APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1988, p. 10–19
0099-2240/88/010010-10$02.00/0

Vol. 54, No. 1

Control of Interspecies Electron Flow during Anaerobic Digestion: Role of Floc Formation in Syntrophic Methanogenesis

JURGEN H. THIELE, M. CHARTRAIN,† AND J. GREGORY ZEIKUS*

Michigan Biotechnology Institute, P.O. Box 27609, Lansing, Michigan 48909, and the Departments of Biochemistry and Microbiology, Michigan State University, East Lansing, Michigan 48824

Received 8 June 1987/Accepted 9 October 1987

The flora of an anaerobic whey-processing chemostat was separated by anaerobic sedimentation techniques into a free-living bacterial fraction and a bacterial floc fraction. The floc fraction constituted a major part (i.e., 57% total protein) of the total microbial population in the digestor, and it accounted for 87% of the total CO_2-dependent methanogenic activity and 76% of the total ethanol-consuming acetogenic activity. Lactose was degraded by both cellular fractions, but in the free flora fraction it was associated with higher intermediary levels of H_2, ethanol, butyrate, and propionate production. Electron microscopic analysis of flocs showed bacterial diversity and juxtapositioning of tentative *Desulfovibrio* and *Methanobacterium* species without significant microcolony formation. Ethanol, an intermediary product of lactose-hydrolyzing bacteria, was converted to acetate and methane within the flocs by interspecies electron transfer. Ethanol-dependent methane formation was compartmentalized and closely coupled kinetically within the flocs but without significant formation of H_2 gas. Physical disruption of flocs into fragments of 10- to 20-μm diameter initially increased the H_2 partial pressure but did not change the carbon transformation kinetic patterns of ethanol metabolism or demonstrate a significant role for H_2 in CO_2 reduction to methane. The data demonstrate that floc formation in a whey-processing anaerobic digestor functions in juxtapositioning cells for interspecies electron transfer during syntrophic ethanol conversion into acetate and methane but by a mechanism which was independent of the available dissolved H_2 gas pool in the ecosystem.

Advanced technologies for treatment of municipal, industrial, or agricultural waste by anaerobic digestion require the formation of microbial aggregates in the form of sludge granules, flocs, or microbial biofilms. Although these aggregates are largely used to maintain high concentrations of active cells in biogas digestors (1, 5, 18, 27, 30), the specialized metabolic functions of microbial aggregates have not been well characterized in methanogenic ecosystems.

It is known (1, 30) that substrate depletion in the center of a microbial aggregate can be caused by reduced diffusional fluxes and that this may impair the overall metabolic rates of the involved microorganisms. On the other hand, several examples exist in nature which show that syntrophic relationships are facilitated by the formation of mixed microbial aggregates or consortia, in which the involved microorganisms share a common spatial microniche (9, 11, 19, 23). Nonetheless, little is known about the inherent biochemical and physical mechanisms that allow for syntrophic interactions in aggregates.

The complete conversion of organic matter into CH_4 and CO_2 in anaerobic digestors requires at least three functionally different trophic groups of bacteria whose coordinate activities are required for the overall process function (7, 8): (i) hydrolytic fermentative bacteria, (ii) syntrophic acetogenic bacteria, and (iii) methanogenic bacteria. Syntrophic acetogenic bacteria or the so-called obligate syntrophic proton-reducing bacteria are generally considered to function by converting ethanol, propionate, butyrate, benzoate, and other reduced intermediary metabolites into acetate and molecular H_2 (2–4, 12, 20, 22) during the anaerobic digestion process. Thus, these conversions are thought to be thermodynamically feasible only at very low H_2 partial pressures (28). Notably, the metabolic efficiency and the growth yield of the syntrophic acetogenic bacteria which produce hydrogen depend strongly on the rate of the H_2 removal by a consuming species such as a methanogen; this process is called interspecies hydrogen transfer (IHT) (4, 25, 31).

A possible involvement of microbial aggregation in the facilitation of interspecies hydrogen transfer during methanogenesis in nature has been assumed by several researchers (12, 15); however, direct experimental evidence is lacking. Recent work in our laboratory (10) demonstrated that less than 6% of all the CO_2-dependent methanogenesis in a sewage sludge digestor was dependent on the dissolved H_2 turnover rate. This led to the hypothesis that most of the hydrogen-dependent methanogenesis occurs as a consequence of direct interspecies H_2 transfer between juxtapositioned microbial associations in flocs.

Our laboratory previously demonstrated the intermediary metabolism, trophic group species enumeration, and composition in a model steady-state whey-processing laboratory chemostat (6–8). The kinetic parameters for the important carbon transformations have been characterized. In this ecosystem, the bacteria existed as free-living cells and flocs which disappeared when methanogens were removed by high dilution rates (6). Furthermore, lactose was principally fermented by *Leuconostoc mesenteroides*, *Klebsiella oxytoca*, and *Clostridium butyricum* into lactate, ethanol, formate, H_2-CO_2, and acetate as intermediary metabolites. In this ecosystem, ethanol was syntrophically transformed into acetate and methane by *Desulfovibrio vulgaris* and *Methanobacterium formicicum* as the principal syntrophic acetogen and CO_2-reducing methanogen, respectively (7; J. H. Thiele, M. Chartrain, and J. G. Zeikus, manuscript in preparation).

The purpose of the present study was threefold. First, to

* Corresponding author.
† Present address: Merck Sharp & Dohme Research Laboratory, Rahway, NJ 07065.

assess whether syntrophic fermentation of ethanol to methane was spatially organized or enriched in the floc versus free flora fractions. Second, to test the suggested hypothesis (10) that in anaerobic digestion processes, CO_2-dependent methanogenesis and interspecies electron transfer were both predominantly located in a floc fraction and independent of the dissolved hydrogen pool in the aqueous phase. Last, to assess the significance of microbial aggregate formation as a mechanism to control electron flow during syntrophic ethanol conversion by *D. vulgaris* and *Methanobacterium formicicum* in a natural, mixed culture floc ecosystem.

MATERIALS AND METHODS

Chemicals and materials. All chemicals were analytical grade and were obtained from Sigma Chemical Co. (St. Louis, Mo.). Gases were obtained from Matheson Scientific, Inc. (Joliet, Ill.). Dried whey was obtained from Lactofrance (Paris, France). Pressure vials were from Wheaton Industries (Millville, N.J.), and butyl stoppers were from Bellco Glass, Inc. (Vineland, N.J.).

Media and chemostat culture conditions. Phosphate-buffered basal (PBB) medium was anaerobically prepared by standard procedures (16) and dispensed into pressure bottles with an N_2-CO_2 (95:5, vol/vol) head phase. Traces of oxygen were removed from the gas by passing it through a furnace containing reduced copper filings heated to 250°C. The medium was autoclaved at 123°C for 15 min. Vitamins, sodium phosphate, and 0.025% cysteine-sulfide reductant were added to the medium after autoclaving. The final medium pH was 7.0. A continuous steady-state anaerobic chemostat ecosystem was operated at 35°C with 1.0% dried whey in PBB medium and under conditions similar to those described elsewhere (8).

Preparation of free flora and floc fractions. All procedures were done under stringent anaerobic conditions. A 125-ml sample was removed from the chemostat and placed into a sterile, N_2-containing, 270-ml centrifugation vial and centrifuged at 410 × *g* for 2 min. The supernatant containing the free-living bacterial flora fraction was carefully decanted, and the soft pellet containing microbial flocs was washed in 10 ml of PBB medium by gentle agitation. The floc fraction was recentrifuged at 920 × *g* for 10 min, and the supernatant was collected. The flocs constituted only a small volume fraction of the digestor contents (1 to 2%). This pellet was resuspended in 10 ml of PBB medium and centrifuged at 410 × *g* for 1 min, and the supernatant was collected. The pellet constituted the washed floc fraction and was suspended in 6.5 ml of PBB medium. The supernatant from the first centrifugation was centrifuged at 2,600 × *g* for 30 min, yielding a clear supernatant and a cell pellet. The supernatant was discarded, and the cell pellet was suspended in 5 ml of PBB medium, yielding a total volume of 6 ml. This suspension constituted the free flora fraction. The free flora fraction contained mostly single cells or cell chains and a few tiny cell clumps. The washed floc fraction contains flocs with an average size of 100 μm in diameter (see Fig. 3A).

All operations were performed aseptically at room temperature in an anaerobic glove bag containing an N_2-H_2 gas mixture (95:5, vol/vol). Centrifugations were done at room temperature in a Sorvall RC2-B preparative centrifuge in gastight Nalgene centrifugation tubes. For the estimation of the total microbial biomass in both fractions, the protein content of the supernatants from the two floc-washing steps was regarded as free flora protein.

Metabolic analysis of chemostat cell fractions. Lactose transformation time courses and the localization and measurement of interspecies H_2 transfer reactions in the two fractions were determined in anaerobic 158-ml serum bottles containing 45 ml of PBB medium and an N_2-CO_2 (95:5, vol/vol) headspace of 2 atm (202.6 kPa). The substrates were added from sterilized concentrated stock solutions to the concentrations indicated. The bottles were warmed to 37°C before inoculation with 0.1 ml of the respective digestor cell fraction. During the experiments the bottles were placed on a Gyrotory water bath shaker (G76; New Brunswick Scientific Co., Inc., Edison, N.J.) at 80 rpm. Liquid-phase samples (1 ml) and gas-phase samples (2 ml) were aseptically taken at the indicated time points and analyzed by gas chromatography. Gas samples were taken with pressure-lock syringes. The pressure in the vials was monitored at the beginning and the end of the experiment, and pressure corrections were applied for the calculations of the gas metabolism data for each time point. All experiments were run in duplicate and repeated at least twice.

Cultivation, purification, and disruption of flocs. Effluent from the whey laboratory chemostat was collected and transferred anaerobically at 37°C into a freshly autoclaved 270-ml centrifuge vial, sealed with a butyl rubber stopper, and incubated at 37°C. During the collection procedure, the centrifuge vial was continuously flushed with oxygen-free N_2 at a rate of approximately 10 ml/min. After one retention time of the chemostat (100 h), the centrifuge vial was removed, flushed with N_2-CO_2 (95:5, vol/vol) for 2 min, and centrifuged at 650 × *g* for 5 min. The centrifuge vial was then transferred into an anaerobic glove bag, the supernatant containing single cells was discarded, and the pellet containing the flocs was gently suspended into 50 ml of PBB medium. The centrifugation step was repeated once, and the pellet was finally resuspended in 10 ml of PBB medium. The washed flocs were transferred into a 158-ml vial containing 50 ml of PBB medium with 0.1% dried whey and were incubated at 37°C for 2 to 3 days. All transfers were performed under aseptic conditions by using syringe techniques and an 18-gauge 1.5-in. (3.8-cm) needle (Becton Dickinson Vacutainer Systems, Rutherford, N.J.). Using these techniques, the flocs could be maintained on 0.1% whey at 37°C with weekly transfers into fresh medium for more than 8 weeks.

The cultured flocs were purified from the whey medium by inverting the pressure vials and allowing the flocs to sediment under unit gravity. The supernatant was then removed with a syringe, and 50 ml of PBB medium was injected into the vial. This washing procedure was repeated twice, and the fresh cultured flocs were finally resuspended into 20 ml of PBB medium. Nutrient-limited cultured flocs were prepared by two additional washing steps followed by incubation in PBB medium without substrates at 37°C for 2 days. Both cultured floc preparations were each anaerobically transferred into two autoclaved 58-ml serum vials containing an N_2-CO_2 (95:5, vol/vol) atmosphere by syringe techniques with an 18-gauge 1.5-in. needle. The content of one vial was disrupted by passage through a 23-gauge 1-in. (2.5-cm) needle with a 10-ml glass syringe (10 to 20 strokes) while monitoring strict anaerobic conditions with resazurin as the redox indicator dye.

Disrupted and intact cultured flocs were anaerobically distributed into freshly prepared sterile 58-ml serum vials containing 5 ml of PBB medium and the indicated substrates (added after autoclaving). The vial head phase contained N_2-CO_2 (95:5, vol/vol) at 1.7 atm (172.2 kPa) pressure. The vials were incubated in a Gyrotory water bath shaker (G76) at 37°C and 100 rpm. The floc preparation protein concen-

tration was between 0.1 and 0.2 g/liter. Each experiment was run in triplicate, and the individual data values were averaged.

To determine the time course for substrate consumption and gas production, we took samples (two 1-ml samples) of the headspace and samples (0.4 ml) of the liquid phase at the indicated times using 23-gauge 1-in. needles. The gas samples were obtained with valve-locked glass syringes to eliminate trace contamination by air, and 0.4 ml was immediately analyzed at atmospheric pressures for its H_2 and CH_4 content.

The volume of the headspace in the test vials was 4,000-fold larger than that of the floc phase (20 μl/mg of protein) and 15 times larger than that of the liquid phase (5 ml), and the solubility of H_2 in the headspace was approximately 50 times larger than in the floc or liquid phase. Thus, these conditions would enable at least 99.999% of net produced H_2 gas to be extracted into the headspace by these procedures.

The pressure in the vials was determined during the time course, and a pressure correction was performed for calculation of produced and dissolved gases. Liquid-phase samples were centrifuged immediately at 5,000 × *g* for 5 min to sediment cells. The supernatant (0.2 ml) was stored at −20°C before metabolite analysis by gas chromatography. The protein content in the vials was determined by using the cell pellet fractions after centrifugation.

Analytical techniques. H_2, CO, CH_4, and CO_2 were separated on a gas chromatograph (series 750; Gow Mac Instruments, Bridgewater, N.J.) with Spherocarb 60/80 (Analabs-Foxboro Co., North Haven, Conn.) as a column material. The column temperature was 150°C, and the carrier gas was N_2 at a flow rate of 20 ml/min. The carrier gas was purified by a CAT-1 catalytic combustion filter on line with a Drierite/molecular sieve filter (Trace Analytical, Menlo Park, Calif.).

After separation, CH_4 and CO_2 were analyzed with a flame ionization detector via a preceding conversion with a ruthenium red-based methanizer (Gow Mac). H_2 and CO were analyzed on an RGD2 reduction gas detector (Trace) based on the mercuric oxide-mercury conversion technique (9). The chromatogram was analyzed with a Hewlett-Packard HP 3390A integrator. Standards were prepared by dilution of gases into N_2. The detection limit was 0.05 ppm (0.05 μl/liter) for H_2, 0.01 ppm (0.01 μl/liter) for CO, and 1 ppm (1 μl/liter) for CH_4 and CO_2. High H_2 gas concentrations above 500 ppm were determined on a Hewlett-Packard 5890A gas chromatograph with a thermal conductivity detector and Porapak N as the column material. The oven temperature was 35°C, and the carrier gas was N_2.

Soluble substrates and fermentation products were acidified with H_3PO_4 (1 N final) and subsequently analyzed on a model 419 Packard gas chromatograph equipped with a Chromosorb 101 (80/100 mesh) column and a flame ionization detector. The column was operated at 180°C. The detection limit was approximately 0.1 mM for ethanol, acetate, propionate, and butyrate.

Lactate was determined enzymatically by standard techniques (13) with L-(+)-lactate dehydrogenase (Sigma). Experimental samples (300 μl) were aseptically taken from the experimental vials, acidified with 100 μl of 7 N $HClO_4$, and stored at −20°C. Samples were thawed and centrifuged, and the supernatant was diluted 10-fold with double-distilled water. Samples were neutralized with K_2CO_3 and used in the enzymatic assay.

The specific volume of cultured floc was determined as follows. Fresh cultured flocs were prepared from 250 ml of digestor content, and 200, 400, and 750 μl of the resulting floc suspension was centrifuged in calibrated microvials at 10,000 × *g*. The supernatant was removed, and the centrifugation was repeated. The resulting pellet was a compact paste, and the volume of the pellet was taken as the volume of the floc fraction in the suspension. The protein content of the original floc suspension was determined, and the specific floc volume was calculated from this value and expressed as microliters per milligram of protein.

Protein was determined by a modification of the Lowry method (14) with bovine serum albumin as a standard.

Gas detection test system capability. A very sensitive and sufficiently rapid measuring system for H_2 and methane at very low concentrations was established for the disruption experiments.

To determine the time resolution of the continuous H_2 extraction, we injected 0.8 ml of PBB medium saturated with 100% H_2 into three 58-ml test vials containing 5 ml of PBB medium. One vial was immediately shaken to extract all H_2 gas from the liquid (100% value), while the two remaining vials were placed into the water bath at 37°C and shaken at 100 rpm identical to test floc preparations. The dissolved H_2 was immediately extracted into the head phase, reaching the 100% value after about 10 min. This continuous H_2 extraction from the liquid phase of the test vials showed a time constant ($1/K$) of approximately 3 min, ensuring a nearly complete extraction from the liquid phase within 10 min. To guarantee the quantitive nature of the continuous gas extraction, the Bunsen coefficients for H_2 and CH_4 in PBB medium at 37°C and the in situ partial pressure of 10^{-3} atm (0.1 kPa) were determined by an extraction technique. The measured coefficients under the experimental conditions were (2.12 ± 0.58) × 10^{-2} ml of gas per ml of liquid for hydrogen and (3.03 ± 0.122) × 10^{-2} ml of gas per ml of liquid) for methane. By using a head phase/liquid phase ratio of 10:1 in this assay system, it was established that more than 99.9% of the H_2 gas was extracted into the headspace.

Calculation of IHT reactions. The significance and the functional localization of the three following microbial reactions were determined:

$$2CH_3CH_2OH + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 4H_2$$
$$\Delta G^{\circ\prime} = +19.2 \text{ kJ per reaction} \quad (1)$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$$
$$\Delta G^{\circ\prime} = -135.6 \text{ kJ per reaction} \quad (2)$$

$$2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$$
$$\Delta G^{\circ\prime} = -116 \text{ kJ per reaction} \quad (3)$$

The IHT ratio was defined as the molar ratio of H_2 used for methane production via CO_2 reduction over the total amount of H_2 produced from the oxidation of ethanol to acetate: IHT ratio = H_2 equivalents consumed for CO_2 reduction to CH_4/(H_2 equivalents consumed + H_2 gas produced). Methane formed from CO_2 reduction was derived for each time interval from the total amount of methane formed in that interval in the presence of ethanol minus the background methane formation in acetate control experiments. The amount of hydrogen equivalents consumed for CO_2 reduction was then calculated by using this value and the stoichiometry of methane formation from H_2 and CO_2. The produced H_2 gas not used for methane formation was calculated from the total amount of dissolved H_2 in the liquid phase and the H_2 content of the gas phase in the presence of ethanol corrected for the background H_2 evolution in acetate control experiments. The IHT ratio was calculated for each time interval.

The following equations were used according to the law of mass conservation for the production of H_2 gas during IHT-dependent conversion of ethanol to acetate and methane. If electron transfer is assumed to occur via the exchange of H_2 gas (reaction 3) in a well-mixed reaction phase in connection with the gas detection system, then:

$$dH_2^{phase}/dt = \text{production rate} - \text{consumption rate} - \text{phase transfer} = r_1 - r_2 - r_3 \quad (4)$$

In a two-phase system with syntrophic ethanol conversion performed by two different bacterial species, the rate terms become:

$$r_1 = V_{max_1} \times [\text{ethanol}]/(K_{s1} + [\text{ethanol}])$$

$$r_2 = V_{max_2} \times [H_2]/(K_{s2} + [H_2])$$

$$r_3 = -[A \times D \times (d\mu H_2/dc) \times (\text{grad } \mu H_2)]$$

where V_{max_1} is the maximum rate of H_2 production from reaction 1 and V_{max_2} is the maximum rate of H_2 consumption from reaction 2; K_{s1} and K_{s2} are the substrate constants of H_2-producing and -consuming bacteria when determined for *D. vulgaris*, *Methanobacterium formicicum*, and the entire digestor population (6); A is the contact surface area of the reaction phase with the neighboring phases; D is the diffusivity of H_2 in the reaction phase; μH_2 is the chemical potential of H_2 gas; $d\mu H_2/dc$ is the concentration dependence of the chemical potential of H_2 in the reaction phase; and grad μH_2 is the chemical potential gradient of H_2 between the reaction phase and the neighboring phases.

For a chemically closed system of two phases, the increase in the H_2 gas in the environment of the reaction phase can be described as:

$$dH_2^{environ}/dt = dH_2^{system}/dt - dH_2^{phase}/dt \quad (5)$$

With $r_1 - r_2 > 0$, the reaction phase constitutes a net source of H_2 gas and for $dH_2^{phase}/dt \ll dH_2^{system}/dt$, equation 5 becomes

$$dH_2^{environ}/dt = dH_2^{system}/dt = -[A \times D \times (d\mu H_2/dc) \times (\text{grad } \mu H_2)] \quad (6)$$

Thus, in the experimental closed-bottle system, a flux of H_2 into the headspace is proportional to the interfacial area and at constant V and D, $d\mu H_2/dc$ and grad μH_2 are a direct measure for the interfacial area of the reaction phase.

Microscopy. Phase-contrast and UV-epifluorescence microscopy were performed with an Olympus model BH2 microscope with a mercury light source and an automatic exposure camera. The fluorescence of the methanogenic factor F_{420} was observed with a B (IF-490) excitation filter and 0.530- or 0.570-mm barrier filters. Floc samples were taken with glass syringes and 18-gauge 1.5-in. needles and were observed within 10 min.

Electron microscopy employed procedures described previously (7, 32). The glutaraldehyde fixative was added before the first centrifugation of the flocs.

RESULTS

Compartmentalization of syntrophic ethanol metabolism. Experiments were initiated to assess whether syntrophic ethanol metabolism and IHT were preferentially localized in the floc versus the free flora fraction. Figure 1 compares lactose intermediary metabolism parameters in the free flora versus the floc fraction. Lactose was rapidly metabolized by both cell fractions into mainly ethanol and acetate, but with lower levels of butyrate and propionate present in flocs than in free flora fractions. Very significant differences, however, were found in the dynamics of gas metabolism displayed by the two fractions. The free flora showed a much higher and transient H_2 production during the early stage of lactose conversion (>2,000 Pa), whereas the flocs produced lower and more stable H_2 levels (<920 Pa). The ratio of H_2/CH_4 formation in the floc preparation during the first 50 h was always <1.5, whereas the free flora showed ratios of >10. Furthermore, the onset of the CH_4 production by free flora

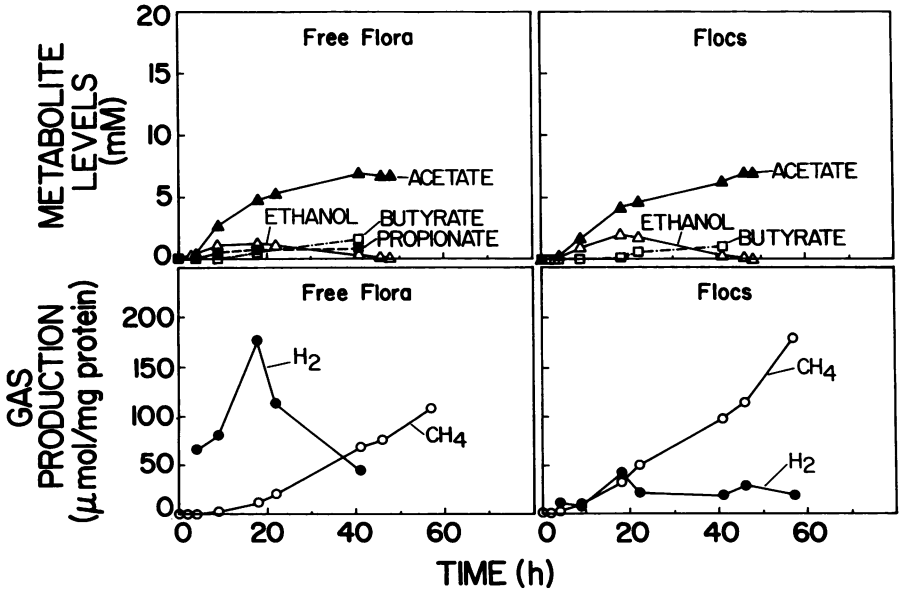


FIG. 1. Comparison of metabolic parameters for anaerobic lactose transformation by free flora versus floc population fractions. The experiments were performed under anaerobic conditions in 158-ml serum vials that contained 45 ml of PBB medium with 0.2% dried whey and an inoculum of either 1.05 mg of free flora protein or 0.58 mg of floc protein.

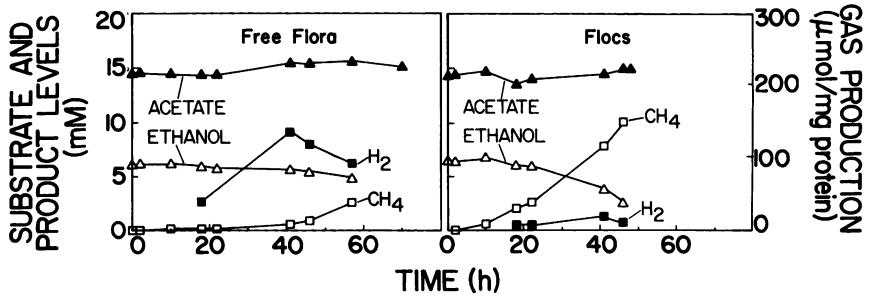


FIG. 2. Comparison of metabolic parameters for syntrophic ethanol transformation by free flora versus floc population fractions. The experiments were performed anaerobically in 158-ml serum vials that contained 45 ml of PBB medium with 5 mM ethanol and 14 mM acetate and an inoculum of either 1.05 mg of free flora protein or 0.58 mg of floc protein.

occurred 10 h later than in the floc preparation and only after the appearance of high H_2 concentrations. At the end of these time course experiments, microscopic observations indicated that free flora was growing in vials initially containing flocs and vice versa.

Figure 2 compares the intermediary metabolism of ethanol by the free flora versus the floc fractions. The floc fraction was more active at conversion of ethanol to acetate and methane than the free flora. However, the specific and total levels of produced hydrogen were lower in the floc fraction than in the free flora. To assess whether this correlated with higher hydrogen-consuming activities in flocs, we performed experiments which compared the specific activities of methanogenic H_2 consumption and acetogenic ethanol consumption by free flora versus floc fractions. Both the total and the specific ethanol oxidation activity and hydrogen consumption activity were much higher in the floc than in the free flora fraction (Table 1). The floc fraction displayed 87% of the total H_2-consuming methanogenic activity and 76% of the total ethanol-consuming activity in the ecosystem. H_2-consuming acetogenesis was insignificant in the flocs. No acetate was formed when flocs were incubated for more than 50 h with 2 kPa of H_2-CO_2 atmosphere (data not shown). Sulfate-reducing activity can be excluded as well owing to the absence of detectable sulfate in the medium. This indicated that flocs functioned to compartmentalize preferentially both syntrophic ethanol metabolism and methanogenic hydrogen consumption within the digestor ecosystem. Notably, microscopic analysis at the end of the experiments indicated that free flora was not formed in the floc vials or vice versa when ethanol was the sole carbon and electron source.

Analysis of IHT inside flocs. To assess the significance of IHT in syntrophic ethanol conversion to methane within the floc compartment, we designed experiments to test the effect of a disruption of cell contact on H_2 gas metabolism, ethanol consumption, and methane production by flocs. It was assumed that disruption would destroy the floc integrity and might uncouple efficient H_2 gas transfer (i.e., H_2 production and consumption) during syntrophic ethanol conversion. If H_2 was exchanged inside the flocs between clusters (microcolonies) of H_2-producing acetogens and H_2-consuming methanogens, then H_2 would accumulate while methane formation would decrease, similar to the ethanol degradation results observed for the free flora preparations. On the contrary, if H_2 was predominantly exchanged via the H_2 gas pool outside the flocs, a disruption should have no effect on the compartmentalization of the interspecies electron transfer.

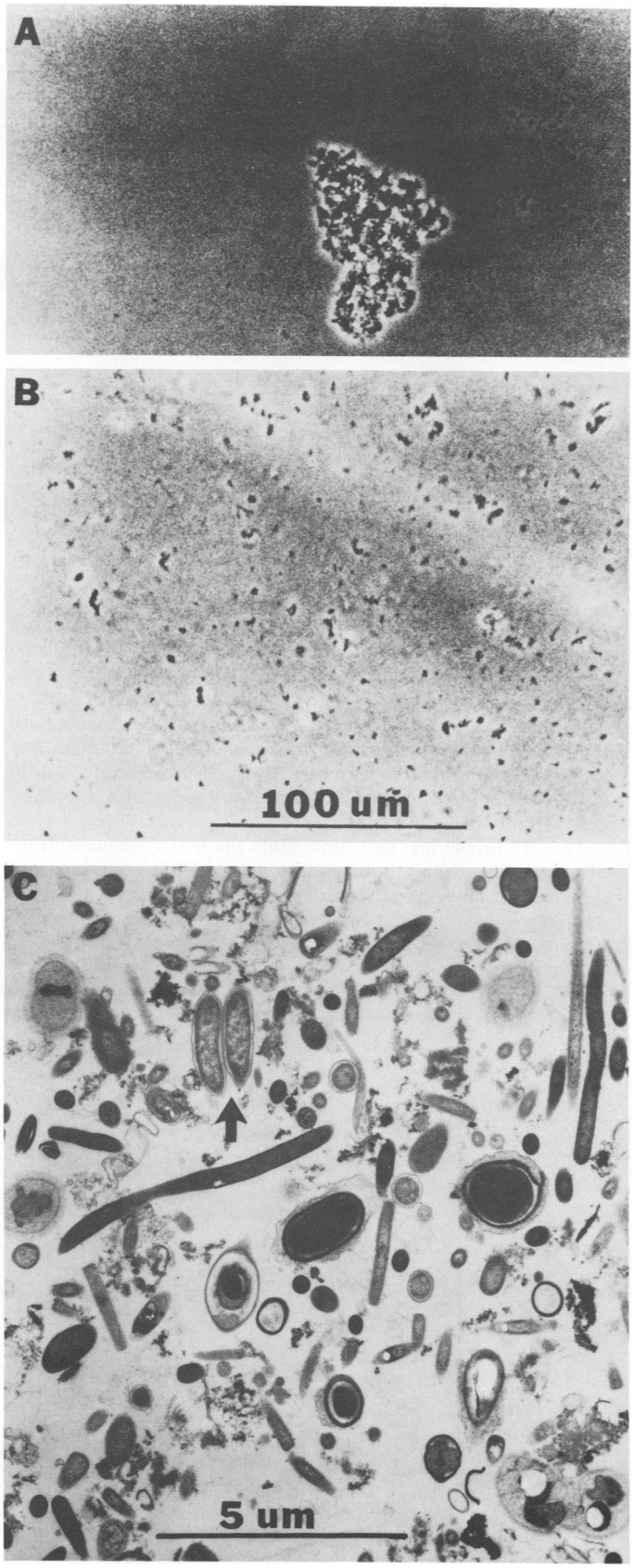
Syringe treatment resulted in a homogeneous population of fragments less than 1/10th of the original size (Fig. 3A and B). This gentle disruption did not cause individual cell injury, as was ascertained by thin-section electron microscopy (Fig. 3C). Vibrios, typical of *D. vulgaris*, and long slender rods, typical of *Methanobacterium formicicum*, were frequently seen juxtapositioned in electron micrographs of floc thin sections (Fig. 3C). Cell pairs of the vibrio were rarely seen, and microcolonies of four or more identical cells were not observed. Large clumps of a sarcina, presumably *Methanosarcina barkeri*, and long filaments similar to *Methanothrix soehngenii* were occasionally found within the flocs. Aside from clumps of *Methanosarcina barkeri*, significant numbers of microcolonies of identical cells were not observed at low magnification. UV-epifluorescence microscopy showed a presumptive homogeneous distribution of a fluorescent rod, by size and shape similar to *Methanobacterium formicicum*, the major hydrogen- and formate-consuming (i.e., CO_2-reducing) methanogen in the digestor ecosystem (7).

Electron microscopic analysis of thin sections did not reveal any significant difference in the general microbial species composition between disrupted and intact floc preparations. Thus, the disruption technique used did not introduce any bias into the general microbial composition during

TABLE 1. Comparison of substrate consumption parameters of syntrophic ethanol metabolism in free flora versus floc population fractions[a]

Population fraction	Total protein (mg)	Acetogenic ethanol consumption		Methanogenic H_2 consumption	
		Sp act (μmol/h/mg)	Total activity (μmol/h)	Sp act (μmol/h/mg)	Total activity (μmol/h)
Free flora	49.2	1.2	58.6	0.18	8.7
Floc	65.3	2.9	188.7	0.88	57.3

[a] Flocs and free flora were freshly prepared from 125 ml of whey-processing digestor contents and were incubated at 37°C in 158-ml serum vials that contained 45 ml of PBB medium with either a mixture of 6 mM ethanol and 14 mM acetate or H_2-CO_2 (80:20, vol/vol). The conversion rates for substrate consumption were determined between 10 and 20 h when all rates were linear.



the experiments. The viability and metabolic activity of acetogens and methanogens was not diminished by this disruption technique (see below).

Figures 4 and 5 compare hydrogen and methane production by intact versus disrupted floc preparations incubated in the presence of ethanol and acetate (Fig. 4) or acetate alone as control (Fig. 5). For these experiments, disrupted and intact samples were prepared from flocs subcultured for 2 days in the absence of lactose to reduce high background methane production. Control experiments (data not shown) with freshly prepared nonsubcultured flocs displayed the same kinetic properties for ethanol metabolism as those presented below. Ethanol addition enhanced both hydrogen evolution and methane production approximately 10-fold over those of the acetate controls. Also, the total rate of methane production was 10-fold higher than the rate of H_2 evolution. Notably, floc disruption enhanced H_2 evolution without changing the rate of methane production or ethanol consumption. The rate of increase and the maximum size of the H_2 gas pool in liquid phase and head phase in disrupted floc preparations at 500 min was approximately twice that of intact floc preparations. Thus, disruption reduced IHT compartmentalization, but this did not significantly alter syntrophic ethanol conversion to methane.

The kinetics of ethanol consumption and methane formation shown in Fig. 4 were consistent with reaction 3, and ethanol was not limiting within intact flocs, as the disruption of flocs did not enhance the kinetics of ethanol conversion. Greater than 95% of ethanol carbon was recovered as acetate, indicating the insignificance of ethanol or acetate conversion to other carbon products (i.e., propionate, lactate, butyrate). Acetate, a product of syntrophic ethanol conversion (Fig. 4), was not significantly converted to methane in these experiments. This is not surprising, as in the complete whey chemostat ecosystem the rate constant for the acetate removal was 70 times lower than for the ethanol conversion (8). The measured H_2 pool partial pressure in the acetate control experiments (Fig. 5) was 10 to 30 Pa, whereas in the presence of ethanol, the H_2 partial pressure was 300 to 600 Pa. Thus, hydrogen was at least one reaction intermediate of syntrophic ethanol conversion to methane.

These results emphasize that syntrophic ethanol conversion to methane (reaction 3) occurred predominantly within the floc compartment and was not mediated by the dissolved H_2 gas pool outside the flocs because disruption altered the kinetics of H_2 evolution but not CH_4 production or ethanol consumption. If H_2 gas was exchanged between acetogens and methanogens via the H_2 gas pool outside the flocs, then disruption itself would not have increased the flux of H_2 into the headspace, because the interfacial area and volume of the liquid and gas phase were exactly identical in experimental vials with either disrupted or intact preparations.

A reassociation of the fragments into larger aggregates was also observed during all experiments after prolonged incubations (>300 min). This reassociation did not influence the difference between intact and disrupted flocs or change

FIG. 3. Microscopic analysis of intact floc fraction versus a disrupted floc preparation. (A) Phase-contrast photomicrograph of a typical intact washed floc fraction before disruption (×380). (B) Phase-contrast photomicrograph of a syringe-disrupted floc preparation (×380). (C) Thin-section electron photomicrograph of a floc preparation after syringe disruption, illustrating normal cell integrity (×7,600. Arrow: Note a curved cell pair typical of *D. vulgaris* and a long, filamentous, slender rod typical of *Methanobacterium formicicum*.

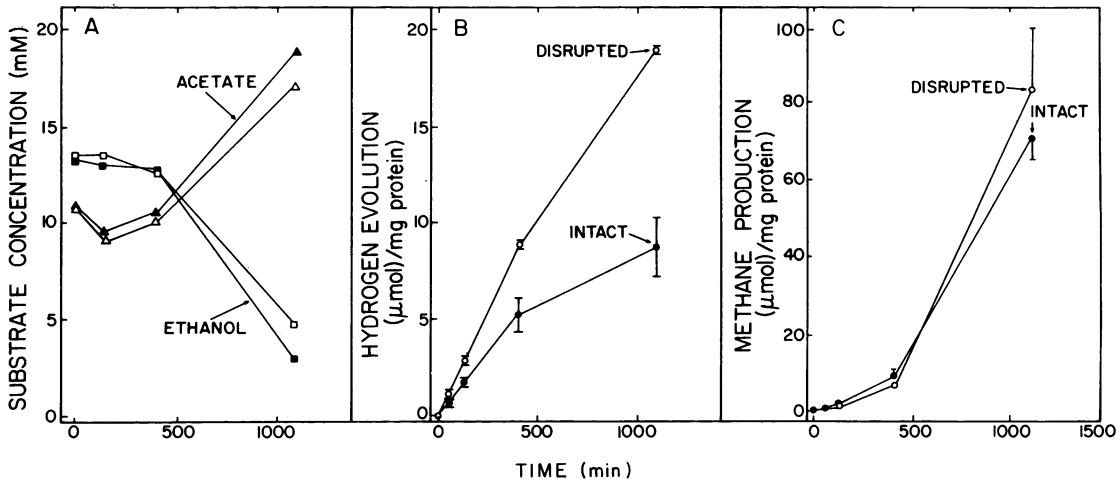


FIG. 4. Comparison of ethanol-dependent H_2 and CH_4 production by intact versus disrupted floc preparations. The experiments were performed in 58-ml serum vials that contained 5 ml of PBB medium, 10 mM acetate, 13 mM ethanol, and either 1.0 mg of intact floc protein or 0.8 mg of disrupted floc protein. The floc preparations were subcultured on whey, washed, and then nutrient limited for 2 days before substrate addition. The data represent the average ± standard deviation (bars) of three separate experiments which varied less than 15%. The protein concentrations and pH remained constant, and carbon and electron recoveries were greater than 95% during these experiments. Symbols: □, △, and ○, disrupted floc preparations; ■, ▲, and ●, intact floc preparations.

the patterns of the interspecies electron transfer. Thus, the initial disruption was the important step to reduce the compartmentalization of interspecies electron transfer.

DISCUSSION

In general, these data demonstrate that juxtapositioning of microbial species within flocs enables enrichment and spatial organization of syntrophic metabolism in methanogenic ecosystems. CO_2-dependent methanogenesis, ethanol consumption, and H_2 consumption were all preferentially localized in the whey-processing digestor flocs examined here. These results support the previous hypothesis of Conrad et al. (10) that CO_2-dependent methanogenesis and IHT are predominantly located in an ecological compartment (i.e., floc) and are independent of the dissolved hydrogen pool in the bulk aqueous phase. These data also show that in this ecosystem, ≤5% of syntrophic ethanol conversion to methane can be directly accounted for by IHT, while the balance (≥95%) depends on some other mechanism for interspecies control of electron flow. In a separate study (29), we demonstrated that XH_2 was formate in the floc ecosystem.

To our knowledge, this represents the first documentation of a specialized metabolic function for cellular aggregation (i.e., floc formation) in anaerobic digestion ecosystems. The floc fraction, separated only by particle size, constituted a

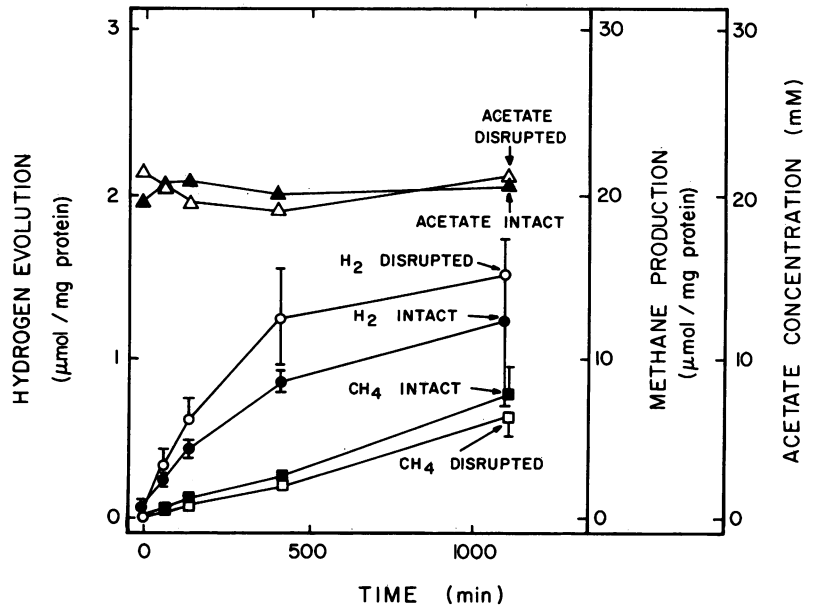


FIG. 5. Comparison of H_2 and CH_4 production in the absence of syntrophic substrate by intact versus disrupted floc preparations. The experimental conditions were the same as described in the legend to Fig. 4 except the PBB medium contained 20 mM acetate alone.

preferred environment for syntrophic acetogens and CO_2-reducing methanogens (i.e., H_2 or formate consuming). Whey was required for the growth of flocs in the free flora culture preparations and of free flora in floc culture preparations. This indicated that an equilibrium exists between the floc state and the free flora state and that this was a property of the bacterial species involved and not an artifact caused by the initial chemostat digestor conditions.

Although 2.5-fold or higher specific reaction rates were observed for the syntrophic acetogenic reaction in flocs (Table 1), the specific formation of free H_2 gas was always at least 80% lower in the floc than in the free flora experiments. Hydrogen production by syntrophic acetogenic bacteria is thermodynamically and kinetically favored at very low H_2 partial pressures, which were apparently maintained in the floc. This was accompanied by lower butyrate production and the virtual absence of propionate in floc preparations actively metabolizing lactose. These results support previous findings that *Clostridium propionicum* and *C. butyricum*, the dominant propionate- and butyrate-producing organisms in the whey digestor, produced mainly acetate when grown as mixed cultures or defined cocultures in the presence of *Methanobacterium formicicum* (Thiele et al., in preparation). Thus, these anaerobic digestor flocs constituted an environment for efficient syntrophic conversions during the biomethanation of whey.

Table 2 assesses the quantitative effect of floc formation and disruption on important H_2 metabolism parameters during syntrophic ethanol conversion to estimate the efficiency of syntrophic methanogenesis by correcting the total CH_4 formed in the presence of ethanol (Fig. 4) for the background methane in the acetate control (Fig. 5). A 1:4 stoichiometry of the methane production from hydrogen was assumed for these calculations (reaction 2). The IHT ratio or the efficiency of IHT is described as the ratio of H_2 flux to CO_2-reducing methanogens over the total flux of H_2 from ethanol oxidation. Disruption of flocs clearly decreased the IHT ratio at all time intervals. This effect was most dramatic during the first 60 min, when a 2.8-fold reduction in efficiency was observed upon disruption (24 versus 68% at 0 to 56 min). These results can be explained by the expected increase in the surface area resulting from floc disruption (equation 6). Based on the geometry of a sphere, a floc of 50-μm radius when disrupted into fragments of 10- to 20-μm radius yields a 2.5- to 5-fold increase in the total surface area. This increase in interfacial area would cause a predictable proportional increase of H_2 flux into the headspace. The decreased H_2 flux observed with prolonged incubations is also predictable because the accumulation of H_2 gas in the headspace would decrease the diffusional driving force for further H_2 excretion by flocs. These data demonstrated that methanogenic H_2 consumption occurred inside the flocs during syntrophic ethanol oxidation.

TABLE 2. H_2 metabolism parameters during syntrophic ethanol transformation by intact versus disrupted cultured flocs[a]

Condition (min)	Dissolved H_2 (μM)	H_2 to methane (μmol)	Produced H_2 gas (μmol)	IHT ratio (%)[b]
0–56				
Intact	0.17	0.90	0.43	68
Disrupted	0.22	0.21	0.67	24
56–136				
Intact	0.48	3.80	0.96	80
Disrupted	0.74	2.84	1.73	62
136–405				
Intact	1.84	22.16	3.01	88
Disrupted	2.18	16.79	5.33	76
405–1,100				
Intact	3.66	225.21	3.01	99
Disrupted	5.14	289.97	9.73	97

[a] The data were taken from the experiment described in the legend to Fig. 4. The dissolved H_2 and CH_4 were calculated from their partial pressure in the headspace and the measured Bunsen coefficients for both gases.
[b] The IHT ratio is defined in the text.

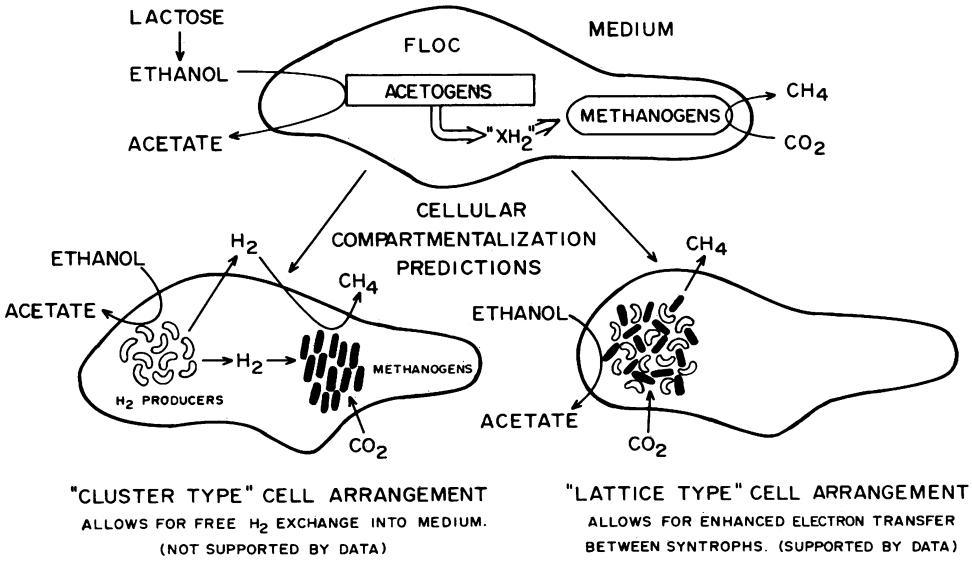


FIG. 6. Predictive model for species compartmentalization and electron flow coupling during syntrophic ethanol metabolism in whey digestor flocs. The data support a lattice-type cell juxtapositioning between *D. vulgaris*, the principal syntrophic acetogen, and *Methanobacterium formicicum*, the principal CO_2-reducing methanogen. The data support a mechanism for coupling electron flow from the acetogen to the methanogen via XH_2, which represents reducing equivalents that are independent of the H_2 pool outside the flocs.

Methanogenic H_2 consumption follows Michaelis-Menten-type reaction kinetics as a function of the dissolved H_2 concentration (17, 26). However, the kinetics observed for the H_2 pool increase outside the flocs and the methane produced from H_2 were totally uncoupled. At an average dissolved [H_2] of 0.48 μM (56 to 136 min), the rate of CH_4 formation from H_2 was 0.012 μmol/min, but at a concentration of 1.84 μM (136 to 405 min) it was only 0.0205 μmol/min. Thus, a 3.83-fold increase in the dissolved [H_2] was accompanied by only a 1.74-fold increase in the rate of the methane formation. A 3.25-fold increase would be expected from Michaelis-Menten-type kinetics with a K_S of 6.5 μM for *Methanobacterium formicicum* (Thiele et al., in preparation). In the second half of the progress curve, where most of the ethanol conversion occurred, a twofold increase in the dissolved [H_2] was accompanied by a fourfold increase in the rate of methane formation (405 to 1,100 min). Exactly the same behavior was observed with the disrupted preparations. This showed clearly that even at low H_2 partial pressures, the rate of methane formation by CO_2 reduction in the flocs did not depend on the dissolved H_2 gas pool.

It is impossible to decide from the kinetic data whether the metabolic coupling of interspecies electron transfer between syntrophic acetogens and methanogens in the flocs occurred through the juxtaposition of single cells in a lattice-type arrangement. This cell arrangement is strongly suggested by the microscopic data, which could not support the existence of separate microcolonies of acetogens and methanogens in the flocs. A dynamic diffusion reaction model, however, supported IHT between closely adjacent single cells, because the model could predict the effect of a floc disruption on the interspecies electron transfer compartmentalization (Fig. 4) within 5% error (24). These simulation studies employed only parameters, which were explicitly measured in the whey digestor floc system (24). The good agreement between experimental data and model predictions supports the hypothesized interspecies electron transfer between single juxtaposed acetogens and methanogens. The model predicted (24) that 100-μm-diameter flocs would develop doubled internal H_2 gas levels, which after disruption were the source of the elevated H_2 outside the floc fragments. Thus, an H_2 mass transfer limitation between the flocs and their environment was the physiological basis for the observed compartmentalization of syntrophic electron transfer at low environmental H_2 concentrations. The disruption destroyed this diffusion barrier and resulted in doubled external H_2 levels.

In any event, the significance of the discovery that syntrophic methanogenic reactions were compartmentalized within the whey digestor flocs by species juxtapositioning cannot be underestimated. Syntrophic growth and energy metabolism of acetogens and methanogens depends on the magnitude of the carbon and electron flux (reaction 3), which is controlled by the interspecies diffusional flux of the syntrophic reduced intermediate XH_2. Fick's first law of diffusion [i.e., $J = -D(dc/dx)$] suggests that either a short distance x or a large XH_2 concentration difference between the acetogen and methanogen cell surface improves the interspecies metabolite transport step of the syntrophic reaction. However, pushing the diffusional metabolite transport by large values for this concentration difference would result in a product inhibition of the acetogenic partial reactions by high XH_2 (30) and thus limit the overall carbon and electron flux. Shorter cell-cell distances, on the contrary, allow for high syntrophic fluxes at proportionally lower XH_2 concentrations. This mechanism is independent of the nature of the transferred intermediate (e.g., H_2 or formate) and depends predominantly on the diffusivity of the transferred compound and the volumetric reaction rates of acetogenesis and methanogenesis (24).

The ecological significance of the present results is represented in a model shown in Fig. 6. This model explains that syntrophic ethanol conversion to CH_4 and acetate occurred by CO_2 reduction independent from the available H_2 pool in this ecosystem and that interspecies electron transfer was kinetically compartmentalized within flocs by the juxtapositioning of *D. vulgaris* and *Methanobacterium formicicum* cells. This results in high intercellular flux rates, which compartmentalize the syntrophic interspecies electron transfer reactions. Therefore, this ecological feature is somewhat analogous to the coimmobilization of two coupled enzyme reactions to gain high substrate turnover rates owing to close reaction centers. High flux rates by enzyme coimmobilization studies enable as high as a 10-fold increase in activity compared with that of the free enzyme (21). Thus, species juxtapositioning could maximize the overall conversion of organic matter under digestor overload conditions, in which the syntrophic reactions become rate limiting, and could avoid mass transfer limitations between the metabolic partners during normal digestor operation. Microbial aggregates that possess these features could provide a mechanism to protect syntrophic methanogenic reactions from potential inhibition by high H_2 concentrations in the aqueous bulk phase.

ACKNOWLEDGMENTS

During the course of these experiments, J.H.T. was supported by a research fellowship of the Deutsche Forschungsgemeinschaft. The project received support from awards made by Institut Pasteur and the Michigan Biotechnology Institute.

We are grateful to Marty Garment for his assistance during the electron microscopy. The important discussions with Sadettin Ozturk, Bernhard Palsson, and Tom Thompson during the preparation of this manuscript are gratefully acknowledged.

LITERATURE CITED

1. **Atkinson, B.** 1984. Consequences of aggregation, p. 351–373. *In* K. C. Marshall (ed.), Microbial adhesion and aggregation. Springer-Verlag, New York.
2. **Boone, D. R., and M. P. Bryant.** 1980. Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogic ecosystems. Appl. Environ. Microbiol. **40**:626–632.
3. **Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. A. Crabill.** 1977. Growth of *Desulfovibrio* in lactate or ethanol medium low in sulfate in association with H_2-utlizing methanogenic bacteria. Appl. Environ. Microbiol. **33**:1162–1169.
4. **Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe.** 1967. "*Methanobacillus omelianskii*," a symbiotic association of two species of bacteria. Arch. Microbiol. **59**:20–31.
5. **Callander, I. J., and J. P. Barford.** 1983. Cheese whey anaerobic digestion. Effect of chemical flocculant addition. Biotechnol. Lett. **5**:153–158.
6. **Chartrain, M., L. Bhatnagar, and J. G. Zeikus.** 1987. Microbial ecophysiology of whey biomethanation: comparison of carbon transformation parameters, species composition, and starter culture performance in continuous culture. Appl. Environ. Microbiol. **53**:1147–1156.
7. **Chartrain, M., and J. G. Zeikus.** 1986. Microbial ecophysiology of whey biomethanation: characterization of trophic populations and prevalent species composition in continuous culture. Appl. Environ. Microbiol. **51**:188–196.
8. **Chartrain, M., and J. G. Zeikus.** 1986. Microbial ecophysiology of whey biomethanation: intermediary metabolism of lactose degradation in continuous culture. Appl. Environ. Microbiol.

51:180–187.
9. **Cheng, K.-J., and J. W. Costerton.** 1980. The formation of microcolonies by rumen bacteria. Can. J. Microbiol. **26:** 1104–1113.
10. **Conrad, R., T. J. Phelps, and J. G. Zeikus.** 1985. Gas metabolism in support of juxtapositioning of hydrogen-producing and methanogenic bacteria in sewage sludge and lake sediments. Appl. Environ. Microbiol. **50:**595–601.
11. **Costerton, J. W., and R. T. Irvin.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35:**299–324.
12. **Ferry, J. G., and R. S. Wolfe.** 1981. Anaerobic degradation of benzoate to methane by a microbial consortium. Arch. Microbiol. **107:**33–40.
13. **Gutmann, I., and A. W. Wahlefeld.** 1974. L-(+)-Lactate determination with lactate dehydrogenase and NAD, p. 1464–1468. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis. Springer-Verlag, New York.
14. **Hartree, E. F.** 1972. A modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48:** 422–427.
15. **Jones, W. J., J.-P. Guyot, and R. S. Wolfe.** 1984. Methanogenesis from sucrose by defined immobilized consortia. Appl. Environ. Microbiol. **47:**1–6.
16. **Kenealy, W., and J. G. Zeikus.** 1981. Influence of corrinoid antagonists on methanogen metabolism. J. Bacteriol. **146:** 133–140.
17. **Kristiansson, J. K., P. Schoenheit, and R. K. Thauer.** 1982. Different K_S values for hydrogen of methanogenic and sulfate reducing bacteria: an explanation for the apparent inhibition of methanogenesis by sulfate. Arch. Microbiol. **131:**278–282.
18. **Lettinga, G., A. F. M. van Vellsen, S. W. Hobma, W. de Zeeuw, and A. Klapwigk.** 1980. Use of the upflow sludge blanket reactor concept for biological wastewater treatment, especially anaerobic treatment. Biotechnol. Bioeng. **22:**699–734.
19. **Marshall, K. C. (ed.).** 1984. Microbial adhesion and aggregation. Life Sciences Research Report 31. Dahlem Workshop Reports. Springer-Verlag, New York.
20. **McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton.** 1981. *Syntrophomonas wolfei* gen. nov. spec. nov., an anaerobic syntrophic fatty acid-oxidizing bacterium. Appl. Environ. Microbiol. **41:**1029–1039.
21. **Mosbach, K.** 1978. The microenvironment of immobilized multistep enzyme systems, p. 381–399. *In* P. Srere and R. W. Eastbrook (ed.), Microenvironments and metabolic compartmentalization. Academic Press, Inc., New York.
22. **Mountfort, D. O., and M. P. Bryant.** 1982. Isolation and characterization of an anaerobic syntrophic benzoate-degrading bacterium from sewage sludge. Arch. Microbiol. **133:**249–256.
23. **Oremland, R. S.** 1983. Hydrogen metabolism by decomposing cyanobacterial aggregates in Big Soda Lake, Nevada. Appl. Environ. Microbiol. **45:**1519–1525.
24. **Ozturk, S. S., B. Palsson, J. H. Thiele, and J. G. Zeikus.** 1987. Modeling of interspecies hydrogen transfer in microbial flocs, p. 142–152. *In* C. S. Ho and J. Y. Oldshue (ed.), Biotechnology processes: scale-up and mixing. American Institute of Chemical Engineers, New York.
25. **Phelps, T. J., R. Conrad, and J. G. Zeikus.** 1985. Sulfate-dependent interspecies H_2 transfer between *Methanosarcina barkeri* and *Desulfovibrio vulgaris* during the coculture metabolism of acetate of methanol. Appl. Environ. Microbiol. **50:** 589–594.
26. **Robinson, J. A., and J. M. Tiedje.** 1984. Competition between sulfate reducing and methanogenic bacteria for H_2 under resting and growing conditions. Arch. Microbiol. **137:**26–32.
27. **Sahm, H.** 1984. Anaerobic wastewater treatment. Adv. Biochem. Eng./Biotechnol. **29:**84–115.
28. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41:**100–180.
29. **Thiele, J. H., and J. G. Zeikus.** 1988. Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. Appl. Environ. Microbiol. **54:**20–29.
30. **Tramper, J., J. W. van Groenstign, K. C. A. M. Luyben, and L. W. Hulshof.** 1984. Some physical and kinetic properties of granular anaerobic sludge, p. 145–155. *In* E. H. Houwink and R. R. van der Meer (ed.), Innovations in biotechnology. Elsevier Biomedical Press, Amsterdam.
31. **Wolin, M. J.** 1975. Interactions between H_2-producing and methane-producing species, p. 141–150. *In* H. G. Schlegel, G. Gottschalk, and N. Pfenning (ed.), Symposium on microbial production and utilization of gases. E. Goltz, Göttingen, Federal Republic of Germany.
32. **Zeikus, J. G., and V. G. Bowen.** 1975. Fine structure of *Methanospirillum hungatii*. J. Bacteriol. **121:**373–380.