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Microcycle Sporogenesis of *Bacillus cereus* in a Chemically Defined Medium

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A chemically defined medium which allowed germination, outgrowth, and subsequent resporulation of *Bacillus cereus* T spores, without intervening cell division (microcycle sporogenesis), is described. No medium replacement was required. The second-stage spores were heat-stable and had similar germination characteristics and dipicolinic acid content to primary spores. Deoxyribonucleic acid (DNA) replication began soon after germination and there was a doubling in the DNA content of the cells within 2 hr.

The phenomenon of microcycle sporogenesis (formation of a spore from a spore without intervening cell division) was first reported with *Bacillus cereus* by Vinter and Slepecky (13), who used a medium replacement technique in which an approximately 100-fold dilution of the original complex medium was made a short time after spore germination was complete. Holmes and Levinson (8) have since reported microcycle sporogenesis of *B. megaterium* in a chemically defined medium, without employing replacement. The present report deals primarily with the description of a chemically defined medium for microcycle sporogenesis of *B. cereus* strain T, also without employing medium replacement. This organism was chosen because of the relative abundance of data concerning outgrowth and sporogenesis of *B. cereus* T (4) and the technical advantages inherent in its use. This approach to the study of sporulation gives a system uncomplicated by cell division for investigating the biochemical processes associated with the conversion of a vegetative cell to a spore.

MATERIALS AND METHODS

Organism. B. cereus strain T was used in this study.

Media and culture conditions. The microcycle medium contained: G-salts (3); the six amino acids present in the chemically defined growth and sporulation medium of Nakata (10); adenosine, 0.05%; $CaCl_2.2H_2O$, 0.008%; and tris(hydroxymethyl)aminomethane (Tris), 0.04 M. The final *p*H was 7.3. It was important to neutralize the amino acid mixture with KOH because if NaOH is used the cells germinate but do not outgrow. The amount of KOH required to neutralize the amino acids gave a final medium concentration of 0.085%. Addition of KCl (0.04%) to medium neutralized with NaOH restored the ability of the spores to undergo microcycle sporogenesis. Either 8×10^{-6} M K_2HPO_4 or 0.002% yeast extract (Difco) plus 10^{-6} M K_2HPO_4 was also added. The medium did not contain glucose because its presence causes a rapid drop in *p*H which prevents subsequent development of the cells. In initial experiments, the medium containing yeast extract was used to stimulate outgrowth. It was subsequently found that the requirement for yeast extract could be satisfied by increasing the phosphate concentration to 8×10^{-6} M, without any adverse effect on outgrowth and microcycle sporulation. Spores for microcycle cultures were suspended in distilled water at 7.5 mg (dry weight)/ml, heat-activated for 2 hr at 65 C, and washed once and suspended in sterile distilled water at 7.5 mg/ml. The medium was inoculated with spores at 75 μg (dry weight)/ml, and almost 100% germination occurred in about 8 min at 30 C. To minimize the problem of spores climbing flask walls, 200-ml volumes of culture were incubated at 30 C in 300-ml Florence flasks on a New Brunswick Rotary Shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 300 rev/min. Under these conditions, aeration of the culture was adequate for resporulation. The fraction of cells completing microcycle sporogenesis was determined by examining wet mounts under phase microscopy and counting the percentage of refractile spores. Viable cells and heat-stable cells (30 min at 65 C) were measured during microcycle sporogenesis by use of standard plating techniques.

Preparation of spores. Two 2-liter Erlenmeyer flasks, each containing 400 ml of G-medium, were inoculated with spores, heat-activated as described above, at 12.5 μg (dry weight)/ml, and incubated at 30 C on a New Brunswick Rotary Shaker at 300 rev/min until most of the cells were in the fourth division cycle. At this point, the contents of the two flasks were transferred to 8 liters of G-medium in a 15-liter carboy and were grown under conditions of forced aeration until a large percentage of free spores was formed. The spores were harvested in a Sharples refrigerated centrifuge, washed 15 to 20 times with

distilled water, and lyophilized and stored at 4 C in a desiccator. Determinations of spore dry weight were carried out by drying concentrated suspensions to constant weight in a hot-air oven at 104 C.

Germination studies. Spores were heat-activated for 3 hr at 65 C in distilled water, washed once, and suspended in 0.05 M Tris, *p*H 8.5. Germination was initiated by the addition of L-alanine to a final concentration of 0.1 M and was carried out at 30 C. Cell samples taken during germination were stabilized with 0.09 M $HgCl_2$ and the percentage of phase-dark spores was counted microscopically.

Chemical estimations. Deoxyribonucleic acid (DNA) was estimated by Burton's diphenylamine method (1). The cells were extracted twice with perchloric acid and herring DNA was used as standard. Dipicolinic acid (DPA) was estimated by the method of Janssen et al. (9). Inorganic phosphate was estimated by a modified Fiske and SubbaRow procedure, with ferrous sulfate as the reducing agent (12).

Chemicals. All chemicals were of reagent-grade purity. L-Amino acids and uracil were obtained from the Sigma Chemical Co. (St. Louis, Mo.), adenosine from Mann Research Laboratories, Inc. (New York, N.Y.), and thymidine from Schwarz Bio Research Inc. (Orangeburg, N.Y.). Tritiated thymidine (methyl-labeled; specific activity, 12.3 c/mmole) was obtained from Tracerlab (Richmond, Calif.), and uracil-5-^3H (specific activity, 6.0 c/mmole) was purchased from Schwarz Bio Research Inc. Mitomycin C was a product of Calbiochem (Los Angeles, Calif.), and actinomycin D was a gift from H. B. Woodruff (Merck, Sharp and Dohme, West Point, Pa.).

Uptake of ^3H-thymidine and ^3H-uracil. Samples (1 to 2 ml) of cells from cultures undergoing microcycle sporogenesis were removed at various times and exposed to ^3H-thymidine or ^3H-uracil in small (25 or 50 ml) Erlenmeyer flasks, in a reciprocating water bath at 30 C for 5 and 10 min, respectively. In separate experiments, the kinetics of incorporation of ^3H-thymidine, by culture samples exposed to the tracer at regular intervals up to 12 hr after germination, remained linear for at least 10 min. The kinetics of incorporation of ^3H-uracil remained linear for at least 15 min. The cells were killed by the addition of an equal volume of ice-cold 10% trichloroacetic acid, containing 0.01% unlabeled uracil or thymidine. The precipitated cells were allowed to stand at 0 C for 30 min, collected by filtration on membrane filters (pore size, 0.45 μ; Millipore Corp., Bedford, Mass.), and washed four times with 4 ml of ice-cold 5% trichloroacetic acid, containing 0.005% unlabeled uracil or thymidine. The filters were transferred to glass scintillation vials, dried in a hot-air oven at 60 C, and 10 ml of a scintillation solution, composed of 100% toluene containing 0.5% PPO (2,5-diphenyloxazole) and 0.01% POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene], was added. The radioactivity in the samples was determined in a Tri-Carb Liquid Scintillation Spectrometer, model 3375 (Packard Instrument Co., Inc., Downers Grove, Ill.).

RESULTS

Description of microcycle sporogenesis. Figure 1 shows the time course of development for refractile spores. In this particular experiment, yeast extract was present in the medium and 90% resporulation occurred after 18 hr. In many cases, resporulation was as high as 98%. Cultures in which the yeast extract was replaced by phosphate gave similar curves, although there was a slightly slower development of refractile spores (90% after 20 hr). A single division in 1 to 2% of the original spores was characteristically observed. Microscopically, there was no obvious cell lysis, an observation confirmed by plate counts on the suspension at various times (Table 1). The reason for the low viable count at 15 min is not known, but microscopic observation indicated that there was no cell division by 4 hr. Clumping was observed in some cultures, especially in those which did not contain yeast extract, from about 8 to 18 hr.

Cells undergoing microcycle sporogenesis showed initial signs of granulation at 4 hr (i.e., about 1 hr before the cells began to emerge, and

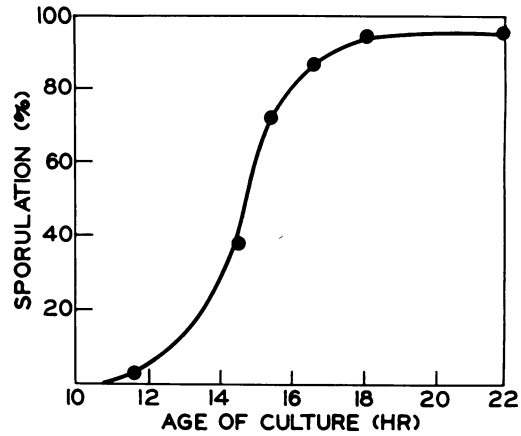

FIG. 1. *Development of refractile spores during microcycle sporogenesis of Bacillus cereus T.*

TABLE 1. *Viable and heat-stable cell counts during microcycle sporogenesis*

Age of culture (*hr*)	Cells/ml of culture	
	Viable count	Spore count
0	Not measured	5.9×10^7
0.25	3.9×10^7	5.4×10^5
4	6.8×10^7	Not measured
25	7.1×10^7	6.9×10^7

became very granulated as the microcycle progressed. There was no *p*H decrease in the cultures at any time. In some cases, the primary spores shed their spore coats into the medium where they remained apparently undigested throughout the cycle; in other cases, the coats remained attached to the vegetative cells and could still be observed there after mature sporangia had formed.

The effect of phosphate concentration on the microcycle process is shown in Table 2. In the absence of phosphate, no second-stage spores were formed, whereas increasing the concentration of phosphate to 10^{-4} M caused significant division. A higher percentage of spores was formed on prolonged incubation of these cultures.

Sadoff (11) reported that addition of gluconate to G-medium accelerated the formation of refractile spores of *B. cereus* T. In our microcycle system, the addition of gluconate up to 0.1% had no such effect.

Characteristics of microcycle spores. The microcycle spores were as heat-stable as primary spores and required heat activation for rapid germination. The optimal time for heat-activation was 3 to 4 hr at 65 C. Heat-activated primary and microcycle spores exhibited virtually 100% germination when exposed to L-alanine, although the response of the microcycle spores was somewhat slower. Primary spores were 90% germinated and microcycle spores 64% germinated after 5 min of incubation in the germination medium. At 10 min, the figures were 99 and 88%, respectively. Unactivated primary and second-stage spores showed only 20 to 50% (depending on the spore batch) and 40% germination, respectively, after 15 min in the germination medium at 30 C. The DPA contents of the microcycle and primary spores were 11 and 10.5% of the spore dry weight, respectively.

DNA synthesis. The pattern of DNA synthesis during microcycle sporogenesis was investigated by direct estimation of DNA and by pulse-labeling experiments with tritiated thymidine.

Direct estimations showed a doubling in DNA within 2 to 3 hr and no further increase up to 6 hr. Cultures in medium containing either 8×10^{-6} M phosphate or 10^{-6} M phosphate + 0.002% yeast extract gave the same result (Fig. 2). Although not shown in Fig. 2, estimations were carried out up to 18 hr under both culture conditions, and there was no further increase or decrease in the DNA content of the cells. The result obtained in pulse-labeling experiments is shown in Fig. 3. There were differences in the pattern of thymidine uptake between cells incubated in the two media. The maximal rate of uptake, especially in the culture containing only phosphate, occurred when DNA synthesis, as measured by chemical estimation, had ceased. In an attempt to understand this apparent discrepancy between the two methods for estimating DNA synthesis, the effects of the antibiotics mitomycin C and actinomycin C on thymidine uptake were investigated. The experiments were performed with cells incubated in the phosphate medium (no yeast extract), and the results obtained are summarized in Table 3. The concentration of actinomycin D employed was that which effectively prevented uptake of tritiated uracil by cells undergoing microcycle sporogenesis (Table 4). Mitomycin C inhibited thymidine uptake at its peaks of incorporation, whereas actinomycin D had little effect. Higher concentrations

TABLE 2. *Effect of phosphate concentration on microcycle sporogenesis of Bacillus cereus T*

Inorganic phosphate (moles/liter)	Response[a]	
	Microcycle[b] (%)	Division (%)
0	—	—
6×10^{-6}	60	1
8×10^{-6}	75	1–2
1×10^{-5}	80	6–8
2×10^{-5}	86	6–8
1×10^{-4}	20	70

[a] Observations were made at 17.5 to 18 hr after inoculation.

[b] Microcycle refers to the percentage of resporulation after 18 hr in sporangia that have not undergone division. The percentage of microcycle spores is greater than 95% after 25 hr in the presence of 8×10^{-6} M phosphate.

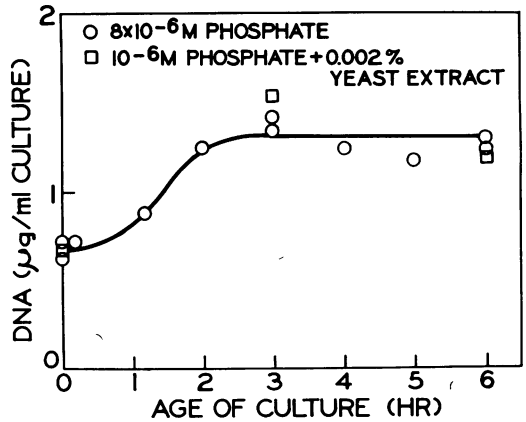

FIG. 2. *DNA content of Bacillus cereus T during microcycle sporogenesis.*

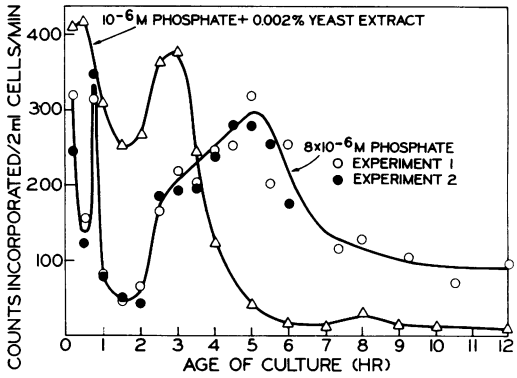

FIG. 3. *Uptake of ^{3}H-thymidine during microcycle sporogenesis of Bacillus cereus T. At the times indicated, 2-ml cell samples were exposed to 0.4 μc of ^{3}H-thymidine, specific activity 0.46 μc/mμmole (605 counts per min per μμmole) for the phosphate-containing cultures and 0.45 μc/mμmole (450 counts per min per μμmole) for the yeast extract culture.*

TABLE 3. *Effect of mitomycin C and actinomycin D on uptake of ^{3}H-thymidine by Bacillus cereus T during microcycle sporogenesis*[a]

Age of culture	Thymidine uptake (counts per 5 min per ml of culture)		
	No inhibitor	Mitomycin C (100 μg/ml)	Actinomycin D (2 μg/ml)
10 min	610	130	430
45 min	900	50	465
4 hr	1,200	—	1,580
5 hr	420	60	630

[a] ^{3}H-thymidine, 0.2 μc, specific activity 0.46 μc/mμmole, was added to 1-ml samples of the culture taken at the times indicated. Mitomycin C was added 10 min before, and actinomycin D, 1 min before, the isotope.

TABLE 4. *Effect of actinomycin D on uptake of ^{3}H-uracil by Bacillus cereus T during microcycle sporogenesis*[a]

Age of culture (min)	Uracil uptake (counts per 10 min per ml of culture) with indicated amt of actinomycin D			
	0 μg/ml	0.5 μg/ml	1 μg/ml	2 μg/ml
10	1,305	1,060	520	70
45	850	450	235	None

[a] At the times indicated, 2-ml cell samples were exposed to ^{3}H-uracil, 0.55 μc, specific activity 0.012 μc/mμmole, and to actinomycin D. Higher concentrations of the antibiotic completely inhibited uracil uptake.

of actinomycin D (20 μg/ml) completely inhibited thymidine uptake.

DISCUSSION

The microcycle spores of *B. cereus* T are similar to primary spores in that they germinate rapidly, have an equivalent DPA content, are refractile and heat-stable, and, as far as can be ascertained in the light microscope, are not markedly different in size or shape. Holmes and Levinson (8) reached similar conclusions about the properties of the second-stage spores produced during microcycle sporogenesis of *B. megaterium*, although they did find that the microcycle spores had slightly altered germination characteristics as compared with primary spores. Both types responded to glucose as a germinating agent, but only primary spores responded to L-alanine. Another similarity to the findings of Holmes and Levinson with *B. megaterium* was our discovery that second-stage spores of *B. cereus* T were unable to complete microcycle sporogenesis in the medium which permitted microcycle sporogenesis of primary spores. It is of interest that the fine structure of *B. megaterium* during microcycle sporogenesis was examined by electron microscopy (2), and that second-stage spores showed no significant structural differences from primary spores. Similar studies with *B. cereus* would be relevant.

Granulation during sporulation of *B. cereus* T in G-medium was correlated with the stage of "commitment to sporulation" (6) and with the induction of the tricarboxylic acid cycle (5). Cells of *B. cereus* undergoing microcycle sporogenesis showed signs of granulation after 4 hr of incubation but they exhibited no commitment to sporulation at this time. In fact, it was observed that transfer of cells to G-medium, as late as 11 hr after inoculation (i.e., 1 hr before refractile spores begin to appear), led to division in approximately 70% of the cells.

The lack of agreement between the two methods of studying DNA synthesis deserves some comment. From the chemical estimations, there seems to be no doubt that DNA replication began shortly after germination and was completed within about 2 hr. This finding differs from the data obtained by Holmes and Levinson (8) with microcycle sporogenesis of *B. megaterium*. By chemical estimation, they observed no DNA synthesis until after 1.5 hr of incubation, with a doubling in DNA content by 4 hr and a tripling by the end of the microcycle.

In contrast to the results of the chemical estimations, the pulse-labeling experiments, carried out in the presence of 8×10^{-6} M phosphate, indicate that there was a large peak of thymidine

assimilation from about 2 to 8 hr. From the area under the curve in Fig. 3 and the specific activity of the thymidine, as well as from the fact that the guanine plus cytosine content of *B. cereus* DNA is approximately 34% (7), it can be calculated that the thymidine uptake from 2 hr → 8 hr, if indicative of DNA synthesis, represents replication of about 9% of the original spore DNA. The fact that, at this time, mitomycin C inhibited thymidine uptake by the cells and actinomycin D did not (Table 3) suggests that thymidine was incorporated into DNA but not RNA. Cell fractionations on cultures pulse-labeled at the three peaks of thymidine incorporation are being carried out to determine the intracellular location of the label. The incorporation of thymidine up to 2 hr represents replication of about 2% of the spore DNA, a figure difficult to reconcile with the chemical estimations. The possibility that endogenous thymidine reduces ^{3}H-thymidine incorporation and that permeability barriers may reduce incorporation of the isotope cannot be excluded. It is also possible that exposure of the cells to thymidine after 2 hr in these experiments caused DNA synthesis which does not normally occur in the microcycle medium.

The pulse-labeling experiments, in the presence of 10^{-6} M phosphate and 0.002% yeast extract, agree somewhat better with the chemical estimations, although there was still considerable thymidine uptake after 2 hr. The isotope assimilated up to 6 hr represents replication of about 13% of the orginal spore DNA.

It appears that variation of the nutritional conditions markedly affects the uptake pattern. If, as seems likely, the chemical estimations are reliable, these experiments question the validity of using ^{3}H-thymidine as an indicator of the timing of DNA synthesis during microcycle sporogenesis.

ACKNOWLEDGMENT

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