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Separation of Spores and Parasporal Crystals of *Bacillus thuringiensis* in Gradients of Certain X-Ray Contrasting Agents

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Spores and parasporal crystals of *Bacillus thuringiensis* can be separated at moderate centrifugation speeds (10,000 to 12,000 rpm) in gradients of Renografin or sodium diatrizoate.

Current studies of parasporal crystals and spores of *Bacillus thuringiensis* require that they be efficiently and completely separated. Their differences in density, surface properties, and solubility, as well as the germination and lysis of spores, have all been used alone and in combination in a number of published procedures to achieve their separation. Cooksey (1) has compared the various techniques.

Recently, Fast (2) described a method of crystal purification using isopycnic density gradient centrifugation in CsCl. This procedure represents a substantial improvement over previous separation techniques because of its high yields and the short time required. This note describes an extension of Fast's technique, using linear, preformed gradients of Renografin-water or sodium diatrizoate-water and lower centrifugation revolutions per minute. We believe this technique to be the most practical technique available for purifying parasporal crystals.

Renografin gradients have been used by Tamir and Gilvarg (4) to separate spores of *Bacillus megaterium* from vegetative cells and by Wise et al. (5) to obtain spores of altered dipicolinic acid content. Renografin is the X-ray contrasting agent methylglucamine 3,5-diacetylamino-2,4,6-triiodobenzoate supplied by E. R. Squibb & Sons. Sodium diatrizoate, also an X-ray contrasting agent, is the sodium salt of 3,5-diacetamido-2,4,6-triiodobenzoic acid, available from Winthrop Chemical as sodium Hypaque.

Renografin gradients were formed by using a Sigmamotor kinetic-clamp pump to transfer a select volume of water (one-half the centrifuge tube volume) from one cylindrical vial to a second vial of equal size. The second vial, the mixing chamber, contained a stirring magnet and an equal volume of Renografin. A duplicate pump transferred the mixed Renografin-water solution from the second vial to a centrifuge tube in such a manner that equal volumes in the two vials were continually maintained. A linear density gradient of 1.0 to 1.4 g/cm^3 is produced, but by using suitable solutions of Renografin-water in either one or both vials the range of the density gradient can be adjusted to any value between 1.0 g/cm^3 at the surface and 1.4 g/cm^3 at the bottom of the centrifuge tube. Both vials are emptied, and no material is wasted. The same equipment will produce exponential gradients if the volume in the mixing vial is maintained constant. A similar system, in which a Pharmacia P-3 peristaltic pump controlled transfer so that the rate of flow from the mixing chamber was twice the rate of flow into it, produced linear gradients of sodium diatrizoate.

Most data presented resulted from Renografin gradients, but one of us (J.N.A.) worked extensively with gradients of sodium diatrizoate and found them to be comparable to Renografin in the purification of *B. thuringiensis* parasporal crystals.

Spores and crystals of *B. thuringiensis* var. *dendrolimus*, grown in medium containing 0.1% glucose, 0.2% yeast extract, 0.2% $(NH_4)_2SO_4$, and 0.05% K_2HPO_4, were washed in water as previously described (3). About 0.3 ml of the final water suspension (ca. 400 mg [dry weight]) was layered onto 11 ml of a linear water-Renografin gradient in thick-walled glass tubes (18 by 103 mm). The samples were placed in a Sorvall HB-4 swinging bucket rotor and centri-

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fuged for 2 h at 10,000 rpm in a Sorval RC2-B centrifuge. Band positions were reproducible and did not change after an additional 2 h of centrifugation. Figure 1 illustrates the band positions obtained in linear 1.0 to 1.4 g/cm^3 Renografin gradients. Bands of crystals and spores were harvested with a Pasteur capillary pipette after first removing the above liquid. Sonication of the spore suspension prior to centrifugation, addition of a small amount of Tween 80, and adjustment to pH 8.0 were all found to help minimize clumping and promote cleaner separations. Renografin was subsequently removed from the purified fractions either by dialysis or by diluting the fraction with water and centrifuging out the particulate matter.

The shape of Renografin gradients can be determined from absorbance data at 260 nm after a dilution of 10^4-fold of small aliquots from the tube (4).

We have already used Renografin gradients to study sporulation and crystal formation in *B. thuringiensis* var. *entomocidus* under a variety of nutritional conditions (3). We used 0 to 100% gradients routinely, because separation of spores and crystals is adequate and several lighter bands resulting from vegetative cells, debris, and sporulation-related granules are demonstrated. Our first buoyant density values (obtained spectrophotometrically) of 1.30 and 1.25 g/cm^3 in Renografin gradients and 1.32 and 1.27 g/cm^3 in sodium diatrizoate for the spores and crystals, respectively, are lower than the values of 1.35 and 1.30 g/cm^3 reported by Fast (2), although buoyant densities are not strictly

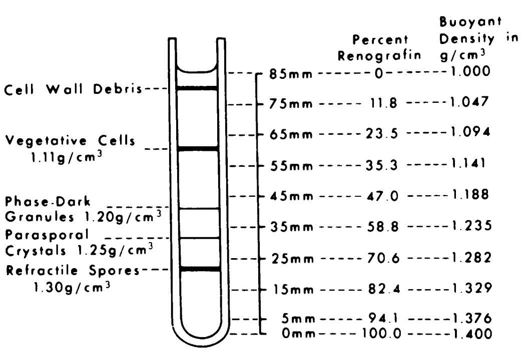

FIG. 1. *Diagram of typical spore-parasporal crystal separation achieved on a 0 to 100% Renografin gradient after density gradient centrifugation at 10,000 rpm for 2 h. B. thuringiensis var. dendrolimus was grown in GYS medium (3).*

comparable between gradients of a different type. Since then, similar preparations of *B. thuringiensis* were centrifuged in both Renografin and CsCl linear gradients at 20,000 rpm for 17 h. Using a direct method of weighing a carefully collected aliquot of each band in a 100-λ tared pipette, it was found that the average buoyant densities of spores and parasporal crystals, respectively, were 1.32 and 1.27 g/cm^3 in both gradient systems. The discrepancy between these values and those of Fast (2) could be due to strain differences, determinations of buoyant density, or lower relative centrifugal force.

In the experiments reported, *B. thuringiensis* spores and crystals were harvested from liquid medium. Gradient centrifugation produced pure crystal bands and nearly pure spore bands. Recently, we have found that spores and crystals of *B. thuringiensis* var. *galleriae* grown on solid medium are more difficult to separate than liquid-grown cultures. Parasporal crystals produced on plates are larger and more irregular in shape, and they tend to clump with spores in gradients and to move with spores in biphasic and flotation separation systems.

The procedure described herein does not require ultracentrifugation; it is rapid, simple, and less expensive than methods using CsCl. In addition, Renografin is not toxic; the purified spores are fully viable, and we found that clumping of spores and paraspores is less troublesome in Renografin than in CsCl gradients.

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