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High Efficiency of Plating of the Thermophilic Sulfur-Dependent Archaebacterium *Sulfolobus acidocaldarius*

E. BÖRJE LINDSTRÖM* AND H. MIKAEL SEHLIN

Unit for Applied Cell and Molecular Biology, University of Umeå, S-901 87 Umeå, Sweden

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A procedure for plating *Sulfolobus acidocaldarius* using Gelrite as the gelling agent is presented. The technique uses a supporting gel of 0.8% (wt/vol) Gelrite and an overlay soft gel of 0.4% (wt/vol) Gelrite, in which the colonies are grown. The plating efficiency was essentially 100%.

In our laboratory we are studying the thermophilic sulfur-dependent archaebacterium *Sulfolobus acidocaldarius* BC (3). This strain can use either iron or a reduced inorganic sulfur compound as an energy source. It is also capable of growing in liquid media supplemented with yeast extract or Casamino Acids (Difco Laboratories, Detroit, Mich.) or both. However, attempts to grow *S. acidocaldarius* on solid media have been troublesome. We have tried agar techniques without success. However, we have obtained colonies by using 12% (wt/vol) starch (Merck & Co., Darmstadt, Federal Republic of Germany) both with and without a 9% (wt/vol) soft layer (W. Zillig, personal communication), but the plating efficiency was low. Moreover, the starch medium dried quickly and often cracked upon prolonged incubation.

Thermophilic bacteria have recently (1, 2) been cultured on solid media by using Gelrite (Kelco, Div. Merck & Co., Inc., San Diego, Calif.) as the solidifying agent. We present here an overlay plating technique for *S. acidocaldarius* BC with Gelrite as the solidifying agent which gives a high plating efficiency.

The final concentrations of the supporting gel and the overlay were 0.8% (wt/vol) Gelrite and 0.4% (wt/vol) Gelrite, respectively. Double-strength Gelrite solutions were prepared in distilled water with 1.6 and 0.8 g of powder per 100 ml of water. The powder was vigorously mixed with water and autoclaved for 20 min at 121°C. These solutions were kept at 70°C after autoclaving until used.

The double-strength supporting gel was diluted with an equal volume of prewarmed (70°C), sterile, double-strength, iron-free mineral medium 9K (pH 2.0) (4) supplemented with 20 mM tetrathionate, 2 mM sodium chloride, and 0.2% (wt/vol) Casamino Acids to 8 mM magnesium chloride (final concentration) as the stabilizing divalent ion.

The mixing was done by stirring the double-strength Gelrite medium continuously and pouring the prewarmed supplemented mineral medium into the Gelrite solution. We experienced difficulties with the solidification of this supporting gel when we poured the Gelrite-water solution (pH ca. 6.5) into the hot mineral medium. This was probably due to partial hydrolysis of the polysaccharide as a result of the rapid drop in pH and of the temperature. After mixing, about 25 ml of the supporting gel was transferred to each of several petri dishes. The supporting gel was almost completely transparent after solidification. The final pH of the gel was between 2.0 and 2.5. The double-strength (0.8%) soft gel was distributed in screw-capped glass tubes, 2.5 ml in each, and stored at 70°C until used.

* Corresponding author.

The viable counts were determined in the following way. *Sulfolobus* cells were grown in a glass reactor which contained 500 ml of growth medium 9K containing 10 mM tetrathionate as an energy source and supplemented with 0.1% (wt/vol) Casamino Acids. The culture was aerated with air enriched with 0.5% (vol/vol) carbon dioxide. Stirring was done with a magnetic stirrer at 300 rpm.

During the growth, samples were withdrawn to record the optical density at 440 nm, total cell counts, and viable counts. The samples for viable counts were diluted in prewarmed (70°C) 9K medium (pH 2.0), and 2.5 ml of the different dilutions was added to the double-strength soft gel tube and after thorough mixing was poured onto the prewarmed supporting medium in the plate. The soft gel was quickly distributed evenly over the gel surface. When the soft layer had solidified, the plates were incubated upside down in a sealed plastic bag at 65°C to minimize evaporation. The generation time for the liquid culture was estimated to be 9 h. When the optical density was plotted against the viable counts, a linear correlation (Fig. 1), with an optical density at 440 nm of 0.1, corresponding to 3.0×10^7 cells per ml, was obtained. Cultures without Casamino Acids gave the same linear correlation and the same result.

A Burker counting chamber and a light microscope with a magnification of ×400 were used to determine total cell counts for three readings of the optical density at 440 nm:

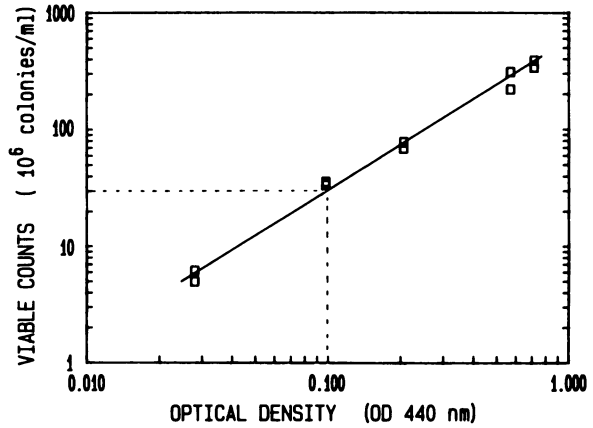

FIG. 1. Growth of *S. acidocaldarius* BC in liquid and on solid media. Viable counts are plotted against the corresponding optical density values during growth. After sampling and appropriate dilution, viable counts were made in duplicate from three consecutive 10-fold dilutions. Only plates which had 5 to 1,000 colonies were used.

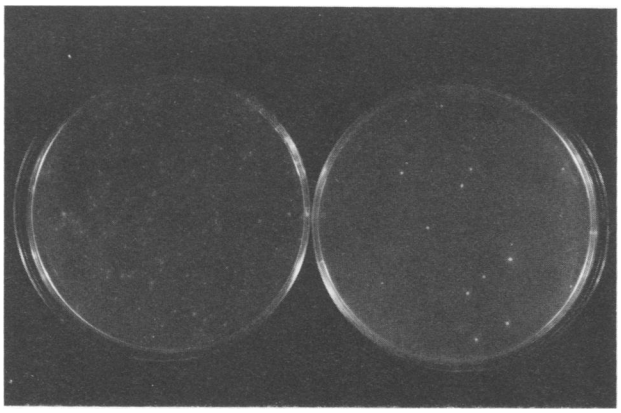

FIG. 2. Colonies of *S. acidocaldarius* BC after 5 days of incubation at 65°C in a 0.4% (wt/vol) soft layer of Gelrite. A 10-fold difference between inocula was used for the two plates (each 9 cm in diameter). Duplicates of the two dilutions shown gave 221 and 223 (left) and 18 and 44 (right) colonies per plate.

0.050, 0.136, and 0.178. The average total cell counts at these readings were 1.78×10^7, 4.70×10^7, and 5.30×10^7 cells per ml, and the average viable counts were 1.35×10^7, 4.60×10^7, and 6.00×10^7 colonies per ml, respectively. The plating efficiency was therefore essentially 100%.

After 3 to 4 days of incubation, tiny transparent colonies were visible. In a few days, the colonies gradually turned yellow in their centers, probably because of deposits of sulfur droplets. Colonies from two different dilutions of a sample are shown in Fig. 2. The incubation period (5 days) was the same for both. The sizes of the colonies obviously vary with the dilution factor. This may be due to limitations of the energy source.

We have also obtained rust-colored colonies on plates with 100 mM ferrous iron as an energy source. The final pH in these plates was kept below 2. Since growing *Sulfolobus* cells autotrophically on ferrous iron gives very low cell densities, we used bacteria grown in a liquid medium with 10 mM tetrathionate. Before being plated, the cells were washed twice and diluted in medium 9K (pH 2).

With this plating technique and the high plating efficiency obtained, one prerequisite has been determined for the selection of mutants resistant to substances such as toxic metals and for classical genetic experiments with *Sulfolobus* cells.

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