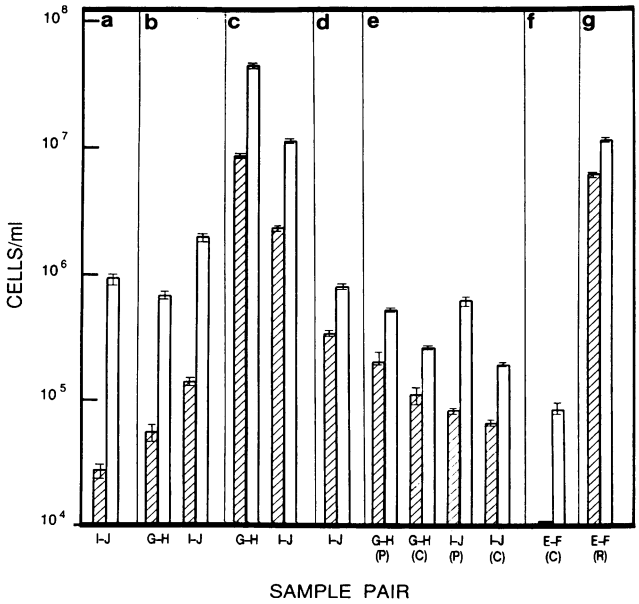

FIG. 5. Comparison of nonformalinized (▨) and formalinized (□) samples. The methanogens shown are those antigenically related to *Methanobrevibacter arboriphilus* AZ (probe no. 21) (a) and DC (probe no. 22) (b), *Methanobrevibacter smithii* ALI (probe no. 10) (c) and PS (probe no. 1) (d), *Methanosarcina barkeri* W (probe no. 19) (e), *Methanosarcina mazei* S6 (probe no. 18) (f), and *Methanosarcina thermophila* TM1 (probe no. 20) (g). P, Packets; C, single cocci; R, rods. Bars represent the numbers of cells (or packets when indicated) per milliliter of sample pellet. Each bar represents the arithmetic mean ± the range (n = 2).

The data obtained by comparing formalinized with nonformalinized samples showed that formalinization helped to preserve the methanogens. About half of the samples that had been stored without formalinization showed lower counts of methanogens than formalinized counterparts.

As a working hypothesis, it is proposed that the rod recognized by the probe for *Methanosarcina thermophila* TM1 is a *Methanothrix* organism of an immunotype different from the reference ones, *Methanothrix soehngenii* Opfikon and *Methanothrix* sp. strain CALS-1 (2, 10, 12). The following arguments lend support to the hypothesis. (i) Some characteristics of the rods resemble those of *Methanothrix soehngenii* Opfikon, such as morphology under phase optics, under bright-field optics in gram-stained preparations, and under epifluorescence optics of unstained and IIF preparations reacted with the probe for TM1. These rods, when reacted with the probe for TM1, resemble Opfikon rods reacted with the probe for Opfikon. (This is the only way to immunologically compare the two organisms, since they do not cross-react with either probe (ii) Some *Methanothrix* immunotypes, perhaps distinct species or strains, might react with probes for *Methanosarcina* species. These organisms are acetoclastic, and in earlier work, it has been observed that they share antigens (14). Regardless of whether the rod recognized by the probe for TM1 (Fig. 1f) in this bioreactor is a *Methanothrix* species, it is clear that it is a distinct immunotype, different from all the known ones. Isolation and characterization will be required to determine whether it is a new species.

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LITERATURE CITED

1. **Aivasidis, A., and C. Wandrey.** 1988. Recent developments in process and reactor design for anaerobic wastewater treatment. Water Sci. Technol. **20**:211–218.
2. **Boone, D. R., and W. B. Whitman.** 1988. Proposal of minimal standards for describing new taxa of methanogenic bacteria. Int. J. Syst. Bacteriol. **38**:212–219.
3. **Chartrain, M., and J. G. Zeikus.** 1986. Microbial ecophysiology of whey biomethanation: characterization of bacterial trophic populations and prevalent species in continuous culture. Appl. Environ. Microbiol. **51**:188–196.
4. **Conway de Macario, E., A. J. L. Macario, and A. Pastini.** 1985. The superficial antigenic mosaic of *Methanobrevibacter smithii*. Identification of determinants and isolates by monoclonal antibodies. Arch. Microbiol. **142**:311–316.
5. **Daniels, L.** 1984. Biological methanogenesis: physiological and practical aspects. Trends Biotechnol. **2**:91–98.
6. **Henson, J. M., P. H. Smith, and D. C. White.** 1985. Examination of thermophilic methane-producing digesters by analysis of bacterial lipids. Appl. Environ. Microbiol. **50**:1428–1433.
7. **Jeris, J. S., and P. L. McCarty.** 1965. The biochemistry of methane fermentation using C-14 tracers. J. Water Pollut. Control Fed. **37**:178–192.
8. **Kobayashi, H. A., E. Conway de Macario, R. S. Williams, and A. J. L. Macario.** 1988. Direct characterization of methanogens in two high-rate anaerobic biological reactors. Appl. Environ. Microbiol. **54**:693–698.
9. **Lettinga, G., and L. H. Pol.** 1986. Advanced reactor design operation and economy. Water Sci. Technol. **18**:99–108.
10. **Macario, A. J. L., and E. Conway de Macario.** 1982. The immunology of methanogens: a new development in microbial biotechnology. Immunol. Today **3**:279–284.
11. **Macario, A. J. L., and E. Conway de Macario.** 1983. Antigenic fingerprinting of methanogenic bacteria with polyclonal antibody probes. Syst. Appl. Microbiol. **4**:451–458.
12. **Macario, A. J. L., and E. Conway de Macario.** 1985. Antibodies for methanogenic biotechnology. Trends Biotechnol. **3**:204–208.
13. **Macario, A. J. L., and E. Conway de Macario.** 1985. Monoclonal antibodies of predefined molecular specificity for identification and classification of methanogens and for probing their ecologic niches, p. 213–247. *In* A. J. L. Macario and E. Conway de Macario (ed.), Monoclonal antibodies against bacteria, vol. 2. Academic Press, Inc., Orlando, Fla.
14. **Macario, A. J. L., and E. Conway de Macario.** 1987. Antigenic distinctiveness, heterogeneity and relationships of *Methanothrix* spp. J. Bacteriol. **169**:4099–4103.
15. **Macario, A. J. L., and E. Conway de Macario.** 1988. Quantitative immunologic analysis of the methanogenic flora of digestors reveals a considerable diversity. Appl. Environ. Microbiol. **54**:79–86.
16. **Novaes, R. F. V.** 1986. Microbiology of anaerobic digestion. Water Sci. Technol. **18**:1–14.
17. **Nyns, E.-J.** 1986. Biomethanation processes, p. 207–267. *In* H. J. Rehm and G. Reed (ed.), Biotechnology, vol. 8. VCH Publishers, Inc., New York.
18. **Parkin, G. F., and W. F. Owen.** 1986. Fundamentals of anaerobic digestion of wastewater sludges. J. Environ. Eng. **112**: 867–920.
19. **Prensier, G., H. C. Dubourgier, I. Thomas, G. Albagnac, and M. N. Buisson.** 1988. Specific immunological probes for studying the bacterial associations in granules and biofilms, p. 55–61. *In* G. Lettinga, A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Proceedings of the Granular Anaerobic Sludge; Microbiology and Technology Workshop. Puduc Wageningen, The Netherlands.
20. **Smith, P. H., and R. A. Mah.** 1966. Kinetics of acetate metabolism during sludge digestion. Appl. Microbiol. **14**:368–371.
21. **Toerien, D. F., and W. H. J. Hattingh.** 1969. Anaerobic digestion. I. The microbiology of anaerobic digestion. Water Res. **3**:385–416.
22. **White, D. C.** 1983. Analysis of microorganisms in terms of quantity and activity in natural environments, p. 37–66. *In* J. H. Slater, R. Whiteenbury, and J. W. T. Wimpenny (ed.), Microbes in their natural environments, Cambridge University Press, Cambridge.
23. **Zinder, S. H., S. C. Cardwell, T. Anguish, M. Lee, and M. Koch.** 1984. Methanogenesis in a thermophilic anaerobic digestor: *Methanothrix* sp. as an important aceticlastic methanogen. Appl. Environ. Microbiol. **46**:796–807.