

GELRITE as an Agar Substitute for the Cultivation of Mesophilic *Methanobacterium* and *Methanobrevibacter* Species

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GELRITE was evaluated as a gelling agent for the growth of mesophilic *Methanobacterium* and *Methanobrevibacter* species. GELRITE was shown to be superior to agar in its gel strength and clarity. Viable cell counts and colony sizes of *Methanobacterium* species were greater on GELRITE-based medium compared with agar-based medium.

The removal of hydrogen by species of methanogenic archaeobacteria belonging to the genera *Methanobacterium* and *Methanobrevibacter* is crucial to the efficient operation of anaerobic digesters (1). Genetic manipulation of these obligately anaerobic organisms should be valuable in understanding and improving the process, and thus suitable methods of cultivation on solid medium are required.

Major advances in anaerobic techniques have led to reproducibly high plating efficiencies for methanogens with short generation times, that is, 90% for the marine species *Methanococcus maripaludis* and *Methanococcus voltae* (5) and greater than 80% for the thermophile *Methanobacterium thermoautotrophicum* (6). Plating abilities of the slower growing mesophilic methanogens have been less well studied, although recoveries of 80% have been reported for *Methanobrevibacter arboriphilus* and *Methanosarcina barkeri* (6). In these previous studies, it was found advantageous to use concentrations of agar of about 1.5% (wt/vol), since higher levels were inhibitory to growth (5), and the overlay method of plating which protected bacterial cells against traces of oxygen and prevented smearing of colonies by condensing water during incubation (6). In this laboratory, an agar concentration of 1% (wt/vol) has proved most suitable for the growth of mesophilic *Methanobacterium* and *Methanobrevibacter* species, but the disadvantages of this plating regimen are severalfold. Firstly, the low gel strength attained with 1% (wt/vol) agar and the wetness of the plates give unsatisfactory replica plating and often result in the formation of gas cavities in the medium during evacuation and repressurization of anaerobic pressure vessels used for incubation of plates. Secondly, colony formation is very slow, with some strains requiring incubation for up to 4 weeks to reach a size suitable for counting, and small colonies are difficult to enumerate against the opaque agar medium. Lastly, viable counts show considerable variability and are thus unreliable. For these reasons, this study has examined the suitability of GELRITE gellan gum (Kelco Div., Merck & Co., Inc., San Diego, Calif.) as an alternative gelling agent for the growth of mesophilic *Methanobacterium* and *Methanobrevibacter* species. GELRITE, previously known as PS-60, is a deacetylated polysaccharide produced by *Pseudomonas elodea* (3) and has already been shown to compare favorably with agar for the growth of clinically important (9) and thermophilic (8) eubacteria.

The organisms studied are shown in Table 1. MET 3 medium was used throughout (4) and supplemented with

2-mercaptoethanesulfonic acid (coenzyme M) to a final concentration of 0.1 µg/ml for growth of *Methanobrevibacter ruminantium*. GELRITE-based medium was prepared in the same way as agar medium (4), but special care was taken to ensure adequate dispersal of the gelling agent before heating, and adjustment of pH and addition of reducing agents was carried out at 70°C since solidification occurred below this temperature. Dispensing of solid medium and all manipulations of cultures were carried out in an anaerobic cabinet (model 1024, Forma Scientific, Mallinckrodt, Inc., Marietta, Ohio) under an atmosphere of N₂-CO₂-H₂ (7:2:1), and equipment for plating of cultures was stored in the cabinet for at least 24 h before use. Agar and GELRITE plates (9 cm diameter) containing not less than 25 ml of medium were allowed to dry inverted at 37°C for 30 min before plating. The substrate for growth of all strains was H₂-CO₂ (4:1) at 203 kPa.

A late-exponential-phase culture of each species incubated static at 37°C (30°C for *Methanobrevibacter arboriphilus*) was mixed vigorously, and a sample was withdrawn for direct estimation of CFU on a Helber counting chamber. Duplicate samples (20 µl) of serial dilutions made in MET 3 broth were spread on the surface of medium solidified with either 1% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) or GELRITE, allowing four samples per plate. Plates were incubated in stainless-steel pressure vessels similar to those of Balch et al. (2) containing 50 g of silica gel to remove condensing water. The number of viable CFU were recorded when all colonies on the highest dilutions were an adequate size for counting. Mean colony size was determined from the diameters of 20 well-separated colonies on the high dilutions and measured with a graduated rule with the aid of a magnifying glass.

Viable cell counts of *Methanobacterium* and *Methano-*

TABLE 1. Mesophilic methanogenic bacteria used in this study

Organism and strain	DSM no. ^a
<i>Methanobacterium bryantii</i> M.o.H.G.	862
<i>Methanobacterium formicicum</i> MF	1535
<i>Methanobacterium</i> sp. strain FR-2	2257
<i>Methanobrevibacter smithii</i> PS	861
<i>Methanobrevibacter ruminantium</i> M1	1093
<i>Methanobrevibacter arboriphilus</i> AZ	744

^a DSM, Deutsche Sammlung von Mikroorganismen Göttingen, Federal Republic of Germany.

TABLE 2. Viable cell counts and colony sizes of *Methanobacterium* and *Methanobrevibacter* species on medium solidified with agar or GELRITE

Organism	Expt	CFU on dilution counted (mean \pm SD)		CFU/ml		Days of incubation	Colony diam (mm) (mean \pm SD)	
		Agar	GELRITE	Agar	GELRITE		Agar	GELRITE
<i>Methanobacterium bryantii</i>	1	1 \pm 0	3.5 \pm 0.5	5 \times 10 ⁶	1.75 \times 10 ⁷	19	0.3 \pm 0.2	0.6 \pm 0.2
	2	20.5 \pm 1.5	3.5 \pm 1.4	1 \times 10 ⁷	1.75 \times 10 ⁸	28	0.8 \pm 0.2	1.2 \pm 0.2
<i>Methanobacterium</i> sp. strain FR-2	1	3.5 \pm 2.5	6.5 \pm 1.8	1.75 \times 10 ⁷	3.25 \times 10 ⁷	14	0.3 \pm 0.2	1.5 \pm 0.1
	2	1 \pm 1	20 \pm 3	5 \times 10 ⁶	1 \times 10 ⁷	14	1.0 \pm 0.1	1.4 \pm 0.1
<i>Methanobacterium formicicum</i>	1	14 \pm 2	7 \pm 5	7 \times 10 ⁶	3.5 \times 10 ⁷	19	0.2 \pm 0.1	0.5 \pm 0.2
	2	2 \pm 1	19 \pm 3	1 \times 10 ⁶	9.5 \times 10 ⁸	14	0.5 \pm 0.3	0.8 \pm 0.2
<i>Methanobrevibacter smithii</i>	1	8.5 \pm 1.5	8.5 \pm 0.5	4.25 \times 10 ⁹	4.25 \times 10 ⁹	14	1.2 \pm 0.2	1.0 \pm 0.2
	2	1 \pm 0	3 \pm 1	5 \times 10 ⁷	1.5 \times 10 ⁸	14	1.2 \pm 0.2	1.0 \pm 0.1
<i>Methanobrevibacter arboriphilus</i>	1	6 \pm 0	2 \pm 0	3 \times 10 ⁸	1 \times 10 ⁸	17	0.3 \pm 0.1	0.4 \pm 0.1
	2	3.5 \pm 3	7 \pm 5	1.8 \times 10 ⁴	3.5 \times 10 ⁹	21	0.2 \pm 0.2	0.9 \pm 0.1
<i>Methanobrevibacter ruminantium</i>	1	2 \pm 0	21 \pm 4	1 \times 10 ⁷	1 \times 10 ⁹	14	1.7 \pm 0.5	1.3 \pm 0.3
	2	7.5 \pm 2.5	12.5 \pm 10.5	3.8 \times 10 ⁴	6.25 \times 10 ⁸	14	1.0 \pm 0.2	1.4 \pm 0.4

brevibacter species on medium solidified with agar or GELRITE at 1% (wt/vol) are shown in Table 2. All *Methanobacterium* species consistently showed greater recoveries on GELRITE-based medium. Viable counts on medium solidified with agar were 0.1 to 54% that on GELRITE, although *Methanobacterium* sp. strain FR-2 showed better recoveries than *Methanobacterium bryantii* and *Methanobacterium formicicum*. Marked differences in plating ability were also observed with the *Methanobrevibacter* species, although recovery was similar for both gelling agents in some experiments. The results with *Methanobacterium formicicum*, *Methanobrevibacter arboriphilus*, and *Methanobrevibacter ruminantium* clearly illustrate the variability of plating efficiencies encountered with agar-based medium.

Calculation of plating efficiencies from total counts of CFU determined microscopically often gave values in excess of 100%. Microscopic counts are subject to several sources of error (7), and it was extremely difficult to make accurate estimations of numbers of CFU for *Methanobrevibacter smithii* and *Methanobrevibacter ruminantium*, which have very small cells. The filamentous growth morphology of all strains may also lead to variability in viable counts, since filaments could break up during dilution and plating. However, comparison of viable counts with total microscopic counts and growth in the serial dilution tubes indicated that plating efficiencies approached 100% for all species on GELRITE-based medium.

Colony sizes of each *Methanobacterium* species were consistently larger on medium solidified with GELRITE (Table 2), by a factor of up to three times. This was not the case with the *Methanobrevibacter* species; colonies of *Methanobrevibacter smithii* were marginally larger on agar-based medium, while those of *Methanobrevibacter arboriphilus* were smaller. *Methanobrevibacter ruminantium* produced large colonies with both gelling agents. Thus, the use of GELRITE as the gelling agent reduced the incubation time necessary for the formation of colonies; for example, viable counts of *Methanobacterium* sp. strain FR-2 on medium prepared with GELRITE could be determined after 7 days of incubation compared with at least 14 days for agar. These results also suggest that the agar type used is inhibitory to growth of the *Methanobacterium* species tested here. Colony size of *Methanococcus voltae* was reported to decrease with increasing agar concentration, while that of *Methanococcus maripaludis* was unaffected (5). The effect of inhibitory compounds present in agar may also account

for the variability in viable counts observed on agar-based medium. GELRITE is highly purified, contains no sulfur, and has been reported to be less inhibitory than agar to the growth of plant tissue cultures (3).

In the course of this study it was noticed that MET 3 medium solidified with GELRITE was optically clearer and drier than agar at an equivalent concentration, thus reducing smearing of colonies by condensing water. Enumeration and measurement was consequently more accurate. The higher gelling temperature of GELRITE reduced the preparation time of plates, and the greater gel strength facilitated spreading of inocula and prevented the formation of gas cavities in the medium. Replica plating was also greatly improved with GELRITE at 1% (wt/vol).

This study has shown that GELRITE is superior to agar as a gelling agent for the cultivation of mesophilic *Methanobacterium* and *Methanobrevibacter* species in several respects. GELRITE decreases preparation time in the solidification and drying of plates and produces an optically clearer, drier medium of high gel strength suitable for replica plating. Increased counts of viable CFU on GELRITE-based medium especially for *Methanobacterium* species will be advantageous in genetical studies, for example, in the isolation of mutants or selection of transformants. The use of GELRITE will also markedly reduce the extended incubation times necessary for colony formation of mesophilic *Methanobacterium* species.

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