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Spontaneous Protoplast Formation in *Methanobacterium bryantii*†

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Methanobacterium bryantii was found to undergo rapid lysis when grown in a prereduced chemically defined medium under H_2–CO_2 (4:1, vol/vol). The addition of 20 mM $MgCl_2$ to the medium gave, rather than rapid lysis, a gradual formation of phase-dark spherical bodies which in thin section appeared as true protoplasts. In general, the protoplasts were stabilized by divalent but not monovalent cations and, unlike whole cells, were sensitive to lysis by Triton X-100. Electron microscopic examination revealed that protoplast formation was preceded by a general breakdown of the cell wall with an apparent squeezing out of the protoplast through the degraded wall. The growth of cells was greatly increased and not accompanied by detectable lysis in a medium modified by elevating the levels of nickel and ammonium.

Methanogenic bacteria comprise an unusual group of obligate anaerobes, most being capable of utilizing CO_2 and H_2 as sole carbon and energy sources (26). Methanogens, together with extreme halophiles and certain thermoacidophiles, are members of the so-called *Archaebacteriae*, a procaryotic kingdom purported to represent a third line of descent distinct from the eubacterial and eucaryotic lines (9). One of the most abundant and widely distributed methanogens appears to be *Methanobacterium bryantii* (4, 11, 23), formerly called *Methanobacterium* strain M.O.H. (1).

It has been observed in this laboratory that routine growth of *M. bryantii* in prereduced S medium (3) under an atmosphere of H_2–CO_2 (4:1, vol/vol) resulted in lysis of the cells after approximately 2 weeks. This occurred when the cells were cultivated in 10-ml volumes in 120-ml serum vials (2) under static conditions with no daily replenishment of H_2–CO_2. In these cell lysates there was observed a very small number of phase-dark spherical bodies. We believed that under these growth conditions *M. bryantii* may have undergone lysis after passing through a protoplast stage. Such a protoplast formation would prove useful in attempts to fractionate the cell wall layers, an experimental procedure required to locate many of the structural and enzymatic components of the cytoplasmic membrane and outer wall, such as the unique ether-linked polyisoprenoid chain lipids (16).

New techniques for spheroplast (or protoplast) formation are necessary for methanogens, because their cell wall chemistry differs from that of other bacteria (15). This report demonstrates the spontaneous formation of protoplasts by *M. bryantii* grown in S medium and the stabilization of these protoplasts by magnesium ions.

(Portions of this work were presented at the 31st Annual Meeting of the Canadian Society of Microbiologists at Ottawa, Canada in June 1981.)

MATERIALS AND METHODS

Organism, media, and culture maintenance. *M. bryantii* (5) was obtained from M. P. Bryant. Cultures were maintained at 35°C with weekly transfers in a carbonate-buffered synthetic medium under an atmosphere of H_2–CO_2 (4:1, vol/vol). This medium (S medium [3]) contained (in milligrams per liter of deionized water): Na_2CO_3, 480; $(NH_4)_2SO_4$, 450; K_2HPO_4, 290; KH_2PO_4, 180; $MgSO_4 \cdot 7H_2O$, 120; $CaCl_2 \cdot 2H_2O$, 60; NaCl, 54; $FeSO_4 \cdot 7H_2O$, 21; $N(CH_2COOH)_3$, 15; $MnSO_4 \cdot H_2O$, 5; $CoCl_2 \cdot 6H_2O$, 1; $ZnSO_4 \cdot 7H_2O$, 1; $CuSO_4 \cdot 5H_2O$, 0.1; $AlK(SO_4)_2 \cdot 12H_2O$, 0.1; H_3BO_3, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.1; pyridoxine-HCl, 0.1; thiamine-HCl, 0.05; riboflavin, 0.05; nicotinic acid, 0.05; *p*-aminobenzoic acid, 0.05; lipoic acid, 0.05; biotin, 0.02; folic acid, 0.02; vitamin B_{12}, 0.005; resazurin, 1. The medium was prereduced (12) by using cysteine-HCl (1.27 mM) and sodium sulfide (0.83 mM). Enriched medium was prepared by adding yeast extract and tryptone (SYT medium), at a final concentration of 2 g/liter, to S medium before reducing with cysteine sulfide. Modified S medium was prepared the same way as regular S medium, with the following changes in composition: the phosphate concentration was increased 10-fold to 30 mM; $NiCl_2 \cdot 6H_2O$ was added to a final concentration of 5 μM; $Na_2MoO_4 \cdot 2H_2O$ concentration was increased to 1.5 μM; and NH_4Cl was added to increase the NH_4^+ concentration to 40 mM. This medium was designated medium JM.

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Growth experiments. Cells were cultivated from a 10% inoculum in 100-ml volumes in modified 1-liter Wheaton bottles (Wheaton Glass Co., Brampton, Ontario) at 35°C under an atmosphere of H_2–CO_2 (4:1, vol/vol). Cells were shaken at 150 rpm, and the bottles were pressurized daily with H_2–CO_2 (4:1, vol/vol) to 170 kPa. Growth of the organisms was followed turbidimetrically at 600 nm by using square cuvettes of 1-cm light path. Methane accumulation in the headspace was followed by gas chromatography. After passage through a Porapak T 50/80 column (3.08 m by 0.64-cm outer diameter; Chromatographic Specialties Ltd.) with helium (20 ml/min) as carrier gas, methane was detected by flame ionization. Oven temperatures were as follows: injector, 100°C; column, 80°C, and detector, 250°C.

Effect of CO_2 and H_2 deprivation on growth of *M. bryantii*. Matched cultures of *M. bryantii* (100 ml of modified S medium in 1-liter bottles) were allowed to grow for 3 days with daily pressurization with H_2–CO_2 (4:1, vol/vol). On day 3, the culture headspaces were completely flushed with either H_2–CO_2 (4:1, vol/vol) or N_2 and then pressurized with the same gas. Growth of the cultures was followed turbidimetrically at 600 nm.

Protoplast formation. *M. bryantii* was grown for 2 days with shaking in 100 ml of S medium before a sterile, anaerobic solution of $MgCl_2$ in S medium was added to give a final $MgCl_2$ concentration of 20 mM. Incubation was continued with shaking at 35°C and daily pressurization of the growth vessel to 170 kPa with H_2–CO_2 (4:1, vol/vol). In some experiments, the $MgCl_2$ was added to S medium before inoculation.

Electron microscopy. Electron microscopy of the *M. bryantii* protoplasts was performed essentially in the same manner as described for *Methanospirillum hungatei* spheroplasts (22).

Atomic absorption spectroscopy. Nickel content of the media was determined by atomic absorption spectroscopy at a wavelength of 232 nm after extraction with ammonium pyrrolidine dithiocarbamate in methyl iso-butyl ketone.

RESULTS

Protoplast formation in *M. bryantii*. *M. bryantii* has an unusual but characteristic growth pattern when cultured in S medium. The cells grow logarithmically for about 3 days, usually reaching an absorbancy at 600 nm (A_{600}) of about 0.9 and then, before entering stationary phase, abruptly lyse (Fig. 1A). The lysis is essentially complete, with microscopic examination of the lysate showing cell debris and only the occasional cell or phase-dark spherical body (Fig. 2A).

The growth of *M. bryantii* in S medium supplemented with 20 mM $MgCl_2$ is shown in Fig. 1A. Similar results were obtained regardless of whether the $MgCl_2$ was added at the same time as inoculation or at times just before expected lysis (i.e., at 2 days). In Mg^{2+}-supplemented medium, *M. bryantii* grew logarithmically to an A_{600} similar to that found in regular S medium. However, in the presence of 20 mM $MgCl_2$, the cells did not abruptly lyse. In some experiments, the A_{600} remained fairly constant for a period of several days, whereas other times there was a steady decrease in the optical density (Fig. 1A). Microscopic examination of the culture revealed a gradual progressive increase in the proportion

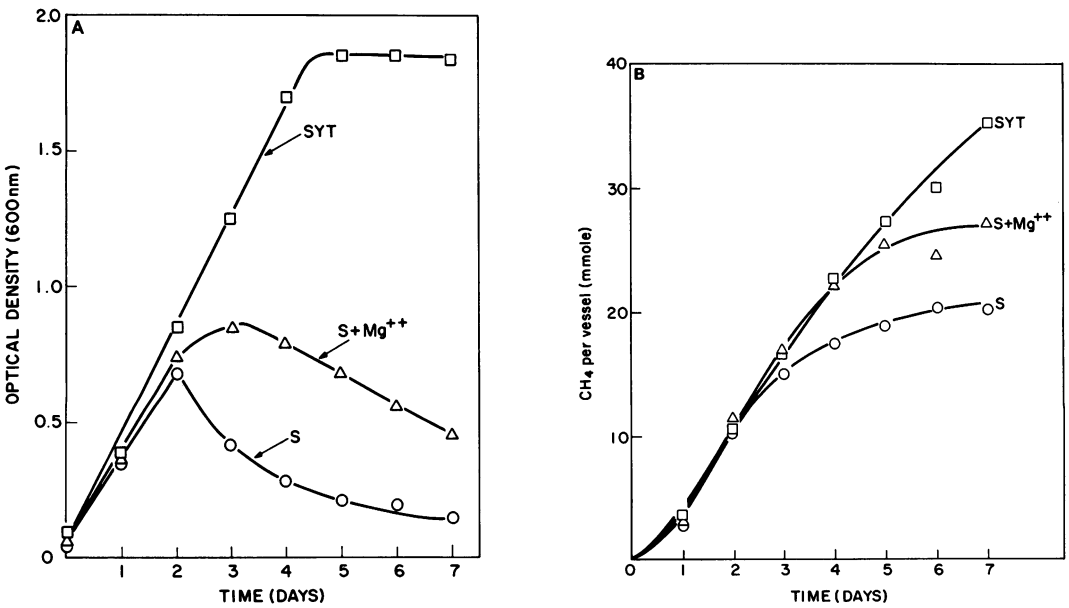

FIG. 1. Growth (A) and methane production (B) by *M. bryantii* in S medium (○), S medium plus 20 mM $MgCl_2$ (△), and SYT medium (□). Cultures were shaken and pressurized daily with H_2–CO_2 (4:1, vol/vol).

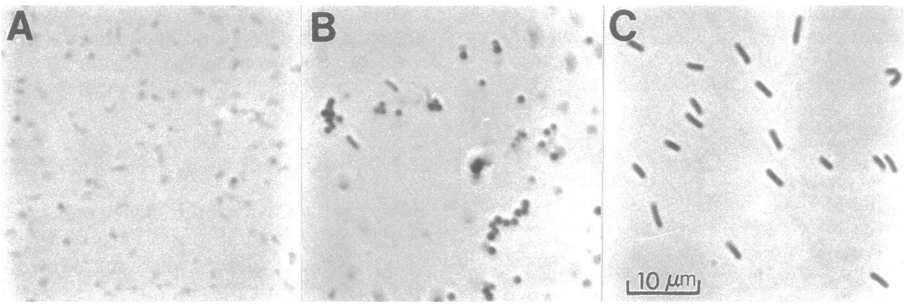

FIG. 2. Phase-contrast microscopy of *M. bryantii* after 1 week of growth in various media. A, S medium; B, S medium plus 20 mM $MgCl_2$; C, SYT medium.

of phase-dark spherical bodies to intact cells, so that usually by day 6, 2 to 3 days after the expected total lysis of the S grown culture, the population grown in Mg^{2+}-supplemented S medium existed almost totally in this protoplast state (Fig. 2B). In addition (Fig. 1B) total methane accumulated in the headspace of Mg^{2+}-supplemented S-grown cells was about 20% higher than that observed in S-grown cells.

The level of magnesium added to the medium to stabilize the protoplasts was optimum at 20 mM. At lower levels (up to 10 mM) the cells lysed, whereas at higher levels (40 to 100 mM) protoplast formation was greatly delayed, but did eventually occur. It should be noted that protoplast formation was sometimes delayed for 2 to 3 weeks even in S medium with 20 mM $MgCl_2$, whereas the control culture in S medium alone lysed after the usual 3 to 4 days. The reason for this is not clear, but it may have to do with small changes in the levels of other trace elements.

The sudden lysis of *M. bryantii* suggested that the cells may be starved of an essential component required for growth. As shown in Fig. 1A, *M. bryantii* grew to a much higher A_{600} (two- to threefold higher than in S medium, with a final cell yield of 4.2 g [wet weight] per liter) in S medium supplemented with 0.2% yeast extract and 0.2% tryptone (SYT medium). Methane accumulation was also substantially higher than in S-grown cells. Furthermore, cells grown in SYT medium did not lyse, even after 2 weeks. Microscopic examination of the culture indicated that the population remained as intact cells throughout the incubation period (Fig. 2C). When examined by fluorescence microscopy, SYT-grown cells were found to be fluorescent, a characteristic of methanogens (8, 19). In addition, the purity of the SYT-grown culture was shown by reversion to the expected pattern of growth and lysis when subcultured into S medium.

Electron microscopic examination of protoplasts. Figure 3 illustrates the phases in protoplast formation of *M. bryantii* grown in S medium supplemented with 20 mM $MgCl_2$. Figure 3A shows a cell with a virtually intact cell wall. Slight wall damage can be observed. Much more extensive damage can be seen in Fig. 3B and C, where the wall is shown separating from the inner membrane, and the protoplast is starting to emerge from the degraded wall. This emergence is continued in Fig. 3D through F. The outer wall is clearly left behind, and no outer wall material is observed on the protruding spherical body, leading us to believe it is a true protoplast. The protoplast seems to emerge wherever the wall damage is greatest—in one case (Fig. 3D) about one-third of the distance from the polar region, whereas in another case (Fig. 3E) apparently in the middle of the cell. Figure 3G shows the final stages of wall-protoplast association with small remnants of the wall still imparting some shape to the sphere. Figure 3H shows a spherical body apparently free of all cell wall attachment. One double-track membrane can be observed surrounding the protoplast.

Stability of protoplasts. When Mg^{2+}-stabilized protoplasts of *M. bryantii* were treated with the nonionic detergent Triton X-100 (final concentration of 0.2%, vol/vol), there was a large decrease in the A_{600}, suggesting lysis of the protoplasts. Immediate microscopic examination of the detergent-treated preparation confirmed that total lysis of the phase-dark protoplasts had occurred. When intact cells were treated in an identical manner, there was no drop in A_{600}, and no detectable changes in cell morphology were observed when the culture was examined in the phase-contrast microscope.

To test the stability of *M. bryantii* protoplasts in the presence of various cations, Mg^{2+}-stabilized protoplasts were centrifuged (10,000 × *g*, 10 min), and the resulting pellet was resuspended in distilled water or in 20 mM solutions (chloride salts) of various monovalent and divalent cations. Examination of the protoplast sus-

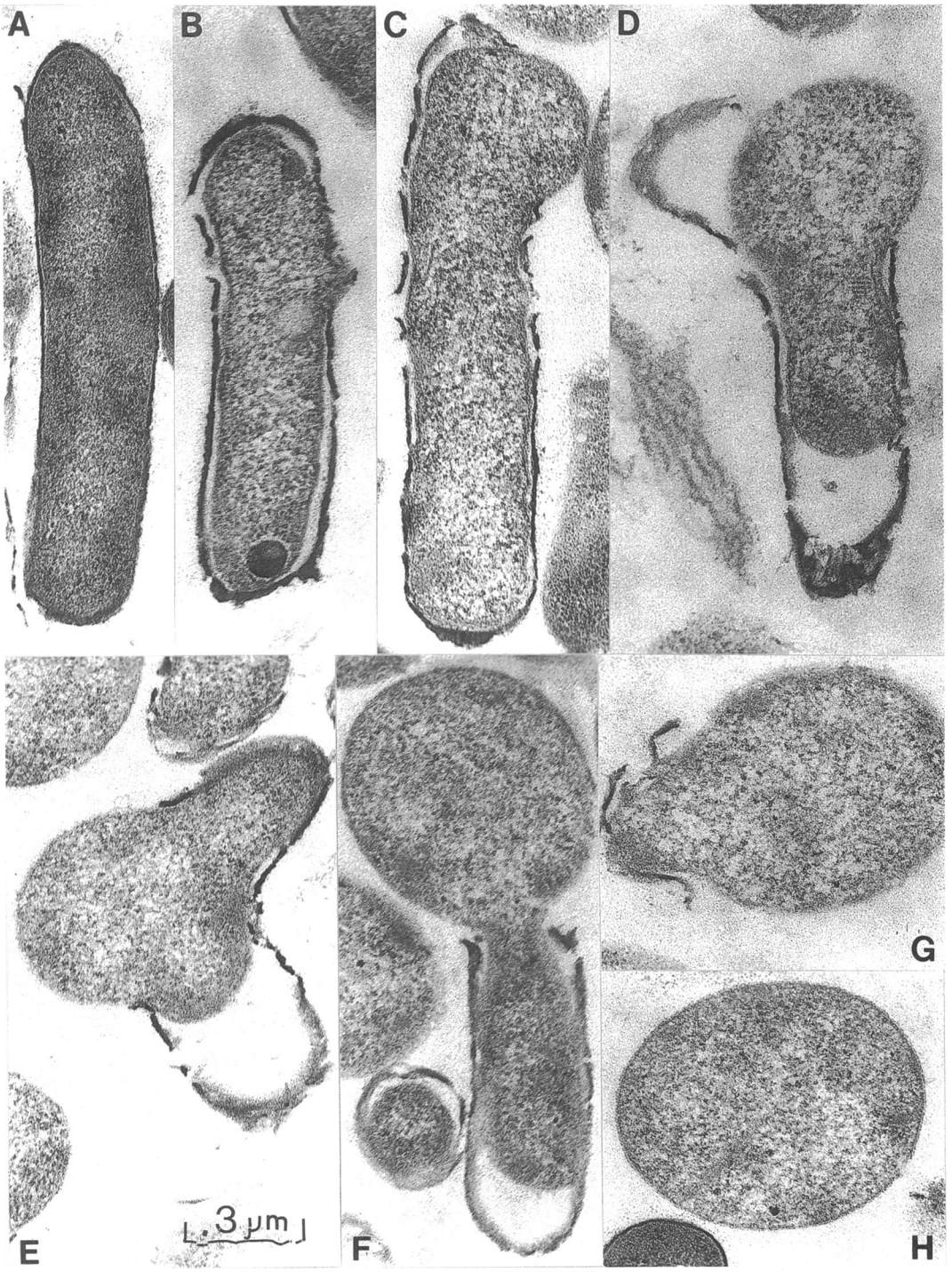

FIG. 3. Process of protoplast formation in *M. bryantii* grown in S medium plus 20 mM $MgCl_2$ as observed in thin sections. The sequence shows the increasing cell wall damage accompanied by the emergence of the internal protoplast (A, B, and C). The wall is clearly left behind (D, E, and F), and as the last cell wall fragments become detached (G), a spherical body free from all observable cell wall material (i.e., a true protoplast) results (H).

pensions after several hours indicated that the spheres were only stable in solutions of divalent cations. In distilled water or in the presence of monovalent cations, the phase-dark spheres were converted, by 6 h, to a mixture of cell debris, irregular-shaped phase-light membraneous bodies, and only a minority of phase-dark protoplasts. This process was noticeably underway even after 1 h. In some cases, especially with sodium, the presence of the monovalent cation seemed to stimulate more lysis than occurred in distilled water alone. In the presence of divalent cations, the suspension consisted mainly of phase-dark protoplasts even after overnight incubation at room temperature. Several of the divalent cations caused considerable aggregation of the protoplasts. This was especially striking in the case of Fe^{2+}, Co^{2+}, and Ni^{2+} and to a slight degree with Mn^{2+}. Mg^{2+} and Ca^{2+} did not cause any noticeable degree of aggregation; consequently, they are considered to be the ions of choice for protoplast stabilization.

Factors affecting the rapid lysis of *M. bryantii*. It was found that a modified S medium containing 30 mM potassium phosphate buffer, 40 mM NH_4Cl, 5 μM $NiCl_2 \cdot 6H_2O$, and 1.5 μM $Na_2MoO_4 \cdot 2H_2O$ resulted in much-improved growth yields (final A_{600}, ≈2.5; 5 g [wet weight] per liter). Furthermore, cells grown in modified S medium did not lyse during 2 weeks of incubation when the culture was shaken and pressurized daily with H_2–CO_2 (4:1, vol/vol).

Experiments were then performed to determine whether any one ingredient of the modified S medium could suffice for increasing the yields and preventing the lysis of *M. bryantii*. Growth of *M. bryantii* in S medium plus 40 mM NH_4Cl or in S medium containing 30 mM potassium phosphate and 1.5 μM $Na_2MoO_4 \cdot 2H_2O$ was essentially the same as that in unmodified S medium. On the other hand, S medium supplemented with 5 μM $NiCl_2 \cdot 6H_2O$ supported increased growth of *M. bryantii*, with an A_{600} of about 1.8, compared with approximately 2.5 in modified S medium. However, the cells still underwent a rapid lysis (Fig. 4). Additions of potassium phosphate, NH_4Cl, or $Na_2MoO_4 \cdot 2H_2O$ were made to S medium containing 5 μM $NiCl_2 \cdot 6H_2O$ to determine which of the three other components was necessary to prevent the rapid lysis of *M. bryantii* cells. It was only in S medium supplemented with $NiCl_2 \cdot 6H_2O$ and NH_4Cl that the *M. bryantii* culture grew to the high A_{600} of modified S medium without undergoing lysis (Fig. 5).

A dependence on nickel for growth is depicted for *M. bryantii* in Fig. 6. Nickel found contaminating the medium was less than 0.1 μmol/liter, the limit of detection of the method. Growth was

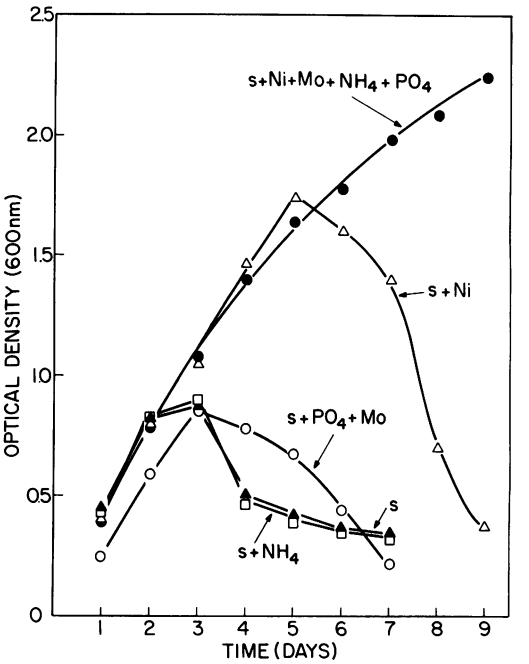

FIG. 4. Effect of various additions to S medium on growth of *M. bryantii*: S medium (▲), S medium plus NH_4 (□), S medium plus PO_4 and Mo (○), S medium plus Ni (△), S medium plus Ni, NH_4, Mo, and PO_4 (●). Cultures were shaken and pressurized daily with H_2–CO_2 (4:1, vol/vol). See text for final concentration of additions.

directly dependent on added nickel, with maximum growth obtained at a final nickel concentration of about 1 μM.

The rapid lysis of *M. bryantii* is not a general response to the limitation of an essential growth factor (Fig. 7). Cultures were grown for 3 days with daily pressurization with H_2–CO_2 (4:1, vol/vol). At this time the headspace was completely flushed and replaced with either H_2–CO_2 (4:1, vol/vol) or N_2. The control culture grew to an A_{600} of 2.9; the CO_2- and H_2-limited cells ceased growing immediately, but did not undergo a rapid lysis. There was only a small decrease in A_{600} over a period of 6 days under N_2.

DISCUSSION

The present report describes the first case of spontaneous protoplast formation in a methanogen. *M. bryantii* grown in S medium plus 20 mM $MgCl_2$ formed stable phase-dark spherical bodies which in thin section appeared to be free of cell wall material, a fact considered as evidence for the term "true protoplast" (17). In the absence of $MgCl_2$, the cells underwent a rapid lysis. Spontaneous spheroplast formation has been reported in the anaerobic gram-negative

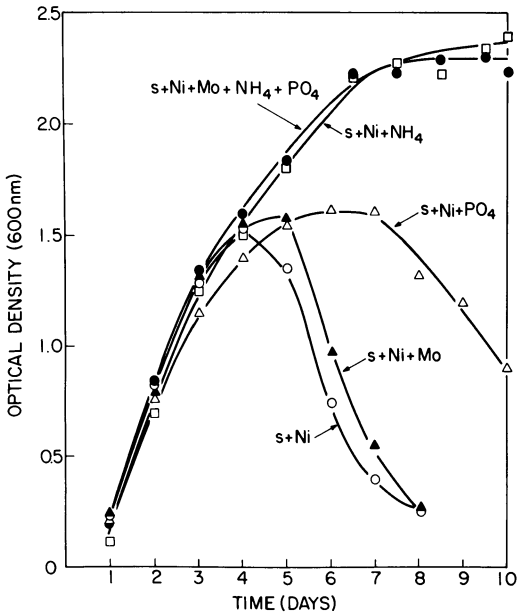

FIG. 5. Requirement for both nickel and ammonium to prevent lysis of *M. bryantii*. Cultures were shaken and pressurized daily with H_2–CO_2 (4:1, vol/vol). The following media were used: S medium plus Ni (○), S medium plus Ni and Mo (▲), S medium plus Ni and PO_4 (△), S medium plus Ni and NH_4 (□), S medium plus Ni, Mo, NH_4, and PO_4 (●). See text for final concentrations of additions.

bacterium *Bacillus ruminicola* (6), but with stationary-phase cells. Spontaneous protoplast formation has also been reported to occur in nature in ponds and as a response to senescence in laboratory culture (13). These are distinct from spontaneous protoplast formation in *M. bryantii*, since the lysis of *M. bryantii* in the absence of $MgCl_2$ and the stabilization of protoplast formation in the presence of $MgCl_2$ occurred before stationary phase was reached.

The growth and lysis of *M. bryantii* in S medium is reminiscent of the growth of a diaminopimelic acid-requiring strain of *Escherichia coli* in media deficient in diaminopimelate (18). In that case, lysis of cells occurred during logarithmic growth when diaminopimelate, an essential cell wall constituent, was exhausted from the medium. Lysis of the culture was hypothesized to occur through protoplast formation. In the case of *M. bryantii*, growth conditions leading to cell wall deterioration resulted in an osmotically labile intermediate state, presumably protoplasts. The presence of $MgCl_2$ in the medium prevented these protoplasts from quickly lysing once formed. Magnesium ions have been used to stabilize protoplasts and spheroplasts of many bacterial species (10, 14). The conversion of cells to protoplasts in S medium plus $MgCl_2$ occurred over a period of several days. This slow process is similar to the case in *Mycoplasma smegmatis* where treatment with lysozyme and methionine led to protoplast formation over a period of 2 weeks (25).

In *M. bryantii*, both NH_4^+ and Ni^{2+} were required to prevent the rapid lysis of the cells in a low-Mg^{2+} medium. Growth of *M. bryantii* in modified S medium deficient in either of these two components resulted in rapid lysis. The dependence on nickel for growth of *M. bryantii* is the second documented case in methanogens, the first being *Methanobacterium thermoautotrophicum* (20). In both cases, optimum growth was obtained at about 1 μM added nickel. The role of NH_4^+ and Ni^{2+} in the prevention of lysis is unknown, but presumably, like diaminopimelate, both are involved in cell wall synthesis. The formation of protoplasts is preceded by a general and extensive breakdown of the cell wall as observed in thin sections. Ammonium, being the sole nitrogen source, is presumably required as a structural component of the wall. A high content of nitrogen-containing compounds has been reported for *M. bryantii* walls (15). Nickel may be involved in the enzymatic synthesis of cell wall precursors, perhaps through factor F_{430}. Nickel is a component of F_{430} in *M. bryantii* (24), and the majority of ^{63}Ni taken up by these cells becomes associated with F_{430} (Jarrell and Sprott, unpublished data). A similar finding has already been reported for *M. thermoautotrophicum* (7). The lack of any essential growth factor did not cause the rapid lysis of *M. bryantii*, since cells starved for CO_2 and H_2 showed only a slow and minor drop in optical density over a period of several days.

The rapid lysis of *M. bryantii* could be pre-

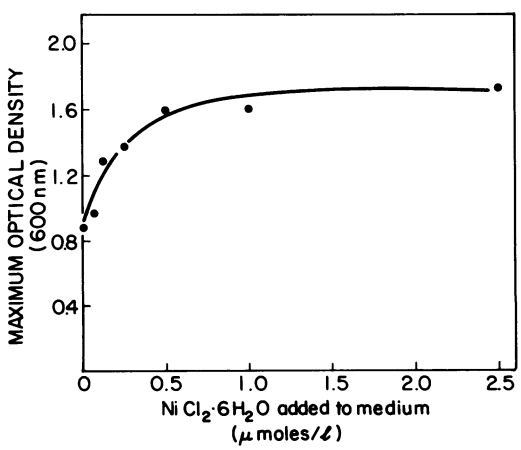

FIG. 6. Effect of added $NiCl_2 \cdot 6H_2O$ on the maximum cell concentration of *M. bryantii*. The contaminating level of nickel in the medium was less than 0.1 μM.

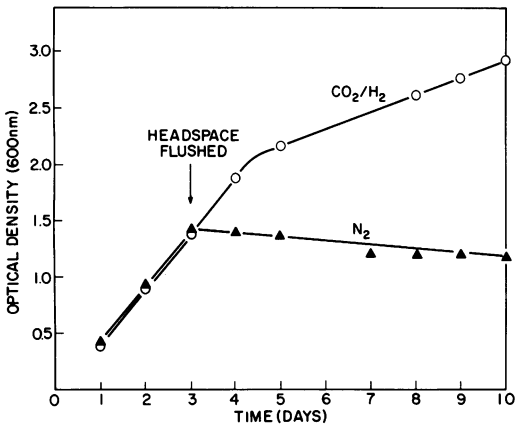

FIG. 7. Effect of CO_2 and H_2 limitation on the lysis of *M. bryantii* grown in S medium plus Ni, NH_4, Mo, and PO_4 (medium JM). After 3 days of growth in an atmosphere of H_2–CO_2 (4:1, vol/vol), the headspaces of the bottles were flushed and replaced with either H_2–CO_2 (4:1, vol/vol) or N_2.

vented by the addition of tryptone and yeast extract. Since *M. bryantii* is commonly grown in a medium containing yeast extract and trypticase (medium one [1]), this lysis phenomenon may not have been previously observed. Early growth studies with *M. bryantii* (4) did not show the lytic pattern of growth either, but the growth of cells in that medium was poor.

M. bryantii is now the second methanogen in which there is a way of releasing the outer cell wall. The other previous report, also from this laboratory (22), concerned the spheroplasting of *Methanospirillum hungatei* through the use of alkaline pH and dithiothreitol. That technique was not effective on any other methanogen genus tested. Lysozyme is ineffective against methanogen cell walls because of their unusual structural composition (15); hence, methanogen protoplasts and spheroplasts cannot be formed using the standard lysozyme-EDTA techniques.

The formation of protoplasts of *M. bryantii* by this simple and gentle technique should allow for the isolation and characterization of methanogen membranes under conditions of minimal damage. Protoplast formation and membrane isolation through osmotic lysis can all be done anaerobically at room temperature and neutral pH. The technique also avoids the use of chelating agents, wall degrading enzymes, and harsh mechanical disruption of cells. Such membrane vesicles should prove useful for chemical composition analysis, and in the localization of enzymatic and structural components of the wall and membrane fractions.

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