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Effect of Magnesium on Methanogenic Subpopulations in a Thermophilic Acetate-Degrading Granular Consortium

JENS E. SCHMIDT,[1,2] ALBERTO J. L. MACARIO,[2] BIRGITTE K. AHRING,[1]* AND EVERLY CONWAY DE MACARIO[2]

The Anaerobic Microbiology/Biotechnology Research Group, Department of Biotechnology, Building 223, Technical University of Denmark, DK-2800 Lyngby, Denmark,[1] and Wadsworth Center for Laboratories and Research and Department of Biomedical Sciences, School of Public Health, New York State Department of Health, Albany, New York 12201-0509[2]

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The effects of Mg^{2+} on thermophilic (55°C) granules grown on acetate in 0.2-liter upflow anaerobic sludge blanket reactors were studied. The methanogens in the granules were identified and counted by using antibody probes and the antigenic fingerprinting method. Packets of large coccoidal cells antigenically related to *Methanosarcina thermophila* TM-1 were scarce in the absence of Mg^{2+} but increased with increasing Mg^{2+} concentrations up to 30 mM; *Methanosarcina* packets immunologically related to *Methanosarcina barkeri* R1M3 showed a similar trend, and their numbers increased up to 100 mM Mg^{2+}. The number of single cells antigenically related to TM-1, R1M3, and *Methanosarcina mazei* S-6 were scarce at low Mg^{2+} concentrations but increased drastically at 30 and 100 mM Mg^{2+}. The number of rod-shaped bacteria antigenically related to *Methanobacterium thermoautotrophicum* GC1 and ΔH was highest with no Mg^{2+} present, and their numbers decreased with increasing concentrations of the cation. These quantitative data, obtained by counting cells in suspensions made from disrupted granules, were confirmed by microscopic observation of the methanogenic subpopulations in thin histologic sections of the granules.

Upflow anaerobic sludge blanket (UASB) reactors are generally used for high-rate anaerobic treatment of wastewaters in western Europe (15). The success of this reactor type depends on the formation of highly flocculated, compact sludge aggregates called granules. The formation of granules allows the active bacterial biomass to be retained in the reactor independently of the flow rate, maintaining a good conversion efficiency even at relatively high flow rates. The flocculation ability of the sludge depends partly on divalent cations (12). For example, a concentration of 4.1 mM Mg^{2+} gives a better sludge settleability than 0.4 mM Mg^{2+} (7, 15).

Microbial methanogenesis from acetate is a quantitatively important terminal reaction in the anaerobic decomposition of organic matter (1, 27), and in anaerobic reactors it has been estimated that approximately 70% of the methane produced is derived from acetate (28).

Two different mechanisms of acetate metabolism have been described: (i) the aceticlastic reaction by which the methyl group of acetate is transformed into methane by acetotrophic methanogens belonging to the genera *Methanothrix* and *Methanosarcina*, and (ii) the syntrophic acetate oxidation reaction in which acetate is first oxidized to hydrogen and carbon dioxide by a nonmethanogenic acetate-oxidizing organism and the products are then metabolized by hydrogenotrophic methanogens (1, 23, 33). Oxidation of acetate by a syntrophic mechanism was found to be of importance in thermophilicly digested sludge under conditions of low acetate concentration (less than 1 mM), indicating that organisms capable of acetate oxidation exist in the digested sludge and that these organisms, together with hydrogenotrophic methanogens, are of importance under certain conditions (23).

* Corresponding author.

Methanosarcinae are acetate-degrading organisms that undergo changes in morphology and growth rate in cultures with different concentrations of cations (2, 5, 13). Usually, a high cation concentration in combination with a high concentration of trimethylamine has been shown to favor the growth of the single-cell form. With low cation concentrations and 50 mM of acetate as substrate, many *Methanosarcina* species (*M. mazei* S-6 and LYC and *M. thermophila* TM-1) will grow as packets of large coccoidal cells (5, 13, 32, 34). Ahring et al. (2) recently showed that individual single cells of *M. thermophila* TM-1 are produced with moderate acetate concentrations (50 mM) by increasing the Mg^{2+} concentration in the medium. When TM-1 is grown with 0.49 mM Mg^{2+}, large aggregates or packets, 1 mm or more in diameter, are formed; in the absence of Mg^{2+}, no growth occurs. A concentration of 30 mM Mg^{2+} is optimal for initial growth and methane production, and when the concentration of Mg^{2+} is increased to 100 mM or greater, the organism grows as small aggregates or single cells; no growth occurs at or above 400 mM Mg^{2+}.

Several studies using transmission electron microscopy revealed that *Methanothrix*-like organisms dominate the granules of mesophilic UASB reactors (9–11, 24). However, methanosarcinae were found to predominate under mesophilic conditions in granules grown in fish-meal-process wastewater, and only small quantities of *Methanothrix*-like organisms were detectable by light microscopy (26). *Methanosarcina* was further found to be the only genus of acetotrophic methanogens present in granules grown on volatile fatty acids in a thermophilic UASB reactor (1, 31).

Studies with mesophilic granules grown in UASB reactors with different substrates showed that the diversity of the methanogenic subpopulations increased with substrate complexity (14). In a subsequent investigation, it was demonstrated that shifting granules from 38 to 55°C caused time-course changes in the methanogenic subpopulations with

distinctive profiles (30). In parallel, the microanatomy of the granules also underwent modifications affecting the inner texture and the spatial arrangement of the microbial colonies (21).

All these studies demonstrate that the microbial consortium in the granules is a dynamic assembly capable of responding to environmental changes. In the present study, we examine the effects of various Mg^{2+} concentrations in the culture medium on the methanogenic subpopulations of granules maintained in 0.2-liter UASB reactors at 55°C. The granules used as the inoculum for these small experimental reactors were obtained from a 5-liter UASB reactor operated at 55°C, with methanosarcinae as the main acetotrophic population. Applying immunologic and histologic methods, we report our observations of methanogenic subpopulations.

MATERIALS AND METHODS

Source of inoculum. Granules for the small experimental reactors were from a 5-liter thermophilic (55°C) UASB reactor originally inoculated with thermophilicly digested sewage sludge and operated for 2 years with a hydraulic retention time of 9 h and a removal efficiency exceeding 95%. This reactor was fed with modified UASB medium (29); $NaHCO_3$ was omitted, and the medium was gassed with N_2 and supplemented with 47 mM acetate and 0.5 mM Mg^{2+}.

Experimental UASB reactors. Granules were studied in five 0.2-liter UASB reactors (25) operated under thermophilic conditions (55°C) with modified UASB medium (29) (see above) supplemented with different Mg^{2+} concentrations and 47 mM acetate. The various Mg^{2+} (added as $MgCl_2 \cdot 6H_2O$) concentrations in the medium for each of the five reactors were 0, 0.5, 10, 30, and 100 mM, and the reactors and corresponding granules were designated A_0, $A_{0.5}$, A_{10}, A_{30}, and A_{100}, respectively. These five reactors were operated for 2 months, with a hydraulic retention time of 9 h. Granules were sampled at the end of the experiment, after the reactors had been functioning at steady state (defined as a constant acetate conversion) for 1 month.

Sample preparation. Wet and dry weights of the granules from the five experimental UASB reactors were determined by using standard methods (3), as previously described (14). For cell counts and identification, 1 g of wet granules was added to 5 ml of phosphate-buffered saline (pH 7.2) and disrupted with a tissue grinder (Tri-R-Instruments, Rockville Center, N.Y.) to obtain a homogeneous suspension. Part of the cell suspension obtained was formalinized and prepared for immunologic testing by following standard techniques (17, 18).

Cell identification and counting. The slide immunoenzymatic assay constellation was applied for the immunologic identification and enumeration of methanogens, as described previously (18, 20). This method involves a series of complementary assays, including indirect immunofluorescence, enzymatic reactions, Gram staining, and microscopic observation by using epifluorescence and phase-contrast, differential interphase-contrast, and bright-field illuminations. Identification of methanogens was done by using the antigenic fingerprinting method (17, 19). Reference methanogens (see below) were used as controls and as reference morphotypes in each test. The antigenic fingerprinting results were compared with fingerprints in our data base (17, 18, 20). Total cell counts were done with a hemocytometer (improved Neuhauser Hemocytometer; Max Levy, Philadelphia, Pa.) by using standard procedures (14, 30). All experiments were run in triplicate or more.

Panel of reference methanogens. Reference methanogens whose physiologic, taxonomic (4, 6), and antigenic characteristics had been previously determined were used in the slide immunoenzymatic assay constellation as positive, negative, and morphotype controls, as previously described (17, 19, 20). These organisms are listed below in the order prescribed by the antigenic fingerprinting method (the number preceding each methanogen defines a position in the fingerprint) (17, 19): 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; 9, *Methanobrevibacter arboriphilus* (*Methanobrevibacter arboriphilicus*) DH1; 10, *Methanobrevibacter smithii* ALI; 11, *Methanobacterium thermoautotrophicum* GC1; 12, *Methanobacterium thermoautotrophicum* ΔH; 16, *Methanosarcina barkeri* 227; 18, *Methanosarcina mazei* S-6; 19, *Methanosarcina barkeri* W; 20, *Methanosarcina thermophila* TM-1; 21, *Methanobrevibacter arboriphilus* (*Methanobrevibacter arboriphilicus*) AZ; 30, *Methanothrix soehngenii* Opfikon; and 31, *Methanothrix* sp. strain CALS-1.

Antibody probes. Calibrated antibody probes were derived from antisera against the reference methanogens listed above and were used for antigenic fingerprinting as described previously (17, 19).

Histochemistry and immunohistochemistry. Granules from the five experimental reactors were preserved in Telly's fixative overnight, placed in 70% alcohol for 6 to 8 h, and then dehydrated by using the automated Ultratechnicon system to embed the granules in paraffin. Thin sections (5 μm thick) were obtained with a 820 Spencer Microtome (American Optical Instrument Co., Buffalo, N.Y.). The sections were collected on 14-mm-diameter glass SIA-slide circles, with heavy teflon coating (19). After the paraffin was cleared from the slide, the thin sections were processed and stained by following standard methods for tissue sections (8, 16). Indirect immunofluorescence was performed with the antibody probes by following current techniques (8, 21). Microscopic observation and photography were done with a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

Performance of the experimental reactors. The average percent conversion and standard deviation (given in parentheses) of acetate in the five small experimental UASB reactors during the last 12 days of the experiment were 92.6 (3.8), 96.5 (2.4), 98.1 (1.5), 98.9 (0.4), and 98.0 (0.9) for UASB reactors A_0, $A_{0.5}$, A_{10}, A_{30}, and A_{100}, respectively. The conversion of acetate increased with increasing Mg^{2+} concentrations up to 10 mM and remained at this level, with both 30 and 100 mM added. Granules A_0 and A_{100} were fluffy and a grey-white color, in contrast to granules $A_{0.5}$, A_{10}, and A_{30}, which were dense and a black-brown color. Precipitation of minerals in the sludge bed in reactors A_{30} and A_{100} was observed.

Microscopic observation of the granular sludge. Microscopic examination, by using both light microscopy and scanning electron microscopy, showed that methanosarcinae were the most abundant microbes in granules $A_{0.5}$, A_{10}, A_{30}, and A_{100}. Microscopic examination also showed both long and short rod-shaped bacteria. Many of the rod-shaped bacteria were autofluorescent under epifluorescent illumination, indicating that they were also methanogenic; in gran-

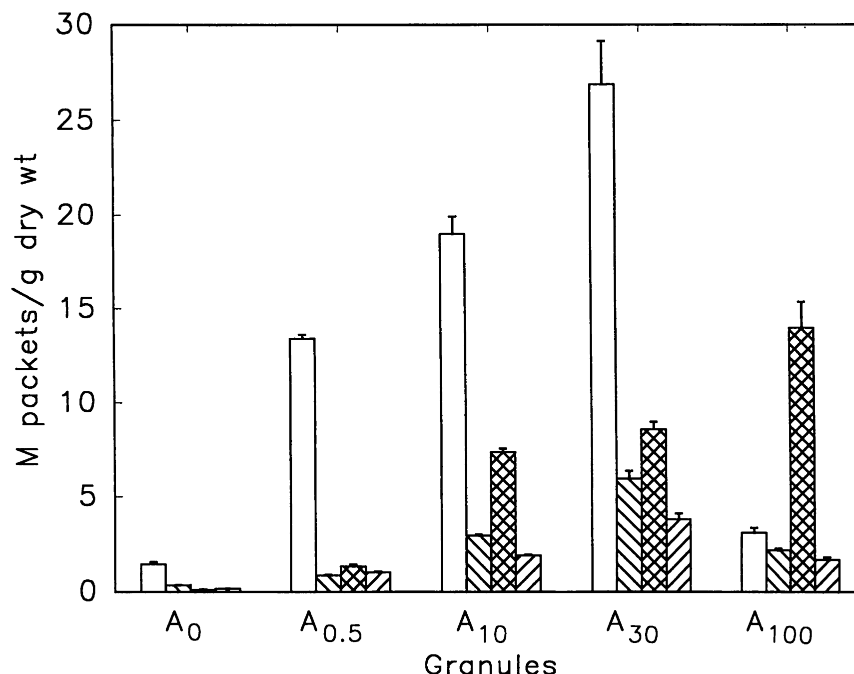

FIG. 1. Concentration (arithmetic mean ± standard deviation; $n = 3$) of *Methanosarcina* packets made of large coccoidal cells antigenically related to *M. thermophila* TM-1 (□), *M. mazei* S-6 (▧), or *M. barkeri* R1M3 (▩) or *M. barkeri* W (▨), expressed as millions (M) of packets per g (dry weight) in granules maintained in the 0.2-liter UASB reactors at the Mg^{2+} concentrations (mM) indicated by the subscript of A.

ules from A_0, the most predominant bacteria were rod shaped, and many of them were autofluorescent (data not shown). *Methanosarcina* packets were also observed.

Methanogenic subpopulations detected immunologically. (i) *Methanosarcina* packets. Figure 1 shows the concentration of *Methanosarcina* packets of large coccoidal cells. Methanosarcinae antigenically related to *M. thermophila* TM-1 increased with increasing Mg^{2+} concentrations up to 30 mM and then decreased almost to the level observed in the absence of Mg^{2+}. A similar trend was observed for the methanosarcinae antigenically related to *M. mazei* S-6 or *M. barkeri* W, but the number of methanosarcinae fell to considerably lower levels. The packets antigenically related to *M. barkeri* R1M3 showed a steady rise in number, paralleling the increase in Mg^{2+} concentration. No *Methanosarcina* packets made of large coccoidal cells immunologically related to *M. barkeri* 227 or *M. barkeri* MS were observed in the granules.

Packets of small coccoidal cells immunologically related to *M. thermophila* TM-1, *M. mazei* S-6, or *M. barkeri* W or R1M3 were observed at low concentrations (less than 2.69×10^6 packets per g [dry weight]) for all granules, except for A_{100} granules in which packets antigenically related to TM-1 or R1M3 reached concentrations of 22.46×10^6 or 7.09×10^6 packets per g (dry weight), respectively.

(ii) *Methanosarcina* single cells. *Methanosarcina* single cells antigenically related to *M. thermophila* TM-1 or *M. mazei* S-6 were scarce in the absence of Mg^{2+} and at 0.5 mM Mg^{2+}, but they increased when the Mg^{2+} concentration reached 10 and 30 mM, and this increase was even more pronounced at 100 mM; the number of *Methanosarcina* single cells immunologically related to *M. barkeri* W was relatively lower for all granules but also increased with higher Mg^{2+} concentrations (Fig. 2). The *Methanosarcina* single cells immunologically related to *M. barkeri* R1M3 showed a different trend; their numbers were practically the same at 30 and 100 mM Mg^{2+}. Single cells antigenically related to *M. barkeri* 227 or MS were not observed.

(iii) Methanogenic rods. Figure 3 shows the concentration of the predominant rod-shaped methanogens (rods) detected immunologically. The number of rods antigenically related to *Methanobacterium thermoautotrophicum* GC1 decreased with increasing Mg^{2+} concentrations up to 10 mM and remained at the same level when the cation concentration was increased to 30 and 100 mM. The *Methanobrevibacter arboriphilus* AZ immunologically related rods showed a similar trend but in considerably lower numbers than the rods immunologically related to *Methanobacterium thermoautotrophicum* GC1. Rods antigenically related to *Methanobacterium thermoautotrophicum* ΔH were present in relatively high numbers in the absence of Mg^{2+} and at 0.5 and 10 mM Mg^{2+} but fell to lower numbers at 30 and 100 mM Mg^{2+}. Very low numbers of a methanogenic rod immunologically related to *Methanobacterium bryantii* MoH were found in all granules in the presence of Mg^{2+}, and the number of these rods also decreased as the cation concentration increased.

(iv) Other methanogens tested. Methanogens immunologically related to *Methanobrevibacter smithii* PS and ALI, *Methanospirillum hungatei* JF1, *Methanobrevibacter arboriphilus* DH1, *Methanobacterium formicicum* MF, *Methanobacterium bryantii* MoHG, *Methanothrix soehngenii* Opfikon, and *Methanothrix* sp. strain CALS-1 were not observed in the samples studied.

Effect of Mg^{2+} concentration on the total methanogenic population detected immunologically and on *Methanosarcina* morphology. Table 1 shows the dry weight of granules and the total number of cells and methanogens detectable with

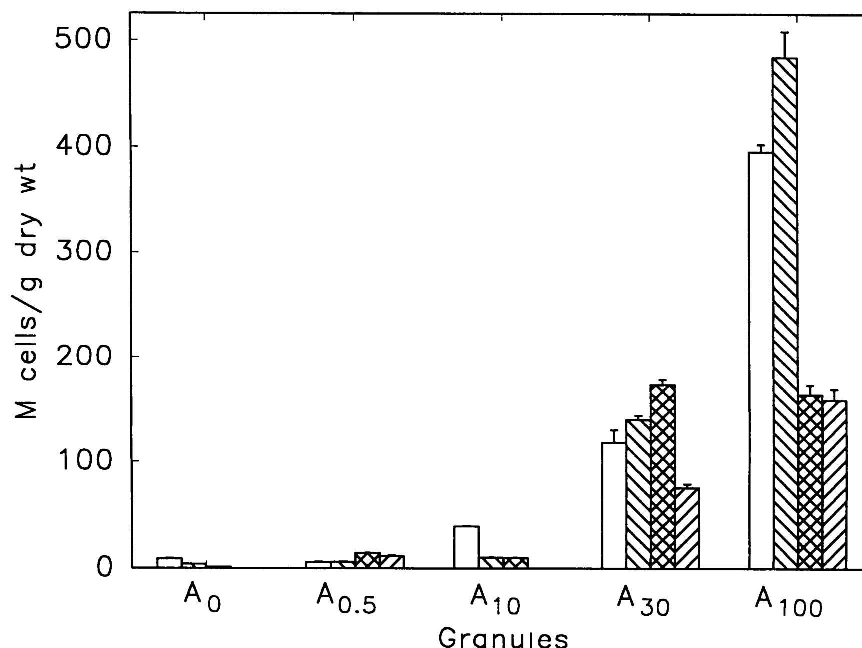

FIG. 2. Concentration (arithmetic mean ± standard deviation; $n = 3$) of *Methanosarcina* single cells antigenically related to *M. thermophila* TM-1 (□), *M. mazei* S-6 (▧), or *M. barkeri* R1M3 (▩) or *M. barkeri* W (▨), expressed as millions (M) of cells per g (dry weight) in the same granules used for Fig. 1.

antibodies. The highest and lowest total cell numbers were found in granules A_{10} and A_{100}, respectively. The total number of rods detected immunologically decreased with increasing Mg^{2+} concentrations. The opposite trend was observed for the number of *Methanosarcina* single cells, which increased with increasing Mg^{2+} concentrations. The same trend was observed for *Methanosarcina* packets, and the proportion of packets made of small coccoidal cells increased with increasing Mg^{2+} concentration. It should be noted that counts of *Methanosarcina* packets underestimate the total cell numbers, since it is not possible to count individual cells in the packets.

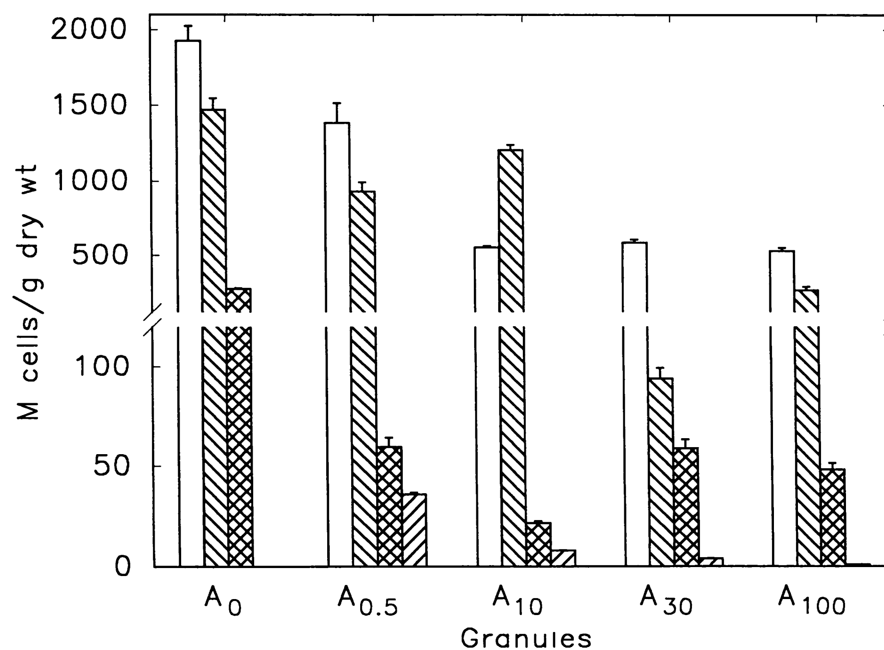

FIG. 3. Concentration (arithmetic mean ± standard deviation; $n = 3$) of the predominant methanogenic rods antigenically related to *Methanobacterium thermoautotrophicum* GC1 (□), *Methanobacterium thermoautotrophicum* ΔH (▧), *Methanobrevibacter arboriphilus* AZ (▩), or *Methanobacterium bryantii* MoH (▨) expressed as millions (M) of cells per g (dry weight) in the same granules used for Fig. 1.

TABLE 1. Microbial composition of the granules at the various Mg^{2+} concentrations

Granule[a]	Dry wt of granules[b] (g/ml)	Total no. of cells[b] (10^9 cells/g [dry wt])	No. of methanogens[c] (10^6 cells or packets/g [dry wt])			
			Rod shaped	Single cell	Packet of large coccoidal cells	Packet of small coccoidal cells
A_0	0.303 (0.027)	19.17 (0.95)	3,674	13.34	1.42	0.64
$A_{0.5}$	0.241 (0.024)	18.77 (1.76)	2,425	37.20	15.85	0.16
A_{10}	0.489 (0.031)	24.71 (0.49)	1,787	58.86	31.44	0.00
A_{30}	0.365 (0.023)	14.68 (1.38)	749	509.26	45.26	2.89
A_{100}	0.301 (0.006)	13.95 (1.25)	849	1,203.83	20.78	31.18

[a] Mg^{2+} concentration in mM is shown by the subscript.
[b] Arithmetic mean (standard deviation) is given for $n = 3$.
[c] Detected immunologically; coefficient of variation was less than 10%.

Histochemistry and immunohistochemistry. Thin sections of all granules examined, A_0, $A_{0.5}$, and A_{30}, stained with hematoxylin-eosin showed rods, long filaments, and aggregates of *Methanosarcina* packets (Fig. 4a). The aggregates were slightly more abundant in the sections of the A_{30} granules, whereas the rods and filaments were more prominent in sections of granules A_0 and $A_{0.5}$. These observations are in agreement with the quantitative data presented in Fig. 1 and 3.

Concurrently with an increase in the Mg^{2+} concentration in the medium, the number of *Methanosarcina* single cells and small packets visible in the sections increased as expected from the results shown in Fig. 2. In histologic sections, these *Methanosarcina* single cells and small packets were close to each other, forming a blanket, usually rolled upon itself. When the blanket roll was seen in sections cut perpendicularly to the axis of rolling, it appeared as a large ring, sometimes hollow and at other times with rods and filaments inside (Fig. 4b and c). The structural features described above were visible not only in hematoxylin-eosin-stained sections (Fig. 4a to c) but also in sections stained with periodic acid-Schiff stain, Gram staining, and the silver impregnation method (data not shown).

Immunohistochemistry with antibody probes for reference methanogens revealed that a considerable proportion of the aggregates of packets depicted in Fig. 4a were antigenically related to *Methanosarcina thermophila* TM-1 (Fig. 4d). The blankets that formed the rings shown in Fig. 4b and c were made of single cells and small packets antigenically related to *Methanosarcina mazei* S-6 and to *M. thermophila* TM-1 (Fig. 4e and f). These observations are in agreement with the counts shown in Fig. 2. Methanogenic rods antigenically related to *Methanobacterium thermoautotrophicum* GC1 and ΔH (Fig. 3) were also visible in thin sections, where they appeared as sparse lawns in contact with methanosarcinae (not shown).

DISCUSSION

In this work, methanogens in cell suspensions of disrupted granules and in situ (in thin histologic sections) were studied. While the former approach allowed us to count cells and determine the concentration of each methanogenic subpopulation identifiable with antibody probes, studies with thin histologic sections provided information on the topography of the same subpopulations in the intact granules.

Study of cell suspensions showed that methanogenic rods predominated in the absence of Mg^{2+}. As the Mg^{2+} concentration was raised, rods became less numerous, in contrast with methanosarcinae, which increased in number.

Two main patterns were evident for methanosarcinae pertinent to the two morphologic forms of these methanogens, i.e., packets and single cells. Packets of large coccoidal cells increased with increasing Mg^{2+} concentrations up to 30 mM, but at 100 mM the number of these packets was lower, similar to that at 10 mM. Single cells were scarce at Mg^{2+} concentrations of 10 mM or less, but increased drastically in number at 30 mM Mg^{2+} and reached very high values at 100 mM Mg^{2+}. Thus, at the highest Mg^{2+} concentrations tested, *Methanosarcina* single cells took the places of packets.

Transformation of the morphology from packets to single cells could present a practical problem in anaerobic reactors since single, free-floating cells may easily be lost in the effluent compared with the bulkier and heavier packets and aggregates. However, the multicellular structure found in the thin sections could work in retaining the biomass in the reactor, but this possibility needs further documentation.

Studies with histologic sections demonstrated that the granules possess structural organization. Filaments, rods, and aggregates of *Methanosarcina* packets are intimately related microanatomically, witnessing to the functional-biochemical interactions that must occur between these different microbes. The structure of the granules was affected by the Mg^{2+} concentration in the culture medium, as was the number of each methanogen (see above). Globular aggregates of *Methanosarcina* packets were more conspicuous in sections of granules grown in the absence of Mg^{2+} or in the presence of the lower Mg^{2+} concentrations tested. In contrast, a blanket formed by *Methanosarcina* single cells and small packets became more and more prominent as the Mg^{2+} concentration was increased in the medium. This blanket resembled a multicellular structure, which we have recently observed, that is formed by methanosarcinae in pure culture and that we called lamina (22). Lamina occurred at certain stages of growth and was formed by single cells and small packets held together by an intercellular connective material, just as are the blankets found in the granules studied here as well as in other UASB granules previously examined (21).

The concentration-dependent effect of Mg^{2+} may be due to the ion itself, but also the changes in the ionic strength might play a role. For instance, increasing the Mg^{2+} concentration from 0.5 to 100 mM will result in a 10-times-higher ionic strength. However, decreasing the Mg^{2+} concentration from 0.5 to 0 mM will not significantly change the ionic strength in the medium. Thus, the observed effect of Mg^{2+} at low concentrations of Mg^{2+} is likely due to the cation itself.

The hydrogenotrophic methanogens detected in the granules could be involved in processes other than the turnover of acetate, e.g., the degradation of dead granular biomass which will increase when the granules get fluffy. The in-

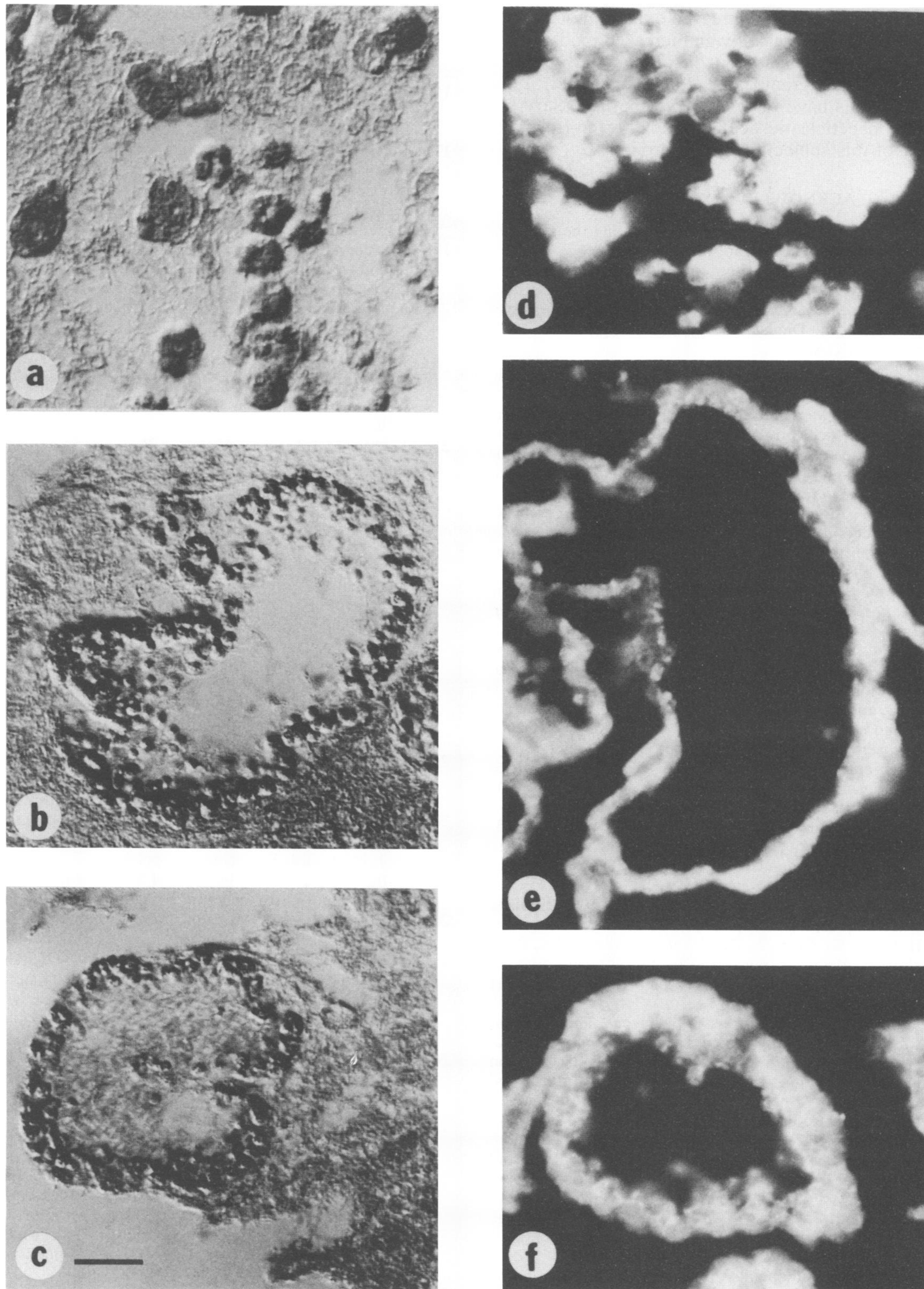

FIG. 4. Thin histologic sections of granules. Aggregates of *Methanosarcina* packets enmeshed in a net of rods and filaments (a) and cross sections of blankets made of *Methanosarcina* single cells and small packets rolled upon themselves so that the blankets appear as rings, either hollow (b) or with rods and filaments inside (c), and stained with hematoxylin-eosin. Reactions of aggregates of packets and blankets (rings) with an antibody probe for *M. thermophila* TM-1 are shown by indirect immunofluorescence (d to f). Bar represents 11.2 μm for all panels.

crease in hydrogen-utilizing methanogens with no Mg^{2+} added might also be due to an increased oxidation of acetate by syntrophs, as indicated also by an increased oxidation of methyl-labelled acetate to carbon dioxide in the granules from A_0 compared with granules A_{30} (data not shown). Low Mg^{2+} concentrations have been shown to be necessary for growth of a nonaceticlastic acetate-oxidizing consortium. Further study of this subject is now in progress.

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REFERENCES

1. **Ahring, B. K.** 1991. Methanogenesis during thermophilic anaerobic digestion with focus on acetate, p. 275–283. *In* H. Verachtert and W. Verstraete (ed.), International Symposium in Environmental Biotechnology, part 1, Oostende (Belgium). Koninklijke Vlaamse Ingenieursvereniging vzw, Antwerp, Belgium.
2. **Ahring, B. K., F. Alatriste-Mondragon, P. Westermann, and R. Mah.** 1991. Effects of cations on *Methanosarcina thermophila* TM-1 growing on moderate concentrations of acetate: production of single cells. Appl. Microbiol. Biotechnol. **35:**686–689.
3. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
4. **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.
5. **Boone, D. R., and R. A. Mah.** 1987. Effects of calcium, magnesium, pH, and extent of growth on the morphology of *Methanosarcina mazei* S-6. Appl. Environ. Microbiol. **53:**1699–1700.
6. **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.
7. **Brunnetti, A., G. Boari, R. Passiono, and A. Rozzi.** 1983. Physico-chemical factors affecting start-up in UASB-digestors, p. 317–334. *In* W. J. van den Brink (ed.), Anaerobic waste water treatment. The Haque, The Netherlands.
8. **Culling, C. F. A., R. T. Allison, and W. T. Barr.** 1985. Cellular pathology techniques, 4th ed., p. 1–642. Butterworth, London.
9. **Dolfing, H. C., A. Griffioen, A. R. W. van Neerven, and L. P. T. M. Zevenhuizen.** 1985. Chemical and bacteriological composition of granular methanogenic sludge. Can. J. Microbiol. **31:** 744–750.
10. **Dubourguier, H. C., D. B. Archer, G. Albagnac, and G. Prensier.** 1988. Structure and metabolism of methanogenic microbial conglomerates, p. 13–25. *In* E. R. Halland and P. N. Hobson (ed.), Anaerobic digestion, 1988. Pergamon Press, Inc., Elmsford, N.Y.
11. **Dubourguier, H. C., G. Prensier, and G. Albagnac.** 1988. Structural and microbial activities of granular anaerobic sludge, p. 18–33. *In* G. Lettinga, A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and technology. Puduc, Wageningen, The Netherlands.
12. **Forster, C. F., and J. Dallas-Newton.** 1980. Activated sludge settlement—some suppositions and suggestions. Water Pollut. Control. **79:**338–351.
13. **Harris, J. E.** 1987. Spontaneous disaggregation of *Methanosarcina mazei* S-6 and its use in the development of genetic techniques for *Methanosarcina* sp. Appl. Environ. Microbiol. **53:**2500–2504.
14. **Koornneef, E., A. J. L. Macario, J. T. C. Grotenhuis, and E. C. de Macario.** 1990. Methanogens revealed immunologically in granules from five upflow anaerobic sludge blanket (UASB) bioreactors grown on different substrates. FEMS Microbiol. Ecol. **73:**225–230.
15. **Lettinga, G., A. F. M. van Velsen, S. W. Hobma, W. de Zeeuw, and A. Klapwijk.** 1980. Use of the upflow sludge blanket (USB) rector concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. **22:**699–734.
16. **Luna, L. G. (ed.).** 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd ed. McGraw-Hill Book Co., New York.
17. **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.
18. **Macario, A. J. L., and E. Conway de Macario.** 1985. Antibodies for methanogenic biotechnology. Trends Biotechnol. **3:**204–208.
19. **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 ecological niches, p. 213–247. *In* A. J. L. Macario and E. Conway de Macario (ed.), Monoclonal antibodies against bacteria, vol. I. Academic Press, Inc., Orlando, Fla.
20. **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.
21. **Macario, A. J. L., F. A. Visser, J. B. van Lier, and E. Conway de Macario.** 1991. Topography of methanogenic subpopulations in a microbial consortium. J. Gen. Microbiol. **137:**2179–2189.
22. **Mayerhofer, L. E., A. J. L. Macario, and E. Conway de Macario.** 1992. Lamina, a novel multicellular form of *Methanosarcina mazei* S-6. J. Bacteriol. **174:**309–314.
23. **Petersen, S. P., and B. K. Ahring.** 1991. Acetate oxidation in a thermophilic anaerobic sewage sludge digestor: the importance of non-aceticlastic methanogenesis from acetate. FEMS Microbiol. Ecol. **86:**149–157.
24. **Prensier, G., H. C. Dubourguier, 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.), Granular anaerobic sludge; microbiology and technology. Puduc, Wageningen, The Netherlands.
25. **Rinzema, A., J. van Lier, and G. Lettinga.** 1988. Sodium inhibition of acetoclastic methanogens in granular sludge from a UASB-reactor. Enzyme Microbiol. Technol. **10:**24–32.
26. **Sandberg, M., and B. K. Ahring.** Anaerobic treatment of fishmeal process waste water in an UASB reactor at high pH. Appl. Microbiol. Biotechnol. in press.
27. **Smith, M. R., S. H. Zinder, and R. A. Mah.** 1980. Microbial methanogenesis from acetate. Proc. Biochem. **14:**368–371.
28. **Smith, P. H., and R. A. Mah.** 1966. Kinetics of acetate metabolism during sludge digestion. Appl. Microbiol. **14:**368–371.
29. **Sørensen, A. H., M. Winther-Nielsen, and B. K. Ahring.** 1991. Kinetics of lactate, acetate, propionate in unadapted and lactate-adapted thermophilic, anaerobic sewage sludge: the influence of adaptation of sludge for start-up of thermophilic UASB-reactors. Appl. Microbiol. Biotechnol. **34:**823–827.
30. **Visser, F. A., J. B. van Lier, A. J. L. Macario, and E. Conway de Macario.** 1991. Diversity and population dynamics of methanogenic bacteria in a granular consortium. Appl. Environ. Microbiol. **57:**1728–1734.
31. **Winther-Nielsen, M., and B. K. Ahring.** 1990. Degradation of volatile fatty acids in a thermophilic UASB-rector, p. 192. *In* C. Christiansen, L. Munck, and J. Villadsen (ed.), 5th European Congress on Biotechnology, Abstract Book. Munksgaard, Copenhagen.
32. **Xun, L., D. R. Boone, and R. A. Mah.** 1988. Control of the life cycle of *Methanosarcina mazei* S-6 by manipulation of growth conditions. Appl. Environ. Microbiol. **54:**2064–2068.
33. **Zinder, S. H., and M. Koch.** 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch. Microbiol. **138:**263–272.
34. **Zinder, S. H., and R. A. Mah.** 1979. Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. Appl. Environ. Microbiol. **38:**996–1008.