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Simplified Procedure for Producing *Bacillus subtilis* Spores for the Guthrie Phenylketonuria and Other Microbiological Screening Tests

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Bacillus subtilis **ATCC 6051 and ATCC 6633 spores used in bacterial inhibition screening assays for genetic metabolism defects in newborn infants were produced by using liquid synthetic replacement sporulation media. These media allowed a high degree of sporulation, as judged by direct cell counts. Sporulation took place within 23 to 27 h with these media. Also, a more rational procedure for selecting the most sensitive clones of these organisms to the various inhibitors used in the microbiological screening assays is presented.**

Bacillus subtilis ATCC 6051 and 6633 spores used in the phenylketonuria bacterial inhibition screening assay (BIA) and other microbiological assays used to screen for inherited metabolic defects in newborns have traditionally been produced by using potato agar slants (3). Recently two other methods have appeared in the literature. The first reported the use of Eagle medium supplemented with bovine calf serum (8), and the second reported the use of esculin agar (heart infusion agar supplemented with esculin) (2). Although these methods work, they all suffer from certain imperfections. The potato agar requires up to 8 days to get sufficient spore formation. Also, it is difficult to separate the spores from the agar, and there are large aggregates of spores formed when agar medium is used (1). Eagle medium with calf serum is a liquid and is therefore more convenient for harvesting spores, and generally there is less aggregation of spores. Eagle medium and calf serum, however, are expensive, and sporulation takes from 4 to 8 days with this medium. Esculin agar, on the other hand, is inexpensive, and spore formation takes place within 24 to 48 h; but again it is difficult to separate spores from agar, and spore aggregation is a problem.

In the past in this laboratory and at present in others, selection of inhibitor-sensitive clones of ATCC 6051 and 6633 was accomplished by the random procedure of picking individual colonies grown on heart infusion agar and testing the subcultured growth from each colony to determine sensitivity to the various inhibitors.

This study reports the use of two chemically defined media, referred to in this paper as synthetic replacement sporulation media (SRSM). These media allow sporulation of vegetative-growth-phase cells without appreciable further vegetative growth. SRSM combine the convenience of liquid media with rapid sporulation (23 to 27 h), low cost, and simple preparation.

This study also reports a more rational method for selecting the most sensitive clones of these organisms to the various metabolic inhibitors used in the various BIAs.

MATERIALS AND METHODS

Bacteria. *B. subtilis* ATCC 6051 (Marburg strain) and ATCC 6633 can be obtained from the American Type Culture Collection, Rockville, Md.

Media. Demain phenylketonuria test broth base (used to grow vegetative-growth-phase cells and used in inhibitor-containing agar plates for replica plating and in the BIAs) was obtained from BBL Microbiology Systems. This medium will be referred to hereafter as Demain for the liquid medium and Demain agar for the solid medium. Nutrient broth (used to make master plates for replica plating) was from Difco Laboratories and was used at one-half the recommended concentration. Difco agar was added to the Demain test broth base and one-half-concentration nutrient broth to make a final concentration of 1.5% (wt/vol) and 1.0% (wt/vol), respectively.

SRSM-1, used to sporulate *B. subtilis* 6051, was originally developed by Sugae and Freese (9) and contains (final concentration per liter in distilled or deionized water) 5.8 g of K_2HPO_4, 9.05 g of KH_2PO_4 (autoclaved), 5 ml of a filter-sterilized metal mixture (containing 0.14 M $CaCl_2$ [0.7 mM], 0.01 M $MnCl_2$ [50 μM], and 0.2 M $MgCl_2$ [1 mM]), 0.5 ml of filter-sterilized 2 mM $FeCl_3$ (1 μM) in 0.01 N HCl, and 20 ml of filter-sterilized 0.5 M monosodium L-glutamate (10 mM). The pH should be 6.5. Components were filter sterilized by using a 0.45-μm filter. Molar concentrations in brackets are the final concentrations per liter of medium.

SRSM-2, used to sporulate *B. subtilis* 6633, was developed by Ramaley and Burden (7) and contains (final concentration per liter in distilled or deionized water) 0.33 ml of H_3PO_4 (85%), 1.0 g of $MgSO_4 \cdot 7H2O$, 0.4 g of NaCl, 0.4 g of KCl, 25 mg of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 10 mg of $CaCl_2$, and 6 mg of $MnSO_4 \cdot H_2O$. The pH is adjusted to 7.2 with concentrated KOH. When autoclaved, this solution becomes turbid. However, this does not interfere with sporulation. After autoclaving, 20 ml of a 0.5 M monosodium L-glutamate solution (final concentration, 10 mM) is filter sterilized by using 0.45-μm filters and added to the 980-ml solution described above.

Growth conditions. Cells of both strains were grown from stock spore cultures. Stock spores are kept in distilled water at an optical density of 1.0 at 550 nm (OD_{550}) and stored at 4°C. Optical density was determined with a Bausch and Lomb Spectronic 20. All cultures were incubated at 37°C. Liquid cultures were shaken at 120 strokes per min in a New Brunswick shaking incubator and were always 25% or less than the volume of the flask.

Replica plating and replacement sporulation. Figure 1 shows a scheme for replica plating and replacement sporulation. Vegetative-growth-phase cells at an OD_{550} of 0.2 grown in Demain were serially diluted 10-fold in Demain and

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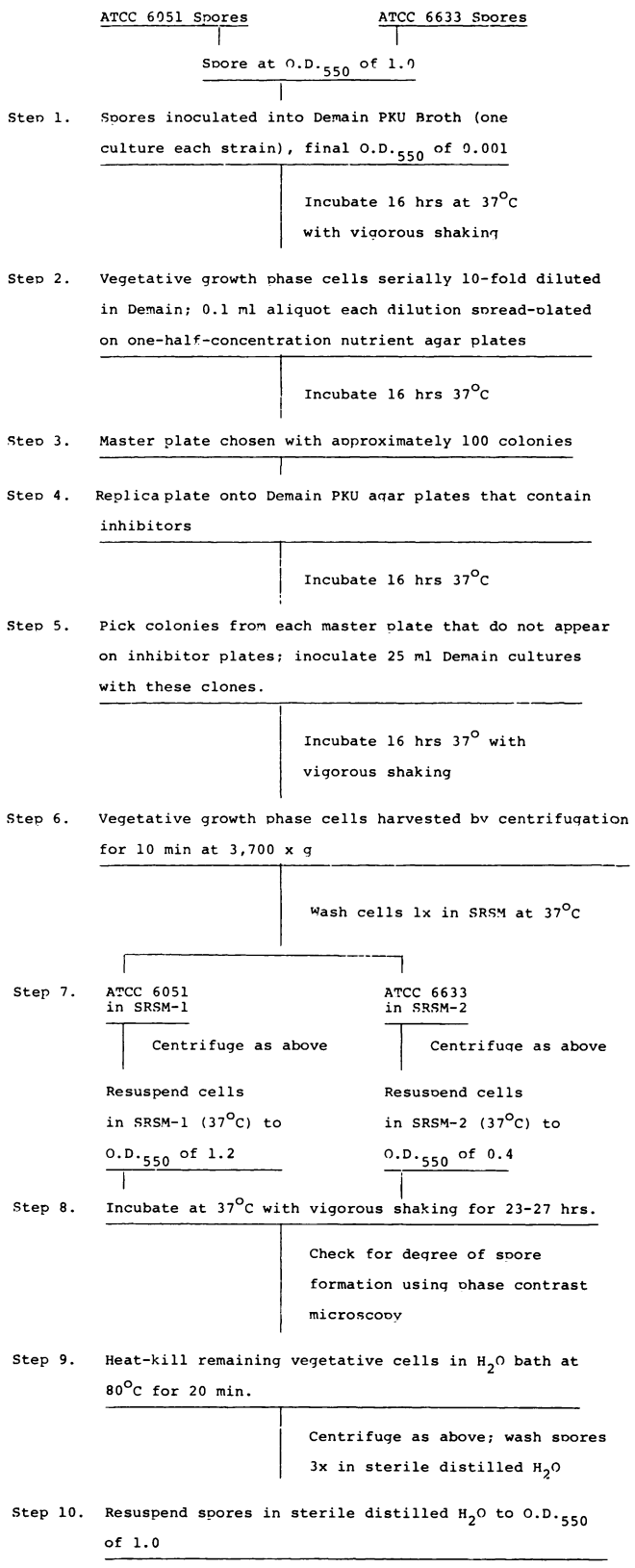

FIG. 1. Procedure for replica plating and replacement sporulation.

spread plated (4) on one-half-concentration nutrient agar plates. Replica plating was accomplished as previously reported (5). Demain agar plates, used in the replica plating procedure, contained one of the inhibitors at the same concentration used in the various bacterial assays (Table 1).

Spores under phase-contrast microscopy are refractile and appear as bright ovoid cells. Small aggregates of spores, when observed, were broken up by using a Biosonik probe-type sonicator (Bronwill Scientific) with the small probe at 50% maximum power for 0.5 to 2 min at 30-s intervals.

Inhibitor sensitivity. To determine maximal sensitivity to the metabolic inhibitors used in the various bacterial assays, spores grown from clones picked from the replica plates were plated in Demain agar medium exactly the same way and at the same concentration as would be done for the various BIAs (i.e., Demain was mixed with autoclave agar solution and cooled to 50°C, spores were added, and the plate was poured). When the agar had gelled, 6-mm Schleicher & Schuell no. 903 filter paper disks, each impregnated with 20 μl of one of the inhibitors (i.e., 1 mM D-tyrosine, 10 mM 4-azaleucine, and 10 mM *S*-2-aminoethyl-L-cysteine [ATCC 6051] and 10 mM beta-2-thienylalanine, 1 mM DL-methionine-DL-sulfoxamine, 1 mM 1,2,4-triazole-3-alanine, and 0.1 mM 5-fluorouracil [ATCC 6633]), were placed on the surface of the agar. After incubation, the zones of inhibition (Fig. 2) were measured, and the clones from each strain that yielded the largest zones of inhibition to the various inhibitors were saved. These maximally sensitive spores were used to grow large batches of vegetative-growth-phase cells in liquid Demain cultures. Large batches of maximally sensitive spores were made following steps 6 through 10 of Fig. 1.

RESULTS AND DISCUSSION

A Demain culture of 50 ml of strain 6633 vegetative cells at an OD_{550} of 2.0 diluted to an OD_{550} of 0.4 in SRSM-2 yielded ca. 500 ml of spores at an OD_{550} of 1.0. It was important that the vegetative cells were diluted to an OD_{550} of 0.4, because if the OD_{550} was somewhat higher, some of the cells lysed and the spore yield was low. However, if the cell concentration was too low, the cells continued slow vegetative growth for an extended period, and sporulation time increased.

A culture of 50 ml of strain 6051 vegetative cells at an OD_{550} of 2.0 diluted to an OD_{550} of 1.2 in SRSM-1 yielded ca. 175 ml of spores at an OD_{550} of 1.0. Various OD_{550}s from 0.2 to 2.2 were tried, and an OD_{550} of 1.2 yielded the best sporulation in the shortest incubation time with this medium.

A spore suspension grown in SRSM-1 and SRSM-2, washed with distilled water, and stored at 4°C in distilled water in convenient volumes in screw-capped tubes will remain viable for years, and that is one of the recommended ways to store *B. subtilis* spores (1).

TABLE 1. Concentrations of inhibitor used in replica plates

B. subtilis strain	Inhibitor	Concn (μM)
ATCC 6051	D-Tyrosine	4.7
	4-Azaleucine	53.0
	S-2-Aminoethyl-L-cysteine	24.0
ATCC 6633	Beta-2-thienylalanine	12.0
	DL-Methionine-DL-sulfoxamine	1.0
	1,2,4,-Triazole-3-alanine	4.3
	5-Fluorouracil	1.0

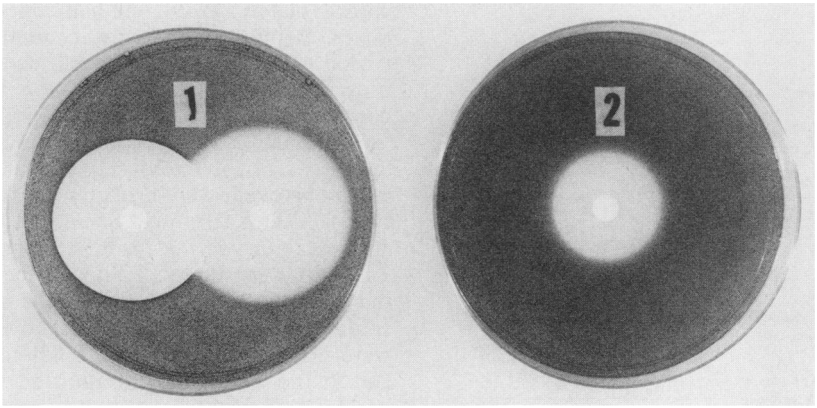

FIG. 2. Zones of inhibition for the three most widely used inhibitors. For ATCC 6633, the left filter paper disk was impregnated with beta-2-thienylalanine, and the right one was impregnated with DL-methionine-DL-sulfoxamine (plate 1); for ATCC 6051, the disk was impregnated with 4-azaleucine (plate 2). Bacterial growth was stained with 0.1% 2,3,5-triphenyltetrazolium chloride for contrast.

TABLE 2. Sporulation of ATCC 6051 in SRSM-1 and ATCC 6633 in SRSM-2

Strain (medium)	Cell counts[a] at h after transfer:								S/V ratio
	0		19		23		27		
	V	S	V	S	V	S	V	S	
6051 (SRSM-1)[b]	3.96×10^8	0	5.94×10^8	3.54×10^8	4.76×10^8	3.32×10^8	4.52×10^8	3.20×10^8	0.71 (at 27 h)
6633 (SRSM-2)[c]	5.56×10^7	0	1.03×10^8	3.83×10^7	1.69×10^8	1.15×10^8	2.89×10^8	2.09×10^8	0.72 (at 27 h)

[a] V, Total cell counts, vegetative and spores; S, total spore counts.
[b] Values indicate the number of cells per milliliter at an OD_{550} of 1.2 at 0 h to an OD_{550} of 1.8 at 27 h.
[c] Values indicate the number of cells per milliliter at an OD_{550} of 0.4 at 0 h to an OD_{550} of 2.0 at 27 h.

Table 2 demonstrates sporulation of both *B. subtilis* strains in SRSM media. The number of all cells (V) in a culture was determined by direct count by using a Petroff-Hausser chamber and phase-contrast microscopy at ×970 magnification. The spore count (S) was similarly ascertained by counting only the refractile spores. The most reliable measure of sporulation was the S/V ratio. Before counting, the cell and spore suspensions were briefly sonicated (see above) to break up small aggregates and chains.

A comparison was made of zones of inhibition. Pour plates were poured by using spores or vegetative-growth-phase cells, and filter paper disks of inhibitor were placed on the agar surface as reported above. The results showed that plates with spores gave well-defined measurable zones of inhibition, whereas plates with vegetative-growth-phase cells gave ill-defined zones that were difficult to measure. This difference was probably due to the fact that vegetative cells first had to deplete their intracellular pool of amino acids and nucleotides before the substrate analog inhibitors could successfully inhibit growth. Spores, on the other hand, contain relatively small intracellular pools of amino acids and nucleotides (6), and therefore vegetative-cell outgrowth from spores was somewhat more sensitive to these inhibitors.

This paper has presented a more rational procedure for selecting clones with maximal sensitivity to the metabolic inhibitors. The new procedure first selected sensitive clones from vegetative cells and then selected a maximally sensitive clone from the spores made from the sensitive vegetative clones. Spores were used for determining maximal sensitivity because we have shown that spores and vegetative cells differ in their response to these inhibitors. Also, ultimately it is spores that are used in the BIAs.

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