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Formation of Fatty Acid-Degrading, Anaerobic Granules by Defined Species

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An endospore-forming, butyrate-degrading bacterium (strain BH) was grown on butyrate in monoxenic coculture with a methanogen. The culture formed dense aggregates when *Methanobacterium formicicum* was the methanogenic partner, but the culture was turbid when *Methanospirillum hungatei* was the partner. In contrast, a propionate-degrading, lemon-shaped bacterium (strain PT) did not form aggregates with *Methanobacterium formicicum* unless an acetate-degrading *Methanosaeta* sp. was also included in the culture. Fatty acid-degrading methanogenic granules were formed in a laboratory-scale upflow reactor at 35°C fed with a medium containing a mixture of acetate, propionate, and butyrate by using defined cultures of *Methanobacterium formicicum* T1N, *Methanosaeta* sp. strain M7, *Methanosarcina mazei* T18, propionate-degrading strain PT, and butyrate-degrading strain BH. The maximum substrate conversion rates of these granules for acetate, propionate, and butyrate were 43, 9, and 17 mmol/g (dry weight)/day, respectively. The average size of the granules was about 1 mm. Electron microscopic observation of the granules revealed that the cells of *Methanobacterium formicicum, Methanosaeta* sp., butyrate-degrading, and propionate-degrading bacteria were dispersed in the granules. *Methanosarcina mazei* existed inside the granules as aggregates of its own cells, which were associated with the bulk of the granules. The interaction of different species in aggregate formation and granule formation is discussed in relation to polymer formation of the cell surface.

The formation of anaerobic granules in upflow anaerobic sludge blanket reactors is important for the reactor to operate at a high chemical oxygen demand (COD) removal rate, and the granule formation phenomenon is believed to be substantially based on microbial self-immobilization (8, 9, 16, 17). Bacterial species that play an essential role in cell-cell aggregation are the key to understanding the phenomenon of granule formation. The key species may be defined as ones which (i) form dense aggregates by themselves in an anaerobic reactor and/or (ii) provide a binding surface for other bacteria which cannot form aggregates and granules by themselves. Therefore, the species having the ability to aggregate are likely to be the prevalent microorganisms in the granules.

Methanogens have already been hypothesized to be key species in granule formation. Several proposals about the microbial mechanisms of granule formation with volatile fatty acids (VFAs) as major substrates can be summarized as follows. (i) The *Methanothrix* species plays an essential role in granulation (3, 5, 13). (ii) The hydrogen-utilizing methanogen *Methanobrevibacter arboriphilicus* AZ produces extracellular polypeptides to induce granule formation under high-H_2 partial pressure conditions (10). (iii) *Methanosarcina* cells produce initial aggregates as nuclei to form granules (2). These proposals and mechanisms, however, have not been validated experimentally.

Microcolonies of syntrophic acetogens in granular structures have been observed within anaerobic granules (3). Syntrophic microcolonies consisting of acetogens and methanogens were major structural components of granules developed on brewery wastewater (15). The role of syntrophic acetogens in granule formation has not been clearly elucidated.

In this study, the aggregate-forming behaviors of various defined methanogenic and syntrophic fatty acid-degrading cultures were analyzed for potential granule-forming syntrophic associations. We report here the formation of anaerobic granules with defined cultures and the stability and VFA-degrading performance of the granules.

MATERIALS AND METHODS

Bacterial strains. All of the methanogens and syntrophic fatty acid degraders utilized in this study were isolated from methanogenic granules developed on a fatty acid mixture containing acetate, propionate, and butyrate (18). The following methanogens were used in this study: *Methanobacterium formicicum* T1N (DSM 6298) and *Methanospirillum hungatei* BD, with either formate or H_2-CO_2 as the substrate; *Methanosaeta* sp. strain M7, a mesophilic acetate-utilizing methanogen; and *Methanosarcina mazei* T18 (DSM 6300), with H_2-CO_2, acetate, and methanol as substrates. The following syntrophic acetogenic cultures were used: an obligate anaerobic, spore-forming, rod-shaped, butyrate-degrading strain, BH, and a lemon-shaped, spore-forming, propionate-degrading strain, PT. Strain BH is morphologically similar to *Syntrophospora bryantii* strains. Both of these syntrophic acetogens were isolated with *Methanobacterium formicicum* T1N as a partner for syntrophic fatty acid metabolism.

Medium. The phosphate-buffered basal medium (6) was used for the growth of cells in anaerobic 158-ml serum vials, which were sealed with butyl rubber stoppers. For the growth of syntrophic fatty acid-degrading cultures, each vial contained 50 ml of phosphate-buffered basal medium supplemented with 15 mM phosphate, 40 mM sodium bicarbonate, 1% vitamin solution (vol/vol) (14), and 1 mM sodium sulfide as a reductant, except for the culture containing *Methanosaeta* sp. strain M7, which contained 0.3 mM sodium sulfide. The substrate concentration was 15 mM sodium butyrate or 15 mM propionate. Before inoculation, the vials were pressurized with 1 atm (101.29 kPa) of N_2-CO_2 (95:5) mixture gas, and the pH in each vial was adjusted to 7.0 to 7.1 with a 2.5 M KH_2PO_4 or K_2HPO_4 solution when necessary.

For reactor experiments, liquid medium (16 liters) was prepared anaerobically in a 20-liter carboy. The medium composition was (per liter of distilled water): $MgCl_2 \cdot 6H_2O$, 0.32 g; $CaCl_2 \cdot 2H_2O$, 0.32 g; NaCl, 0.8 g; NH_4Cl, 0.8 g; KH_2PO_4, 0.32 g; $Na_2S \cdot 9H_2O$, 0.08 g; resazurin, 0.002 g; and trace element solution, 5 ml (6). The concentrations of acetate, propionate, and butyrate were adjusted by addition of the mixtures of the sodium salt and acid forms of the volatile acids. The medium pH was 4.7.

Aggregate formation by defined cultures. A methanogenic culture (*Methanobacterium formicicum* T1N), two syntrophic butyrate-degrading cocultures (butyrate-degrading strain BH plus *Methanobacterium formicicum* T1N and

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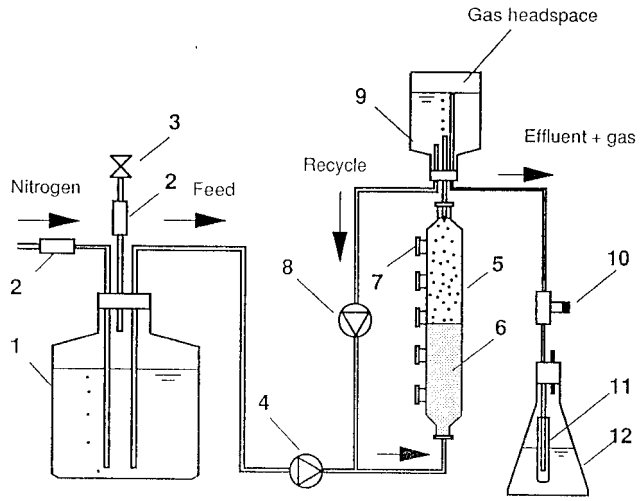

FIG. 1. Schematic diagram of upflow reactor system. System parts: 1, medium reservoir; 2, sterile gas filter; 3, gas valve; 4, feed pump; 5, column reactor; 6, granule bed; 7, sampling port with a butyl rubber stopper; 8, recycle pump; 9, settler; 10, glass T with a butyl rubber stopper; 11, water seal; 12, effluent collecting flask.

strain BH plus *Methanospirillum hungatei* BD), one syntrophic propionate-degrading coculture (propionate-degrading strain PT plus *Methanobacterium formicicum* T1N), and one syntrophic propionate-degrading triculture (strain PT, *Methanobacterium formicicum* T1N, and *Methanosaeta* sp. strain M7) were examined for cell aggregation. To prepare the triculture of strain PT, *Methanobacterium formicicum*, and *Methanosaeta* sp. strain M7, a serum bottle containing a coculture of strain PT and *Methanobacterium formicicum* was inoculated with 2% of *Methanosaeta* sp. strain M7 culture as the third partner to utilize acetate. To obtain the coculture of strain BH and *Methanospirillum hungatei*, the coculture of strain BH plus *Methanobacterium formicicum* was heat treated at 80°C for 20 min to kill the cells of *Methanobacterium formicicum*. The heat-treated culture was then inoculated (2% [vol/vol]) into a bottle containing fresh medium. A 2% inoculum of late-exponential-phase *Methanospirillum hungatei* cells was added.

All of the defined cultures were incubated at 37°C. Initially, culture transfer was done with syringes equipped with 22-gauge needles. When aggregates or clumps were formed in the bottles, culture transfer was performed with syringes equipped with 18-gauge needles in order to protect the structure of the aggregates or clumps from any damage during transfer.

Upflow reactors. A laboratory-scale upflow reactor made of a glass column and a glass bottle was operated at 35°C to develop fatty acid-degrading granules. The total volume of the reactor was 0.22 liter, including a 0.12-liter settler. All tubing used was either stainless steel or black butyl rubber with a thickness of 0.25 in. (0.64 cm). A schematic diagram of the reactor system is presented in Fig. 1. During continuous operation, oxygen-free N_2 (at 40 kPa passed through a sterilized glass wool filter) was bubbled into the medium reservoir to keep a constant positive pressure in the headspace and to maintain anaerobiosis. The medium was pumped into the bottom of the reactor with a peristaltic pump (Gilson Medical Electronics, Inc., Middleton, Wis.). After gas-liquid-solid separation in the settler, the effluent was allowed to leave the reactor through a water seal made of a 5-ml glass tube installed inside the effluent-collecting flask in order to keep the reactor free of oxygen. Some of the liquid in the settler was recycled back to the reactor by another peristaltic pump, at a flow rate of 1 liter/h, to neutralize the feed and to maintain the desired hydraulic loading rate (ca. 2.8 $m/m^2 \cdot h$). The inlet end of the effluent tube in the settler allowed gas and effluent to pass together (Fig. 1). Biogas samples for methane and hydrogen determinations were withdrawn under sterile conditions from the glass "T" tube on the effluent tubing with a 1-ml glass syringe. The glass T tube was positioned in a manner such that the effluent liquid did not touch the inside of the butyl rubber stopper. This facilitated the gas sampling for analysis. Liquid samples were withdrawn aseptically through the sampling port on the reactor.

Development of syntrophic VFA-degrading granules. To ensure successful start-up, anaerobic conditions were established in the reactor system prior to inoculation. The reactor, including feed pump tubing and recycle pump tubing, was autoclaved at 121°C for 30 min. Initially, an HCl solution (1 N) was added to the water seal installed in the effluent flask, and the reactor was flushed with oxygen-free nitrogen gas for 30 min. Subsequently, the basal medium (pH 7.0 to 7.1), prereduced with 0.5 mM Na_2S and buffered with sodium bicarbonate (30 mM) and potassium phosphate (30 mM), was added to fill the reactor. Recycling of the medium was started, and N_2 flushing was stopped. Anaerobic conditions in the reactor were monitored with the redox potential indicator resazurin. Finally, the carboy containing the feed medium was connected to the reactor under aseptic conditions. The reactor was inoculated through the sampling port, and feeding was initiated. Monocultures and syntrophic cultures were used as the inoculum. The different species were inoculated in the following sequence. Initially, actively growing cells of *Methanobacterium formicicum* T1N (40 ml, 0.22 g [dry weight]) were inoculated, and about 10 mM sodium formate was added to the reactor; after 8 h, aggregates of *Methanosarcina mazei* T18 (0.5 ml) and cells of the *Methanosaeta* sp. strain M7 culture (30 ml, 0.05 g [dry weight]) were inoculated, and then the reactor was fed continuously at 7 ml/h. Finally, a coculture of strain BH and *Methanobacterium formicicum* (30 ml, 0.025 g [dry weight]) and a triculture of propionate-degrading strain PT, *Methanobacterium formicicum* T1N, and *Methanosaeta* sp. strain M7 (30 ml, 0.06 g [dry weight]) were added into the reactor one after another at an interval of 12 h. After inoculation, pH and fatty acid concentrations in the reactor were determined initially on alternate days and then were determined daily. Methane and hydrogen contents in biogas were determined every other day.

At the time of start-up and during the operation of the reactor, adequate precautionary steps were taken to ensure that the reactor operated free of contamination. These included sterilization of the reactor, connecting tubes, media, and other solutions and operation of the reactor in a very clean, temperature-controlled room. A simple microscopic examination technique was used to detect the presence of morphologies of cells other than those belonging to the five microbial species that were inoculated in the reactor. Contamination in the feed carboy and the reactor was monitored carefully. The carboy for each medium, after sterilization, was sealed under nitrogen gas and held for 5 days to monitor development of any microbial growth before it was connected to the reactor. Samples from influent media, reactor contents, and the effluent were withdrawn at least twice a week for microscopic examination. Each sample was examined through 30 to 40 microscopic fields for a potential contaminant morphology. In addition, the gas phase and aqueous phase of the reactor were analyzed for any change in the metabolic intermediates and end products that might signal the presence of a contaminant in the reactor.

Substrate conversion activities. To determine the maximum VFA degradation rates of the granules that developed from the defined cultures, another reactor of the same size was prepared and filled with prereduced basal medium (pH 7.0) as described above. The basal medium in the reactor contained acetate (5 mM), propionate (5 mM), and butyrate (5 mM). The granules (free of flocs) from the initial reactor were transferred anaerobically with a 10-ml glass syringe with an 18-gauge needle into another reactor. This inoculum contained 5 ml of compact granules. After inoculation, the new reactor was continuously fed with a medium containing propionate (30 mM) and butyrate (30 mM). The maximum degradation rates for propionate and butyrate were determined on the basis of the following day's operational data (VFA concentrations in the reactor and feed rate) with the following equations:

$$dS/dt = V_R \cdot (S_{t1} - S_{t2})/(t_2 - t_1) + Q \cdot [S_0 - 0.5 \cdot (S_{t1} + S_{t2})] \quad (1)$$

where dS/dt is the degradation rate of propionate or butyrate (millimoles per hour), V_R is the liquid bulk volume of the reactor system including the recycle tubing (0.22 liter in this experiment), S_{t1} and S_{t2} are propionate or butyrate concentration (millimolar) at times t_1 and t_2, respectively, Q is feed rate (liters per hour), and S_0 is the propionate or butyrate concentration (30 mM) in feed. The acetate degradation rate was calculated as follows:

$$dS_a/dt = V_R \cdot (S_{at1} - S_{at2})/(t_2 - t_1) + Q \cdot [S_{a0} - 0.5 \cdot (S_{at1} + S_{at2})] + dS_p/dt + 2 \cdot dS_b/dt \quad (2)$$

where dS_a/dt, dS_p/dt, and dS_b/dt are degradation rates (millimoles per hour) for acetate, propionate, and butyrate, respectively. In equation 2, acetate produced from syntrophic propionate and butyrate degradation is included, but valerate formed in the reactor was ignored for this purpose, since valerate concentrations were low (<0.1 mM).

The apparent half-velocity coefficients (K_m) for propionate and butyrate were determined from an integrated solution to the Michaelis-Menten equation. Determination of apparent K_m was made at 35°C in separate batch experiments by using 158-ml serum bottles as described previously (19).

Chemicals, gases, and analytical methods. The chemicals and gases used were described previously (15). Methane was analyzed with a Hewlett-Packard gas chromatograph equipped with a flame ionization detector, whereas H_2 was analyzed with a Hewlett-Packard gas chromatograph equipped with a thermal conductivity detector, as described previously (15). VFA concentrations in the liquid samples were determined with a gas chromatography-flame ionization detector system (15). The turbidities of the bacterial cultures and reactor effluents were determined with a UV-160 spectrophotometer (Shimadzu Corp., Kyoto, Japan) at 660 nm. The specific gravity of the granules was determined in accordance with the standard method 213E (1). The size of the granules was determined from photographs and calculated as described elsewhere (11). The microscopic examination was made with an Olympus model BH2 microscope. The sample preparation and observation by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed as described elsewhere (15).

RESULTS

Evaluation of aggregation behavior. The aggregation behaviors of various syntrophic fatty acid-degrading cultures and pure cultures of methanogen were estimated on the basis of the optical density at 660 nm of liquid and by microscopic examination. The optical density at 660 nm of pure cultures of *Methanobacterium formicicum* and *Methanosarcina mazei* was very low (<0.02), because almost 100% of the cells formed aggregates. Most cells of *Methanosaeta* sp. strain M7 formed rosette clumps, but some remained as dispersed cells. *Methanospirillum hungatei* grew as dispersed cells only.

Cells of *Methanobacterium formicicum* and of the butyrate-degrading strain BH in coculture formed dense aggregates. Some of the aggregates were as large as 0.2 to 0.4 mm in diameter after two transfers. Microscopic examination indicated that the cells of the two strains grew homogeneously (Fig. 2A). When the aggregates formed, the medium appeared clear (optical density at 660 nm, <0.04), and only a very few cells were found free in the liquid. In contrast, no aggregates were observed in cocultures of strains BH and *Methanospirillum hungatei*. Microscopic examination revealed that *Methanospirillium hungatei* grew as single filaments, while strain BH formed small clumps consisting of only a few cells. It was also observed during microscopic observation that the cells of the two strains were always separated from each other (Fig. 2B). This coculture did not undergo aggregation. The clump-forming characteristic of strain BH indicates the possibility of the presence of adhesive cell surface polymers on the surface of strain BH cells.

Cells of propionate-degrading strain PT and *Methanobacterium formicicum* did not grow homogeneously in coculture. Microscopic observation revealed that cells of strain PT were always found outside the clumps of *Methanobacterium formicicum* cells (Fig. 2C). However, in the triculture consisting of strain PT, *Methanobacterium formicicum*, and *Methanosaeta* sp. strain M7, a larger number of aggregates formed. These aggregates were homogeneously composed of cells from the three strains (Fig. 2D). Some free cells were also observed.

Granule formation by defined species. Operational results, including the concentrations of fatty acids in the influent and effluent, VFA degradation rates per reactor, COD volumetric loading rates, and COD volumetric removal rates, are presented in Fig. 3. The operational conditions of the reactor and the results obtained are summarized in Table 1. Feeding of acetate, butyrate, and propionate was controlled during the operation of the reactor (Fig. 3A).

After start-up, acetate was degraded immediately. The butyrate concentration in the effluent remained at 10 mM for about 10 days before significant butyrate degradation occurred. Significant decrease in effluent propionate concentrations and propionate degradation occurred only after 37 days (Fig. 3B). This indicates that the order of growth of different fatty acid degraders was as follows: acetate-degrading methanogen → butyrate degrader → propionate degrader. Fatty acid degradation rates in the reactor increased almost logarithmically during the operational period (Fig. 3C). The increase in the COD volumetric removal rates was proportional to the increase in the COD volumetric loading rates. At the beginning, the COD removal rate (total reactor system) was less than 0.1 g of COD/liter of reactor volume/day, with a COD removal of less than 3%. At the end of the operation, the volumetric COD removal rate had increased to 22.5 g of COD per liter/day, with COD removal efficiencies of 80 to 83% (Fig. 3D). An attempt to monitor the specific volumetric loading rate and specific COD removal rate on the basis of the biomass of the granules was impossible, since both flocs and biofilms were present in the reactor system.

At the beginning, the reactor contained about a 5-ml volume of bacterial bed consisting of inoculated cultures of apparently low-density aggregates. After 17 days of continuous operation, most of the light fraction of the aggregates was washed out of the column reactor into the settler. A few dense small aggregates with a brownish yellow color remained at the bottom of the reactor. The granule-like aggregates were observed at the bottom of the reactor after 20 days of operation. These gradually developed to form a granule bed in the reactor. By day 82, about 7 ml of the granules had developed in the reactor. During this period (after about 50 days), attachment of bacteria to the reactor wall became visible. This process of attachment and subsequent growth of biofilms continued until at the termination of the experiment at day 82, roughly 50% of the total biomass on (the basis of dry weight) was attached to the reactor wall and the rest was in the granule bed. The attached biofilms were yellowish, while the color of the granules was brownish yellow.

Microbial structure of granules. The granules developed by the defined species were of irregular shapes and were about 1 mm in diameter (Fig. 4). Microscopic examination revealed that both granules and biofilms had the same microbial composition, and both had all five of the species inoculated into the reactor at readily observable numbers. Under SEM examination, the cells of *Methanosaeta* sp. strain M7, *Methanobacterium formicicum*, propionate-degrading strain PT, and butyrate-degrading strain BH were frequently observed on the surface of granules (Fig. 4). The cells of *Methanosarcina mazei* were observed only occasionally. The five cultures can be clearly identified in the TEMs on the basis of their distinct morphotypes (18). TEM cross thin sectioning revealed that *Methanobacterium formicicum, Methanosaeta* sp. strain M7, strain PT and strain BH (except, *Methanosarcina mazei*) grew homogeneously within the granules (Fig. 4). *Methanosarcina mazei* cells grew as individual aggregates inside the granules but did not appear to mix with other bacteria. On the outer surface of the aggregates, *Methanosarcina mazei* and *Methanobacterium formicicum* grew in what appeared to be an attached layer (Fig. 4). This layer of *Methanobacterium formicicum* cells appeared to serve as a bridge between *Methanosarcina mazei* aggregates and the other syntrophic bacteria growing together. The overall cell density of *Methanosarcina mazei* in these granules was hard to determine by electron microscopy because of aggregation and because the size of the *Methanosarcina mazei* cells was irregular.

Metabolic performance. The conversion rates of acetate, propionate, and butyrate by syntrophic anaerobic granules are presented in Table 2. The granules had high fatty acid degradation rates, a specific gravity of 1.023, and a density of 58 g (dry weight)/liter. On the basis of the stoichiometry of complete oxidation of acetate, propionate, and butyrate to CO_2, the maximum COD removal rate of the granules was 3.22 g of COD per g (dry weight) per day at 35°C. These granules had a normal distribution, with 0.8 mm as the mean diameter on the basis of the total number of granules (Fig. 5). The volume of the granule bed was principally due to the granules with a size not larger than 1.0 mm in diameter. This indicates that the granules had a relatively uniform size distribution. The apparent half-velocity coefficients (K_m) of the granules were determined to be 0.038 ± 0.007 mM for propionate and 0.19 ± 0.03 mM for butyrate. These values are the same as the apparent K_m values for propionate and butyrate in granular sludge, from which strains PT and BH were isolated (19). These values can

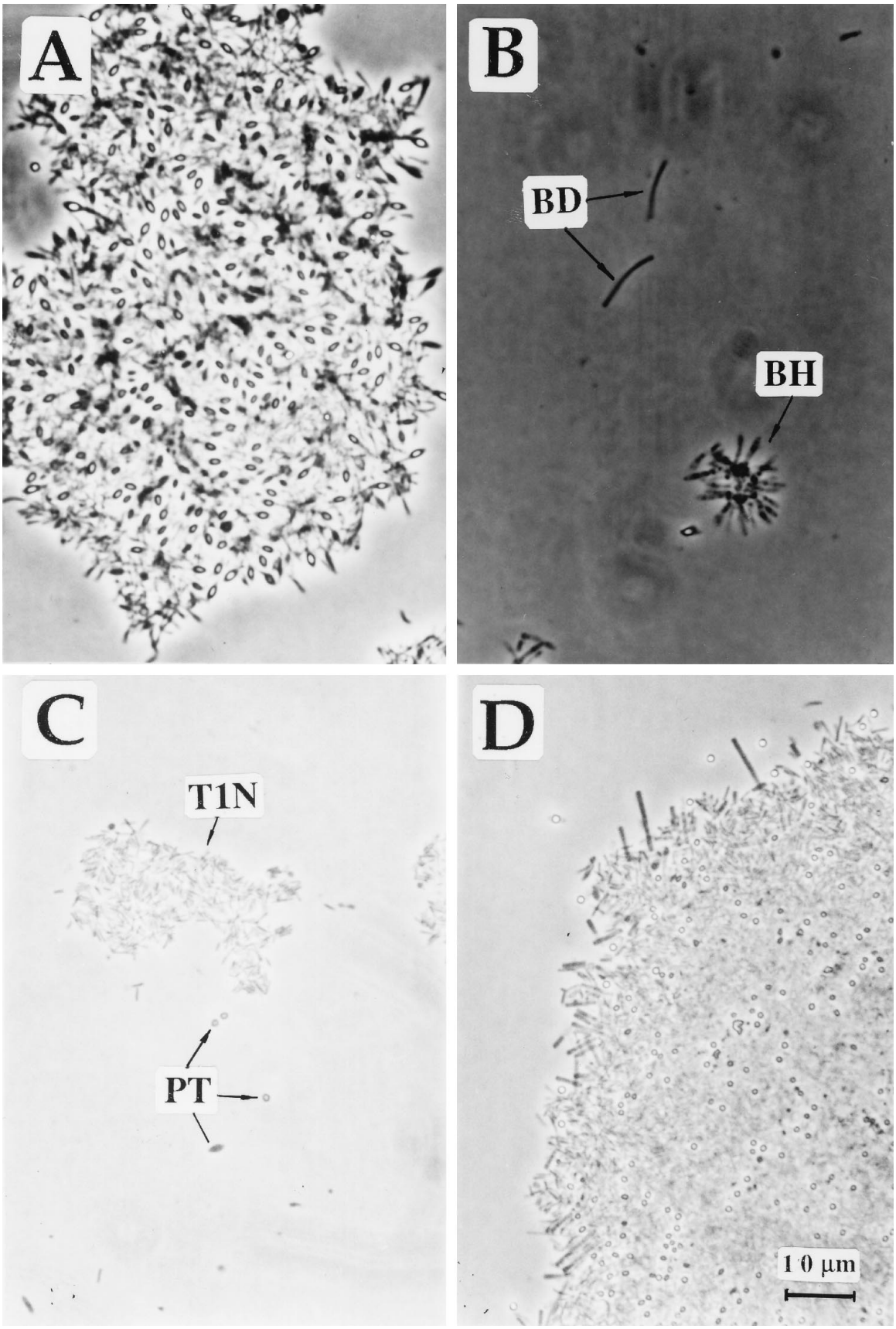

FIG. 2. Absence or presence of aggregate formation by syntrophic methanogenic cocultures and triculture. (A) Aggregate formation by *Methanobacterium formicicum* T1N and butyrate-degrading strain BH. (B) Absence of aggregation between *Methanospirillum hungatei* BD and strain BH. (C) Absence of aggregation between propionate-degrading strain PT and *Methanobacterium formicicum* T1N. (D) Aggregate formation by strain PT, *Methanosaeta* sp. strain M7, and *Methanobacterium formicicum* T1N.

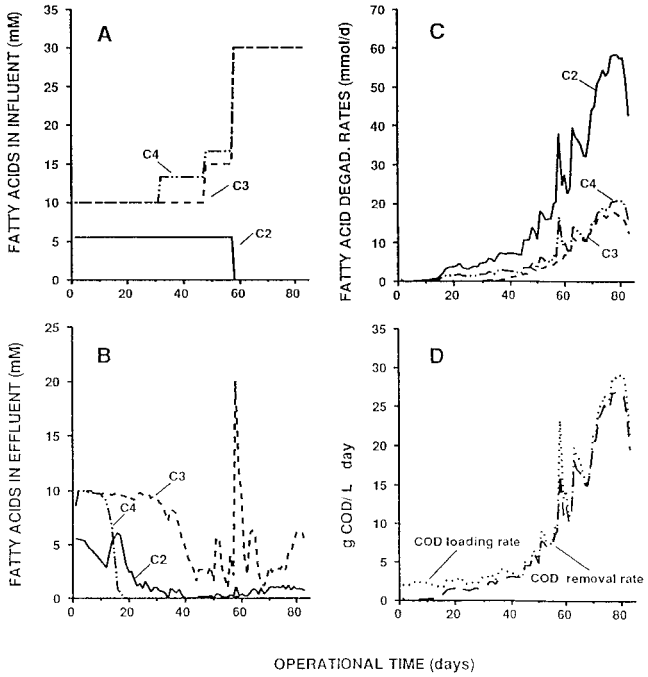

FIG. 3. Operational results of formation of VFA-degrading granules by defined species. (A) VFA concentrations in the influent. (B) VFA concentrations in the effluent. (C) VFA degradation (DEGAD.) rates. C2, acetate; C3, propionate; C4, butyrate. (D) Volumetric COD loading rates and volumetric COD removal rates achieved.

be considered the respective kinetic parameters of propionate-degrading strain PT and butyrate-degrading strain BH.

DISCUSSION

Granule formation in anaerobic wastewater treatment systems is a complicated process involving ecophysiology of bacterial cells, physicochemical reactions among cells, existing inert materials, and environmental or operational parameters (e.g., pH, nutrients, turbulence by hydraulic force, and biogas production). However, knowledge about the granulation mechanism is very limited. The development of granules by defined microbial species thus expands our understanding of the granulation process and the possibility of developing different granules for different wastes.

Contamination is a common problem in continuous operation of the reactors. However, during this study, no contaminant morphologies were detected during the microscopic examination of the samples from the influent media, the reactor, and the effluent. This may have happened because of the precautionary steps that were employed in anticipation of the system becoming contaminated. In addition, maintenance of strict anaerobic conditions and the presence of only VFAs as substrates might have made it difficult for contaminants to grow. Simple microscopic examination is admittedly not a rigorous technique and suffers from the absence of a scientific validation unless supported by other techniques, and yet it still is the most commonly used technique in every laboratory. Since only five microbial species of distinctive morphology (three methanogens and two fatty acid degraders) were employed, it was easy to distinguish these from the contaminant. The microscopic observations were validated by electron micrographs in that the TEM results with these granules showed only those five microbial species that were inoculated in the reactor. Even if the contaminant escaped detection during microscopic examination, then its population was so low that it would not have played any role in the granulation process. Furthermore, the absence of any shift in metabolic intermediates and end products confirmed that the reactor was operated as a contamination-free system.

Methanobacterium formicicum has been observed in methanogenic granules developed on various wastewaters under mesophilic temperatures (7, 12). In this study, the genus *Methanobacterium* was identified as one of the essential organisms in formation of the syntrophic VFA-degrading granules, because *Methanobacterium formicicum* formed aggregates together with butyrate-degrading strain BH, it formed homogeneous aggregates with propionate-degrading strain PT and *Methanosaeta* sp. strain M7 in the triculture, and it grew as the major species in the granular matrix together with all other species. Particularly, this species had a special affinity for *Methanosarcina mazei* as was seen by its attachment on the surface of the aggregates of *Methanosarcina mazei* (Fig. 4). Thus, *Methanobacterium formicicum* played an important role in bridging the gap between the aggregates of *Methanosarcina* sp. and the aggregates formed by other species. This revealed that *Methanobacterium formicicum* was not only a predominant methanogen in the granular structure observed in this study, but it also served as a key link between *Methanosarcina* sp. and other microbial species within the granular structure.

The essential role of *Methanosaeta* sp. (*Methanothrix* sp.) in granular structure has been reported by several investigators (3, 5) and is further recognized in this study. In the granules formed by the defined species, *Methanosaeta* sp. strain M7 formed a network together with *Methanobacterium formicicum* to build a granular structure. Particularly, it has been demonstrated that this strain possessed novel binding properties which allowed it to make aggregates with *Methanobacterium formicicum* and strain PT; the latter two species, otherwise, could not form syntrophic methanogenic aggregates. This char-

TABLE 1. Operational conditions and reactor performance of the reactor inoculated with defined syntrophic methanogenic culture

Operation date (day)[a]	Feed VFA (mM)[b]			Feed rate (ml/h)	Hydraulic retention time (h)	pH	COD		% CH_4	Amt of H_2 (Pa)
	C_2	C_3	n-C_4				Load (g/liter/day)[c]	Removal (%)		
1 to 31	5	10	10	5.3–8.0	42–28	6.2–7.0	2.0–3.0	57–59	70–75	5–9
32 to 47	5	10	13.3	7.5–11.3	29–19	7.0–7.2	3.2–5.0	64–80	74–75	3–5
48 to 57	5	15	16.6	11.3–16.8	19–13	7.0–7.1	6.5–9.7	78–80	74–75	3–4
58 to 82	0	30	30	13.2–29.0	17–7.6	7.3–7.4	13–29	61–83	79–80	3–4

[a] The date sequence begins on the first day when all starter cultures were added to the reactor.
[b] C_2, acetate; C_3, propionate; n-C_4, butyrate.
[c] COD load was calculated on the basis of the following stoichiometries of complete oxidation of substrates to CO_2 (grams of COD per mole): acetate, 64; propionate, 112; and butyrate, 160.

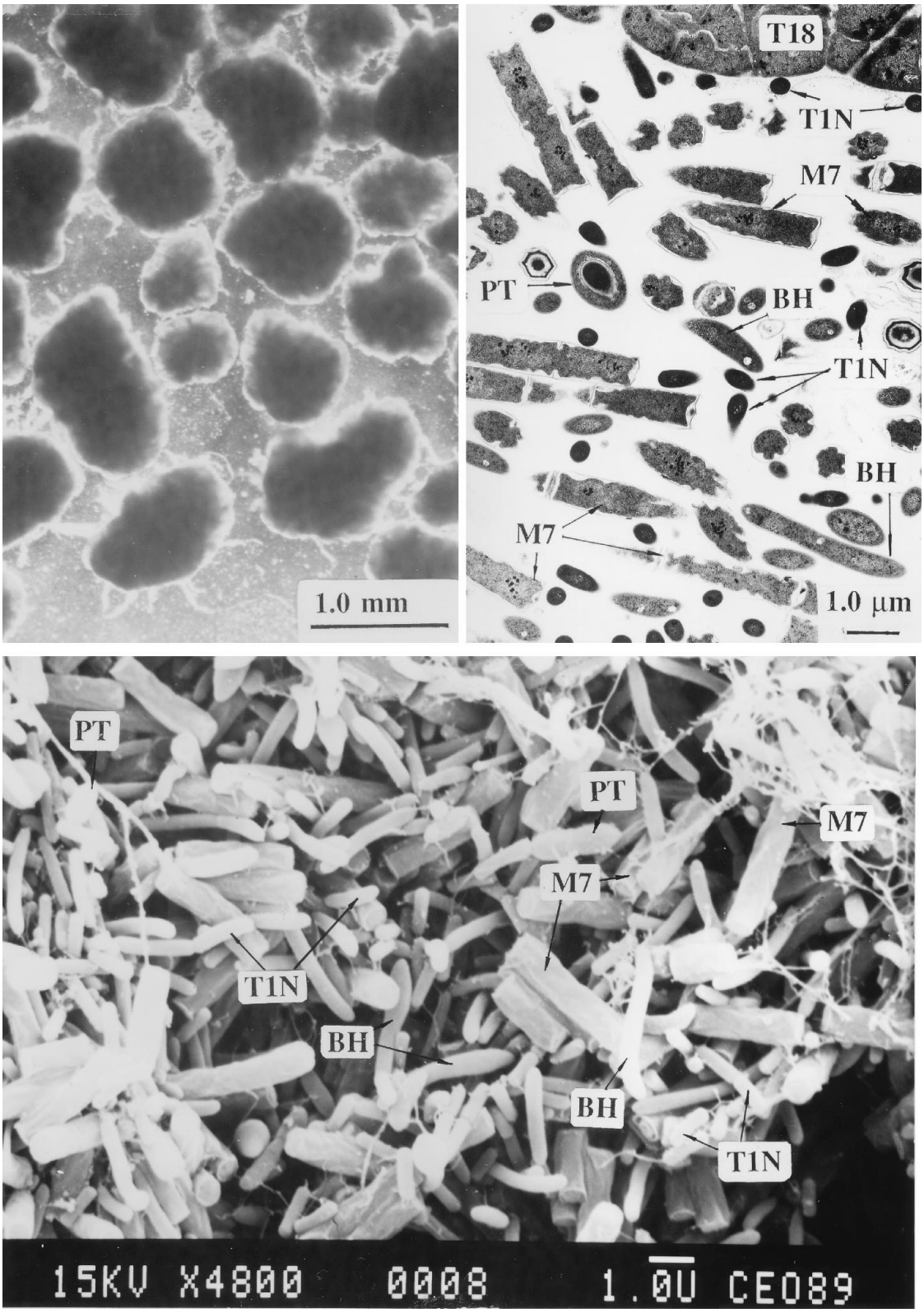

FIG. 4. Syntrophic VFA-degrading granules formed by defined species. (Left) Overview of the granules. (Bottom) The surface of a granule under SEM. BH, butyrate-degrading strain BH; M7, *Methanosaeta* sp. strain M7; PT, propionate-degrading strain PT; T1N, *Methanobacterium formicicum* T1N. (Right) Homogeneous growth of *Methanobacterium formicicum* T1N, *Methanosaeta* sp. strain M7, strain BH, and strain PT formed the bulk of the granules on a TEM thin section. Individual aggregates of *Methanosarcina mazei* T18 were associated with the granule bulk through *Methanobacterium formicicum* T1N cells.

TABLE 2. Physicochemical, metabolic, and microbial characteristics of the granules developed by defined species

Characteristic	Value
Physicochemical	
Density (g of suspended solids/liter)	58
Volatile suspended solids/suspended solids	0.88
Sp grav	1.023
Specific fatty acid conversion rate (mmol/g of suspended solids/day)[a]	
Acetate	43.1
Propionate	8.96
Butyrate	17.1
Apparent K_m (mM)[b]	
Propionate	0.038
Butyrate	0.189
Specific COD removal rate (g of COD/g of suspended solids/day)	3.22

[a] Determined in a reactor fed with VFA mixture continuously at 35°C. Acetate, propionate, and butyrate concentrations in the reactor were 5, 6, and 1 mM, respectively. The feed contained 0.3 mM sodium sulfide as a reductant.

[b] K_m was determined from an integrated solution to the Michaelis-Menten equation.

acter is of substantial importance in the formation of syntrophic VFA-degrading granules.

The *Methanosarcina mazei* strain had a strong aggregation character similar to that of other *Methanosarcina* strains. However, the role of the *Methanosarcina* strain in granulation was questioned (5, 8) except when the granules developed in a high-acetate-concentration environment, which mainly consisted of *Methanosarcina* cells. This study showed that *Methanosarcina mazei* contributed significantly to the granulation of the defined microbial species. TEM observation revealed that cells of *Methanosarcina mazei* T18 were present as individual aggregates in granules and were bound to the bulk matrix of granules through *Methanobacterium formicicum* cells. Addition of *Methanosarcina mazei* appeared to enhance the granulation process. It was possible that aggregates of *Methanosarcina* cells provided growth nuclei through attachment of *Methanobacterium formicicum* to other bacteria. Whether this role was essential for granule formation and whether this role could be adequately filled by inert materials could not be determined from this experiment.

Methanospirillum hungatei did not grow together with butyrate-degrading strain BH in syntrophic methanogenic aggregates, although the latter has strong aggregation character. *Methanospirillum hungatei* cells were observed occasionally inside the granules grown on brewery wastewater (15). This indicated that *Methanospirillum hungatei* was not an essential species for the structure of the granule. Therefore, we did not use it as starter culture for development of granules. The role of spore-forming, propionate-degrading strain PT did not seem to be important in the granulation process, but it was essential for propionate metabolism.

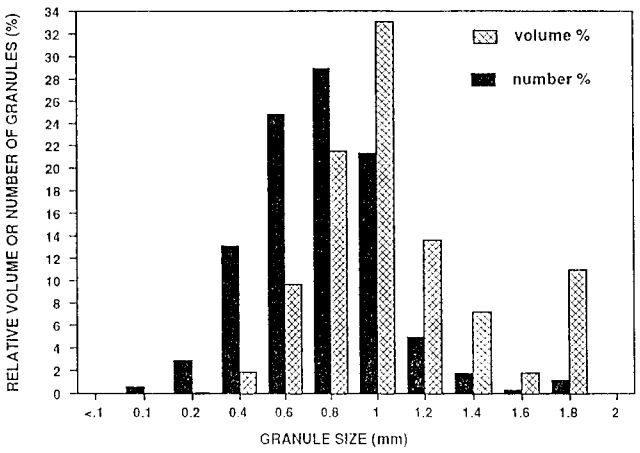

FIG. 5. Size distribution of the granules formed by defined species.

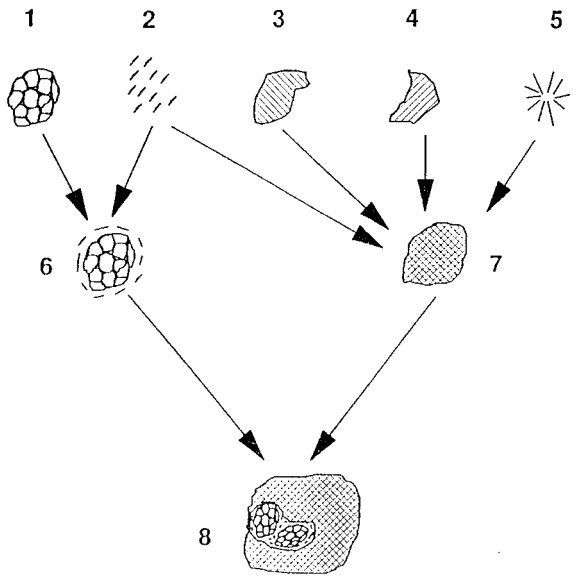

FIG. 6. Proposed route for the formation of VFA-degrading granules in this study. Steps: 1, *Methanosarcina mazei*; 2, *Methanobacterium formicicum* T1N; 3, coculture of strains T1N and BH; 4, triculture of strains T1N, M7, and PT; 5, strain M7; 6, microbial aggregate of strains T1N and T18; 7, microbial aggregate of strains T1N, BH, PT, and M7; and 8, VFA-degrading granules.

Formation of the syntrophic VFA-degrading granules in this study was the result of comprehensive physiological contributions by all species used as an inoculum. Aggregate formation is considered to be the essential step in granule formation. Aggregate formation by cells of one species or multiple species is related to the characteristics of the cell surface, such as extracellular polymer formation and hydrophobicity (4, 12). Extracellular polymer formation by the key species which induce aggregation is very important. We have observed that *Methanobacterium formicicum* T1N, *Methanosaeta* sp. strain M7, *Methanosarcina mazei* T18, and strain BH produce extracellular polymers, and thus, these organisms may play a key role in the granule formation process (11a). *Methanospirillum hungatei* BD did not produce a polymer, and in this case, it seems of less importance in granule formation.

In accordance with observations about aggregate formation and the structure analysis of the syntrophic VFA-degrading granules, a hypothesis for the granule formation with these defined species is proposed as described in Fig. 6. The following five microbial components used for granulation were (i) *Methanosarcina mazei* T18, (ii) *Methanobacterium formicicum* T1N, (iii) coculture of *Methanobacterium formicicum* and strain BH, (iv) triculture of *Methanosaeta* sp. strain M7, *Methanobacterium formicicum* T1N and strain PT, and (v) aggregates and dispersed cells of *Methanosaeta* sp. strain M7. During continuous operation, the cells of *Methanobacterium formicicum* T1N initially adhered on the surface of *Methanosarcina mazei* T18 aggregates (component 6), while components 2, 3, 4,

and 5 formed syntrophic propionate-butyrate-degrading aggregates (component 7). Finally, several pieces of components 6 and 7 combined to form a granule (component 8) through the binding properties of strain T1N.

This study demonstrates that the syntrophic fatty acid-degrading granules can be produced with a limited number of key microbial species which possess essential aggregate-forming ability, and their association can completely mineralize the major intermediates of anaerobic digestion, i.e., acetate, propionate, and butyrate. Careful selection of the key species is very critical for granulation, as are the inoculation sequence and the operation of the reactor. Granule formation has proved that *Methanosaeta* sp. and *Methanobacterium formicicum* play a key role in granulation and has revealed the importance of *Methanosarcina* sp. in the structure of the granules. These data provide evidence that suggests that methanogens are the key microbial group involved in the granulation process. An aggregate-forming, syntrophic, butyrate-degrading strain, BH, also contributed to granule formation. The size analysis indicated that these syntrophic granules were not limited in substrate diffusion, because their average diameter was 0.8 mm and major parts of granules were not larger than 1 mm. Therefore, the granules formed by the defined species are highly active.

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