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PHYSIOLOGY OF THE SPORULATION PROCESS IN *CLOSTRIDIUM BOTULINUM*[1]

II. MATURATION OF FORESPORES

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ABSTRACT

DAY, LAWRENCE E., (Michigan State University, East Lansing) AND RALPH N. COSTILOW. Physiology of the sporulation process in *Clostridium botulinum*. II. Maturation of forespores. J. Bacteriol. **88**:695-701. 1964.—*Clostridium botulinum*, strain 62-A, did not sporulate endotrophically, but forespores matured to refractile, heat-resistant spores when replaced in solutions containing L-alanine and L-proline, L-isoleucine and L-proline, or L-alanine and L-arginine. Solutions of L-arginine or L-citrulline would not support the maturation process. Acetate, CO_2, and δ-amino valeric acid were produced during sporulation in a replacement solution of L-alanine and L-proline, indicating the operation of the Stickland reaction. There was no large uptake of either exogenous L-alanine or acetate during this process. Similarly, there was no apparent protein or nucleic acid synthesis, since high levels of chloramphenicol, 8-azaguanine, or mitomycin C failed to inhibit, and no significant amount of P^{32} was incorporated into the spore nucleic acids. Dipicolinic acid (DPA) was synthesized during forespore maturation. It is believed that these final steps in sporulation of *C. botulinum* require only an exogenous source of energy which can be obtained via the Stickland reaction system, and that the synthesis of DPA and other unknown materials relies primarily on endogenous substrates.

The sporulation of *Clostridium botulinum* has been sufficiently well synchronized in complex media to correlate some morphological and functional changes (Day and Costilow, 1964). The next step was to attempt to study these changes in more simple environments. The technique of replacement (Hardwick and Foster, 1952) has been utilized widely with aerobic bacilli to study sporulation in an environment which will not support growth. However, preliminary studies (Day and Costilow, 1961) indicated that cells of this anaerobe obviously committed to sporulation would not complete the process endotrophically after replacement into water or buffer.

The present study was designed to determine the exogenous nutrient requirements for the maturation of forespores, and to investigate the nature of the physiological events occurring during this process.

MATERIALS AND METHODS

Organism and medium. C. botulinum 62-A, obtained from the American Type Culture Collection (ATCC 7948), was used in these studies. The medium and procedures for handling and sporulating the culture were described by Day and Costilow (1964).

Replacement of cells. All replacement operations were carried out in a nitrogen atmosphere in a Plexiglas chamber. The cells to be replaced were removed from an activated culture by centrifugation, washed three times with sterile distilled water, and resuspended in a volume of replacement solution equivalent to the volume of the original medium from which the cells were removed. In most instances, 10 ml of replacement solution were contained in 15-ml screw-cap vials, and the replaced cultures were incubated at 37 C in a Brewer jar flushed with natural gas. In those experiments in which C^{14} was present in the replacement solutions, the cultures were in flasks

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and were constantly agitated by bubbling with natural gas. The effluent gas was passed through 20% KOH to collect any CO_2 evolved. The cultures were acidified at the termination of the experiment so that all of the CO_2 could be trapped.

Cell counts. Refractile spores were counted with a Petroff-Hausser counting chamber. Heat-resistant spores were counted by heat-shocking samples at 80 C for 10 min, and culturing appropriate dilutions in yeast extract-starch-bicarbonate agar (Wynne, Schmieding, and Daye, 1955) modified by the deletion of glucose. Four oval tubes were used per dilution. Colonies were counted after 48 hr at 37 C.

Isolation and identification of end products. The Celite column described by Wiseman and Irvin (1957) was used for the isolation of acetic acid from replacement cultures. 3-(4-Anilino, 1-naphthlazo) 2,7-naphthylene disulfonic acid monoammonium salt was used as the internal indicator in the column.

Amino acids were identified by descending paper chromatography with the use of a solvent system of isopropanol-acetic acid-water in a ratio of 3:1:1 by volume. A 0.01-ml sample was used.

Assay of C^{14} activity. Alkaline samples were placed in the gel scintillation mixture of Gordon and Wolfe (1960), and the disintegrations were counted with a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., La Grange, Ill.).

Nucleic acid synthesis assay. The method of Hanawalt (1959) was used to assay nucleic acid synthesis in sporulating cells of *C. botulinum*. The P^{32} as $H_3P^{32}O_4$ was added to the culture at the desired time, and subsamples were removed and fractionated. The activities of the various fractions were measured with a Geiger-Mueller counter (Radiation Counter Laboratories, Inc., Skokie, Ill.) with an end-window tube (model 3; 1.6 mg/cm^2). The counts were recorded with a Berkeley Decimal Scaler (model 100; Berkeley Scientific Corp., Richmond, Calif.), with a high-voltage setting of 1,250 v.

Dipicolinic acid (DPA) assay. The DPA content of spores was assayed by the method of Janssen, Lund, and Anderson (1958).

RESULTS

Sporulation after replacement in new menstrua. Initial attempts to produce spores after harvesting and replacing cells of *C. botulinum* into new menstrua failed. It was quickly discovered that the replacement had to be conducted in the absence of oxygen. With the use of a chamber filled with nitrogen, cells were replaced into fresh sporulation medium at a number of stages of growth and sporulation proceeded without any effect on the rate or extent of sporulation. However, no sporulation occurred when the cells were replaced in distilled water, irrespective of the stage of development. In fact, only limited spore formation occurred when a complex mixture of all the common amino acids was used as the final medium. Further observations with more highly synchronized cultures demonstrated that only those cells which were at the forespore stage developed refractile spores on replacement in the mixture of amino acids.

Thinking that perhaps an energy source was the only exogenous requirement for the maturation of the forespores, we replaced cells from a culture which had initiated sporulation into solutions of various amino acids known to provide energy. The oxidation of either L-alanine or L-isoleucine in the presence of L-proline, or of L-alanine in the presence of L-arginine, would support the formation of refractile spores (Table 1). L-Ornithine is apparently not a very good electron acceptor for the Stickland reaction system in this organism, because it would not substitute for L-proline. Neither L-proline, acetate, L-arginine, nor L-citrulline alone supported spore maturation.

The rates of development of both refractile and heat-resistant spores after replacement in the L-alanine–L-proline solution, as compared with controls which were replaced in fresh sporulation medium, are shown in Fig. 1. The refractile and heat-resistant spore data are from two separate experiments and, thus, are not directly comparable. However, it is evident that a significant number of the forespores do develop heat resistance in the presence of L-alanine and L-proline. The initial rate of development of refractile spores in the solution of amino acids was the same as the control. In fact, when the culture was replaced at a later time when essentially all cells had reached the forespore stage, the rate and extent of refractile spore formation was the same in an L-alanine–L-proline solution as in an unreplaced control. The initial rate and the extent of heat-resistant spore formation was considerably less in the simple system than in the control.

TABLE 1. *Formation of refractile spores after replacement in various amino acid solutions**

Expt no.	Replacement medium	Time of replacement	Total population		Spore population	
			Initial	Final	Initial	Final
		hr	$\times 10^{-8}$	$\times 10^{-8}$	$\times 10^{-8}$	$\times 10^{-8}$
1	Control	10†	8.6	5.1	0.2	4.7
	L-Alanine + L-proline	10	7.7	5.2	0.1	1.5
	L-Isoleucine + L-proline	10	7.5	4.7	0.2	1.6
2	Control	11†	8.0	3.7	0.2	3.3
	L-Alanine + L-arginine	11	8.1	5.9	0.2	1.6
	L-Alanine + L-ornithine	11	8.2	6.0	0.3	0.5
3	Control	13†	—	—	0.5	1.7
	L-Alanine + L-proline	13	—	—	0.5	1.7
	L-Proline	13	—	—	0.5	0.5
	Acetate	13			0.5	0.5
4	Control	13†	14.0	5.1	0.1	4.8
	L-Arginine	13	12.0	7.0	0.1	0.2
	L-Citrulline	13	9.6	5.0	0.1	0.1

* Replacement solutions were prepared in 0.067 M phosphate buffer (pH 7.2). The concentration of the amino acids in experiments 1 and 3 was 2 mg/ml each; in experiment 2, 1 mg/ml; and in experiment 4, 5 mg/ml each. The solutions were in 10-ml volumes in screw-cap vials. Counts were determined by the use of a Petroff-Hausser counting chamber. Final populations were determined after 12 to 15 hr of incubation at 37 C.

† The controls were not replaced; time of initial count.

Earlier studies (Day and Costilow, 1964) demonstrated that acetate is utilized by *C. botulinum* early in the sporulation process, and that acetate is produced from the oxidation of L-alanine by this organism via the Stickland reaction (Clifton, 1940). Acetate accumulated rapidly during spore maturation in L-alanine–L-proline replacement cultures; but there was no evidence of significant acetate incorporation during this period, since the level of acetate-*1*-C^{14} free in solution remained constant (Fig. 2).

Further evidence that the L-alanine in replacement solutions is used primarily as an energy source for spore maturation is given in Table 2. Essentially all of the radioactivity from DL-alanine-*1*-C^{14} added to an L-alanine–L-proline replacement solution was accounted for in the KOH in which the CO_2 was trapped plus that free in the solution. Thus, there appears to be no large amount of this amino acid incorporated during this process.

A paper chromatogram of the supernatant fluid of an L-alanine–L-proline solution after sporulation demonstrated the presence of δ-amino valeric acid in addition to the amino acids added. No other amino acids were present in sufficient quantities to be detected by this technique.

Protein synthesis. Such data indicate that protein synthesis is not operating during the maturation process, and conflict with preliminary data reported by Day and Costilow (1961) indicating that chloramphenicol interrupted the sporulation process at all stages. Since these results were obtained with less highly synchronized cultures, the experiments were repeated. When the chloramphenicol was added prior to the time that a significant number of forespores were present, no sporulation occurred. However, the maturation of forespores in a control (not replaced) culture to which the antibiotic was added was identical with the maturation of a culture replaced in an L-alanine–L-proline solution; and the addition of the chloramphenicol to the latter culture had no effect on the development of refractile spores (Fig. 3).

DPA synthesis and the development of heat resistance occurred in cultures with high levels of chloramphenicol. The final DPA levels in cul-

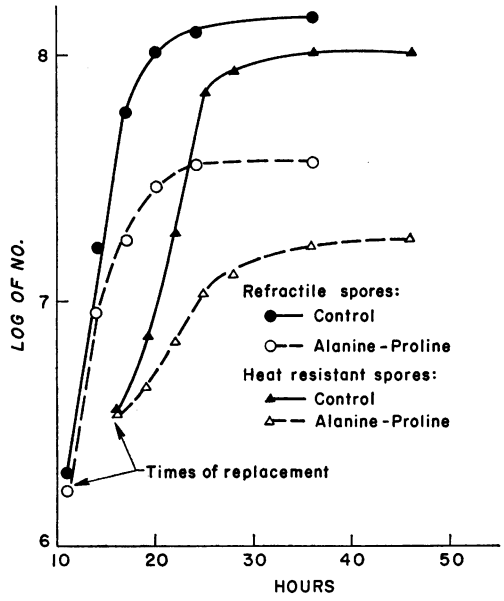

FIG. 1. *Formation of refractile and heat-resistant spores of Clostridium botulinum in a replacement solution containing* L-*alanine and* L-*proline. The concentration of each amino acid was 2 mg/ml in 0.06* M *phosphate buffer (pH 7.2). Control cultures were replaced in fresh sporulation medium. The data are from two separate experiments. Cells were replaced at 11 hr in the experiment measuring refractile spores, and at 16 hr when heat-resistant counts were followed. Cultures were incubated at 37 C.*

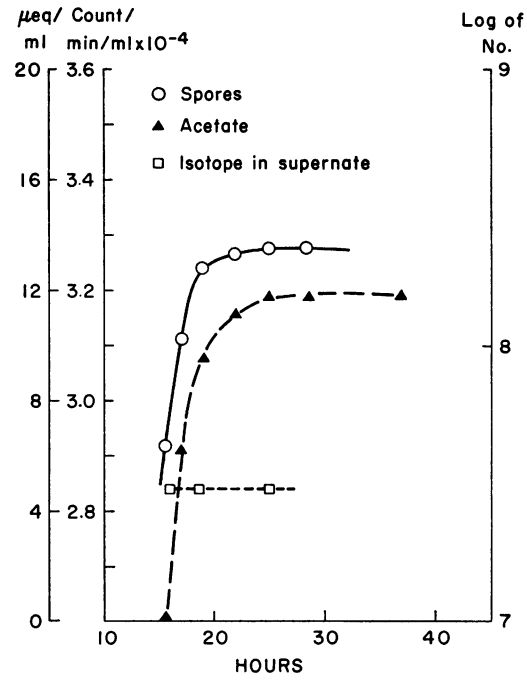

FIG. 2. *Production of acetic acid during formation of refractile spores in a replacement solution containing* L-*alanine and* L-*proline. The replacement solution and procedure were as outlined in Fig. 1, except that 0.01 μc/ml of acetate-1-C^{14} was added to the solution. Subsamples were analyzed for acetic acid and isotope as described in Materials and Methods.*

tures containing 100 μg/ml of this agent added after 14, 16, 18, 20, and 24 hr of incubation were 8.9, 11.3, 18.1, 18.8, and 22.8 μg/ml, respectively, as compared with an initial DPA level of less than 1 μg/ml at 14 hr and a final level of 27.1 μg/ml in a control culture. Heat-resistant spore counts increased up to 50 × after addition of the antibiotic, and ranged from 34 to 100% of the heat-resistant spore counts of the control. These data indicate that protein synthesis is not a requirement for the maturation of forespores of *C. botulinum*.

Nucleic acid synthesis. P^{32} as $H_3P^{32}O_4$ was added to an activated culture of *C. botulinum* at 8 hr, and its incorporation into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was followed during sporulation (Fig. 4). Only a very small amount of isotope appeared in the DNA fraction, and this did not increase at all after the first refractile spores were observed. Conversely, P^{32} was incorporated into RNA at a much greater rate,

TABLE 2. *Distribution of C^{14} in a replacement culture of Clostridium botulinum in* L-*alanine,* L-*proline, and* DL-*alanine-1-C^{14}**

Determination	Initial	Final
Total cell count per ml	1.1×10^9	4.40×10^8
Spores per ml	7.4×10^7	2.70×10^8
Isotope count per min:		
Supernatant fluid	3.57×10^6	2.40×10^6
KOH	2.52×10^3	1.11×10^6
Totals	3.57×10^6	3.51×10^6
Per cent C^{14} recovery		98.4

* The replacement medium contained 2 mg/ml each of L-alanine and L-proline, and 5 μc of DL-alanine-1-C^{14} in 200 ml of 0.01 M phosphate buffer (pH 7.4). The replaced cells were incubated for 13 hr in this mixture at 37 C. Cell and spore counts were made with a Petroff-Hausser chamber. KOH was used to trap the CO_2 evolved.

and the rate was constant through the initial stages of sporulation. Some increase was evident until essentially all cells had attained at least the forespore stage. When the same type of experiment was conducted with the use of the replacement technique, it was found that no P^{32} was incorporated into the RNA fraction during spore maturation in a solution containing L-alanine and L-proline (Fig. 5). No isotope was recovered in the DNA fraction in either the control or replaced culture in this experiment.

These data indicate that there may be no significant DNA synthesis involved in the sporulation process, and that RNA synthesis occurs only during the early stages. This was further substantiated by inhibitor data. Mitomycin C (40 μg/ml), an inhibitor of DNA synthesis, completely inhibited vegetative growth but had no effect on the rate and extent of sporulation when added at the onset of this process. Conversely,

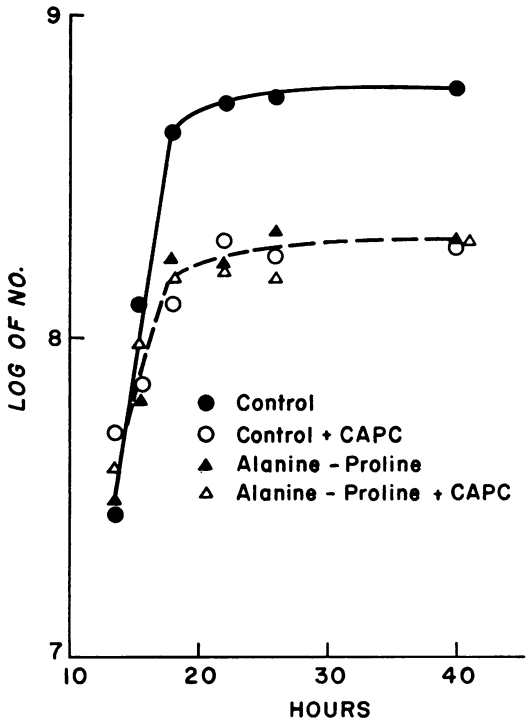

FIG. 3. *Effect of chloramphenicol (CAPC) on the formation of refractile spores in control cultures and cultures replaced in L-alanine–L-proline solution. The replacement solution and procedure were as in Fig. 1, except that 100 μg/ml of CAPC were added to the flasks indicated at the time of replacement. The controls were not replaced.*

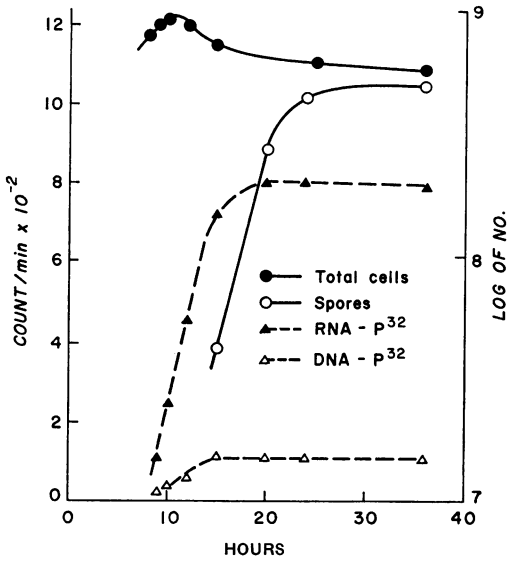

FIG. 4. *Nucleic acid synthesis during the sporulation of Clostridium botulinum. An activated culture was inoculated into 4% Trypticase broth containing 1 ppm of thiamine·HCl, and approximately 0.5 μc/ml of P^{32} as $H_3P^{32}O_4$ was added after 8 hr. Subsamples were removed at the indicated intervals, total and spore counts were made with a Petroff-Hausser counting chamber, cell samples were fractionated by the method of Hanawalt (1959), and the number of counts per minute of the P^{32} incorporated into RNA and DNA was determined.*

8-azaguanine, a general inhibitor of nucleic acid synthesis, partially inhibited spore formation when added at the onset. This compound also inhibited vegetative growth completely.

DISCUSSION

An exogenous energy source is required for the maturation of forespores of *C. botulinum*. This is in contrast to a number of species of *Bacillus* which will complete the sporulation process from the granular stage in distilled water (Hardwick and Foster, 1952; Foster and Perry, 1954; Perry and Foster, 1954; Black, Hashimoto, and Gerhardt, 1960). Collier (1956) observed a similar phenomenon in *C. roseum*. The principal difference may lie in the fact that the aerobic bacilli studied contained sufficient poly-β-hydroxybutyrate to supply the necessary energy. Although this compound is not necessary for sporulation in the aerobes, it is frequently present and, when present, is utilized during sporulation (Tinelli, 1955;

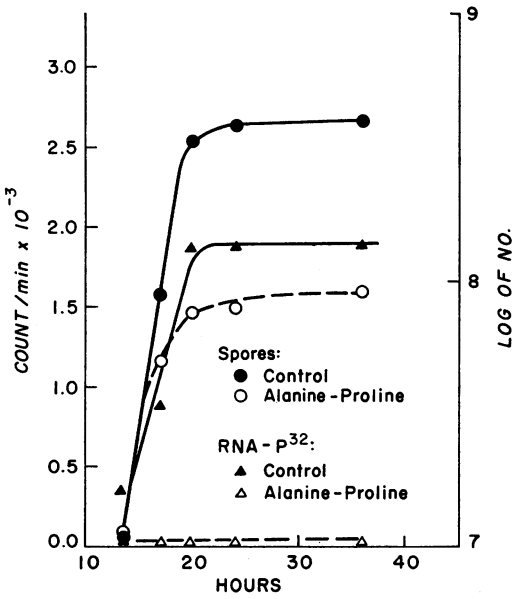

FIG. 5. *RNA synthesis during refractile spore formation in Clostridium botulinum in a control culture and after replacement in a solution containing* L*-alanine and* L*-proline. The replacement solution and procedure were as outlined in Fig. 1. Approximately 2 μc/ml of* P^{32} *as* $H_3P^{32}O_4$ *were added to the indicated cultures at the time of replacement. The control was replaced in fresh sporulation medium.* P^{32} *incorporation was measured as indicated in Fig. 4. There was no radioactivity in the DNA fraction of either the control or the* L*-alanine–*L*-proline replacement solution.*

Slepecky and Law, 1961; Stevenson et al., 1962; Nakata, 1963). No evidence of accumulation of poly-β-hydroxybutyrate was found in *C. botulinum* (Day and Costilow, 1964).

It was somewhat surprising that neither L-arginine nor L-citrulline would support the spore maturation process. Perkins and Tsuji (1962) observed that high levels of L-arginine stimulated sporulation of this same strain of *C. botulinum* in a synthetic medium and that these amino acids were utilized primarily as energy sources. Also of interest is the fact that L-ornithine would not substitute for L-proline or L-arginine in a replacement solution, since L-ornithine is believed to be the true electron acceptor in Stickland systems in which L-arginine is present as the electron acceptor (Woods, 1936). These phenomena may result from the relative availability of energy from the various systems. Thus, L-proline may be a more efficient electron acceptor for this organism than L-ornithine, and the Stickland system may provide more energy to the cell than the arginine dihydrolase system at this stage of development. With L-alanine and L-arginine in the same solution, energy could be available from both systems. Previous data obtained with germinating spores of this culture indicated that this may be true (Costilow, 1962).

The possibility exists that some of the products of cell lysis are utilized during the maturation process as suggested by Powell and Hunter (1953) and Black et al. (1960). If so, the cells were depending on nutrients from very dilute solutions, because in some instances the lysis was limited to 2.2×10^8 cells per ml, and no amino acids resulting from lysis were detectable in 0.01 ml of the replacement solution by a paper chromatogram.

The fact that there was no significant effect of high levels of chloramphenicol on the maturation of spores after replacement in simple systems, whereas the antibiotic reduced the extent of sporulation in a nonreplaced culture to the same level as that in a culture replaced in an L-alanine–L-proline solution, indicates that protein synthesis is not required for this phase of the process. There is the possibility that the forespore is impermeable to chloramphenicol, but Gerhardt and Black (1961) demonstrated that spores of *B. cereus* were readily permeable to compounds of much higher molecular weight.

The cessation of nucleic acid synthesis after forespore formation, as indicated by the absence of P^{32} incorporation and the lack of inhibition by either 8-azaguanine or mitomycin C, also indicates that these prime synthetic processes of the cell are not required for the development of a refractile, heat-resistant spore. However, it is apparent that protein and RNA syntheses are necessary through the initial phases of sporulation of *C. botulinum*, whereas no significant DNA synthesis is apparent after the cells are obviously swollen. These results are in reasonable agreement with results reported for aerobic sporeformers (Delaporte, 1950; Robinow, 1956; Fitz-James and Young, 1959; Hodson and Beck, 1960; del Valle and Aronson, 1963).

The fact that DPA synthesis occurs during forespore maturation in the presence of high levels of chloramphenicol indicates either that the enzyme(s) required for this synthesis is already

present, or that the forespore is impermeable to the antibiotic.

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