Spontaneous Protoplast Formation by *Methanosarcina barkeri*

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*Methanosarcina barkeri* strain FR-19 lysed spontaneously in substrate-depleted cultures. The addition of 0.3 M sucrose prevented complete lysis and resulted in the formation of osmotically sensitive UV-fluorescent spheres. Electron microscopic examination showed that complete degradation of the cell wall occurred before the release of true protoplasts, which were stabilized by sucrose and glucose (0.2-0.3 M) but not by glycerol. Exponentially increasing methane production and regeneration of protoplasts could not be demonstrated.

**INTRODUCTION**

*Methanosarcina barkeri* has an unusual morphology, growing as aggregates of randomly dividing daughter cells each surrounded by a cell wall which is continuous with the outer wall of the aggregate (Zeikus & Bowen, 1975). The cell wall is extremely thick (approximately 200 nm) and may be laminated. Chemical analysis of two strains of *M. barkeri* has indicated that heteropolysaccharide is the major structural component of the cell wall (Kandler & Hippe, 1977). The formation of viable single cells of *M. barkeri* during growth has not been reported and dispersal occurs by breakdown to small aggregates.

Genetic studies with *M. barkeri* are greatly hindered by its morphological characteristics, and by the lack of a cell wall degrading enzyme. The formation of viable single cells is needed so that pure clones of mutant strains can be selected, and the enzymic degradation of the cell wall would facilitate DNA extraction and protoplast formation, which are essential for developing artificial methods of gene exchange.

Lysis of *M. barkeri* in stationary phase cultures has been observed (Smith & Mah, 1978; Scherer & Sahm, 1981). The spontaneous release of osmotically sensitive cells resembling protoplasts in a stationary phase culture was reported for a gas vacuolate strain of *M. barkeri* (Archer & King, 1984). We describe here the spontaneous formation of protoplasts in substrate-depleted cultures of a poorly vacuolate strain of *M. barkeri*, and studies on the stabilization and regeneration of the protoplasts.

**METHODS**

**Bacterial strain.** *Methanosarcina barkeri* strain FR-19 was a derivative of *M. barkeri* strain FR-1 (DSM 2256) (Archer & King, 1983) which had arisen on repeated subculturing. FR-19 was poorly vacuolate and formed larger aggregates than the parent strain.

**Media and culture conditions.** All anaerobic media, supplements and solutions were prepared by the method of Hungate (1969). *M. barkeri* FR-19 was grown in MET 3 medium which contained (g l−1): Oxoid yeast extract (5), sodium formate (2), K2HPO4 (0-9), NaCl (0-9), (NH4)2SO4 (0-9), KH2PO4 (0-45), CaCl2.6H2O (0-2), MgSO4.7H2O (0-2), FeSO4.7H2O (0-01), resazurin (0-001) and 10 ml trace mineral solution (Archer & King, 1983). The medium was purged with N2/CO2 (4:1) for 5 min before being boiled under the same gas for 5 min. After cooling, anaerobic Na2CO3 (8%, w/v) was added to a final concentration of 0-2% (w/v) and the pH was adjusted to 6-7. For growth in small volumes, samples (5 ml) were dispensed under N2/CO2 (4:1) into screw-capped glass tubes fitted with butyl rubber septa (Belco, Vineland, NJ, USA), autoclaved, and reduced before inoculation by the addition of 0-1 ml of a solution containing Na2S.9H2O (1.7%, w/v) and cysteine hydrochloride (1.7%, w/v). Larger volumes of medium (50 or 200 ml) were prepared in Duran bottles (0-2 or 1 litre

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capacity (respectively) fitted with a septum and screw cap, and were reduced before autoclaving by the addition of Na₂S, 9H₂O (0.4 g l⁻¹) and cysteine hydrochloride (0.4 g l⁻¹). SM3R medium was MET 3 containing Casamino acids (2 g l⁻¹), tryptose (2 g l⁻¹), vitamin solution (10 ml l⁻¹) (Balch et al., 1979), gelatin (5%, w/v), MgCl₂, 6H₂O (10 mM) and sucrose (0.3 M); the latter was added after autoclaving. Solid SM3R medium was prepared by the addition of Difco agar (1%, w/v) and was reduced before autoclaving. Plates were incubated in stainless steel pressure vessels similar to that described by Balch et al. (1979). Methanol was added to media before inoculation to a final concentration of 1% (v/v). For growth on H₂ plus CO₂, the atmosphere of broth cultures and pressure vessels was replaced with H₂/CₐO₂ (4:1) at 202-65 kPa pressure. Media supplements and solutions of osmotic stabilizers were prepared in glass distilled water and filter sterilized under anaerobic conditions.

All manipulations were done in an anaerobic cabinet (Forma model 1024, Marietta, Ohio, USA) containing a N₂/CₐO₂/H₂ (7:2:1 by vol.) gas atmosphere. All apparatus was placed in the cabinet 24 h before use to remove all traces of oxygen. An inoculum of 2% (v/v) was necessary to ensure growth and incubation was static at 37 °C.

Measurement of growth. The production of methane, estimated as described by Archer & King (1983), was used as a measure of growth.

Measurement of methanol. The concentration of methanol in the culture medium was measured with a Pye 104 gas chromatograph equipped with a flame ionization detector. The 1-5 m column was packed with Chromosorb 101 (50-100 mesh) and was used at 165 °C; argon was the carrier gas and the detector temperature was 250 °C.

Light microscopy and cell counts. A Leitz Ortholux II microscope (Leitz, D-6330 Wetzlar FRG) was used for phase contrast and fluorescence microscopy (excitation at 420 nm). Numbers of aggregates, and of UV-fluorescent and non-fluorescent spheres, were calculated from triplicate counts on Thoma counting slides.

Electron microscopy. Samples for transmission electron microscopy were fixed overnight at 4 °C in anaerobic 2.5% (v/v) glutaraldehyde in cacodylate buffer (0.1 M, pH 7-2) containing 0.3 M sucrose. Post-fixation and embedding were as described by Lund et al. (1978). Thin sections were stained with 2% (w/v) uranyl acetate and examined on an AEI 801 electron microscope.

Stabilization of protoplasts. A freshly autolysed culture was passed through a Millipore filter with a pore size of 3 µm to remove remaining aggregates, and the number of UV-fluorescent protoplasts was counted. Samples (0-5 ml) of the suspension were centrifuged in sterile tubes in an Eppendorf centrifuge and resuspended in 0.25 ml of solutions of osmotic stabilizers. Counts of UV-fluorescent protoplasts were recorded after 0 and 24 h incubation at 37 °C in duplicate experiments.

RESULTS

Growth cycle of M. barkeri FR-19

Microscopic examination showed that the size of the aggregates increased markedly during exponential growth to the extent that a pellet was observed at the bottom of the culture vessel. Upon depletion of the methanol and the onset of the stationary phase (Fig. 1), autolysis of the aggregates commenced. An increase in the turbidity of the culture medium accompanied the disintegration of the aggregates, and was maximal when autolysis was complete at approximately 6 d after the stationary phase was reached. The resulting suspension in MET 3 medium consisted of single, phase-dark, UV-fluorescent spheres and phase-light, non-fluorescent vesicles. Aggregates were not observed, although due to the limitations of the counting procedure they could have been present at less than 6 × 10⁴ ml⁻¹. The phase-dark spheres were 1 to 1.5 µm in diameter and resembled protoplasts, but they were unstable in MET 3 medium and became phase-light and non-fluorescent. The phase-light spheres were, therefore, thought to be empty, membrane-bound vesicles.

The osmotic fragility of M. barkeri FR-19 at different stages in the growth cycle was determined by resuspending cells in either anaerobic glass distilled water or MET 3 medium, and subsequently comparing viability by measuring methane production from methanol in serial dilutions in MET 3 medium. Exponentially growing cells were unaffected by dilution in water, but stationary phase cells sampled before lysis were killed. This may suggest that cell wall degradation before lysis renders the cells osmotically sensitive, although lysis was not observed.

Stability of UV-fluorescent spheres

Filtering freshly autolysed cultures yielded a suspension of 10⁷ UV-fluorescent spheres ml⁻¹ in which no aggregates were observed, although they may have been present at a low concentration. Dilution of these suspensions with anaerobic, glass distilled water or addition of sodium dodecyl sulphate (1%, w/v) caused immediate lysis. Tests to identify osmotic stabilizers
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![Graph 1](image1)

![Graph 2](image2)

**Fig. 1.** Growth of *M. barkeri* FR-19 in 50 ml MET 3 medium containing methanol as substrate. ●, Methanol in culture medium; ■, total methane produced. Results are the means of duplicate experiments.

**Fig. 2.** Spontaneous protoplast formation by *M. barkeri* FR-19 in 200 ml MET 3 medium containing 0·3 M-sucrose. ●, Total methane produced; □, aggregates; ▲, protoplasts. Results are the means of duplicate experiments. Sampling times for the examination of cells by electron microscopy are indicated by arrows.

<p>| Table 1. Effect of osmotic stabilizers on protoplasts of <em>M. barkeri</em> FR-19 |</p>
<table>
<thead>
<tr>
<th>Stabilizer</th>
<th>Conc (M)</th>
<th>UV-fluorescent protoplasts*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h†</td>
</tr>
<tr>
<td>None (MET 3 medium only)</td>
<td></td>
<td>59-4</td>
</tr>
<tr>
<td>Glucose</td>
<td>0·1</td>
<td>6-6</td>
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<tr>
<td></td>
<td>0·2</td>
<td>56-5</td>
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<tr>
<td></td>
<td>0·3</td>
<td>58-0</td>
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<tr>
<td>Sucrose</td>
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<td>18-8</td>
</tr>
<tr>
<td></td>
<td>0·2</td>
<td>55-4</td>
</tr>
<tr>
<td></td>
<td>0·3</td>
<td>56-2</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0·1-0·3</td>
<td>0</td>
</tr>
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</table>

* Expressed as a percentage of the number of protoplasts in the original suspension which was prepared by filtering a freshly autolysed culture grown in MET 3 medium.
† Protoplasts were counted immediately after centrifugation and resuspension in the appropriate solution.

showed that centrifugation and resuspension caused a decrease in the number of UV-fluorescent spheres, even in effective osmotic stabilizers (Table 1). Incubation in MET 3 medium resulted in gradual lysis over 24 h, while glucose and sucrose were suitable osmotic stabilizers at 0·2 and 0·3 M (Table 1). Glycerol was not effective at 0·1 to 0·3 M. Stabilization of the UV-fluorescent spheres in MET 3 was achieved by the addition of 0·3 M-sucrose to this medium: 50% of the UV-fluorescent spheres were still present after incubation for 3 months at 37 °C.
Protoplast formation

MET 3 medium containing 0.3 M-sucrose was not inhibitory to growth and stabilized UV-fluorescent spheres during autolysis, thus allowing their formation to be monitored (Fig. 2). UV-fluorescent spheres were first observed in the late exponential phase of growth and release proceeded rapidly, with numbers increasing from 10^6 to 10^8 ml^-1 in 24 h. The formation of UV-fluorescent spheres was initially accompanied by an increase in the concentration of aggregates as breakdown to smaller units occurred, but was followed by a rapid decrease in numbers. Light microscopy showed that this was due to complete dissolution of the aggregates. No decrease in the number of UV-fluorescent spheres present in a filtered, autolysed culture grown in MET 3 medium containing 0.3 M-sucrose was observed during incubation at 37°C for 7 d, and 80% remained after incubation for 4 months.

Electron micrographs of cells sampled at different stages of the growth cycle (Fig. 2) clearly illustrate the lytic process. The thick heteropolysaccharide wall separating individual cells and surrounding aggregates in exponentially growing cultures (Fig. 3a, b) appeared to break down in stationary phase cultures and could be seen separating from the cell membrane (Fig. 3c, d). The osmotically sensitive UV-fluorescent spheres released were surrounded only by a cell membrane (Fig. 3e, f) confirming that they were true protoplasts. In electron micrographs the cytoplasm of protoplasts appeared to be more granular than that of exponential or stationary phase cells.

Regeneration of protoplasts

In order to establish the conditions needed for regeneration of protoplasts, growth of M. barkeri FR-19 was tested in MET 3 broth containing 0.3 M-sucrose with various additional supplements known to promote protoplast regeneration in other micro-organisms. Gelatin alone was inhibitory to growth at 5% (w/v) and in the presence of 20 mM-CaCl_2, NaCl and KCl, but not with added MgCl_2 (20mM). No inhibition was caused by the addition of Casamino acids, tryptase and vitamins and thus a rich, hypertonic medium, SM3R, was developed (see Methods). Normal growth and protoplast formation occurred in SM3R broth, and protoplasts were osmotically stable for 3 months at 37°C. SM3R agar also supported colony formation by aggregates of M. barkeri FR-19, although plating efficiency was variable (0 to 90%).

Prolonged incubation (up to 4 months) of protoplasts from MET 3 containing 0-3 m-sucrose or SM3R grown cultures diluted in fresh SM3R broth, with either methanol or H_2/CO_2 as substrate, never resulted in an exponentially increasing rate of methane production or the formation of aggregates of new cells. Methanogenesis was detected after incubation for 6 h and occurred at a constant rate for up to 14 d but then ceased. The number of UV-fluorescent protoplasts remaining at the end of the 4 month incubation period was approximately 10^8 ml^-1 in all treatments. Similarly, colonies were not formed from 10^8 protoplasts on the surface of SM3R agar, or in soft SM3R agar (0.75%) overlays, with either substrate. Addition of bovine serum albumin (1%, w/v) to SM3R medium was also unsuccessful in promoting regeneration.

DISCUSSION

Protoplast formation from M. barkeri has not been achieved by artificial means. This study has confirmed that true protoplasts are released during autolysis of substrate-depleted cultures of M. barkeri FR-19, and has shown that complete degradation of the thick cell wall occurs. Isolation of a cell wall autolysin might facilitate artificial protoplast formation, but cell wall lytic activity could not be demonstrated in supernatants of autolysed cultures of M. barkeri FR-19 (unpublished results) or the parent strain FR-1 (D. B. Archer, unpublished results). Such enzymes may, however, be unstable or tightly bound to their substrate. The extraction of walls of late exponential phase cells of M. barkeri FR-19 with LiCl, which has been shown to release wall bound autolysins from Streptococcus faecalis (Pooley et al., 1970) and Bacillus subtilis (Fan, 1970), was also unsuccessful (unpublished results).

Thiol-enhanced spheroplast formation has been reported for the methanogen Methanospirillum hungatei (Sprott et al., 1979) and for protoplast formation from Saccharomyces fragilis (Davies & Elvin, 1964). Several thiols (β-mercaptoethanol, dithiothreitol and cysteine) caused
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Fig. 3. Electron micrographs of cells of *M. barkeri* FR-19 at different stages of the growth cycle (see Fig. 2). (a, b), Exponentially growing cells; (c, d), stationary phase cells; (e, f), protoplasts. Bars, 0.5 μm; CW, cell wall; CM, cell membrane.
release of protoplasts from exponential phase cells of *M. barkeri* FR-19 but this effect was not reproducible, and regeneration of the protoplasts was not achieved (unpublished results).

Spontaneous protoplast formation has been reported for only one other species of methanogen, *Methanobacterium bryantii* (Jarrel et al., 1982), and was induced in the exponential phase by limiting NH$_4^+$ and Ni$_{2+}$ in the growth medium. For this species divalent, but not monovalent, cations were effective osmotic stabilizers and sugars were not tested. Spheroplasts of *M. hungatei* released following treatment with dithiothreitol at pH 9 were stable in 0.5 M-sucrose for several hours (Sprott et al., 1979).

Stabilized protoplasts of *M. barkeri* FR-19 retained their UV-fluorescence and were metabolically active (as measured by production of methane) for at least one week. This may suggest that the protoplasts were viable but were not able to regenerate in the conditions tested here. Both gelatin and bovine serum albumin have improved the regeneration of protoplasts of eubacterial species, for example *Bacillus subtilis* (Gabor & Hotchkiss, 1979) and the anaerobe *Clostridium acetobutylicum* (Allcock et al., 1982); however, in view of the unique characteristics of the methanogenic archaea bacteria, in particular the unusual biochemical structure of the cell wall and membrane, special requirements may be necessary for regeneration (Balch et al., 1979). On the other hand, the granular appearance of the cytoplasm in electron micrographs of thin sections, and the large proportion of protoplasts lysing during centrifugation and resuspension in osmotic stabilizers, may suggest senescence of the cells concomitant with the induction of cell wall autolysins.

Viable single cells of *M. barkeri* are needed for genetic studies. Although protoplasts of *M. barkeri* FR-19 were not proven to be viable, osmotic stabilization of protoplasts in the genus *Methanosarcina* has been demonstrated and the technique has provided a means of gentle lysis of cells for the isolation of high molecular weight DNA (J. E. Harris, unpublished results).

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REFERENCE


