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Formation and Regeneration of *Methanococcus voltae* Protoplasts†

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Methanococcus voltae **cells were converted into protoplasts by suspension in anaerobic 0.1 M Tris-HCl buffer containing 0.4 M sucrose and 0.05 M NaCl as osmoprotectants. Protoplast formation was monitored microscopically by observing the conversion of the typical irregularly shaped (uneven peripheries) coccoid whole cells to rounded forms with smooth peripheries. Although the procedure resulted in about 50% lysis of the initial number of cells, the remainder were converted to the rounded form. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electron microscopy of negatively stained cell preparations indicated that the treatment removed the wall layer from whole cells to yield protoplasts. Protoplast regeneration was evaluated by using optimized plating conditions and an anaerobic microplating technique. Between 50 and 63% of the initial number of protoplasts regenerated as colonies on agar medium (35°C, 7 days). The colony and cell morphologies of the regenerated protoplasts were indistinguishable from those of whole cells plated under identical conditions.**

Methanococcus voltae is a moderately halophilic marine methanogen. It is attractive for genetic studies because it grows relatively fast, grows in defined media, and can be plated with consistently high efficiencies (14, 26). It is well characterized with respect to its nutritional requirements and its biochemical pathways (for a review, see reference 12); unlike many methanogens, it is sensitive to certain antibiotics at low concentrations (22). However, in the genetic studies to date, the observed transformation frequencies of *M. voltae* are very low (3, 8) compared with those of eubacteria, although an increase of at least 1 order of magnitude was recently achieved (20) by electroporation.

Genetic studies in many bacteria for which transformation systems are unavailable or are difficult may be made possible or improved by regeneration of transformed protoplasts or spheroplasts (10). However, because of the unique nature of the cell envelopes of methanogens (17), the methods developed for protoplast and spheroplast formation in eubacteria (10, 19) are generally not effective in methanogens.

There are several specific cases of protoplast formation in methanogens. Protoplasts form spontaneously during growth of *Methanobacterium bryantii* (11) and *Methanosarcina barkeri* (6) in specific media. Dithiothreitol at moderately alkaline pH can be used effectively to induce spheroplast formation by *Methanospirillum hungatei* (24) and, less efficiently, *Methanosaeta concilii* (4). Protoplasts of two unidentified coccoid methanogens form upon treatment with pronase (15), and *Methanobacterium thermoautotrophicum* forms protoplasts upon treatment with lytic enzyme from *Methanobacterium wolfei* (16). Induction of protoplast formation by *M. voltae* by 15 min of treatment with trypsin was reported (9); however, no evidence was presented to demonstrate that the method indeed yielded true protoplasts, and the efficiency of protoplast formation was not indicated. Importantly, in none of the above cases were the osmotically sensitive cells successfully regenerated.

Protoplasts in *Halobacterium* spp. have been obtained by exposing cells to a specific balance of osmoprotectants and salts (13). Since *Halobacterium cutirubrum* and *M. voltae* cells appear similarly sensitive to lysis by suboptimal concentrations of salts, we developed a protoplasting procedure based on that found effective for the extreme halophile. It is expected that this strategy may have general application to protoplast formation within numerous species of osmotically sensitive bacteria with S-layer envelopes. Methods for high-efficiency regeneration of *M. voltae* protoplasts to colonies composed of normal cells are also presented.

MATERIALS AND METHODS

Stock cultures. *M. voltae* PS (= NRC 2854) was obtained from L. Hook (Ohio State University) in 1983 and deposited in the National Research Council culture collection. Working cultures were maintained (35°C, static incubation) by weekly transfers (5%, vol/vol) into complex BV medium. The headspace of the 10-ml cultures in 60-ml serum vials (80% H_2–20% CO_2 gas phase) was repressurized to 10 lb/in^2 (69 kPa) every alternate day. The composition of the BV medium was similar to that of medium 3 described by Balch et al. (1), except that we used Bacto-Tryptone (Difco) in place of Trypticase and Na_2CO_3 in place of $NaHCO_3$ and altered the amounts of L-cysteine HCl, Na_2S, and molybdate. We did not supplement calcium pantothenate, but we did add tungstate, nickel, and selenite. The composition of BV broth medium was as follows (milligrams per liter): KCl, 340; $MgCl_2 \cdot 6H_2O$, 2,750; $MgSO_4 \cdot 7H_2O$, 3,480; NH_4Cl, 250; $CaCl_2 \cdot 2H_2O$, 140; K_2HPO_4, 140; NaCl, 18,000; $CH_3COONa \cdot 3H_2O$, 1,000; yeast extract, 2,000; Bacto-Tryptone, 2,000; $Na_2WO_4 \cdot 2H_2O$, 1.65; Na_2SeO_3, 1.5; $NiCl_2 \cdot 6H_2O$, 1.19; $Na_2MoO_4 \cdot 2H_2O$, 2.5; nitrilotriacetic acid, 15; $MnSO_4 \cdot H_2O$, 5; $FeSO_4 \cdot 7H_2O$, 6; $CoCl_2 \cdot 6H_2O$, 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; biotin, 0.02; folic acid, 0.02; pyridoxine-HCl, 0.1; thiamine-HCl, 0.05; riboflavin, 0.05;

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nicotinic acid, 0.05; vitamin B_{12}, 0.005; lipoic acid, 0.05; resazurin, 1; Na_2CO_3, 600; $Na_2S \cdot 9H_2O$, 220; L-cysteine-HCl, 220. The medium was reduced and anaerobically dispensed into serum vials (under an 80% H_2–20% CO_2 gas phase) as previously described (21). The postautoclaving pH of this medium after 24 h of equilibration at room temperature was 6.5 ± 0.1. Unless stated otherwise, the gas phase used in this study was 80% H_2–20% CO_2.

Protoplasting buffer preparation. The most suitable protoplasting procedure was obtained by using 0.1 M Tris-HCl buffer containing 0.4 M sucrose and 0.05 M NaCl. The Tris-HCl buffer (pH 7.3), containing 1 mg of resazurin per liter as an oxidation-reduction dye indicator, was dispensed (under N_2) into an Erlenmeyer flask modified so that it could be sealed with a butyl rubber stopper and an aluminum crimp. This and similarly dispensed stock solutions of 2 M sucrose and 1 M NaCl in pH 7.3 Tris-HCl buffer were autoclaved (121°C, 15 min). To 15 ml of the Tris-HCl buffer in a sterile 60-ml serum vial (N_2 gas phase) were added 4 ml of the sucrose stock solution and 1 ml of the NaCl stock solution. A 0.2-ml aliquot of H_2S gas was injected into the vial, and the contents were shaken vigorously to reduce the buffer (resazurin turned colorless). The vial headspace was flushed for 5 min with N_2 (to remove excess H_2S and thus avoid its precipitation as sulfide salts). The buffer could be stored at room temperature for several weeks. All gases used in the study were scrubbed free of traces of oxygen by passage over either hot copper or a chemical scrubber (Supelpure, Catalog no. 02-2450; Supelco Canada) in the case of H_2S.

Protoplasting. *M. voltae* cells were grown statically in BV medium (24 to 30 h, 35°C) to an A_{660} of 0.5 to 0.8 (1-cm cuvette, Perkin Elmer Coleman 575 spectrophotometer). The cells grew as a soft sediment that was easily dispersed by gentle shaking. This growth protocol avoided formation of clumps of cells that were otherwise difficult to disperse and caused problems in enumeration. The cells were harvested (7,700 × *g*, 10 min) aseptically and anaerobically (H_2-CO_2 gas phase) in 15-ml glass centrifuge tubes fitted with serum enclosures. Protoplasts were obtained by gently resuspending the cell pellet (H_2-CO_2 gas phase) into anaerobic protoplasting buffer. A control cell pellet was similarly dispersed into an identical volume of BV medium. The resuspended cells were diluted in BV medium supplemented with 1% (wt/vol) bovine serum albumin (BSA) for determining direct microscopic counts and for plating. The conversion of the irregularly shaped (uneven peripheries) coccoid cells to the rounded (spherical) form with smooth (even) peripheries was quantitated by direct microscopic counts (under phase-contrast optics) with a Petroff-Hausser counter (A. H. Thomas Co., Philadelphia, Pa.).

Methane production from H_2-CO_2 by whole cells and by protoplasts resuspended in 10 ml of BV medium supplemented with 1% (wt/vol) BSA was determined by headspace gas analysis (21). The headspaces of the 60-ml vials were repressurized to 10 lb/in^2 with H_2-CO_2 at hourly intervals during the 6-h incubation (35°C, static).

SDS-PAGE. Pellets from identical amounts of mid- to late-growth-phase cultures were resuspended into BV medium (control, whole cells) and protoplast buffer (protoplast cells) and centrifuged (7,700 × *g*, 10 min) aerobically. The supernatants were dialyzed overnight in distilled water (5°C) and then lyophilized. The whole-cell and protoplast cell pellets were resuspended into distilled water (causing rapid lysis) containing 50 μg of DNase per ml. The lysates were lyophilized, resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, loaded onto 12% acrylamide gels, and electrophoresed on a Mini-Protean II dual slab cell (Bio-Rad Laboratories, Richmond, Calif.). The gels were stained (45 min) at room temperature with 0.25% Coomassie blue in 50% methanol–10% acetic acid and destained in 30% methanol–10% acetic acid in distilled water.

The protein contents of lyophilized samples were quantitated by a modification (2) of the Lowry et al. assay with BSA as the standard.

Electron microscopy. Whole cells and protoplasts were fixed with glutaraldehyde and negatively stained with sodium phosphotungstate. A drop of whole cells in BV medium or protoplasts in protoplasting buffer was put on a Formvar-coated copper grid (200 mesh) for 30 s, and excess fluid was removed with the edge of a filter paper. The specimen was fixed (5 min) with 1% glutaraldehyde in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) and washed twice with the HEPES buffer. The wet specimen was then stained (1 min) with 1% sodium phosphotungstate (pH 7.2). The grids were observed in a Siemens 101 electron microscope (Siemens Electric) operating at 80 kV.

Growth in petri plates. For growth on solid media, the BV medium was supplemented with 1% (wt/vol) Noble agar (Difco Laboratories) and 20-ml aliquots of reduced medium were dispensed into 60-ml serum vials (H_2-CO_2 gas phase). The autoclaved medium was stored at room temperature. The medium was melted (5 min in the autoclave, 121°C, slow exhaust), cooled to 50°C, and introduced into an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.; gas phase of 5% CO_2–10% H_2–85% N_2) containing a palladium catalyst to remove any traces of oxygen. The vials were opened, and the agar (20 ml each) was poured into sterilized, disposable plastic petri dishes (100 by 15 mm; Fisher Scientific) that had been introduced into and exposed to the chamber atmosphere at least 2 days before the agar was poured. The lids of the poured plates were left partially off for 2 h, and then the plates were stored inverted (stacked four high) in the anaerobic chamber and used the following day. This 18- to 24-h storage and dehydration in the chamber atmosphere (30°C) before plating was ideal for maximum recovery of viable cells.

All plating was done inside the anaerobic chamber by dispensing 0.1-ml aliquots of appropriately diluted suspensions on the agar surface with sterile Eppendorf pipets. The inoculum was gently spread with sterile, bent glass rods (shaped like hockey sticks). The plates were inverted after 15 min and stacked 10 each into Brewer anaerobic jars. The plates in the jar were held in a specially constructed removable metal holder so that the stacked plates were not directly resting on one another. The sealed jar was removed from the anaerobic chamber and flushed out (5 min) with 79% H_2–20% CO_2–1% H_2S via two valves fitted into the lid. The jars were incubated at 35°C inside the anaerobic chamber. After 4 days of incubation, the negative pressure in the jars was equilibrated to the anaerobic chamber atmosphere; the sealed jars were brought out, flushed out with H_2-CO_2-H_2S and reincubated in the chamber to complete the 7-day incubation. This flushing procedure protected the palladium catalyst in the chamber from being poisoned by the H_2S.

The plating efficiency of whole cells was determined on the basis of the direct microscopic counts and the viable counts. The percentage of protoplasts that could regenerate as colonies was based on the direct microscopic count of the protoplast suspension and the number of colonies obtained

TABLE 1. Effects of sucrose and NaCl on *M. voltae* cells in 0.1 M (pH 7.3) Tris-HCl buffer[a]

Concn (M) of:		% Lysis	% Protoplasts[b]
Sucrose	NaCl		
0.200	0.000	>85	<15
0.400	0.000	50	10–20
0.500	0.000	50	10–20
0.000	0.100	>90	<5
0.000	0.500	>70	<10
0.400	0.010	50	30–40
0.400	0.025	50	50–60
0.400	0.050	50	>99
0.400	0.070	50	>99

[a] Cultures were harvested aerobically. Cell pellets were gently resuspended in buffer with the indicated supplements. Observations made after 15 min.
[b] The indicated percentages of the surviving cells were round with even peripheries (protoplasts), and the remainder appeared as typical irregular, coccoid whole cells with uneven peripheries.

by plating on agar, as well as by an anaerobic slide culture technique described below.

Growth on agar slides. An optimized method for plating of obligate anaerobes on microscope slides with BV agar medium (see above) was developed from the slide culture concept of Postgate et al. (23). Aliquots of 1 ml of melted medium were gently dispensed over the surfaces (ca. 38 by 26 mm) of presterilized slides (76 by 26 by 1 mm; Johns Scientific) inside the anaerobic chamber. The agar was allowed to dry for 15 min (chamber temperature, 30°C); then appropriately diluted aliquots of protoplast preparation (or whole cells) in BV–1% BSA were gently spread on the agar surface by slowly going back and forth two times with a bent glass rod. The slide was put inside an empty petri plate (like those used for other plating) containing a 70-mm-diameter Whatman no. 1 filter paper wetted with 0.75 ml of sterile, anaerobic water just before use. The petri plates containing the agar slides were placed in the Brewer anaerobic jars (without inverting) and incubated under H_2-CO_2-H_2S as described above for growth in petri dishes. At various time intervals, triplicate slides were brought out of each jar and observed under phase-contrast microscopy (Zeiss Universal Research microscope; Zeiss, Germany). The total number of single and multiple protoplasts (i.e., dividing protoplasts) and the number of protoplasts forming microcolonies were counted in several fields in each of the triplicate slides. These were used to determine the percentage of protoplasts that were regenerating as colonies on agar. Whole cells similarly inoculated on agar slides were used as a control for comparison purposes.

RESULTS

Development of protoplasting buffer. The effect of sucrose and NaCl on whole cells of *M. voltae* was determined aerobically in Tris-HCl buffer at pH 7.3. The results (Table 1) indicated that a combination of 0.4 M sucrose and 0.05 M NaCl produced an almost instantaneous conversion of the typical irregularly shaped (uneven peripheries) coccoid whole cells to those which were rounded (spherical) and had even (smooth) peripheries (i.e., protoplasts). These two forms were easily distinguishable by phase-contrast microscopy. Sucrose was required in the protoplasting buffer as an osmoprotectant (it could not be replaced by 0.5 M NaCl), and protoplast formation in the presence of 0.4 M sucrose

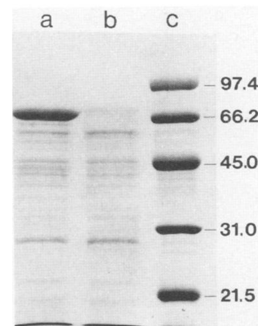

FIG. 1. SDS-PAGE analysis of the proteins from whole cells (lane a, 42.5 μg of protein) and protoplast buffer-treated cells (lane b, 27.5 μg of protein) of *M. voltae*. Lane c contains molecular mass standards (numbers to the right indicate kilodaltons).

was NaCl dependent (Table 1). Although the best salt-sucrose combination resulted in about 50% lysis of whole cells, those surviving were almost exclusively protoplasts. Protoplasts could also be obtained at pH 7.3 when the Tris-HCl buffer was replaced with either HEPES or morpholinoethanesulfonic acid buffer.

When the N_2 headspace gas in the anaerobic protoplasting buffer (0.1 M Tris-HCl, 0.4 M sucrose, 0.05 M NaCl, H_2S reduced) was replaced with 80% H_2–20% CO_2, the pH dropped gradually from 7.3 to a stable value of 6.6 in 45 min. However, buffer in the pH range of 5.5 to 8.0 did not have any observable affect on the amount of lysis or on the protoplast yield. Anaerobiosis per se also did not affect protoplast formation. Unless stated otherwise, all treatments to convert cells into protoplasts were henceforth done with anaerobic protoplasting buffer under the same 80% H_2–20% CO_2 gas phase (working pH, 6.8 ± 0.2) used for growth of *M. voltae*.

SDS-PAGE. The predominant protein that forms the regularly structured surface layer of *M. voltae* has a molecular mass of 76 kDa (18). This protein is easily detected by SDS-PAGE of the total extracts of whole cells (Fig. 1), but it is barely visible in extracts of the protoplasts. Also, the 76-kDa protein band was clearly visible in the supernatant of the protoplast buffer after removal of the protoplasts but was absent in the supernatant from control whole cells in BV medium (data not shown). This shows that the buffer treatment causes the removal of the regularly structured surface layer from whole cells, resulting in the formation of round cells with even peripheries (i.e., protoplasts). The faint band at 76 kDa in the protoplast extract (Fig. 1) could be due to contaminating wall material from the protoplasting buffer or to whole cells that may have remained unaltered by the buffer treatment.

Electron microscopy. The electron microscopic observations of negatively stained preparations of whole cells and buffer-treated cells (protoplasts) are shown in Fig. 2. Both cell preparations tended to lyse on the grids during the staining procedure; nevertheless, the wall layer is readily seen in whole cells (Fig. 2A) and is not discernible in the buffer-treated cells. These results confirm the SDS-PAGE data indicating that the buffer treatment results in the removal of the cell wall layer.

Stability of protoplasts. Leaving the protoplasts in the protoplasting buffer for prolonged periods resulted in extensive lysis of the protoplasts (15 and 60% in 0.5 and 2.5 h, respectively). Resuspending the protoplasts in BV medium

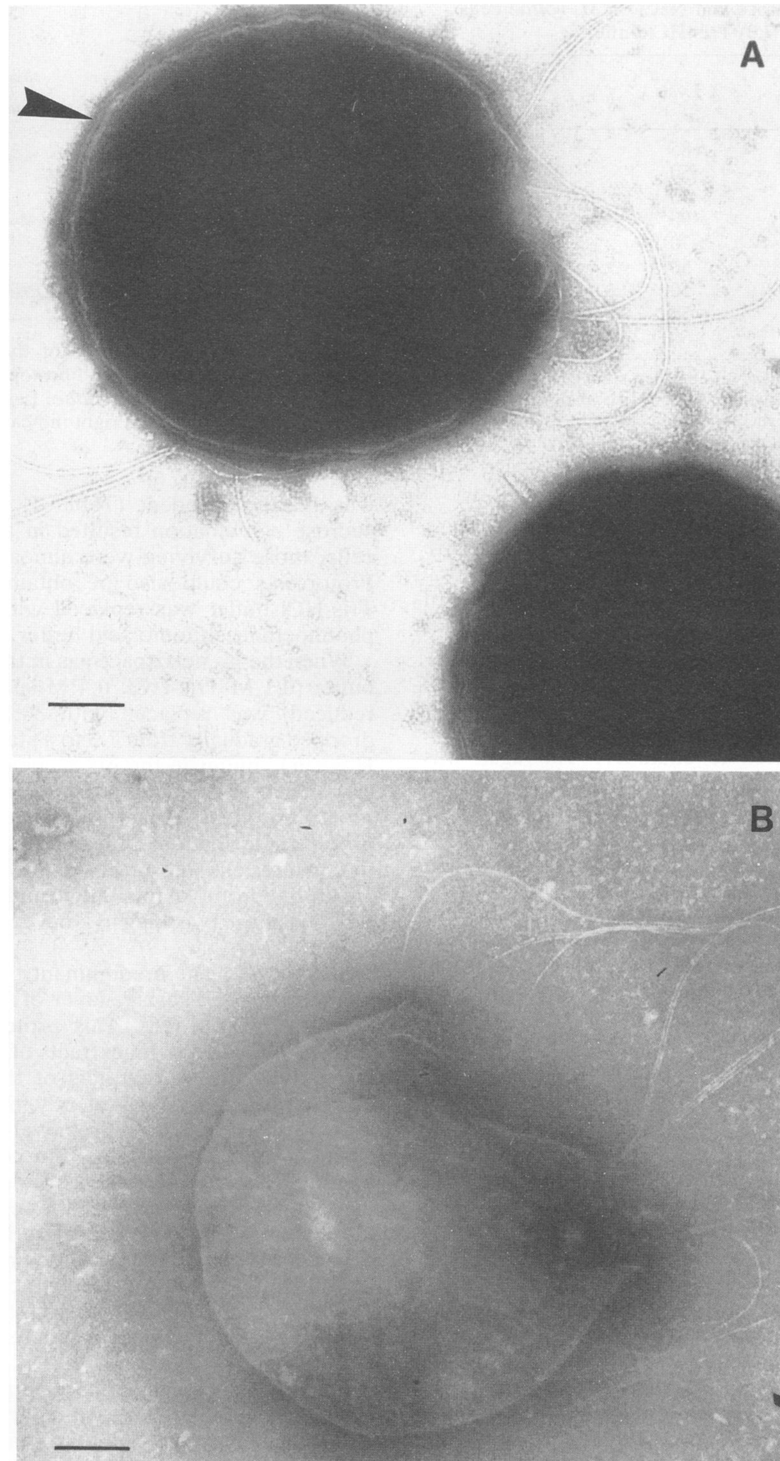

FIG. 2. Electron micrographs of preparations of negatively stained whole cells (A) and protoplast buffer-treated cells (B). Bars, 200 nm. Arrow, wall layer.

resulted in about 30% lysis within 0.5 h, and supplementing with 0.4 M sucrose did not prevent this lysis. However, inclusion of 1% BSA in the BV medium resulted in no observable decline in the number of protoplasts over a 2.5-h incubation at room temperature. Interestingly, the inclusion of 1% BSA in the buffer during the protoplasting procedure did not increase the yield of protoplasts. Therefore, to protect the protoplasts from lysing, they were resuspended into BV–1% BSA within 15 min of suspension in the protoplasting buffer. For the same reasons, BSA-supplemented

BV medium was used for dilution of the protoplasts for direct microscopic counts and for plating.

The metabolic activity of protoplasts was determined by monitoring methane production in BV–1% BSA, with whole cells under identical conditions as the control. The A_{660} of the protoplast suspension increased from 0.149 to 0.198, whereas that of whole cells increased from 0.297 to 0.405, in 6 h. The maximum methane production rate (0 to 2 h of incubation) per unit of initial A_{660} for the protoplasts was 96% of that (58.0 μmol/h/10 ml of culture) for whole cells.

Plating whole cells. The plating of *M. voltae* whole cells at high efficiencies has been reported (3, 14). However, these procedures were less than satisfactory in our laboratory. In our experience, the efficiency of plating *M. voltae* by an agar overlay technique with BV–0.6% agar for the overlay over BV–1% agar medium was only about 50%. Also, as with the soft agar pour plate technique (3), the average colony diameter was less than 0.5 mm (7 days, 35°C). Swirling the inoculum (14) placed on the surface of prepoured solidified agar plates was also found to be unsatisfactory. The colonies did not develop as well-isolated entities, tended to grow into one another (although the number of cells plated per plate was not sufficient to cause overcrowding), and yielded smaller colony sizes because of crowded growth.

The protocol described in Materials and Methods, spreading cells gently with a sterile bent glass rod, gave consistently greater than 90% plating efficiency for *M. voltae* whole cells, with colony diameters of 1 to 2 mm (7 days at 35°C). When the prepoured plates were stored in the anaerobic chamber for more than 24 h before use, the recovery of viable cells was lower and inconsistent, probably because of excessive drying affecting the initiation of growth on agar. The stacking of the plates in the jars in a special holder to prevent them from resting on one another during incubation was necessary for consistent recovery of viable cells and colony size from the top plates to the bottom plates. Our protocol would facilitate freer access of the H_2-CO_2-H_2S to the cells by minimizing the risk of the lower plates being sealed by a combination of the weight of plates on top and accumulating moisture on the petri dish lids caused by medium dehydration.

Protoplast regeneration. The procedures for regeneration of protoplasts on agar medium were the same as those for plating of whole cells. However, the regeneration was poor (<5%) when the protoplasts from the protoplasting buffer were directly plated on the agar (or plated after dilution in the same buffer). This problem was alleviated by stabilizing the protoplasts by diluting or resuspending them in BV medium supplemented with 1% BSA before plating. Also, the regeneration of protoplasts formed with buffer that was not reduced but only stored under oxygen-free N_2 was not possible. For high regeneration efficiencies, the protoplasting had to be done with buffer reduced with H_2S, so that the redox potential was at least sufficiently low (−110 mV) to keep the resazurin dye indicator colorless. With these precautions, the regeneration efficiency of the protoplasts, based on the direct microscopic count and the viable count in the agar plates, was between 50 and 63%. The colony and cell morphology (7 days, 35°C) of regenerated protoplasts were similar to those of the whole cells. The major protein band at 76 kDa (representative of the regularly structured wall layer), which was barely discernible in SDS-PAGE gels of the protoplast cell preparations, was clearly evident in protoplasts that had been allowed to regenerate for 24 to 36 h in BV broth containing 1% BSA (data not shown). This indicates that the regularly structured wall layer is present in regenerated protoplasts.

Although we did not see any shapes representative of typical whole cells with phase-contrast microscopy of the protoplast buffer-treated cells, it is possible that some whole cells were present. Since we were unable to develop an osmotic shock treatment that would lyse the protoplasts but not affect whole cells, we were unable to use such a differential plating procedure to confirm that all colonies from the protoplast buffer-treated cell suspension were indeed arising from protoplasts. Therefore, the regeneration efficiency was redetermined by using an agar slide technique to allow enumeration by microscopically observing the formation of microcolonies from protoplasts. For the slide culture technique to work, the protocol outlined should be strictly adhered to because deviations will affect agar thickness, medium dehydration, and hence plating efficiency.

Development of microcolonies from protoplast buffer-treated cells and from a control consisting of whole untreated cells by slide culture is shown in Fig. 3. At 0 h the whole cells on the agar slide appear slightly oval with uneven peripheries, whereas the protoplasts were round with smooth peripheries. At 6 h, the whole cells were more advanced toward microcolony formation than were protoplasts (numerous cells had apparently divided more than once). After 24 h, microcolonies were predominant in both preparations, except that the microcolonies from the whole-cell control samples were generally much larger than those from the protoplasts. The percentages of cells in the whole and protoplast cell preparation that were dividing (two or more cells) at each time period are given in Table 2. Compared with the protoplast preparation, a larger proportion of cells in the whole cell control sample were dividing at each observation period. After 30 h, the experiments were terminated because of zones of confluent growth in both preparations and because the agar on the slides was showing signs of dehydration. The cells in microcolonies derived from the protoplast and whole-cell preparations (24 h) were indistinguishable from one another as judged by phase-contrast microscopy. They appeared like typical whole cells.

DISCUSSION

We describe relatively mild and rapid conditions for obtaining protoplasts of *M. voltae*, and we demonstrate the regeneration of protoplasts at high efficiencies on agar media. Although protoplast and spheroplast formation in other methanogens has been reported (4, 6, 9, 11, 15, 24), to our knowledge the current work demonstrates the first successful regeneration of such cell forms in methanogens.

The cell wall of *M. voltae* is a regularly structured layer in a hexagonal array, and it consists of a major 76-kDa protein (18). SDS-PAGE showed that our buffer treatment removed this major protein from the regularly structured layer of *M. voltae*. Electron microscopy of negatively stained samples confirmed that the wall layer had been removed in our treated cells, resulting in formation of true protoplasts. Although the regular structure of the wall layer cannot be seen in negatively stained envelopes obtained from whole cells of *M. voltae*, the wall layer itself can be visualized at the edge of negatively stained specimens (18).

The procedure for protoplast formation in *M. voltae* described herein differed somewhat from other procedures. In contrast to the situation with the salt dilution method used for *Halobacterium* species (13), the pH of the protoplasting buffer (5.5 to 8.00) had no observable effect on protoplast

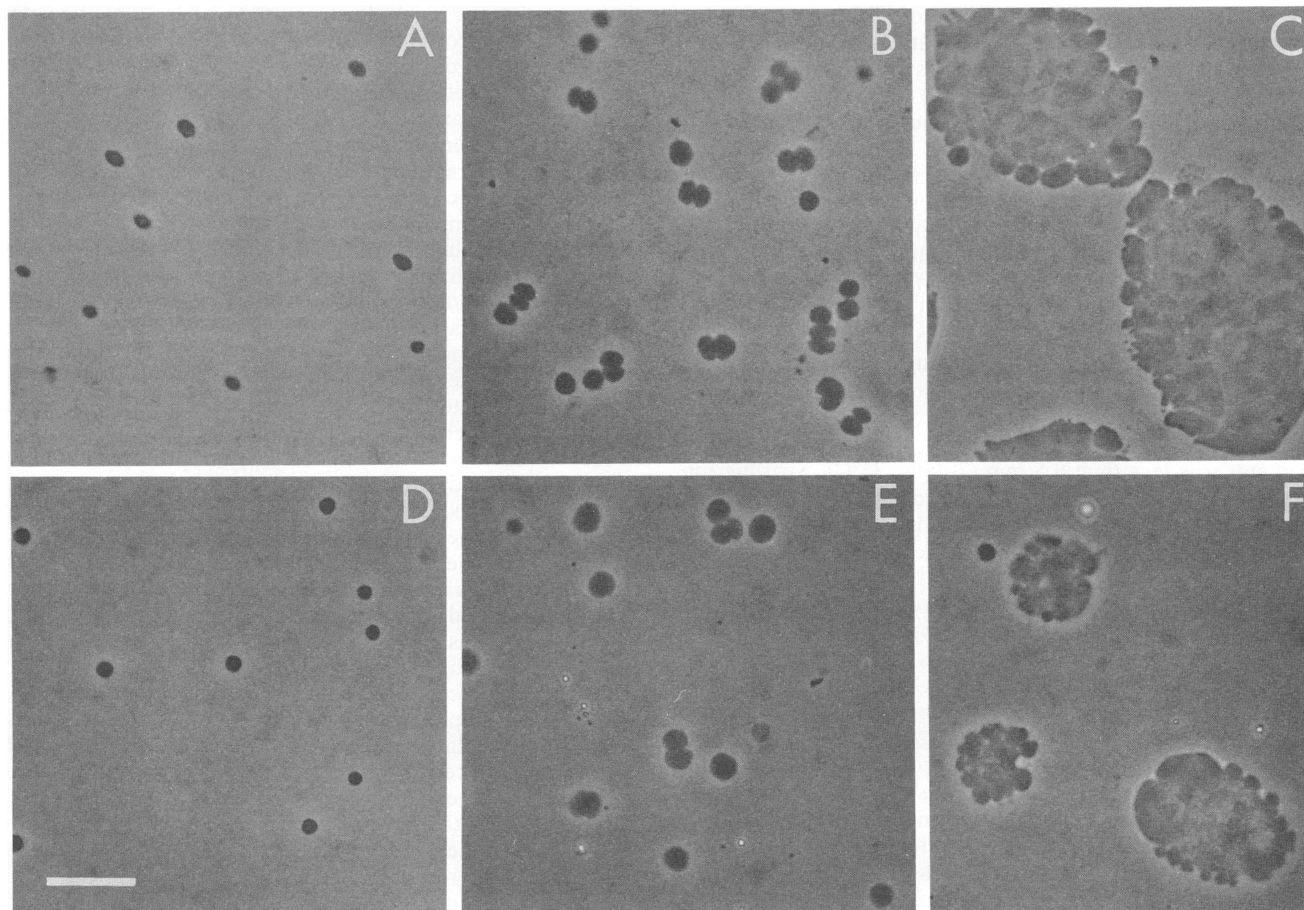

FIG. 3. Development of microcolonies of whole cells (A through C) and protoplast buffer-treated cells (D through F) on slide cultures after 0 h (A and D), 6 h (B and E), and 24 h (C and F) of incubation at 35°C. Bar, 10 μm.

formation in *M. voltae*. Also, comparison with other methods for protoplasting, such as treatment with lysozyme in rumen anaerobes (5), with pronase in methanogenic cocci (15), and with trypsin in *M. voltae* (9), revealed that *M. voltae* cells converted relatively rapidly to protoplasts. Finally, unlike the significant differences in lysis of *Fusobacterium varium* attributed to aerobic versus anaerobic conditions of lysozyme-EDTA treatment (5), the extent of lysis and protoplast formation per se in *M. voltae* was not affected by the aerobic or anaerobic status of the protoplasting buffer.

BSA has been used in protoplasting buffers for several microorganisms (13, 19). In *M. voltae*, BSA was not required in the protoplast buffer, but 1% BSA in BV medium stabilized the protoplasts. Such stabilized protoplasts were evaluated for methanogenic activity. In contrast to spheroplasts of *M. hungatei* (25) and protoplasts of methanococcal strains Göl and AJ2 (15), which produced methane at rates of 50, 55, and 8% of that of whole cells, respectively, the rates of methane production by *M. voltae* protoplasts (per unit of A_{660}) were nearly 100% of the whole cell rates.

TABLE 2. Growth of protoplasts and whole cells (controls) on agar slides

Time (h)	% Dividing cells ± SE (total no. counted)	
	Protoplasts	Whole cells
0.0	0 (99)	10 ± 2 (197)
6.0	15 ± 6 (104)	41 ± 6 (290)
24.0	63 ± 4 (129)	90 ± 3 (95)

We could not obtain well-isolated colonies of *M. voltae* when the inoculum was spread on the agar surface simply by swirling the petri plate (14). In contrast to the common expectation, *M. voltae* cells are rather resistant to shearing forces (12); we concur with this because we obtained >90% viable cell recovery from inocula spread on agar surface by means of bent glass rods. The lower-than-maximal recovery of *M. voltae* observed by Bertani and Baresi (3) could be attributable to factors other than the physical stress caused by using a glass rod to spread the inoculum. As with the soft agar pour plate method (3), colonies obtained in our agar overlay attempts were tiny (<0.5 mm) and difficult to visualize. Our unsatisfactory viable cell recovery with the agar overlay method could be due to a higher temperature of the overlay when it was used. Maintaining melted agar at a precise temperature inside the anaerobic chamber is cumbersome and difficult.

The plating technique reported here for whole cells of *M. voltae* was simplified from that of Jones et al. (14) to minimize manipulations required in an anaerobic chamber

(e.g., medium preparation). Unlike Jones et al. (14), who incubated plates under an H_2 gas phase and additionally supplemented it with H_2S, we used a premixed gas mixture consisting of 79% H_2–20% CO_2–1% H_2S.

The viability and recovery of isolated colonies of *M. voltae* whole cells have been shown to be severely affected by even trace levels (5 μl/liter) of oxygen in the anaerobic chamber (14). Our results indicate that the recovery of protoplasts is also severely affected by traces of oxygen, since it was impossible to regenerate protoplasts that were made with protoplast buffer that was only stored under N_2 rather than previously reduced with H_2S.

The amount of dehydration of agar in regeneration plates has been shown to be critical for successful protoplast regeneration in many species (10, 19). This is true for plating of protoplasts as well as whole cells of *M. voltae*.

In most published work, the proportion of normal cells present after the protoplasting treatment is stated to be less than 2% of the total (10). In *M. voltae*, we could not identify, by phase-contrast microscopy, any typical whole cells (i.e., irregularly shaped cocci with uneven peripheries) after treatment in the protoplast buffer.

BSA is often essential or beneficial in protoplast (7, 10, 19) and spheroplast (13) regeneration of many microorganisms. Successful regeneration of *M. voltae* protoplasts required the presence of 1% BSA in the diluent used for inoculation of the agar medium but not elsewhere in the protocol. *M. voltae* protoplasts plated directly from the protoplast buffer suspension did not grow as colonies.

The regeneration frequencies of protoplasts from different species have been shown to vary from almost undetectable to 50%, with most being in the 10 to 30% range (5, 10, 19). Up to 100% regeneration of protoplasts is rarely achieved (7). The 50 to 63% regeneration of *M. voltae* protoplasts to normal cells is not only the first successful regeneration in methanogenic bacteria but also a higher percentage than those normally achieved with many other microorganisms. The anaerobic slide culture method used for visualization of developing microcolonies from *M. voltae* protoplasts confirmed the high percentage of regeneration observed with the conventional plating technique.

The production of protoplasts in *M. voltae* could be helpful in the isolation of plasma membrane free of wall contamination and for studies on membrane-bound enzymes. The regeneration of protoplasts of *M. voltae* may be a useful tool in increasing the rates of transformation over the low natural transformation efficiencies obtained with whole cells. Protoplast regeneration may also be useful for studying the synthesis and reassembly of the regularly structured wall layer in *M. voltae*.

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