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Spontaneous Disaggregation of *Methanosarcina mazei* S-6 and Its Use in the Development of Genetic Techniques for *Methanosarcina* spp.

JANE E. HARRIS

Agricultural and Food Research Council Institute of Food Research, Norwich Laboratory, Norwich NR4 7UA, United Kingdom

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When monomethylamine was the growth substrate, spontaneous disaggregation of *Methanosarcina mazei* S-6 commenced at the mid-exponential phase and resulted in the formation of a suspension containing 10^8 to 10^9 free cells per ml. Free cells were osmotically fragile and amenable to extraction of DNA. Hypertonic media for the manipulation and regeneration of free cells into aggregates were developed, and plating efficiencies of 100% were achieved for *M. mazei* S-6 and LYC. Free cells of strain S-6 required $MgCl_2$ (10 mM) for growth, whereas aggregates did not. Specific growth rates of strains S-6 and LYC were increased by $MgCl_2$. Treatment with pronase caused sphere formation and removal of the protein wall of cells of strain S-6, but protoplasts could not be regenerated. The disaggregating enzyme produced by strain S-6 facilitated the preparation of suspensions of free cells of some strains of *Methanosarcina barkeri*. Although this provided a means of extracting high-molecular-weight DNA from *M. barkeri*, less than 0.1% of free cells were viable.

The activity of acetotrophic methanogens accounts for up to 70% of the methane produced in the degradation of organic material (11). Genetic manipulation of the strictly anaerobic, methanogenic archaebacteria offers a means of studying and improving the efficiency of mesophilic anaerobic digestion of commercial wastes, and thus gene transfer systems are needed for both acetotrophic and hydrogenotrophic methanogens (4).

Acetotrophic methanogens occurring in digestors are from the genera *Methanosarcina* and *Methanothrix*, but only *Methanosarcina* species are sufficiently amenable to laboratory culture for genetic studies to be attempted. The aggregating morphology and thick heteropolysaccharide cell wall typical of *Methanosarcina* species (5) are considerable barriers to the isolation of mutant strains, the extraction of DNA, and the development of transformation systems. However, *Methanosarcina mazei* (originally named *Methanococcus mazei* [10]) is characterized by a life cycle in which the sarcinal aggregates mature into cysts containing coccoid cells; when ruptured, the cysts release these single cells, which lack the thick aggregate wall but are capable of growing into aggregates (9).

This is the first study of complete spontaneous disaggregation of *M. mazei* S-6 and the exploitation of this process in the development of methods for the preparation, maintenance, and efficient recovery of free cells of this species. I also describe the production of single cells from aggregates of *Methanosarcina barkeri* facilitated by a disaggregating enzyme produced by *M. mazei* S-6. This study is a significant contribution toward the development of genetic studies with acetotrophic methanogens.

MATERIALS AND METHODS

Culture and growth conditions. *M. mazei* S-6 (DSM 2053) (9) and *M. barkeri* MS (DSM 800), 227 (DSM 1538), and Fusaro (DSM 804) were obtained from the German collection of microorganisms. *M. barkeri* W was provided by R. A. Mah, University of California, Los Angeles, and *M. mazei* LYC was provided by R. Sleat, Biotechnica Ltd., Cardiff, United Kingdom. All strains were grown in MET3 medium (5-ml cultures) as described previously (3). Monomethylamine (124 mM) was the substrate for *M. mazei* S-6 unless stated otherwise. *M. mazei* LYC and the *M. barkeri* strains were cultured on H_2-CO_2 (4:1) at 203 kPa. Incubation was at 37°C and without shaking except for growth on H_2-CO_2. All anaerobic manipulations were carried out in an anaerobic cabinet (Model 1024; Forma Scientific, Div. Mallinckrodt, Inc., Marietta, Ohio) under an atmosphere of N_2-CO_2-H_2 (7:2:1). SM3 medium was MET3 medium containing (per liter) 2 g of tryptose (Difco Laboratories, Detroit, Mich.), 2 g of Casamino Acids (Difco), and 10 ml of vitamin solution (2). Solid MET3 and SM3 media were prepared by the addition of 1% (wt/vol) agar (Difco), and plates were incubated under a gas atmosphere of N_2-CO_2-H_2 (7:2:1) or H_2-CO_2 (4:1) as appropriate. Anaerobic sterile solutions of substrates were added after the medium was autoclaved. The final pH of MET3 medium was 7.0; medium of different pH was obtained by adjusting to the appropriate value with HCl or NaOH before dispensing. FC buffer was 0.1 M TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 7.5) containing (per liter) 0.9 g of NaCl, 0.9 g of $(NH_4)_2SO_4$, 0.45 g of KH_2PO_4, 0.01 g of $FeSO_4 \cdot 7H_2O$, 10 ml of a trace mineral solution (1), and 103 g of sucrose. FC buffer was prepared under N_2, and sterile $MgCl_2 \cdot 6\ H_2O$ (final concentration, 20 mM) was added after autoclaving. The final pH was 7.0 to 7.2.

Transmission electron microscopy. Cells were fixed initially by suspension in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), followed by incubation for 2 h in a mixture containing equal parts of buffered 5% glutaraldehyde and 0.15% aqueous ruthenium red. After three washes in the buffer and postfixation for a further 2 h in a mixture of equal parts of 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) and 0.15% ruthenium red, the cells were embedded in

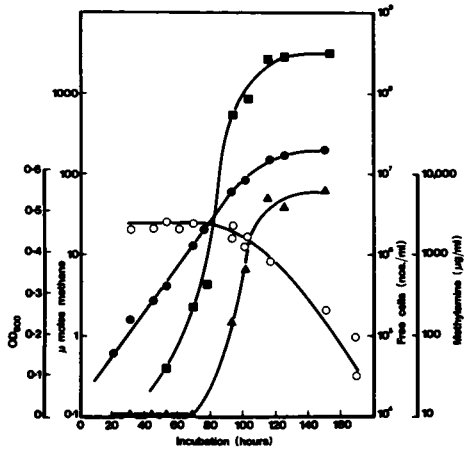

FIG. 1. Growth cycle of *M. mazei* S-6 in MET3 medium with monomethylamine (124 mM) as substrate. Symbols: ▲, optical density at 600 nm (OD_{600}); ●, micromoles of methane produced; ■, numbers of free cells per milliliter; ○, methylamine concentration (micrograms per milliliter).

1% low-melting-point agarose (Sigma Chemical Co., St. Louis, Mo.). Blocks containing the cells were dehydrated in an ethanol series and then transferred to acetone before being infiltrated and embedded in Spurr resin (16). Thin sections were stained sequentially with saturated uranyl acetate in 50% ethanol and lead citrate (12) and examined in an AEI 801 electron microscope.

Analytical methods. Optical density was measured with a Pye Unicam SP600 spectrophotometer. Methane concentration was determined with a Pye 104 gas chromatograph equipped with a flame ionization detector as described previously (1). A Leitz Ortholux II microscope was used for phase-contrast and fluorescence microscopy (excitation at 420 nm). The concentration of monomethylamine in culture supernatants was determined by high-performance liquid chromatography after derivatization with dabsyl chloride by the method of Lin and Lai (6). A Spherisorb 50DS column (25 cm by 6 mm [inside diameter]) and a mobile phase of 95% ethanol–acetonitrile–water (6:6:7, vol/vol/vol) were used. Cell suspensions for microscopic counts were fixed in glutaraldehyde (2.5%), and counts were made in duplicate in Helber counting chambers.

Chemicals. The sources for chemicals were as follows: pronase and proteinase K, BDH, Poole, United Kingdom; subtilisin and *Streptomyces caespitosus* type IV and *Streptomyces griseus* type XIV proteases, Sigma Chemical Co.

RESULTS

Spontaneous disaggregation of *M. mazei* S-6. During growth of aggregates in MET3 medium with monomethylamine (124 mM) as the substrate, single cells of *M. mazei* S-6 were first detected microscopically in the mid-exponential phase (Fig. 1), although the lower limit of the counting technique was 2.5 × 10^4 cells per ml. Aggregates were observed in cultures up to the late exponential phase and were often large enough to be visible to the naked eye and to settle to the bottom of the tube. Cysts which could be disrupted by physical pressure were present from the mid-exponential phase, but most aggregates spontaneously dispersed as the culture entered the stationary phase, with the number of single cells increasing from 10^5 to 10^8 per ml during an incubation period of about 30 h. This was accompanied by an increase in optical density, and complete disaggregation could be assessed by the naked eye as the formation of a homogeneously turbid suspension. The medium remained at pH 6.8 to 7.0 throughout growth. Single cells were phase dark and irregular in shape, becoming spherical in older cultures. Aggregates fluoresced strongly at 420 nm, but single cells were only weakly fluorescent. Although incubation times varied depending on the growth conditions, spontaneous disaggregation also occurred in the following instances: with an inoculum of aggregates or single cells, at all temperatures in the growth range (25 to 40°C), in medium without yeast extract, and with monomethylamine (10 to 200 mM), methanol (0.1 to 2%, vol/vol), or acetate (100 to 200 mM) as the substrate for methanogenesis. With H_2-CO_2 (4:1) as the substrate, large rafts of loose aggregates were eventually formed which were ruptured by pressure but spontaneous release of single cells occurred only partially or not at all. *M. mazei* S-6 could be maintained as aggregates by culturing on H_2-CO_2.

Spontaneous disaggregation also occurred throughout the pH range for growth, but at pHs 6 and 6.5, larger aggregates developed before dispersal.

Preparation and plating of free cells of *M. mazei* S-6. A suspension containing 10^7 to 10^8 free cells per ml and no remaining aggregates could be prepared by passage of a disaggregated culture though a membrane filter (pore size, 5 μm; Millipore Corp., Bedford, Mass.). Free cells were stable for at least 10 days in spent growth medium but were osmotically fragile, lysing in anaerobic glass-distilled water and on addition of sodium dodecyl sulfate (1%, vol/vol). Since the viability in fresh MET3 broth containing monomethylamine was variable due to lysis of free cells, effective osmotic stabilizers were sought. Sucrose, glucose, mannitol, and sorbitol (each at 0.3 M) and the divalent cations Mg^{2+} and Ca^{2+} (each at 20 mM) prevented lysis. Microscopic

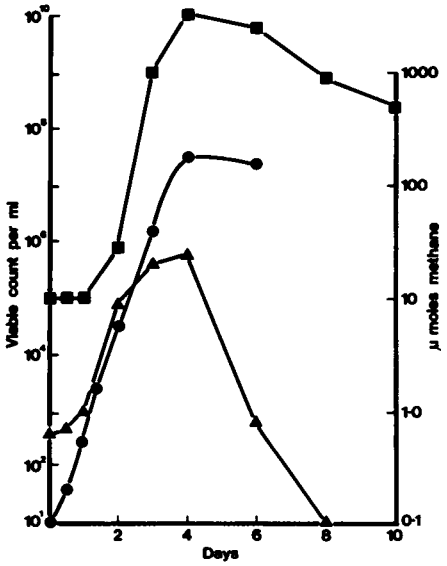

FIG. 2. Life cycle and osmotic sensitivity of free cells. Symbols: ■, viable cells per milliliter; ▲, viable cells per milliliter after dilution in anaerobic glass-distilled water; ●, micromoles of methane produced.

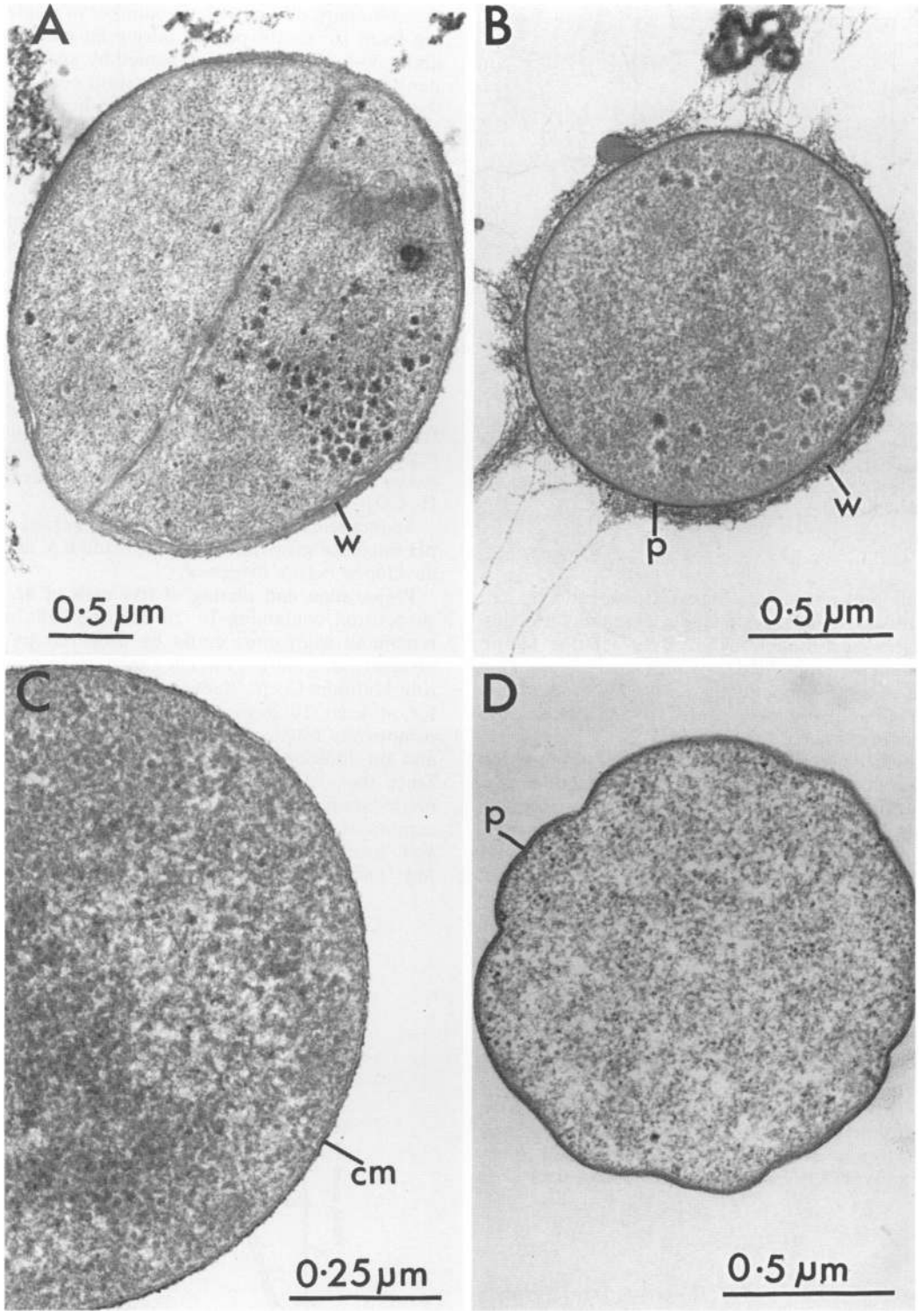

FIG. 3. Ultrastructure of free cells. (A) *M. mazei* S-6, after regeneration for 24 h, showing aggregate wall (w). (B) Freshly released *M. mazei* S-6 showing adhering aggregate wall material (w) and protein wall (p). (C) Pronase-treated cell of *M. mazei* S-6 bounded by cell membrane (cm) only. (D) *M. barkeri* 227 showing protein wall (p).

counts of free cells of strains S-6 and LYC suspended in hypertonic FC buffer remained constant for 5 days at 4°C.

No growth occurred when free cells were plated on the surface of MET3 agar containing monomethylamine in the presence or absence of osmotic stabilizers, but colonies were formed when inoculated plates were overlaid with molten agar (4 ml) at 52°C.

With this plating procedure, regeneration of single cells

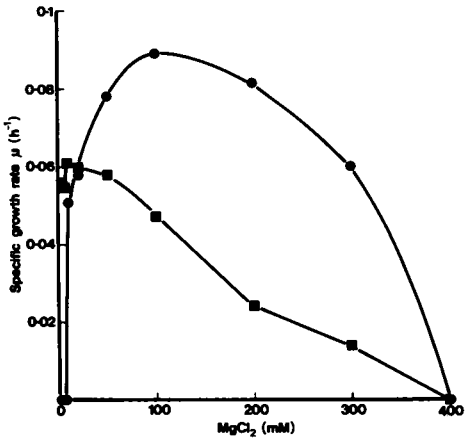

FIG. 4. Effect of $MgCl_2$ on growth on monomethylamine. Symbols: ■, inoculum of aggregates; ●, inoculum of free cells.

into aggregates on solid medium was shown to increase by up to 10,000-fold on addition of osmotic stabilizers, but $CaCl_2$ caused a heavy precipitate and made counting inaccurate. The addition of gelatin (1%, wt/vol) or bovine serum albumin (1%, wt/vol) did not enhance recovery. When single cells were allowed a period of regeneration in liquid medium before being plated, during which they produced methane and became phase bright, recoveries increased further by up to 100-fold. Optimum plating conditions which consistently gave 70 to 100% recovery of free cells of strains S-6 and LYC were on SM3 agar containing sucrose (0.3 M) and $MgCl_2$ (20 mM) after regeneration of the cells for 24 h at 37°C in MET3 broth containing sucrose (0.3 M) and $MgCl_2$ (20 mM). Colonies reached a suitable size for counting after incubation for 3 weeks.

Growth cycle of free cells. The life cycle, viability, and longevity of free cells of *M. mazei* S-6 were monitored by using the overlay plating method and SM3R medium (SM3 medium containing sucrose [0.3 M] and $MgCl_2$ [20 mM]). Free cells were inoculated into MET3 broth containing $MgCl_2$ (20 mM) and monomethylamine, and at each sampling time viability was determined before and after dilution in anaerobic glass-distilled water (Fig. 2). Regeneration, demonstrated by an increase in the number of osmotically stable free cells, was complete after about 48 h. During this period an aggregate wall had been laid down at the cell surface (Fig. 3A). Disaggregation was initially accompanied by a further small increase in the number of osmotically stable cells. This was expected since electron microscopy showed that freshly released cells often carried fibrillar aggregate wall material which could confer rigidity (Fig. 3B). Gradual loss of this residual material was reflected in the increased numbers of osmotically fragile cells appearing in the stationary phase. Samples diluted into anaerobic glass-distilled water containing sucrose (0.3 M) and $MgCl_2$ (20 mM) gave counts of viable cells similar to those for the undiluted controls, indicating that death was due to osmotic effects. Approximately 10% of free cells remained viable after 4 days in the stationary phase, but cells washed and suspended in FC buffer showed no change in viability when stored at 4°C for 5 days.

Effect of $MgCl_2$. Growth from an inoculum of single cells was variable in MET3 broth, and repeated subculturing resulted in the emergence of nondisaggregating variants. Since the addition of $MgCl_2$ (20 mM) prevented the formation of variants and improved the viability of single cells, its effect during growth on monomethylamine was investigated (Fig. 4). The optimum specific growth rates of cultures inoculated exclusively with aggregates or free cells occurred at 10 and 100 mM $MgCl_2$, respectively, and aggregates were inhibited at approximately 50 mM $MgCl_2$ compared with 200 mM for free cells. The most striking difference was the requirement of free cells for 10 mM $MgCl_2$ for growth to occur. The addition of sucrose (0.3 M) had no effect on the response of free cells to $MgCl_2$ concentration. In view of these results, *M. mazei* S-6 was routinely cultured on monomethylamine in the presence of $MgCl_2$ (20 mM).

The specific growth rates of *M. mazei* S-6 and LYC on H_2-CO_2 with inocula of free cells were increased from 0.061 per h for both strains to 0.069 and 0.077 per h, respectively, by $MgCl_2$ (20 mM). Without added $MgCl_2$ both strains developed into aggregates which grouped in large rafts, and only strain LYC eventually disaggregated. The addition of $MgCl_2$ promoted rapid disaggregation of both species before raft formation.

Formation and regeneration of protoplasts. Pronase, proteinase K, subtilisin, and *S. griseus* type XIV protease (each at 100 μg/ml) caused sphere formation by free cells during incubation in FC buffer for 1 h at 37°C, but the viability of the treated suspensions was reduced at least 100-fold. Treatment with *S. caespitosus* type IV protease had no effect on morphology or viability. Further investigation of the effect of pronase showed that sphere formation reached a maximum of 85% after 2 h, and spheres were stable in FC buffer. Electron micrographs revealed that protoplasts had been formed by removal of the protein wall of free cells (Fig. 3C). Although pronase-treated cells suspended in hypertonic growth medium produced methane at a constant rate for approximately 14 days, their viability in liquid and solid SM3 medium with sucrose (0.3 M), $MgCl_2$ (20 mM), $CaCl_2$ (1 to 20 mM), gelatin (1%, wt/vol), or bovine serum albumin (1%, wt/vol) was less than 1%. The fraction of cells capable of regeneration may be unaffected by pronase.

Formation and viability of free cells of *M. barkeri*. Filter-sterilized supernatants of disaggregated cultures of *M. mazei* S-6 catalyzed the dispersal of viable aggregates of *M. barkeri* W, MS, 227, and Fusaro. Supernatants of freshly disaggregated cultures effected the most rapid release of single cells, which was maximal after 1 to 2 h at 37°C, although small aggregates still remained. Oxidized or stored (24 h at 4°C) supernatants showed reduced activity, and boiled supernatants (10 min) were completely inactive.

Filtration (pore size, 5 μm) of dispersed aggregates yielded suspensions containing 10^6 to 10^8 free cells per ml. Single cells of each strain of *M. barkeri* were osmotically sensitive and possessed a cell wall similar to that of single cells of *M. mazei* S-6 (Fig. 3D).

The viability of free cells was examined by using the plating method developed for *M. mazei* S-6 with SM3R medium. Although aggregates of each *M. barkeri* strain formed colonies, plating efficiencies of single cells never exceeded 1%. Cells incubated in SM3R broth showed a similar level of viability.

DISCUSSION

Complete spontaneous disaggregation of *M. mazei* S-6 in liquid culture has not previously been described. In his characterization of this strain, Mah (9) identified the various morphological types seen during the life cycle of colonies in roll tubes but reported that mechanical action was needed to

disrupt aggregates and that cocci were insensitive to sodium dodecyl sulfate. *M. mazei* LYC was recently isolated and distinguished from strain S-6 by its ability to disaggregate spontaneously during exponential growth without cyst formation (7). It is not understood why strain S-6 exhibits several characteristics of strain LYC in this laboratory, namely complete spontaneous disaggregation with the release of osmotically sensitive free cells, production of a disaggregating enzyme, and rapid growth on H_2-CO_2. The culture conditions used are essentially identical to those of other workers. There is some evidence to suggest that induction of the disaggregating enzyme depends on growth rate, since stimulation of growth on H_2-CO_2 by $MgCl_2$ promotes disaggregation. The levels of disaggregating enzyme affect the rate of removal of residual heteropolysaccharide material from free cells and may account for differences in the osmotic sensitivity of free cells observed by other workers.

The growth requirement of free cells for $MgCl_2$ is similar to that reported for cocci of *Methanosarcina acetivorans* (14) and is further evidence that Mg^{2+} functions in maintaining the integrity of protein cell walls of *Methanosarcina* species. This function may also explain the stimulatory effect of Mg^{2+} on the growth rates of strains S-6 and LYC on H_2-CO_2.

The preparation of concentrated suspensions of free cells has provided a means of extracting high-molecular-weight DNA from *M. mazei* S-6 and *M. barkeri* W, MS, 227, and Fusaro. Plasmid DNA was not detected in any of these strains (unpublished results).

Development of a hypertonic buffer, regeneration medium, and plating regime for free cells was badly needed for isolation of mutants of *M. mazei* S-6. Despite the presence of a protein wall, single cells may be amenable to genetic transformation or fusion, but care must be taken to allow complete degradation of aggregate wall material adhering to single cells which may be a barrier to gene transfer.

Pronase-induced protoplasts of *M. mazei* remained capable of methanogenesis, but no evidence for regeneration was obtained. Similarly, the viable fraction of free cells of *M. barkeri* strains is likely to represent cells which retain some aggregate wall material. Ultrastructural studies have shown that microtubular structures are located on the protein wall of individual cells of *M. mazei* S-6 both before and after disaggregation (13). It is possible that damage or loss of such organelles during pronase treatment of *M. mazei* or induced disaggregation of *M. barkeri* may prevent regeneration. The use of purified disaggregating enzyme may be beneficial in the latter instance.

Previous evidence suggested that free cells of *M. mazei* S-6 were protoplasts (J. E. Harris, Abstr. EMBO Workshop Mol. Genet. Archaebacteria, 1985, B6), but it has since been found that ruthenium red, which reacts strongly with negatively charged molecules (8), allows effective visualization of the protein wall in electron micrographs. This is consistent with the finding that protein walls of methanogens contain large amounts of negatively charged amino acids (5, 14, 15). The results suggest that a primary negatively charged protein cell wall is common to both disaggregating and nondisaggregating *Methanosarcina* species.

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