APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1988, p. 2064–2068
0099-2240/88/082064-05$02.00/0

Vol. 54, No. 8

Control of the Life Cycle of *Methanosarcina mazei* S-6 by Manipulation of Growth Conditions

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Received 7 March 1988/Accepted 18 May 1988

The morphology of *Methanosarcina mazei* was controlled by magnesium, calcium, and substrate concentrations and by inoculum size; these factors allowed manipulation of the morphology and interconversions between pseudosarcinal aggregates and individual, coccoid cells. *M. mazei* grew as aggregates in medium with a low concentration of catabolic substrate (either 50 mM acetate, 50 mM methanol, or 10 mM trimethylamine) unless Ca^{2+} and Mg^{2+} concentrations were high. Growth in medium high in Ca^{2+}, Mg^{2+}, and substrate (i.e., 150 mM acetate, 150 mM methanol, or 40 mM trimethylamine) converted pseudosarcinal aggregates to individual cocci. In such media, aggregates separated into individual cells which continued to grow exclusively as single cells during subsequent transfers. Conversion of single cells back to aggregates was complicated, because conditions which supported the aggregated morphology (e.g., low calcium or magnesium concentration) caused lysis of coccoid inocula. We recovered aggregates from coccoid cells by inoculating serial dilutions into medium high in calcium and magnesium. Cells from very dilute inocula grew into aggregates which disaggregated on continued incubation. However, timely transfer of the aggregates to medium low in calcium, magnesium, and catabolic substrates allowed continued growth as aggregates. We demonstrated the activity of the enzyme (disaggregatase) which caused the dispersion of aggregates into individual cells; disaggregatase was produced not only during disaggregation but also in growing cultures of single cells. Uronic acids, the monomeric constituents of the *Methanosarcina* matrix, were also produced during disaggregation and during growth as coccoids.

Methanosarcina mazei is an aceticlastic methanogen important in methanogenesis during anaerobic degradation of organic material. *M. mazei* normally grows as large aggregates of cells, which may be several millimeters in diameter (8). The large size of these aggregates may be an important factor allowing *M. mazei* to settle and remain in O_2-free sediments or anaerobic digestors. Furthermore, the large size of the aggregates may protect interior cells from exposure to O_2 and other environmental extremes. In certain applications, it may be desirable to induce these methanogens to grow as individual cells (e.g., to mobilize them from a reactor for use as inocula). In the laboratory, growth as coccoid units may allow cell contents to be easily obtained or permit genetic studies for elucidation of biochemistry (5, 7, 11). For these reasons, the life cycle of *M. mazei* attracts the attention of both applied and basic researchers.

Mah (8) isolated *M. mazei* in an axenic culture and described its life cycle. *M. mazei* S-6 forms small aggregates of pseudosarcina in young cultures which grow and form cysts in older cultures. The cysts have an outer, cellular wall which encloses a central area containing free, coccoid units. When the cysts are physically disrupted, many coccoid cells are released. Spontaneous disaggregation of *M. mazei* LYC is due to one or more enzymes (called disaggregatase) excreted by the cells (7). Electron microscopy reveals that *M. mazei* S-6 cells are bound together in pseudosarcinal colonies by a fibrous matrix up to 60 nm thick and that this matrix material dissolves during disaggregation (10).

M. mazei S-6 grows as single cells when media contain high concentrations of catabolic substrate (40 mM trimethylamine, 124 mM monomethylamine, 100 mM acetate, or 100 mM methanol), Ca^{2+} (2.7 mM), and Mg^{2+} (4.9 mM) (2, 5). This report describes how culture conditions can be used to manipulate the morphology of *M. mazei*, allowing growth as cocci or pseudosarcinal aggregates.

(Portions of these data were presented previously at the International Conference on Water and Wastewater Microbiology, Newport Beach, Calif., 1988.)

MATERIALS AND METHODS

Cultures and growth conditions. *M. mazei* S-6, *M. mazei* LYC, *M. barkeri* 227, *M. barkeri* MS, *M. barkeri* UBS, and *M. thermophila* TM1 were from our collection. Experiments with *M. mazei* were always done with strain S-6 unless otherwise indicated. Alpha medium was the same as the alpha medium previously described (3) but with the following changes: the Ca^{2+} concentration was reduced to 0.27 mM (by reducing $CaCl_2 \cdot 2H_2O$ from 100 to 40 mg/liter of medium), and 2 g of yeast extract, 2 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), and 0.5 g of L-cysteine hydrochloride were added per liter of medium. The Mg^{2+} concentration of alpha medium is 0.49 mM. *Methanosarcina* (MS) medium had 10-fold-higher concentrations of divalent cations (2.7 mM Ca^{2+} and 4.9 mM Mg^{2+}). It was the same as alpha medium, except that the $MgCl_2 \cdot 6H_2O$ concentration was increased from 0.1 to 1.0 g/liter and the $CaCl_2 \cdot 2H_2O$ concentration was increased from 0.04 to 0.4 g/liter. Media with other concentrations of Ca^{2+} and Mg^{2+} were prepared by additions from sterile, O_2-free solutions. MS minimal medium was the same as MS medium but without yeast extract and Trypticase peptone (the only organic additions other than substrate were 0.5 g of cysteine

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and 0.4 mg of EDTA per liter of medium). Cultures were grown in 5 ml of medium in serum tubes (27-ml volume) or 20 ml of medium in serum bottles (67-ml volume). These vessels were closed with butyl rubber stoppers. The pH of each medium was 7.0 after autoclaving; each had a gas atmosphere of N_2 and CO_2 (7:3). Cultures were incubated at 37°C without shaking. The inoculum volume was 0.1 ml for 5-ml cultures or 0.2 ml for 20-ml cultures. Unless otherwise indicated, inocula of coccoid cells were prepared by growth to late exponential phase in MS medium with 40 mM trimethylamine, and inocula of aggregates were prepared by growth to late exponential phase in alpha medium with 50 mM acetate.

Measurement of disaggregatase activity. Disaggregatase was assayed by measuring its ability to produce turbidity from large, heat-treated aggregates of *M. mazei* (7). The aggregates used as the substrate in this assay were grown in 20 ml of alpha medium with 50 mM acetate as the substrate and inactivated by submersing the culture vessel in boiling water for 10 min. We used a 1-ml syringe with a 21-gauge needle to transfer several inactivated aggregates to 5 ml of sterile MS medium while avoiding exposure of the aggregates to O_2. The sample containing disaggregatase was filter sterilized in the absence of O_2, and 0.5 ml of filtrate was added to the tube containing the aggregates. Turbidity increase was measured as an indication of disaggregatase activity (7).

Analytical methods. Optical density (540 nm) was determined with a spectrophotometer (Perkin-Elmer Junior model 35; The Perkin-Elmer Corp., Norwalk, Conn.). Methane was analyzed by gas chromatography and thermal conductivity detection (1). Specific growth rate was calculated from methane production by including in calculations the methane produced during growth of the inoculum (9). Specific growth rate was determined in triplicate vessels; the standard deviation among replicates was always less than 0.002/h. Experiments were always repeated and gave similar results. Production of uronic acids was determined after centrifugation of cultures (2,000 × *g* for 20 min). The culture supernatant fluids were analyzed by the carbazole method (4).

RESULTS

Production of single cells of *M. mazei* from aggregates. *M. mazei* grew as aggregates in alpha medium with 50 mM acetate or 50 mM methanol. When 0.2 ml of such acetate-grown aggregates was inoculated into 20 ml of alpha or MS medium with 40 mM trimethylamine, the aggregates separated into individual cells late during the exponential phase of growth. This disaggregation, occurring when about two-thirds of the stoichiometrically expected methane had been produced, was accompanied by disappearance of visible aggregates and by accumulation of turbidity in the liquid. In such cultures growing in alpha medium, the turbidity declined rapidly and cultures were again clear within a day. No aggregates were visible, and microscopic examination revealed no intact cells. Methanogenesis by these cultures ceased shortly after disaggregation, even though the methane produced was less than the amount expected on the basis of stoichiometry. Thus, a portion of the catabolic substrate apparently remained unused in these alpha medium cultures, and neither addition of more trimethylamine nor transfer to fresh medium resulted in further methane production.

On the other hand, when similar experiments were done with MS medium (with elevated calcium and magnesium concentrations), the released coccoid cells did not lyse. The turbidity persisted, and the released cells continued to grow until the expected amount of methane was produced. When these cells were inoculated into fresh medium, they continued growth as a turbid suspension.

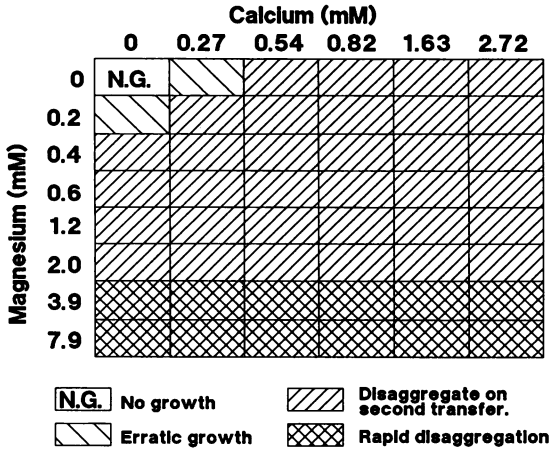

FIG. 1. Effect of calcium and magnesium on disaggregation of *M. mazei*. Aggregates were inoculated into alpha medium with various Ca^{2+} and Mg^{2+} concentrations, and disaggregation was monitored during successive growths in each medium.

Because high concentrations of calcium and magnesium were reportedly required for disaggregation of *M. mazei* S-6 (2), we investigated the minimum concentrations necessary for disaggregation. We transferred 0.1 ml of *M. mazei* aggregates from alpha medium with 50 mM acetate to 5 ml of alpha medium with 40 mM trimethylamine and various concentrations of calcium and magnesium. We examined the cultures microscopically and measured turbidity to determine the morphology of the cells. In media with 3.9 mM Mg^{2+} or more, aggregates dispersed during growth, as in alpha medium and MS medium (see above). When aggregates grew and did not disperse into coccoid units, we transferred these cultures to identical media. In most cases, such aggregates dispersed into individual cells during this second growth (Fig. 1). Disaggregation was not detected in only three media, each of which was low in or lacked both calcium and magnesium. Growth in these media was slow or absent.

At a lower concentration of catabolic substrate (10 mM trimethylamine) in alpha medium (0.27 mM Ca^{2+} and 0.49 mM Mg^{2+}), aggregates of *M. mazei* did not disperse but continued to grow as aggregates. However, aggregates separated into single cells in MS medium, even with only 10 mM trimethylamine as substrate.

Growth requirement of *M. mazei* aggregates for calcium and magnesium. *M. mazei* required calcium and magnesium for growth. Although slow growth often occurred when cells were first inoculated in alpha medium without calcium and magnesium, no growth occurred after three or four transfers (Table 1). When either of these ions was present, growth occurred; however, the requirement for one of these divalent cations may have been met by traces present in the other or in the Trypticase peptone or yeast extract. When *M. mazei* aggregates were inoculated into minimal medium (i.e., medium with no peptone or yeast extract) without calcium and magnesium, growth was not complete; not all of the 40 mM trimethylamine was converted to methane, and no growth occurred in subsequent transfers. Also, in minimal medium

TABLE 1. Effects of Ca^{2+} and Mg^{2+} on specific growth rates of *M. mazei* individual coccoids[a]

Mg^{2+} concn (mM)	Specific growth rate (per h) at a Ca^{2+} concn (mM) of:					
	0	0.27	0.54	0.82	1.6	2.7
0	0	0	0	0	0	0.060
0.2	0	0	0	0	0	0.059
0.4	0	0	0	0	0	0.063
0.6	0	0	0	0	0.059	0.061
1.2	0	0	0	0	0.062	0.064
2.0	0	0	0	0.062	0.063	0.065
3.9	0	0.052	0.061	0.062	0.063	0.064
7.9	0.062	0.061	0.062	0.063	0.063	0.064

[a] Inocula were late-exponential-phase coccoids grown in MS medium with 40 mM trimethylamine as the substrate, and the medium for determination of specific growth rate was alpha medium with various Ca^{2+} and Mg^{2+} concentrations.

with 4.9 mM Mg^{2+} and no Ca^{2+} or with 2.7 mM Ca^{2+} and no Mg^{2+}, growth was not complete, and no growth occurred on subsequent transfers. *M. mazei* grew in MS mineral media.

Growth requirement of *M. mazei* single cells for calcium and magnesium. The single cells released from aggregates had a requirement for greater concentrations of divalent cations than did the aggregates, apparently for stabilization of the protein cell walls. Coccoid cells continued to grow as individual cells when inoculated (5%, vol/vol) into MS medium with 40 mM trimethylamine (or 100 mM acetate or 150 mM methanol). Microscopic examination of cultures during various portions of the growth cycle revealed no formation of aggregates. When single cells were inoculated into alpha medium with 40 mM trimethylamine, no methane was detected after 5 days of incubation, and microscopic examination revealed no intact cells (i.e., the cells had lysed). In order to determine what concentrations of calcium and magnesium are required to prevent the lysis of single cells and to support their growth, we measured the specific growth rates of the single cells growing on trimethylamine (40 mM) with various concentrations of calcium and magnesium (Table 1). Either calcium or magnesium was always required for growth of single cells, and the action of these cations appeared to be synergistic. Media with low concentrations of calcium and magnesium did not support the growth of *M. mazei* single cells (Table 1), which lysed under these conditions. Many of these media in which individual cells lysed were otherwise suitable for growth; aggregates grew in these media (Fig. 1). In each medium tested (Table 1), pseudosarcinal aggregates were never detected when the inoculum was coccoid, individual cells.

In each medium which supported growth of individual cells, sequential transfers of *M. mazei* continued to grow as single cells. *M. mazei* also grew continuously as single cells in MS minimal medium with 40 mM trimethylamine, but the specific growth rate was much slower (0.022/h) than that in complex media.

Formation of aggregates from single cells. *M. mazei* single cells were serially diluted and inoculated into 5 ml of MS medium with 40 mM trimethylamine. When inocula were large (greater than 50 μl of the original culture), single cells always grew as single cells, with no pseudosarcinal aggregates detectable by phase-contrast microscopy. However, when higher dilutions were inoculated (i.e., 5 μl or less of original culture), pseudosarcinal aggregates were seen. An inoculum containing 5 μl of original culture grew into small pseudosarcinal aggregates (each aggregate containing 4 to 20 cells) which subsequently disaggregated within 3 or 4 days after inoculation (Fig. 2). When inocula contained 500 or 50 nl of original culture, aggregates grew to a size visible to the unaided eye before disaggregating within 4 to 5 and 5 to 6 days, respectively, after inoculation (Fig. 2). When such aggregates were obtained before disaggregation and inoculated into alpha medium with either 10 mM trimethylamine, 50 mM acetate, or 50 mM methanol, the aggregates grew and did not disaggregate. In this way, the morphology of trimethylamine-grown, single-cell cultures could be changed to the aggregate form. Similarly, single cells growing on 150 mM acetate or 150 mM methanol also formed aggregates during the early phase of logarithmic growth when small inocula (less than 5 μl) were used.

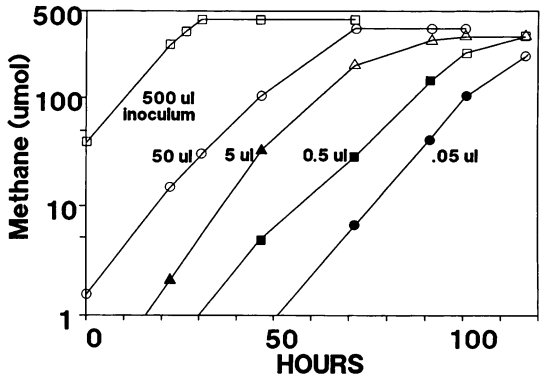

FIG. 2. Effect of inoculum size on growth of *M. mazei* in MS medium with 40 mM trimethylamine. Solid symbols indicate cultures which consisted of aggregates, and open symbols indicate coccoid units.

Stability of single cells in medium supplemented with other divalent cations. We examined the abilities of other earth-abundant cations to stabilize *M. mazei* single cells by suspending cells in alpha medium, MS medium, and alpha medium with each of the following cations (5 mM) added: Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, or Mn^{2+}. The actual activities of the cations in these media were not known because precipitates formed. Similar results were obtained when experiments were repeated in media with lower phosphate concentration (0.8 g of K_2HPO_4 per liter) to minimize precipitation. When *M. mazei* single cells were suspended in alpha medium and alpha medium with cadmium, zinc, or copper added, the turbidity decreased drastically within 24 h, and microscopic examination revealed no intact cocci. Cell suspensions in MS medium and alpha medium with magnesium, calcium, iron, or manganese maintained turbidity for at least 24 h, and intact cocci were visible by microscopy. We also tested the stabilization of cells in the presence of NaCl. Even 400 mM NaCl had no detectable effect on stability of coccoid cells.

Equivalence of roles of Ca^{2+} and Mg^{2+} in supporting growth and viability of single cells. Because a high concentration of either calcium or magnesium is required for single-cell growth, we investigated whether the effects of these ions were exerted by a common mechanism. We grew *M. mazei* single cells on 40 mM trimethylamine either with 7.9 mM Mg^{2+} and no Ca^{2+} or with 2.7 mM Ca^{2+} and no Mg^{2+}. After several transfers, the single cells adapted to either medium could grow equally well in the other, without a detectable lag before growth. In all cases, the specific growth rate was between 0.058 and 0.064/h. We tested the mechanism for prevention of lysis by these two cations by washing the Mg^{2+}-adapted cells twice in medium with 2.7 mM Ca^{2+} and no Mg^{2+}. The suspended single cells did not

lyse, and the absorbance increased from 0.34 to 0.62 within 24 h, apparently due to growth on trimethylamine. Ca^{2+}-adapted cells behaved similarly in medium with Mg^{2+} and no Ca^{2+}. Cells adapted to 2.7 mM Ca^{2+} or to 7.9 Mg^{2+} and suspended in medium without either of these cations lysed.

Effects of other cations and compounds on the morphology of *M. mazei*. We examined the effects of other earth-abundant cations on the ability of *M. mazei* to grow as single cells. We prepared alpha medium with $ZnSO_4 \cdot 7H_2O$, $CuCl_2 \cdot 2H_2O$, $FeCl_2 \cdot 4H_2O$, $Cd(NO_3^-)_2 \cdot 4H_2O$, or $MnSO_4 \cdot H_2O$ added at concentrations of 0.1, 0.2, or 0.4 g/liter. The actual activities of these cations were not known because precipitates formed in the medium. *M. mazei* single cells were used as the inoculum (10 μl was added to 5 ml of each medium). Growth occurred in all media except those with copper, but only in media with $MnSO_4 \cdot H_2O$ (0.2 to 0.4 g/liter) did the aggregates disperse into coccoid cells (specific growth rate, 0.059/h).

M. mazei single cells grew well when inoculated into MS medium with added sucrose (5.4 or 13.4% [wt/vol]) but did not grow in alpha medium with these sucrose amendments.

Morphology of other *Methanosarcina* strains and species in MS medium. When grown in MS medium with 40 mM trimethylamine, *M. mazei* LYC exhibited morphological changes similar to those of strain S-6, including transitory growth as aggregates when inocula were small. However, the tested strains of *M. barkeri* (e.g., 227, MS, and UBS) differed. Strain UBS always grew as single cells, even with very small inocula (50 nl). *M. barkeri* MS and 227 always grew as pseudosarcinal aggregates. *M. thermophila* TM1 at 52°C grew as aggregates after repeated transfer in MS medium with 40 mM trimethylamine or 150 mM acetate. Recent data of Sowers and Gunsalus (11) indicate that the conditions triggering disaggregation of *M. thermophila* TM1 may differ from those causing disaggregation of *M. mazei*.

Disaggregatase activity in *M. mazei* cultures. The growth of *M. mazei* as detergent-sensitive, coccoid cells implied that there was no net matrix synthesis. We measured disaggregatase activity of growing cultures to determine whether the lack of matrix was due to inhibition of its synthesis or to continuing hydrolysis by disaggregatase. We grew a coccoid culture of *M. mazei* in MS medium with 40 mM trimethylamine and prepared serial, 10-fold dilutions in the same medium. From each dilution, 0.5 ml was inoculated into 5 ml of the same medium. Cultures were incubated; each day we measured growth and disaggregatase activity and examined the cultures by phase-contrast microscopy. Disaggregatase activity was detected immediately after transfer in cultures inoculated with 0.5 ml and 2 days after transfer in cultures inoculated with 50 μl; microscopic examination never revealed aggregates in these cultures. In cultures inoculated with higher dilutions, no activity was detected until after disaggregation, at which time activity was always detected. Activity could be detected at all times in coccoid cultures in MS medium maintained by a 10% (vol/vol) inoculum (0.5 ml in tubes).

Enzymatic activity in culture filtrates was fairly stable when O_2-free conditions were maintained. When 0.5 ml of coccoid culture was filtered into 5 ml of sterile MS medium, activity remained for 5 days. Therefore, the lack of aggregates during the growth after transfer of *M. mazei* coccoid cells could have been due to carry-over of disaggregatase rather than its production. We centrifuged (1,500 × *g* for 60 min) *M. mazei* individual cells and washed them twice in an equal volume of MS medium in order to remove disaggregatase from the medium. The washed cells were serially diluted and inoculated into MS medium, cultures were incubated, and the disaggregatase activity was measured. When the inoculum was 0.5 ml, the cells remained as individual cocci even though no disaggregatase activity was detected until growth was complete. With inocula smaller than 50 μl, aggregates formed and dispersed as before, and disaggregatase activity was measured only after disaggregation. Thus, cells could grow for about 5 or 6 generations as individual cocci in the absence of measurable disaggregatase in the medium.

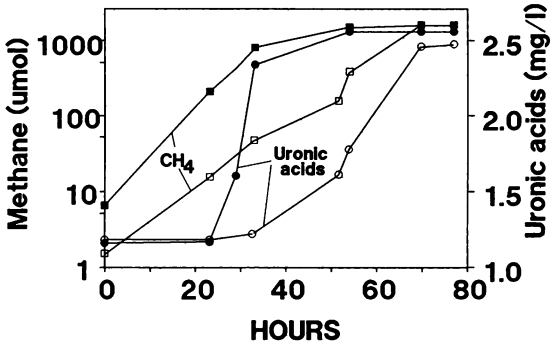

FIG. 3. Production of uronic acids and methane during growth and disaggregation. Aggregates (solid symbols) or coccoid cells (open symbols) were inoculated into MS medium and grown; in the culture inoculated with aggregates, turbidity increased rapidly between 24 and 31 h after inoculation, indicating that disaggregation corresponded with the release of uronic acids.

We also tried to quantify the action of disaggregatase by measuring uronic acids, which are released by the activity of disaggregatase on the matrix of *M. mazei*. In cultures growing as single cells, the uronic acid concentration increased gradually (Fig. 3). The high background of uronic acids in the culture medium made it difficult to measure production of small amounts of uronic acid during the early part of growth. We also examined uronic acid production by *M. mazei* coccoid cells growing in MS mineral medium and found an exponential increase in uronic acid concentration during growth (from 0.16 mg of uronic acids per liter after inoculation to 1.3 mg/liter at the end of growth). The production of uronic acids by single cells of *M. mazei* contrasts with the lack of uronic acid production during single-cell growth of *M. thermophila* (11). However, in cultures of aggregates which dispersed during the growth phase, the uronic acid concentration increased dramatically at the time of disaggregation (Fig. 3).

Stability of washed, coccoid cells in medium with a low concentration of calcium and magnesium. The inability of *M. mazei* coccoid cells to grow in alpha medium was probably due to the lysis of these single cells. To test this, we grew *M. mazei* coccoid cells in MS medium to late log phase; we washed the cells once and suspended them in media with various concentrations of Ca^{2+} and Mg^{2+}. The single cells lysed in alpha medium, but not in alpha medium supplemented with 2.7 mM Ca^{2+} or 3.9 mM Mg^{2+}.

DISCUSSION

M. mazei required calcium or magnesium for growth. When these cations were absent or in low concentration (combined concentration less than 0.3 mM), neither coccoid cells nor aggregates grew. The slow growth of aggregates which occurred initially in complex media without added

calcium or magnesium was probably due to carry-over from the inoculum and to traces contained in the Trypticase peptone or yeast extract, and the absence of growth after three transfers indicates a requirement for calcium or magnesium. Media with moderate amounts of either of these cations (e.g., 0.5 mM Ca^{2+} or 0.4 mM Mg^{2+}) allowed growth of aggregates but not growth of cocci, which lysed under these conditions. Because the calcium and magnesium salts used in this study each contained traces of the other, we cannot rule out a possible growth requirement for at least traces of both cations.

The morphology of *M. mazei* during growth is controlled by divalent cation concentration and quantity of catabolic substrate (2). This study shows that much higher concentrations of divalent cations (2 to 4 mM) were necessary to prevent lysis of coccoid cells. The different concentrations which elicit each of these effects imply that the mechanism of each of these actions may be different.

Divalent cation concentrations also affect growth rate (2), but this may be due predominantly to their effect on morphology. In the previous study (2), aggregates were used as inocula. When cocci were used, growth did not occur in media with low Ca^{2+} and Mg^{2+} concentrations, but Ca^{2+} and Mg^{2+} concentrations within the range allowing growth had little effect on growth rate (Table 1).

A high concentration of catabolic substrates is required for disaggregation and for growth of single cells. The concentrations used in this study far exceed the K_m of cells, so the effect of additional substrate may be on the extent of growth rather than on the rate of growth. In some cases, additional growth may be necessary before aggregates can disperse to cocci, as is shown in Table 1 when disaggregation occurred only after the second transfer in a medium. However, with other media, such as alpha medium with 50 mM acetate, we have made as many as 100 sequential transfers (data not shown) and never observed the turbidity indicative of growth as individual cells. Yet, simply increasing the substrate concentration in this medium to 150 mM (or changing the substrate to 40 mM trimethylamine) caused cells to disaggregate. Clearly, cultures which have grown on 50 mM acetate continue to catabolize acetate and grow exponentially when they are transferred to fresh media, just as cultures in media with 150 mM acetate which have catabolized 50 mM of the acetate continue to grow exponentially when incubation continues. There are several possible explanations for disaggregation of a culture growing in medium with 150 mM acetate while another culture transferred three times in medium with 50 mM acetate remains as aggregates. The factors which may trigger disaggregation include exhaustion of some nutrient or accumulation of some product in the medium with 150 mM acetate. Another possibility is that aggregates must reach a minimum size before disaggregation can take place; when cultures are transferred, larger aggregates may be sheared into smaller ones during passage through the syringe needle, allowing continued growth as aggregates. However, the production of disaggregatase and soluble uronic acids during growth of individual coccoid units supports the argument that disaggregatase production is a function of physiological conditions rather than size of aggregates.

The rapid release of soluble uronic acids, which occurred during disaggregation (Fig. 3), suggests that the uronic acid is released by the action of the disaggregatase. The gradual increase of the soluble uronic acid during the growth of single cells implies that the production of galacturonic and glucuronic acids, the major building units of the matrix material (6), continued during growth of the single cells. Aggregates did not form, perhaps due to the presence of disaggregatase or to inhibition of the synthesis of matrix polymers from uronic acid monomers.

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