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Composition and Role of Extracellular Polymers in Methanogenic Granules

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***Methanobacterium formicicum* and *Methanosarcina mazeii* are two prevalent species isolated from an anaerobic granular consortium grown on a fatty acid mixture. The extracellular polysaccharides (EPS) were extracted from *Methanobacterium formicicum* and *Methanosarcina mazeii* and from the methanogenic granules to examine their role in granular development. The EPS made up approximately 20 to 14% of the extracellular polymer extracted from the granules, *Methanobacterium formicicum*, and *Methanosarcina mazeii*. The EPS produced by *Methanobacterium formicicum* was composed mainly of rhamnose, mannose, galactose, glucose, and amino sugars, while that produced by *Methanosarcina mazeii* contained ribose, galactose, glucose, and glucosamine. The same sugars were also present in the EPS produced by the granules. These results indicate that the two methanogens, especially *Methanobacterium formicicum*, contributed significantly to the production of the extracellular polymer of the anaerobic granules. Growth temperature, substrates (formate and H_2-CO_2), and the key nutrients (nitrogen and phosphate concentrations) affected polymer production by *Methanobacterium formicicum*.**

The performance of an upflow anaerobic sludge blanket reactor depends on the formation of granules of high settleability (13). The physicochemical factors that influence granulation have been studied extensively (12). Methanogens were suggested to be key species in the formation of the granules on volatile fatty acids (VFAs) as major substrates: (i) *Methanosaeta* (*Methanothrix*) species play an essential role in granulation (7, 12); (ii) *Methanobrevibacter arboriphilicus* produces extracellular polypeptides to induce granulation (18); and (iii) *Methanosarcina* species produce initial aggregates as nuclei to form granules (4a). We recently confirmed the role of methanogens in formation of granules by using defined microbial cultures consisting of *Methanosaeta* sp., *Methanobacterium formicicum*, *Methanosarcina mazeii*, and two syntrophic fatty acid degraders (27). However, the role of individual microbial species in the development of polymeric structure in the granules is not well understood.

Bacterial extracellular polymer (ECP) contributed to the adhesion between different species of methanogens and syntrophic acetogenic bacteria present in the granules, improving their long-term stability (2, 8, 17). The ECP has been observed in different types of granules by scanning electron microscopy and transmission electron microscopy (TEM) (5, 9, 10, 17). The ECP has been shown to be composed of extracellular polysaccharides (EPS) (5, 7) and polypeptides (18). In this study, we focused on the composition of ECP from anaerobic methanogenic granules and the EPS from two prevalent methanogens isolated from the granules (25). We show that the ECP of *Methanobacterium formicicum* and *Methanosarcina mazeii* is similar in composition to the ECP from the granules, thus probably playing an important role in the development of granules.

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MATERIALS AND METHODS

Methanogenic granules and methanogen cultures. The methanogenic granules used in this study were obtained from a laboratory-scale upflow reactor fed with a fatty acid mixture consisting of acetate, propionate, and butyrate. These granules consisted mainly of methanogenic and syntrophic acetogenic bacteria (22, 25). *Methanobacterium formicicum* T1N (DSM 6298) and *Methanosarcina mazeii* T18 (DSM 6300) were isolated from these granules (25).

Media and growth conditions. *Methanobacterium formicicum* T1N and *Methanosarcina mazeii* T18 were grown in a basal medium (15) buffered with potassium phosphate (20 mM) plus 0.01% (vol/vol) vitamin solution (23) under anaerobic conditions at 37°C unless otherwise stated. For the growth of *Methanobacterium formicicum*, the medium was supplemented with 1 mM sodium sulfide and 4 mM sodium acetate. Either formate (40 mM) or a gas mixture of H_2 plus CO_2 (80:20, 1.5 atm in the headspace) was used as carbon and energy source. *Methanosarcina mazeii* was grown in the basal medium supplemented with 30 mM sodium acetate and 12 mM sodium bicarbonate. The vials (excluding those using H_2-CO_2 as substrate) were pressurized to 0.3 atm with a mixture of N_2 and CO_2 (95:5). The pH of the medium was 7.0.

TEM. The cells collected on a Millipore filter were placed in a fixative consisting of 3% glutaraldehyde plus 0.5% Alcian blue in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed six times for 1 h in 0.2 M cacodylate buffer (pH 7.2). The rinsed samples were postfixed with 1% OsO_4 plus 1% $LaNO_3$ in 0.2 M *s*-collidine (pH 7.2) at room temperature and dehydrated through a graded series of ethanol solutions followed by propylene oxide. The samples were embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, Pa.). Thin sections were cut with a diamond knife mounted in an LKB ultratome and then stained with uranyl acetate and lead citrate as described by Shea (19). TEM examination was performed on a Philips CM-10 electron microscope at an acceleration voltage of 80 kV.

Extraction of extracellular polymers. The cells were harvested from stationary-phase cultures by centrifugation (10,000 × *g* at 4°C) for 20 min. The polymer was not removed by shaking the cells in phosphate saline solution (11). Therefore, phenol (20% vol/vol) was used to extract the ECP from the cells, while leaving the cells intact. The cells were extracted with 150 ml of 20% (vol/vol) phenol in double-distilled water at 50°C with shaking and intermittent sonication for 45 min and then centrifuged at 4°C (12,000 × *g* for 20 min). The aqueous layer was removed and saved, the same volume of water was added to the phenol layer, and the sample was again extracted at 50°C for 45 min. The aqueous layer was removed and combined with that from the first extraction. The supernatant was placed in dialysis bags (molecular weight cutoff, 12,000 to 14,000) and was extensively dialyzed against water for 2 days. The supernatant was lyophilized to dryness and designated crude ECP.

Polysaccharide isolation and purification. The polymer was purified by gel filtration on a Bio-Gel P100 column (Bio-Rad Laboratories, Richmond, Calif.) and eluted with 0.1% formic acid at room temperature. Carbohydrates in the

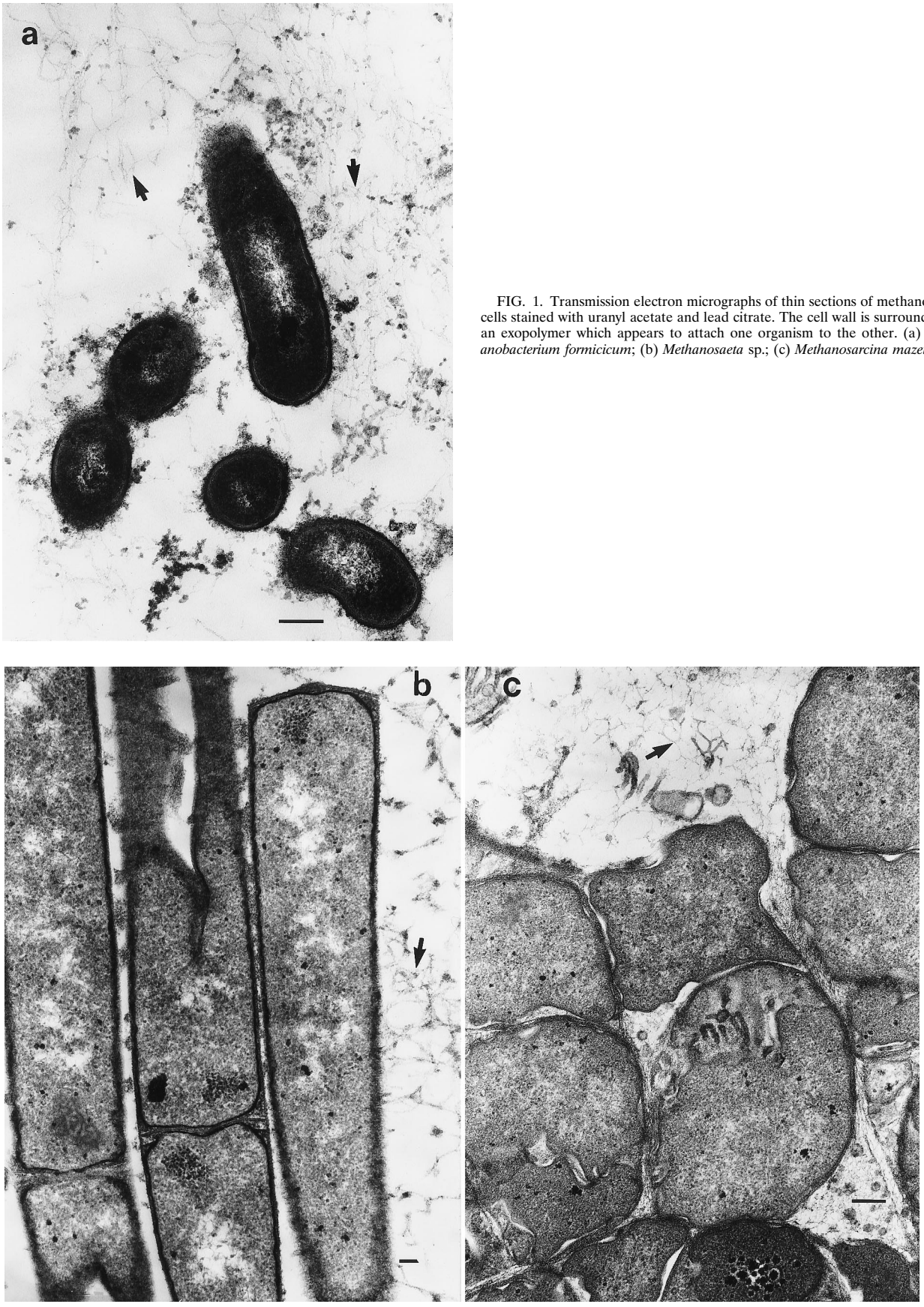

FIG. 1. Transmission electron micrographs of thin sections of methanogenic cells stained with uranyl acetate and lead citrate. The cell wall is surrounded by an exopolymer which appears to attach one organism to the other. (a) *Methanobacterium formicicum*; (b) *Methanosaeta* sp.; (c) *Methanosarcina mazeii*.

TABLE 1. ECP and EPS in methanogenic granules and two methanogenic species

Organism	Growth substrate	Amt (mg/g of VSS) of[a]:		% of EPS in ECP
		Total ECP	Total EPS	
Granules	VFA[b]	90.5	18.0	19.9
M. formicicum	Formate	85	21.8	25.6
	H_2-CO_2	77	16.2	21.0
M. mazeii	Acetate	58.5	8.0	13.7

[a] The data are mean values of duplicate determinations. VSS: volatile suspended solids, which was used as an indicator of the volatile fraction of biomass.
[b] The VFA mixture contained acetate, propionate, and butyrate (2:1:1, mo/mol).

collected fractions were determined by the phenol-sulfuric acid assay (6). One eluting peak, containing carbohydrates, was obtained, pooled, and lyophilized.

Determination of the composition of isolated polysaccharide. The neutral-sugar composition of the purified EPS was determined by gas-liquid chromatography of alditol acetates, which were prepared as described by Albersheim et al. (1). They were prepared by hydrolysis of the oligomer derivatives with trifluoroacetic acid followed by reduction with sodium borohydride and then paracetylation with acetic anhydride-pyridine. The mixture was analyzed by gas-liquid chromatography–mass spectrometry.

Gas-liquid chromatography–mass spectrometry. Electron impact and chemical ionization mass spectra of alditol acetates were obtained with a JEOL AX505H mass spectrometer in the electron impact mode at 70 eV. The column temperature on injection was 206°C. This temperature was maintained for 5 min after injection and then raised by 2°C per min to 230°C.

Analytical methods. Sugars were measured by the phenol-sulfuric acid method as described by Dubois et al. (6) with glucose as the standard. Protein was determined by the method of Lowry et al. (16) with bovine serum albumin as the standard. Uronic acids were determined by the *m*-phenylphenol method (3) with galacturonic acid as the standard. The viscosity was determined in a capillary viscometer at 24°C (4). The elemental analysis was performed with a Perkin Elmer 2400 elemental analyzer.

Chemicals and gases. All the chemicals and gases used were of analytical grade and were obtained from Sigma Chemical Co., St. Louis, Mo., or from Mallinckrodt, St. Louis, Mo. Gases and gas mixtures were supplied by Union Carbide Corp., Linde Division, Warren, Mich., and were passed through heated (370°C) copper filings to remove traces of O_2.

RESULTS AND DISCUSSION

Observation of ECP by TEM. Significant amounts of extracellular polymers were observed around the cells of methanogens (Fig. 1) by TEM. In some cases, the polymers extended from the outer surface of one cell to adhere to another cell. More polymers were observed in the micrographs of *Methanobacterium formicicum* and *Methanosarcina mazeii* than in that of the *Methanosaeta* sp.

The ECP were observed in anaerobic granular sludges that apparently formed a strong matrix for the embedding of cells in granules (5, 7, 10). Our study has confirmed the production of ECP by granules and demonstrated that the two prevalent methanogens isolated from the granules produced the ECP. Based on our TEM examination, methanogens and other prokaryotes in the granules were surrounded by the ECP and attached to each other by fibrous polymers.

Chemical composition of the ECP. The amounts of total ECP and EPS extracted from the cells of *Methanobacterium formicicum*, *Methanosarcina mazeii*, and the methanogenic granules are presented in Table 1. More ECP and EPS was associated with the cells of *Methanobacterium formicicum* than with the cells of *Methanosarcina mazeii*. The granules contained similar amounts of ECP and EPS to those in the cells of *Methanobacterium formicicum* grown on formate. However, the production of ECP and EPS was lower when *Methanobacterium formicicum* was grown on H_2-CO_2 and *Methanosarcina mazeii* was grown on acetate. The EPS produced by these two methanogens had a molecular weight greater than 100,000. The polysaccharide content in the ECP of *Methanobacterium formicicum* was similar (ca. 20%) to that in anaerobic granules and was 13.7% in *Methanosarcina mazeii*.

TABLE 2. Percentages of sugars and amino sugars in EPS extracted from methanogenic granules, *Methanobacterium formicicum* T1N, and *Methanosarcina mazeii* T18

Sugar or amino sugar	% of sugar in organisms on growth substrate			
	Granules (VFAs)	*M. formicicum*		*M. mazeii* (acetate)
		Formate	H_2-CO_2	
Rhamnose	15.0	29.0	25.8	0.6
Fucose	10.7	0	0	0
Ribose	tr[a]	0	0	0.6
Mannose	12.9	29.0	25.0	tr
Galactose	16.1	15.6	17.7	32
Glucose	19.3	9.1	12.9	10.4
Glucosamine	8.6	tr	tr	56.5
Galactosamine + mannosamine	6.4	17.3	18.5	tr

[a] tr, trace amount.

The viscosity of the EPS at concentrations of 0.1% (wt/vol) polymer was 1 and 2 centistoke for *Methanobacterium formicicum* and *Methanosarcina mazeii*, respectively. Elemental analysis indicated that the EPS of *Methanobacterium formicicum* contained 32.1% C, 4.1% H, and 5.3% N.

The major sugar components in EPS extracted from the granules were rhamnose, fucose, mannose, galactose, glucose, glucosamine, galactosamine, and mannosamine (Table 2). A trace amount of ribose was also detected in the EPS. Rhamnose and mannose made up more than 50% of the sugars produced by *Methanobacterium formicicum*. The remaining sugars were galactose, glucose, mannosamine, and galactosamine. The EPS of *Methanosarcina mazeii* did not contain mannose but contained glucosamine, galactose, and glucose as major sugar components with minor amounts of rhamnose and ribose. Uronic acids and fucose were not observed in the EPS of these methanogens, but they were detected in the EPS of the granules. Unlike the strain used in this study, another strain of *Methanosarcina mazeii* has been reported to produce a different type of matrix material that was rich not only in *N*-acetylglucosamine but also in uronic acids (16a).

In this study, the amount of EPS extracted from the methanogenic granules, *Methanobacterium formicicum*, and *Methanosarcina mazeii* ranged from 1.6 to 2.2% of the volatile fraction of the cells. These values are similar to the EPS values obtained from other anaerobic granular sludges (5, 9). The major sugar components in the EPS from the granules were similar to those obtained by Dolfing et al. (5) and Harada et al. (10). The EPS from the methanogenic granules contained all the sugars found in the polymers of *Methanobacterium formicicum* and *Methanosarcina mazeii*. This indicates that the ECP of these two methanogens contributed to the polymer matrix of the granules. Of the two methanogenic species examined, *Methanobacterium formicicum* seems to play a greater role, because it contributes more sugars and amino sugars to the EPS of the granules than *Methanosarcina mazeii* does. These results also validate our earlier results on the development of granules by defined cultures in which *Methanobacterium formicicum* acted as a precursor and a backbone for other species to embed (27). On the other hand, fucose was found in the EPS of the granules but was not present in the EPS of the two methanogens. It is possible that fucose was a component of

TABLE 3. Influence of temperature on polymeric composition of *Methanobacterium formicicum* T1N

Temp (°C)	Amt (mg/g of VSS) of[a]:		% of EPS in ECP
	ECP	EPS	
30	77.8	17.4	22.4
37	77.0	16.2	21.0
42	49.8	10.9	21.9

[a] The data are the mean values of duplicate determinations. Cultures were grown in the same medium.

TABLE 4. Effect of nutrients on EPS production by *Methanobacterium formicicum* T1N

Medium conditions	Amt (mg/g of VSS) of[a]:		% of EPS relative to control
	ECP	EPS	
Control (regular medium)	77.0	16.2	100
Reduced nitrogen	110.4	27.3	168.5
Reduced phosphorus	113.0	28.2	174.1
Excess magnesium	80.0	20.5	126.5

[a] The data are the mean values of duplicate determinations.

EPS produced by other major organisms in the granules. For example, *Methanosaeta* sp. was observed as a major organism in the granules and also produced ECP (25). The role of individual sugars and amino sugars, including fucose, in the EPS of granules needs to be examined by using cultures that are deficient in specific sugars and amino sugars.

Since EPS constituted only about 20% of the ECP, the remaining fraction of ECP probably contained significant amounts of extracellular proteins and other compounds, as reported by other investigators (8, 9, 14, 17, 18). However, we did not analyze the protein content in the ECP.

Effect of substrates on the ECP produced by *Methanobacterium formicicum*. *Methanobacterium formicicum* utilizes formate or H_2-CO_2 as its energy source. The cells grown on formate produced more ECP with a higher polysaccharide content than did the cells grown on H_2-CO_2 (Table 1). The addition of yeast extract to the medium containing formate did not increase the amount of ECP in the volatile fraction (data not shown). The sugar compositions of EPS extracted from the cells grown on formate and H_2-CO_2 are almost identical. The major sugars were rhamnose and mannose (Table 2).

The results suggested that *Methanobacterium formicicum* in the granules may produce more ECP when grown on formate-containing media. During anaerobic digestion, hydrogen and formate can be produced as intermediates during fermentation and syntrophic degradation of fatty acids and ethanol (21, 24). It was difficult to determine the ratio of formate to hydrogen produced as intermediate. On the other hand, *Methanobacterium formicicum* can perform a reversible reaction, i.e., production of hydrogen from formate and synthesis of formate from hydrogen plus bicarbonate. Higher levels of formate were observed in the medium containing higher bicarbonate levels (50 to 80 mM) when H_2-CO_2 was utilized as the substrate (26). Therefore, a high-bicarbonate buffered environment, as is present in many anaerobic reactors, may have been beneficial for the formation of ECP and granulation.

Effect of temperature on the ECP produced by *Methanobacterium formicicum*. *Methanobacterium formicicum* T1N grows optimally at 40°C and does not grow above 45°C (25). The cells grown on H_2-CO_2 at 30, 37, and 42°C were harvested for polymer extraction. At 42°C, *Methanobacterium formicicum* T1N grew mainly as a cell suspension, and few clumps were observed. At 30 and 37°C, this organisms grew as clumps. This suggested that fewer polymers were produced at 42°C, resulting in poor aggregation at higher temperatures than at lower temperatures. This hypothesis was confirmed by the results of the ECP analysis (Table 3). At higher growth temperatures, fewer polymers were extracted from the cells. However, the sugar composition of EPS remained essentially the same, irrespective of the temperature (data not shown).

It has been reported that some bacteria produce more ECP when they are grown at temperatures below their optimal growth temperature. In this study, *Methanobacterium formicicum* produced very similar amounts of ECP when grown at 30 and 37°C, which are below the optimal conditions (40°C). In mesophilic wastewater treatment processes, the reactors are operated at slightly lower temperatures (ranging from 28 to 35°C), which are beneficial for ECP production by *Methanobacterium formicicum*. At 42°C, the amounts of polymers formed by *Methanobacterium formicicum* were significantly reduced. A decrease in ECP production was observed to be related to poor clump formation. These results suggest that a proper operational temperature below that for optimal growth should be used for enhancement of ECP production and growth of granular sludge in an anaerobic reactor.

Effect of nutrient supply on the ECP produced by *Methanobacterium formicicum*. The basal medium used for the growth of *Methanobacterium formicicum* contained (per liter) 1 g of NH_4Cl, 20 mM PO_4-P, and 0.2 g of $MgCl_2 \cdot 6H_2O$. Three modified media were used to examine the effect of nutrients on extracellular polymer production by *Methanobacterium formicicum*: medium with reduced nitrogen (0.2 g of NH_4Cl per liter), medium with reduced phosphorus (5 mM PO_4-P), and medium with excess magnesium (1 g of $MgCl_2 \cdot 6H_2O$ per liter). The amounts of ECP and EPS extracted from the cells grown on these media are presented in Table 4. The reduction in the concentrations of nitrogen and phosphate resulted in the production of significantly increased amounts of ECP and EPS from the cells. In the presence of excess magnesium, the cells produced about 25% more EPS without any change in ECP production.

The polymer production by *Methanobacterium formicicum* was enhanced under reduced nutrient (N and P) conditions. Similar observations were made for other bacterial cultures (20), showing that the carbon utilization shifts toward EPS production when the C/N and/or C/P ratio is greater. The EPS production was slightly higher under low-phosphate conditions than under low-nitrogen conditions. This suggested that operation of a reactor on a low-nitrogen- and low-phosphate-containing medium may be beneficial for bacteria to produce more ECP, which may help in the formation and maintenance of a granular structure.

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