

## Simple, Inexpensive, and Rapid Way to Produce *Bacillus subtilis* Spores for the Guthrie Bioassay

MARTHA L. FRANKLIN AND WILLIAM A. CLARK\*

Laboratory Section, Georgia Department of Human Resources, Atlanta, Georgia 30334

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Esculin agar has been found to be a simple, inexpensive, rapid, and reliable means to promote production of spores of inhibitor-sensitive clones of *Bacillus subtilis* strains ATCC 6051 and 6633 for use in the Guthrie bioassay screening tests for genetic metabolic disorders.

Sporulating cells of *Bacillus subtilis* strains ATCC 6051 and 6633, selected for maximal sensitivity to certain amino acid analogs, are used in the Guthrie bioassay screening tests for neonatal genetic disorders (2). Potato starch agar (Guthrie, personal communication) and Eagle growth medium (1, 3) have been used successfully to produce spores for this purpose. However, both media require from 4 to 8 days to induce the required degree of sporulation in the two strains, and the latter medium is expensive (also, one of the ingredients, calf serum, has become difficult to obtain).

We have found that esculin agar, a medium which is simple and inexpensive to prepare, when inoculated with *B. subtilis* strains ATCC 6051 and 6633, induced the production of spores in large numbers within 24 to 48 h. Spores produced on this medium were easy to harvest in large quantities and gave excellent results when used routinely in the Guthrie bioassay screening tests.

Esculin agar was prepared from 1.0 g of esculin (6,7-dihydroxycoumarin 6-glucoside) (ICN Nutritional Biochemicals, Cleveland, Ohio), 0.5 g of ferric citrate, 40 g of heart infusion agar (Difco), and 1 liter of distilled water. The medium was heated to dissolve the agar, and the pH was adjusted to 7.0. The medium was dispensed in screw-cap tubes (20 by 150 mm), 8 ml per tube, and sterilized by autoclaving at 121°C for 15 min. After their removal from the autoclave, tubes were cooled in a slanted position.

Stock cultures (vegetative cells) of *B. subtilis* ATCC 6051 and 6633 were selected by the method of Robert Guthrie for maximal sensitivity to the various inhibitors used in the Guthrie bioassays (R. Guthrie, personal communication), as follows. ATCC strains 6051 and 6633 were derived from spore suspensions obtained from BBL Microbiology Systems, Cockeysville, Md. These were streaked on heart infusion agar,

and when growth developed, individual colonies were numbered and subcultured on slants of the same medium. Subcultures were harvested with 0.5 ml of Demain's PKU Test Broth Base (BBL Microbiology Systems) with 1% glucose, mixed with 20 ml of warm (50°C) liquid Demain's PKU Test Broth Base with 1.5% agar, and poured into Petri dishes. Prelabeled S & S 903 filter paper disks (6-mm diameter; Schleicher and Schuell) were wetted with measured (10- $\mu$ l) amounts of several inhibitor solutions (DL-beta-2-thionyl-alanine, 1 mM; L-methionine-DL-sulfoximine, 1 mM; 4-aza-leucine dihydrochloride, 10 mM; and D-tyrosine, 1 mM) and then dried. Separate disks labeled P (for phenylalanine) or M (for methionine) were placed on the strain 6633 clone plates, and L (for leucine) and T (for tyrosine) disks were put on the strain 6051 plates for overnight incubation. The culture (clone) showing the widest zone of sensitivity for each inhibitor was preserved for use in Guthrie assays.

Test cultures were suspended in defibrinated sheep blood (to the best of our knowledge, any kind of defibrinated red blood can be used for this purpose) and frozen for preserving as follows. Cultures were grown on heart infusion agar slants at 35 to 37°C for 18 to 24 h, suspended in blood, dispensed into presterilized plastic test tubes (6 by 50 mm; catalog no. 701, Sarstedt Inc., Princeton, N.J.) and frozen by placing the tubes directly into a Revco electric freezer held at around -70°C. Coded labels, typewritten on adhesive tape, were affixed to the tubes (before freezing) for identification.

To reconstitute a frozen culture, it was partially thawed by rotating the tube between thumb and forefinger. A small portion of the thawed culture (2- to 3-mm column in a Pasteur pipette) was inoculated into a tube of heart infusion broth (Difco) and incubated at 35 to 37°C for 18 to 24 h. (The frozen stock tube was returned immediately to the freezer to prevent

further thawing). When the frozen stock was nearly depleted, a new stock could be prepared directly from an agar subculture of the frozen and thawed material, then retested for sensitivity.

To initiate a spore crop, one drop of an 18- to 24-h heart infusion broth culture of inhibitor-sensitive strain 6051 or 6633 was spread over a slant of esculin agar. This was incubated, with the cap loose, at 37 to 40°C for approximately 24 h or until spores appeared in good numbers. The growth was suspended in 1.0 to 1.5 ml of sterile distilled water and heated in a 70 to 80°C water bath for 12 to 15 min to kill the vegetative cells present, leaving only viable spores.

The heat-treated spore suspension was reinoculated onto esculin agar slants (1 or 2 drops per slant) and incubated at 37 to 40°C, with caps loose, for 1 to 2 days, until maximum sporulation had taken place. Twelve slants per strain yielded enough spores to make about 500 ml of spore suspension diluted to an optical density of 1.0. Degree of sporulation was determined by examining Gram-stained smears made periodically during incubation of cultures. When maximum sporulation had occurred (Fig. 1), 1 ml of sterile distilled water was added to each slant, and the growth was scraped off with a loop and deposited in a 500-ml sterile flask with glass beads covering the bottom. The residual spores on each slant were washed off with an additional 1 to 2 ml of sterile distilled water. Harvested spores from a given strain were pooled into the same flask.

Growth of *B. subtilis* on esculin agar tends to form a "skin" which is adherent and difficult to break up. This skin was emulsified by repeatedly drawing up and forcibly ejecting the suspension, using a plugged 10-ml pipette. After the skin had been emulsified, the flask was immersed in an 80°C water bath and shaken continuously for 15 min, allowing the beads to further break up the spore masses, and at the same time killing any remaining vegetative cells.

The heated spore suspension was distributed in 50-ml screw-cap centrifuge tubes and centrifuged at 4,000 rpm for 20 min in an International centrifuge size 1 model SBU (International Equipment Co., Boston, Mass.). The supernatant was discarded, and sterile distilled water was added to wash the spores. (After each pouring off, the sedimented spores were shaken well with the residual drop or two of supernatant fluid remaining with the sediment to obtain a smooth mix. Thereafter, the wash water was added gradually, with mixing after each addition). Spores were washed and centrifuged a total of three times in this manner.

The washed spores were finally suspended in

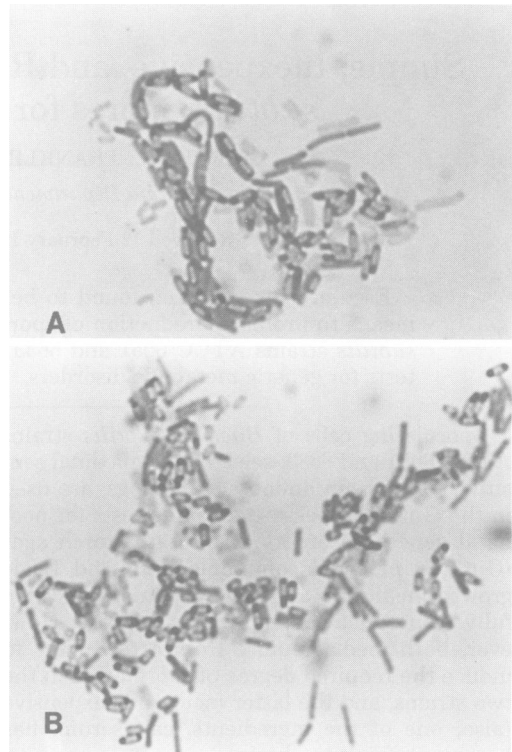


FIG. 1. Gram stains of *B. subtilis* cultures derived from ATCC 6051 (A) and 6633 (B) and selected for maximum sensitivity to various inhibitors after 48 h of incubation (37°C) on esculin agar ( $\times 1,700$ ).

a sufficient volume of distilled water to obtain an optical density of 1.0 for strain 6051 and 0.85 for strain 6633 at a wavelength of 550 nm with a Coleman Junior IIA Linear Absorbance Spectrophotometer, model 6/20A). Large clumps of spores were allowed to settle out for 5 min before the final optical density of the suspension was measured.

The supernatant spore suspension was pipetted off and dispensed, 8 ml each, into 14-ml sterile screw-cap plastic tubes (catalog no. 60-540, Sarstedt Inc.). These were then cooled to  $-70^{\circ}\text{C}$  in a Revco freezer and stored there.

For use, a tube of the frozen spore suspension was quick-thawed by shaking in a 37°C water bath until all the ice had disappeared. The spore suspension then was shaken until it was uniformly turbid. Only enough spore suspension was thawed for the week's tests. When not in use the thawed suspension was kept at 4 to 8°C to prevent deterioration.

Spores of *B. subtilis* strains ATCC 6051 and 6633, selected for maximum sensitivity to various inhibitors and produced in 24 to 48 h with

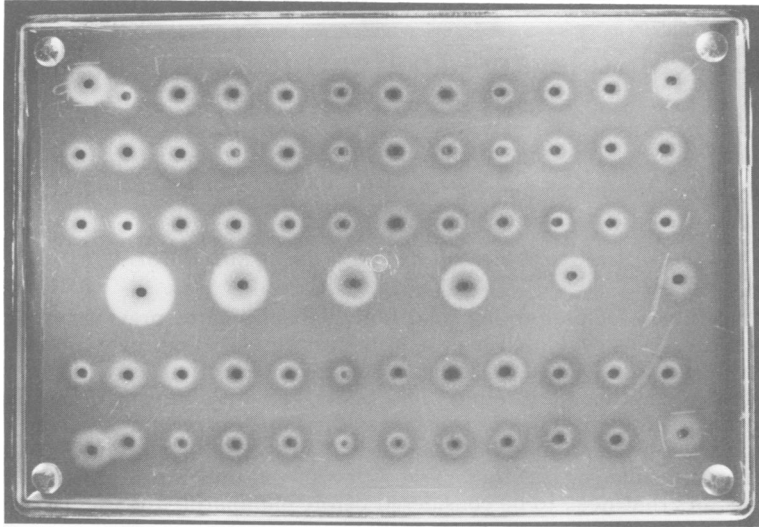


FIG. 2. Guthrie bioassay for methionine with *B. subtilis* ATCC 6633 spores propagated on esculin agar. Corner disks are screening level controls; in rows 1 (top), 2, 3, 5, and 6 are patients' blood; in row 4 are controls for increasing levels of methionine.

esculin agar as a substrate, gave satisfactory Guthrie test results (Fig. 2). Spores thus produced have been used successfully and routinely for metabolic screening in our laboratory.

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