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Propagating *Bacillus subtilis* Spores in a Liquid Medium for the Guthrie Bioassay

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Eagle growth medium was used successfully for the production of spores of *Bacillus subtilis* for the Guthrie bioassay screening tests for genetic metabolic disorders.

I am reporting a simplified method for producing spores of *Bacillus subtilis* ATCC 6051 and ATCC 6633 used in the Guthrie bioassay screening tests for neonatal genetic disorders (2). We have discovered that Eagle growth medium (1), a liquid, is excellent for promoting sporulation in these strains.

Eagle growth medium was prepared with 50 ml of fetal bovine calf serum (Microbiological Associates, Walkersville, Md.; we have substituted rabbit serum successfully, but with lowered spore yield) inactivated for 30 min at 56°C; 50 ml of Eagle essential medium (Flow Laboratories, McLean, Va.); 390 ml of sterile, double-distilled water; 5 ml of 1.75% (wt/vol) L-arginine (Difco; filter-sterilized); 5 ml of L-glutamine (200 mM, Flow Laboratories, presterilized); and 13 ml of 7.5% (wt/vol) sterile $NaHCO_3$. The medium was dispensed in 32-ounce (ca. 0.946-liter) prescription bottles. These were incubated with caps tight at 37°C for 3 days to check sterility.

Stock cultures of *B. subtilis* were maintained in cotton-plugged 5- by 50-mm glass tubes as frozen (−79°C) defibrinated blood suspensions of vegetative cells propagated on heart infusion agar. To reconstitute the culture, the tube was rotated between thumb and forefinger to thaw blood at the meniscus, and a small portion (1/8-inch [ca. 0.32-cm] column in a Pasteur pipette) was spread on a heart infusion agar slant. After

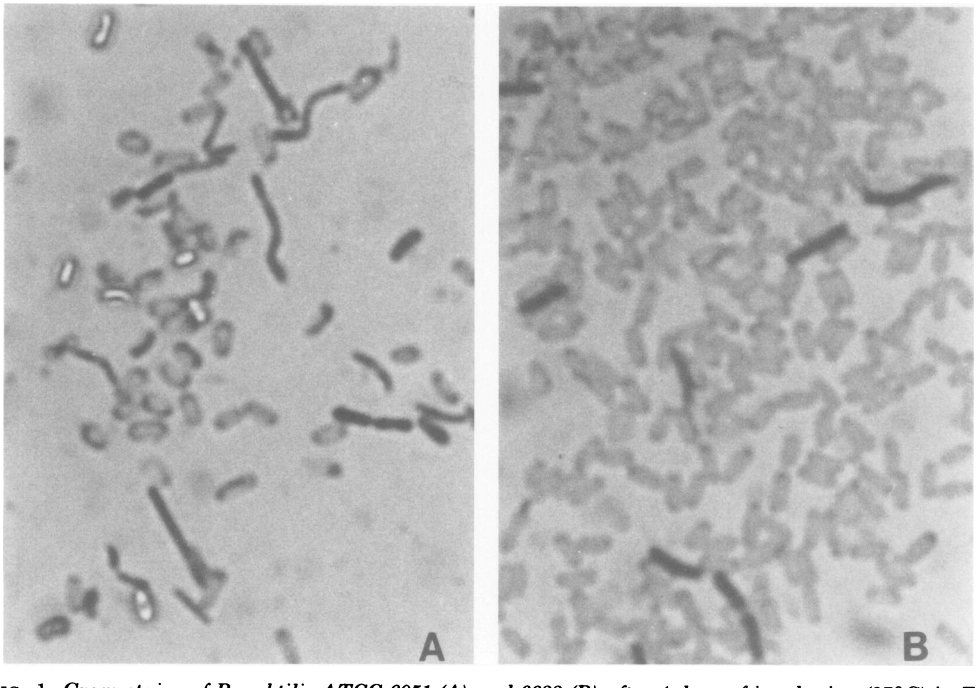

FIG. 1. *Gram stains of B. subtilis ATCC 6051 (A) and 6633 (B) after 4 days of incubation (37°C) in Eagle growth medium (×2,500).*

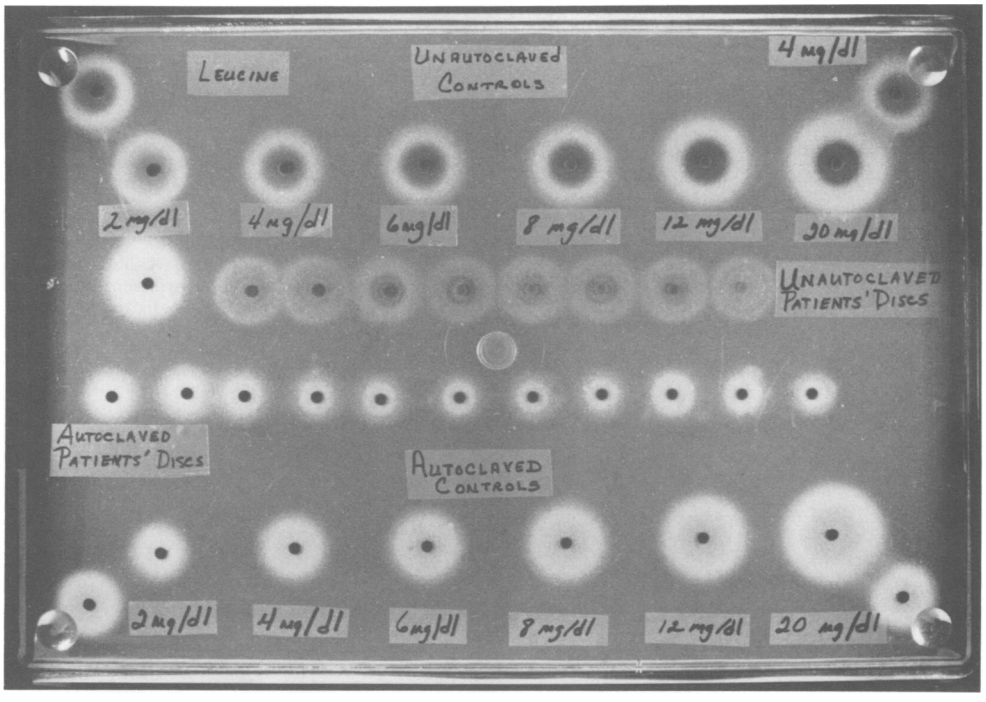

FIG. 2. *Guthrie bioassay for leucine with B. subtilis spores propagated in Eagle growth medium. Corner disks are screening level controls; row 1 (top) and 4 are controls for increasing levels of leucine; rows 2 and 3 are patients' blood (first disk in row 2 is positive; all others are negative).*

TABLE 1. *Comparison of sporulation of two strains of B. subtilis ATCC 6051 and ATCC 6633, grown on Eagle growth medium and on potato agar*

Day	Growth on:			
	Eagle medium		Potato agar	
	6051	6633	6051	6633
1	5[a]	0[a]	0[a]	0[a]
2	35	40	30	0
3	90	70	70	35
4	95	90	NT[b]	30
5	60[c]	95	75	60
6	75	85[c]	90	65
7	60	70	95	75
8	55	75	95	90

[a] Figure represents an estimate of percent sporulation from observation of Gram-stained smears prepared from the two strains after specified incubation on two media.

[b] NT, Not tested.

[c] The decrease in percent sporulation reflects germination of spores already formed, followed by vegetative cell division and (not shown in this time frame) a repeat of the sporulation cycle.

overnight incubation at 37°C, the culture was suspended in 1 ml of sterile Eagle growth medium and placed into a 32-ounce prescription bottle containing 50 ml of Eagle growth medium; this was incubated at 37°C with the flat surface of the bottle down and the cap loose. The bottle was shaken every 24 h to prevent the culture from forming a sheet of growing cells.

A good spore crop was produced in 4 days, but longer incubation yielded even more spores, because the spores that formed germinated, the resultant vegetative cells multiplied, and the sporulation cycle repeated.

The spores were harvested by pouring the contents of the prescription bottle into a sterile, screw-capped 50-ml centrifuge tube and centrifuging at 2,000 rpm for 10 min. The supernatant was discarded. To wash the spores, an additional 50 ml of water was added, and the contents were blended in a Vortex mixer and centrifuged. The spores were washed a total of three times. A sufficient amount of water was added to the washed spores to give a spectrophotometer reading of optical density 0.85 for strain 6633 and a reading of 1.0 for strain 6051 at a wavelength of 550 nm.

After 8 days of incubation, *B. subtilis* 6633 treated as described produced about 20 ml of spore suspension, and strain 6051 produced 10 ml of spore suspension.

Trials comparing the relative effectiveness of potato agar (the recommended medium) and

Eagle growth medium showed the latter to promote sporulation as effectively as the former (see Table 1 and Fig. 1).

Spores produced in Eagle medium gave satisfactory Guthrie test results (Fig. 2).

Eagle growth medium promotes excellent sporulation in 4 days, is easy to work with, reduces the possibility of contamination, and saves time.

Spores produced with Eagle growth medium now are being used routinely for metabolic screening in our laboratory.

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