Effects of Calcium, Magnesium, pH, and Extent of Growth on the Morphology of *Methanosarcina mazei* S-6

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***Methanosarcina mazei* S-6 grew faster and its morphology changed to individual coccoid cells in medium with elevated concentrations of divalent cations and a large amount of catabolic substrate.**

In 1936 Barker (1) observed cocci and large aggregates in an acetate-degrading, methanogenic enrichment culture and named this methanogen *Methanococcus mazei*. Mah (4) repeated the enrichment technique described by Barker (1) and isolated *M. mazei* S-6, later transferred to the genus *Methanosarcina* as *Methanosarcina mazei* (5). *M. mazei* is an important acetate-utilizing methanogen in anaerobic digestors, and the morphology of this bacterium is important for several reasons. The most important feature of its morphology is made use of in digester designs such as anaerobic filters or anaerobic contact beds. These systems allow development of slow-growing organisms such as methanogens by retention of flocculating or rapidly settling bacteria. The size of *M. mazei* cysts (which may be 1 cm or more in diameter) is crucial to the retention of cells in such systems. On the other hand, individual cells have a much greater surface-to-volume ratio, and these may be preferred under certain circumstances. Also, the ability to grow *Methanosarcina* strains as individual cells may lead to applications in studies of the molecular biology of methanogens. Many *Methanosarcina* strains can grow on H_2 - CO_2 , methanol, or acetate; this substrate range has generated interest in the genetics of these organisms, but experiments have been hampered by their growth as aggregates. We determined conditions which allow growth as individual cells.

Growth of *M. mazei* S-6 on acetate in complex liquid medium (2) yields mainly aggregates and, in old cultures, a small number of individual cells. In MET-3 medium (3), *Methanosarcina* sp. strain FR-19 (3) and *M. mazei* S-6 (J. E. Harris, Abstracts, XIV Congress of Microbiology, Manchester, Great Britain, abstr. no. P.G3-7, 1985) disaggregate during growth and the resultant coccoid units of strain FR-19 lyse.

By comparing our culture medium with that of Davis and Harris (3), we identified major differences and tested these factors on the morphology of *M. mazei*. Disaggregation was detected by unaided visual observation and phase-contrast microscopy. Undisaggregated cultures contained large aggregates (1 mm or more in diameter) in a clear liquid; microscopic examination revealed no free cells. Disaggregated cultures were visibly turbid, and individual coccoid cells were observed by microscopy.

One major difference of the medium of Davis and Harris (3) was its higher quantity of catabolic substrate. During growth in our medium with 20 mM trimethylamine, cultures grew as aggregates, although after growth was complete a very small number of free coccoid cells were observed near

the periphery of large aggregates. When cultures were grown on 40 mM trimethylamine, aggregates present during early phases of growth gave rise to coccoid units during the mid- to late-logarithmic growth phase. Because of lower growth yields on these substrates, higher concentrations of acetate and methanol were tested. When 50 mM acetate or 50 mM methanol was used as a substrate, little or no disaggregation occurred. Complete disaggregation during later stages of growth occurred with 100 mM acetate or methanol. The reason for a large quantity of catabolic substrate was not clear, but we suspect that the aggregates must reach a certain size to disaggregate. Normally our inocula consisted of large aggregates of cells which always broke up into coccoid units when grown in medium with 0.5 g each of $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$ per liter. However, when we used logarithmically growing, individual coccoid units for inocula, they grew but did not disaggregate. After growth was complete, many small aggregates consisting of about 10 to 30 cells were observed. This number of cells per aggregate was consistent with each individual coccoid unit having grown into a single, small aggregate of cells.

A second major difference in the medium of Davis and Harris (3) was its higher concentrations of divalent cations and its lower pH value. We tested the growth rate (2) and morphology of *M. mazei* in media with various concentrations of Ca^{2+} and Mg^{2+} (Fig. 1). In our normal medium (pH 7.0) and with 0.2 g $MgCl_2 \cdot 6H_2O$ per liter of medium (0.99 mM), cells grew fastest when the concentration of $CaCl_2 \cdot 2H_2O$ was above 0.3 g l^{-1} (2.0 mM). Medium at a lower pH value was prepared with 100% CO_2 gas and an $NaHCO_3$ concentration adjusted to give a medium pH of 6.7. In this medium, only 0.1 g of $CaCl_2 \cdot H_2O$ per liter (0.68 mM) was required for fastest growth. In medium with 0.2 g of $CaCl_2 \cdot H_2O$ per liter (1.4 mM), *M. mazei* grew fastest with the highest concentration of Mg^{2+} tested (1.0 g $MgCl_2 \cdot 6H_2O$ per liter; 4.9 mM) at pH 7.0, but at pH 6.7 it grew best with 0.5 g of $MgCl_2 \cdot 6H_2O$ per liter (2.5 mM) (Fig. 1B). The morphologies of these cultures were the same whether the medium pH was 6.7 or 7.0. Calcium was required for disaggregation. In the presence of 0.2 g of $MgCl_2 \cdot 6H_2O$ per liter, cells grew only as aggregates without added calcium, and when $CaCl_2 \cdot H_2O$ was between 0.1 and 0.3 g l^{-1} disaggregation took place only near the end of the growth phase or after growth was complete. At higher concentrations of Ca^{2+} , disaggregation occurred early or near the middle of the logarithmic phase of growth. Magnesium enhanced disaggregation, but it was not required. With 0.2 g of $CaCl_2 \cdot 2H_2O$ per liter and low $MgCl_2 \cdot 6H_2O$ concentrations (0, 0.1, or 0.2 g l^{-1}), disaggregation occurred

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FIG. 1. Effect of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ on the growth rate of *M. mazei*. (A) Growth rate with 0.2 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per liter and various Ca^{2+} concentrations. (B) Growth rate with 0.2 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per liter and various Mg^{2+} concentrations.

only after growth was complete. At higher magnesium concentrations (0.3, 0.5, or 1.0 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per liter), disaggregation took place in the mid-logarithmic phase of growth. Controls grown in medium with added NaCl did not disaggregate.

When cells disaggregated during growth, the optical density of cultures first increased and then decreased rapidly after growth was complete, indicating lysis of cells (Fig. 2). Viable cell numbers could not be used to quantify survival of released coccoid units because we could not measure the total (viable plus nonviable) released cells. Measurements were further complicated by the presence of the large aggregates themselves. To quantify the viability of released cells, we measured total viable biomass of cultures in the following way.

A sample was inoculated into fresh medium, and we determined the time required for it to grow to late-logarithmic phase. Assuming no lag and equivalent growth yields, the amount of viable biomass in the inoculum determines how many times the culture must double to reach late-logarithmic phase. We calculated this from the time required to reach late-logarithmic phase and the growth rate of *M. mazei* in that medium. To determine whether released coccoid units remained viable, we measured the viable biomass of samples taken throughout a batch growth cycle by inoculating 0.25-ml portions into 5 ml of fresh medium containing 40 mM trimethylamine. These tubes were incubated until 150 μmol of CH_4 was produced. From the known doubling time of *M. mazei* and the time required to produce 150 μmol of CH_4 , we were able to calculate the amount of viable biomass present in each of these inocula. The amount, an arbitrary value proportional to the viable biomass, was converted to a fraction of the maximum viable biomass. We tested viability of biomass in the normal medium and medium with increased levels of divalent cations (0.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 3.0 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per liter). Both media for testing viable biomass gave similar values during the early portion of growth, when cells were growing as

FIG. 2. Growth of *M. mazei* on 40 mM trimethylamine. Symbols: ●, methane production; ○, turbidity; ■, viable biomass determined in medium with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, each at 0.2 g l⁻¹; □, viable biomass determined in medium with 0.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 3 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per liter of medium.

aggregates. After disaggregation started (as indicated by the turbidity increase at about day 4), the medium with elevated divalent cation concentrations gave higher values for viable biomass than did medium with lower concentrations. Thus, elevated concentrations of divalent cations appeared to increase the viability of the coccoid units. These units may have been protected from lysis by divalent cations or restored from some physiological injury. After disaggregation was complete (about day 7), turbidity decreased slightly, probably because of lysis of cells; viable biomass decreased more rapidly than turbidity, suggesting that many unlysed cells were unable to grow.

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LITERATURE CITED

1. Barker, H. A. 1936. Studies upon the methane-producing bacteria. *Arch. Mikrobiol.* 7:420-438.
2. Boone, D. R., J. A. G. F. Menaia, J. E. Boone, and R. A. Mah. Effects of hydrogen pressure during growth and pregrowth with hydrogen on acetate degradation by *Methanosarcina* species. *Appl. Environ. Microbiol.* 53:83-87.
3. Davis, R. P., and J. E. Harris. 1985. Spontaneous protoplast formation by *Methanosarcina barkeri*. *J. Gen. Microbiol.* 131: 1481-1486.
4. Mah, R. A. 1980. Isolation and characterization of *Methanococcus mazei*. *Curr. Microbiol.* 3:321-326.
5. Mah, R. A., and D. A. Kuhn. 1984. Transfer of the type species of the genus *Methanococcus* to the genus *Methanosarcina*, naming it *Methanosarcina mazei* (Barker 1936) comb. nov. et emend. and conservation of the genus *Methanococcus* (Approved Lists 1980) with *Methanococcus vannielii* (Approved Lists 1980) as the type species. *Int. J. Syst. Bacteriol.* 34: 263-265.