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Methanogenesis from Ethanol by Defined Mixed Continuous Cultures

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Methanogenesis from ethanol by defined mixed continuous cultures was studied. Under sulfate-free conditions, a *Desulfovibrio* strain was used as the ethanol-degrading species producing acetic acid and hydrogen. In a two-membered mutualistic coculture, the hydrogen was converted to methane by a *Methanobacterium* sp. and pH was maintained at neutrality by the addition of alkali. Introduction of a third species, the acetate-utilizing *Methanosarcina mazei*, obviated the need for external pH control. Methanogenesis by the co- and triculture was studied at various dilution rates in the steady state. The mutualistic coculture performed like a composite single species, as predicted from the theory of mutualistic interactions. Coupling between the mutualistic coculture and the acetate-utilizing methanogen was less tight. Increasing the dilution rate destabilized the triculture; at low dilution rates, instability was soon recovered, but at higher dilution rates imbalance between the rates of production and removal of acetic acid led to a drop in pH. Flocs formed in the triculture. An annulus of the *Methanobacterium* sp. and *Desulfovibrio* sp. was retained around the *Methanosarcina* sp. by strands of material probably derived from the *Methanosarcina* sp.

The anaerobic degradation of organic matter to methane requires a mixed culture of anaerobic bacteria. Acetic acid and hydrogen are important intermediates in the degradative process which can be useful indicators of process performance (2). Regulation of the levels of hydrogen and acetic acid is essential in the anaerobic reactor, and it is the methanogenic bacteria which remove both hydrogen and acetic acid. Much of the flux of hydrogen is between mutualistic partners where theory predicts that steady-state levels are closely regulated (19, 25, 26). The coupling between acetogenic bacteria and acetotrophic methanogens is less tight, although regulation of pH through acetotrophic methanogenesis is essential in anaerobic digestion and the levels of acetic acid affect the thermodynamics of acetogenesis (2, 16).

A variety of defined methanogenic mixed cultures have been studied, although little emphasis has been placed on the dynamics of methanogenesis by mutualistic cocultures in continuous culture (1, 8, 36, 37). Continuous culture provides the means of studying methanogenesis under steady-state conditions and of studying the response to perturbations from the steady state. In this study, we take as our model the growth of the mutualistic coculture of a *Desulfovibrio* sp. and a *Methanobacterium* sp. degrading ethanol to methane in sulfate-free medium (5, 7). Addition of the acetate-utilizing *Methanosarcina mazei* to the mutualistic coculture facilitates the complete conversion of ethanol to methane and carbon dioxide. The system is in many ways analogous to anaerobic digestion because organic matter is converted to methane and carbon dioxide via hydrogen and acetic acid.

(Preliminary results of part of this work were presented at a conference [27].)

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MATERIALS AND METHODS

Bacterial strains and culture. *Desulfovibrio* strain FR17 (NCIB 12086) and *Methanobacterium* strain FR2 (DSM 2257) have been described previously (3). FR17 is probably a strain of *Desulfovibrio desulfuricans* and FR2 is probably a strain of *Methanobacterium bryantii* (17). *Methanosarcina mazei* S-6 (DSM 2053) was also used.

Cocultures of *Desulfovibrio* and *Methanobacterium* spp. were grown in sulfate-free medium containing 50 mM ethanol (5). *M. mazei*, adapted to utilize acetic acid as the sole carbon and energy source, was added to an established coculture in the chemostat for studies of the triculture. A 1-liter stirred (800 rpm) chemostat, equipped with pH monitoring and control, and E_h monitoring was used (LH Engineering, Stoke Poges, United Kingdom). Prereduced and sterilized medium was pumped from a 20-liter aspirator to the chemostat by a peristaltic pump (Watson-Marlow, Falmouth, United Kingdom). Two filters (3- and 0.45-μm pore size) were arranged in series between the pump and chemostat. All tubing used was made of butyl rubber, and all joints and gaskets were sealed with silicone jointing compound.

Microscopy. No flocs were detected in the coculture of the *Desulfovibrio* sp. and the *Methanobacterium* sp., but flocs were formed upon the addition of *M. mazei*. The flocs studied were taken from the chemostat at a dilution rate of 0.8 day^{-1}, although similar flocs were also present at lower dilution rates. The density of bacteria around and away from the edge of a clump of *M. mazei* was estimated by counting in a Thoma (Weber, Lancing, United Kingdom) counting slide. An annulus of bacteria at high density (approximately 2×10^9 cells per ml) existed around the clumps. At a distance of approximately 0.2 mm from the edge of a *M. mazei* clump, the bacterial density (entirely *Methanobacterium* and *Desulfovibrio* cells) was the same as that of the coculture (lacking *M. mazei*), approximately 4×10^8 cells per ml. Determination of the cell density and phase-contrast microscopy required that the *M. mazei* clumps were compressed between the slide and the cover slip. This practice

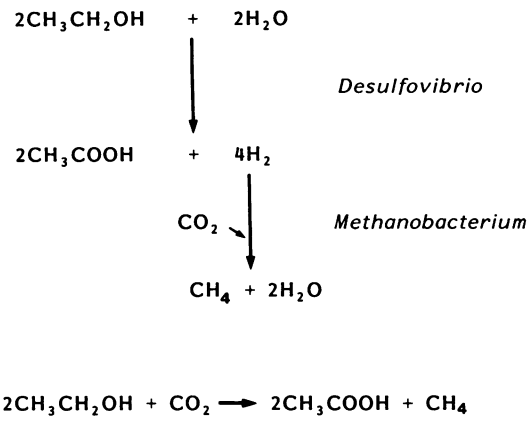

FIG. 1. Methane production from ethanol by a coculture of *Desulfovibrio* and *Methanobacterium* strains in sulfate-free medium.

disturbs the actual structure of the floc so the results do not accurately represent the structure in vivo. Conditions were then chosen which did not require the flocs to be compressed.

The flocs were fixed for 2 h in equal volumes of glutaraldehyde (6%, wt/vol) in 0.05 M cacodylate buffer (pH 7.4) and ruthenium red (0.15%, wt/vol) (21). The flocs were postfixed in equal volumes of aqueous osmium tetroxide (2%, wt /vol) and ruthenium red (0.15%, wt/vol). The flocs were then dehydrated in a graded ethanol series, transferred to acetone, and infiltrated and embedded in the resin described by Spurr (32). Throughout the procedure, the flocs were allowed to sediment under their own weight and were never centrifuged. For light microscopy, sections approximately 1 μm thick were stained in toluidine blue (1%, wt/vol) in borax (1%, wt/vol; pH 11). For transmission electron microscopy, sections approximately 70 nm thick were stained sequentially in saturated uranyl acetate in 50% ethanol and lead citrate as described by Reynolds (28) and examined in a JEOL 1200EX electron microscope.

Analytical methods. Gas produced from the chemostat was collected over 4 M NaH_2PO_4 (pH 2) and its composition was determined by gas chromatography (18). Acetic acid was assayed by an enzymatic kit (BCL, Lewes, United Kingdom). Ethanol was determined by gas chromatography, using a Pye 104 chromatograph fitted with ionization detection. The column was 162 by 0.6 cm, packed with Carbowax 20M (20%; 100/120 mesh) (JJ's, Kings Lynn, United Kingdom), with argon as the carrier gas (40 ml/min), and was operated at 108°C. Hydrogen at levels below 1,000 ppm (ca. 101 Pa) was assayed in a hydrogen monitor (Gas Measurement Instruments Ltd., Renfrew, United Kingdom).

RESULTS

Coculture methanogenesis. The anticipated route of methanogenesis is given in Fig. 1, showing that the expected methane yield is 0.5 mol of CH_4 produced from each mole of ethanol under conditions when all the ethanol is used for energy. Steady states were obtained at dilution rates of up to approximately 2.1 day^{-1}, after which the coculture was progressively washed out (Fig. 2). The methane yield at the dilution rate of 1 day^{-1} was approximately 0.45 mol of CH_4 per mol of ethanol. The concentration of ethanol in the chemostat was very low until high dilution rates were obtained (Fig. 2).

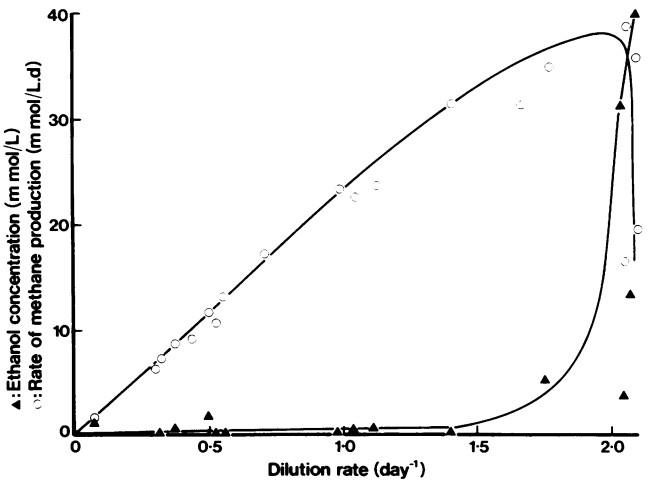

FIG. 2. Dependence of the steady-state ethanol concentration and the rate of production of methane from ethanol in the absence of sulfate by a continuous coculture of *Desulfovibrio* and *Methanobacterium* strains on the dilution rate. Mean values are plotted. Abbreviations: m mol, millimoles; L, liter; d, day.

Triculture methanogenesis. Figure 3 depicts the route of methanogenesis in the triculture. The expected yield of methane is 1.5 mol of CH_4 per mol of ethanol. Addition of *M. mazei* to an established coculture in the chemostat (D = 0.27 day^{-1}) reduced the acetic acid concentration with a consequent rise in the rate of methane production (Fig. 4). Once the acetic acid in the chemostat was used up, the system resumed steady-state methane production at an increased rate compared with that of the coculture. At this point, external regulation of pH was unnecessary. Steady states were obtained up to a dilution rate of 0.8 day^{-1} (Fig. 5). The methane yield over the range of dilution rates was approximately 1.42 mol of CH_4 per mol of ethanol. Steady states were not obtained beyond a dilution rate of 0.8 day^{-1}, despite several attempts over a period of 18 months. As the dilution rate increased, the triculture became more sensitive to perturbation from the steady state. This was characterized by a rise in acetic acid level and a drop in pH. The steady-state hydrogen level in the gas produced at the dilution rate of 0.8 day^{-1} was over 300 ppm, whereas at other steady states, it was between 40 and 100 ppm.

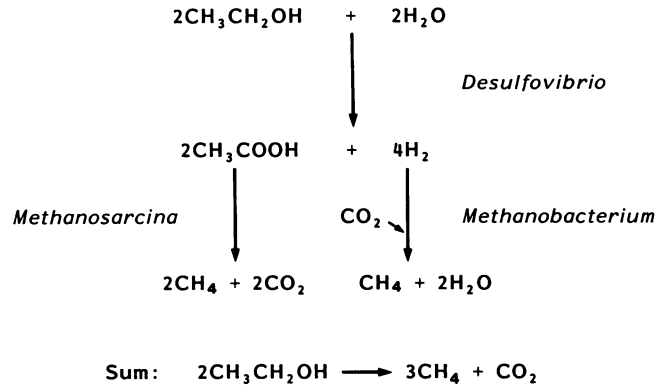

FIG. 3. Methane production from ethanol by a triculture of a *Desulfovibrio* sp., a *Methanobacterium* sp., and *M. mazei* in sulfate-free medium.

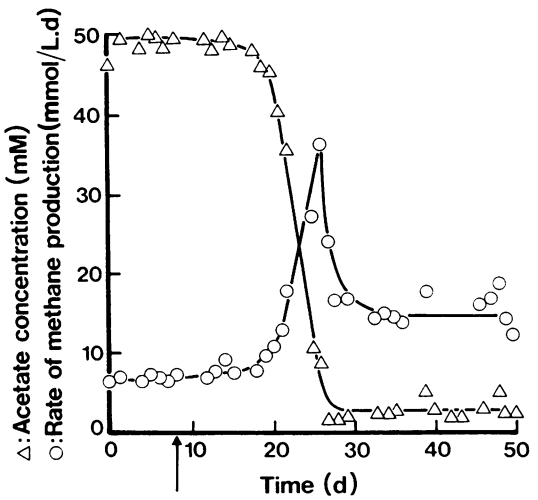

FIG. 4. Addition of *M. mazei* (on day 8, indicated by arrow) to a chemostat coculture of *Desulfovibrio* and *Methanobacterium* strains degrading ethanol to methane in sulfate-free medium. Abbreviations: L, liter; d, day.

Floc formation. Association of the *Desulfovibrio* and *Methanobacterium* strains with *M. mazei* is clearly shown by light microscopy of a compressed floc (Fig. 6a) and an uncompressed floc (Fig. 6b) and by electron microscopy of sectioned flocs (Fig. 6c and d). Any bacteria which were not physically attached to the floc were lost during the sample preparation (unlike the situation in Fig. 6a); this indicates that the annulus of bacteria was retained by physical attachment to *M. mazei*. *Desulfovibrio* and *Methanobacterium* cells were seen to be enmeshed in a network of fibrillar ruthenium red-stained material presumed to originate from *M. mazei* (Fig. 6d).

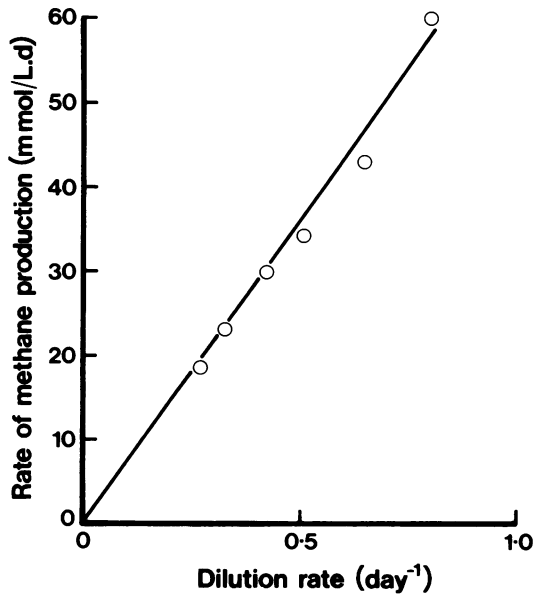

FIG. 5. Dependence of the methane production rate of a chemostat triculture on the dilution rate. The triculture contained a *Desulfovibrio* sp., a *Methanobacterium* sp., and *M. mazei* and degraded ethanol to methane in sulfate-free medium. Mean values are plotted. Abbreviations: L, liter; d, day.

DISCUSSION

Interspecies hydrogen transfer between mutualistic bacterial species is a key event, accounting for much of the hydrogen flux, in the anaerobic degradation of organic matter (15). Obligately proton-reducing acetogenic bacteria are known which degrade volatile fatty acids when cultured in association with hydrogen-utilizing methanogenic species (6, 14, 23). Other obligately mutualistic acetogens are known and, in the absence of sulfate, *Desulfovibrio* spp. metabolize ethanol acetogenically in coculture with methanogens (7). This coculture system is convenient for experimental purposes and has been exploited in the present study to examine some of the mathematical predictions of mutualism (25, 26). Theory predicts that the interdependent coupling of the mutualistic partners is so tight that, dynamically, the coculture will behave as a single composite species. The experimental system studied should behave as a theoretical composite species degrading ethanol to acetic acid and methane with measurable indices typical of pure cultures (24). The results presented for the continuous coculture are consistent with the theoretical predictions confirming the tight coupling between mutualistic species.

Introduction of the acetotrophic methanogen *M. mazei* to the mutualistic coculture resulted in a triculture capable of degrading ethanol to methane and carbon dioxide. The triculture models the important characteristics of the anaerobic digestion process in which neutral organic material is degraded to methane and carbon dioxide via acid intermediates and hydrogen. Levels of volatile fatty acids and the concentration of hydrogen are two important indices of process performance in the anaerobic digester (2, 15). The triculture differed from the mutualistic coculture in having increased methane yields and in regulating its own pH.

M. mazei is capable of producing methane from acetic acid or, poorly, by the reduction of carbon dioxide by hydrogen (22). In mixed cultures, however, *Methanosarcina* spp. probably compete ineffectively for hydrogen with other hydrogenotrophic methanogens because of their relatively high K_s (H_2) (20, 29) and are therefore effectively restricted to acetic acid as the energy-yielding substrate. Introduction of *M. mazei* to the coculture certainly reduced the level of acetic acid in the chemostat and the concentration remained low at all steady states. It proved impossible to maintain steady states with the triculture above a dilution rate of 0.8 day^{-1}. Further increases in dilution rates resulted in a buildup of acetic acid and a drop in pH. If the pH was then restored to neutrality by addition of alkali and if the dilution rate was also reduced, recovery of the triculture was slow and it was usually quicker to begin afresh. Reported values for μ_{max} of *Methanosarcina* spp. utilizing acetic acid as the sole energy source vary (34), but 0.8 day^{-1} compares with the highest of published values. The μ_{max} of the acetotrophic methanogen abruptly limits the methanogenic potential, a problem in industrial digestion which can be overcome by retention of biomass (33).

Aggregation of bacteria in flocs, granules, or films has been exploited in the development of anaerobic digesters treating wastewater at high rates (11, 33). Interspecies interactions which are necessary in the degradation of organic matter can be facilitated by the close proximity of bacteria in an aggregate (10, 13, 31, 35). There is, thus, a metabolic advantage for interacting species to aggregate which may be selected for by appropriate operating conditions within an anaerobic digester (4). Formation of an aggregate also requires a mechanism for the physical association of different

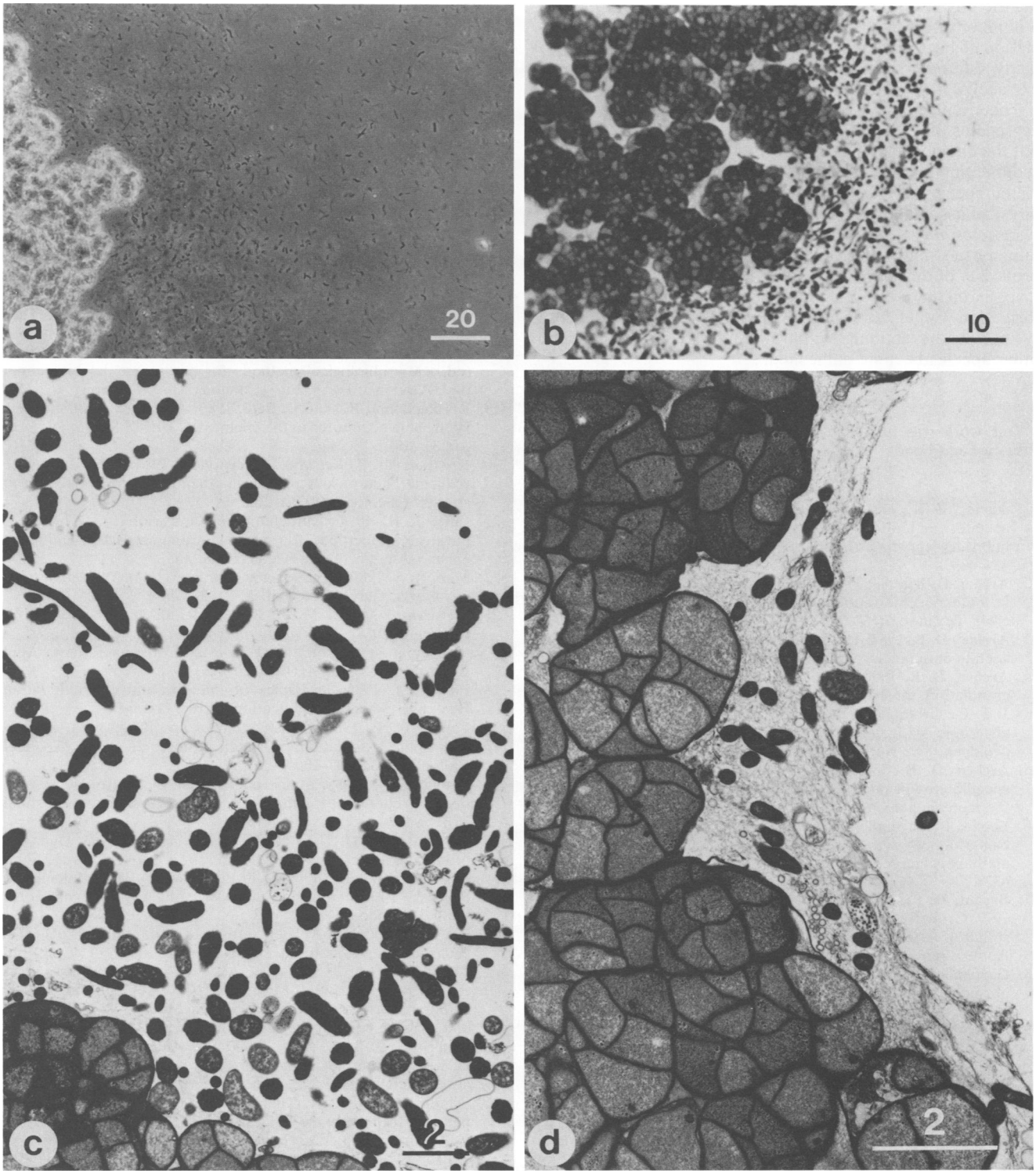

FIG. 6. Phase-contrast micrograph of a compressed triculture clump (a) and light micrograph section (b) of the triculture without compression, showing the annulus of bacteria around *M. mazei*. Electron micrograph sections of the flocs, showing bacteria trapped within a fibrillar network around *M. mazei*, which stained with ruthenium red (c and d). Bar markers are measured in micrometers.

bacterial species to each other or to inert material. Studies of methanogenic granules have revealed the presence of extracellular material which is probably responsible for maintaining the physical structure of the granule (12). There may be a number of mechanisms responsible for the physical association of bacteria and, in methanogenic flocs and granules, many different species are responsible for elaboration of extracellular material (9, 12, 13, 30).

The triculture was continuously stirred, and the visible aggregates of *M. mazei* were distributed uniformly throughout the chemostat vessel. There was no selection for rapidly sedimenting flocs, although *M. mazei* did have closely associated species of *Desulfovibrio* and *Methanobacterium*. No physical association of the *Desulfovibrio* sp. with the *Methanobacterium* sp. was detected in the coculture even though interspecies hydrogen flux would be enhanced by close proximity of the member species (4, 13, 35). In the triculture, it appeared that *M. mazei* provided the physical basis for floc formation by elaborating a network of fibrillar material (visualized by staining with ruthenium red) in which the other species became enmeshed.

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