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Method for Obtaining Free Bacterial Spores of *Bacillus subtilis* var. *niger*

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In attempting to develop a bioluminescent assay of adenosine triphosphate (ATP) for the detection of extraterrestrial life, it was necessary to measure the ATP content of bacterial spores. Such an analysis required that the spores be clean, i.e., free of vegetative cells and debris. Several methods have been reported for separation of spores from vegetative cells and debris, but they are either tedious and only partially successful or they require specialized equipment. Separation of layers in centrifuged pellets (S. K. Long and O. B. Williams, J. Bacteriol. **76**:332, 1958) was only partially successful in this laboratory with *Bacillus subtilis* var. *niger* (*B. globigii*) and a high loss of material resulted. Other methods which have been reported include lysis of vegetative cells (B. D. Church, H. Halvorson, and H. O. Halvorson, J. Bacteriol. **68**:393, 1954; W. L. Brown, Z. J. Ordal, and H. O. Halvorson, Appl. Microbiol., **5**:156, 1957), sonic treatment augmented by lytic enzyme (N. Grecz, A. Anellis, and M. D. Schneider, J. Bacteriol. **84**:552, 1962), differential sedimentation in water (B. T. Stewart and H. O. Halvorson, J. Bacteriol. **65**:160, 1953), froth flotation (W. A. Boyles and R. E. Lincoln, Appl. Microbiol. **6**:327, 1958; S. H. Black, R. E. MacDonald, and P. Gerhardt, Bacteriol. Proc. p. 41, 1958; A. M. Gaudin, A. L. Mular, and R. F. O'Connor, Appl. Microbiol. **8**:84, 1960), centrifugation in dense sucrose solutions (B. D. Church and H. Halvorson, Nature **183**:124, 1959; B. J. Krask and G. E. Fulk, Arch. Biochem. Biophys. **79**:86, 1959), separation in a two-phase aqueous polymer system containing polyethylene glycol 4000 and potassium phosphate (L. E. Sacks and G. Alderton, J. Bacteriol. **82**:331, 1961) and separation, in a single layer system, of 25% carbowax 4000 in phosphate buffer (R. Irie and H. Uchiyama, J. Gen. Appl. Microbiol. (Tokyo) **10**:237, 1964).

This paper describes a simple reproducible method, which does not require specialized equipment, for purifying spores.

Preparation of spore crops. All procedures were performed under sterile conditions. *B. globigii* was grown on thin layers of Tryptic Soy Agar (Difco), in Roux bottles for 10 days at 35 C, and was examined microscopically. Approximately 85 to 90% of the cells were free spores, which were washed from the agar surface with 20 ml of cold distilled water and glass beads and then washed three times in cold distilled water by centrifugation at 7,000 rev/min for 5 min at 3 C. The supernatant fluid and loosely packed cells were poured off and discarded, and approximately 1 g (wet weight) of cells was obtained from each Roux bottle by this procedure.

Purification of spore crops. From 0.5 to 1.5 g (wet weight) of cells was suspended in 20 ml of 0.1% lysozyme (Sigma Chemical Co., St. Louis, Mo.), made up with filter-sterilized 0.066 M phosphate buffer (K_2HPO_4) at *p*H 6.24. The cells were then shaken gently at 3 C for 1 hr, and a heavy precipitate, consisting of clumped cells, formed. The suspension was then filtered through a sterile 10-μ polypropylene membrane filter (Gelman, Instrument Co., Ann Arbor, Mich.), after which the filtrate was collected and examined microscopically. Staining (simple and spore stains) and examination of unstained preparations by phase contrast revealed repeatedly that the filtrate (spore suspension) was free from vegetative cells and debris. Microscopic examination of material retained by the filter showed largely vegetative cells and some spores, usually in clumps. Finally, the spores were sedimented and then washed four times by centrifugation in cold distilled water at 7,000 rev/min for 5 min at 3 C. The cleaned spores did not clump or precipitate when resuspended in lysozyme.

By the above procedure, 500 mg (wet weight) of starting material yielded concentrations of 10^{10} viable spores and 8×10^{10} total spores. The clean spores were packed by centrifugation and stored at − 72 C or they were freeze-dried and stored at the same temperature for weeks, without any significant decrease in the cell count. The procedure was repeated numerous times and always resulted in suspensions containing practically 100% free, refractile, unstained spores.

Our early attempts to rid the suspension of vegetative cells and debris by lysis with lysozyme

at 3, 25, 35, or 45 C were unsuccessful. Perhaps, as suggested by Grecz et al. (1962), spore-bearing cells require an inherent autolytic protease system for lysozyme to be effective.

The clumping of cells in the presence of lysozyme is not yet understood. It could be due to a binding of vegetative cells to the enzyme at 3 C. It is possible that *N*-acetylglucosamine (NAG), part of the large substrate molecule found in bacterial cell walls and attacked by lysozyme, binds to the enzyme in the same way that part of the substrate does. At low concentrations, NAG may act as a competitive inhibitor rather than as a substrate which is broken down. This hypothesis is based on an earlier report (L. N. Johnson and D. C. Phillips, Nature **206:** 761, 1965) which stated that NAG competitively inhibits lysozyme, probably by binding specifically to the site on the enzyme which is responsible for its activity. Johnson and Phillips presented evidence that the inhibitor molecules lie embedded in a crevice or cleft in the surface of the enzyme molecule, which is probably formed by the three dimensional structure of the lysozyme molecule (C. C. F. Blake et al., Nature **206:**757, 1965). It is possible that NAG does not completely fill the crevice and more sugar residues are required to fill the cleft and form a reactive enzyme-substrate complex.

The fact that the bacterial spore coat is composed largely of structural protein rather than of the peptide-amino-sugar complex found in the walls of vegetative cells (M. R. J. Salton, p. 129, *in* I. C. Gunsalus and R. Y. Stanier [ed.], *The Bacteria*, vol. 1, Academic Press, Inc., New York, 1960), may explain why clumping was not observed with pure spore suspensions. This lends further support to the proposed explanation for the clumping of cells in the presence of lysozyme.