PHYSIOLOGY OF THE SPORULATION PROCESS IN 
CLOSTRIDIUM BOTULINUM

I. CORRELATION OF MORPHOLOGICAL CHANGES WITH CATABOLIC
ACTIVITIES, SYNTHESIS OF DIPICOLINIC ACID, AND
DEVELOPMENT OF HEAT RESISTANCE

LAWRENCE E. DAY1 AND RALPH N. COSTILOW

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan

Received for publication 13 May 1964

ABSTRACT

DAY, LAWRENCE E. (Michigan State University, East Lansing), and RALPH N. COSTILOW. Physiology of the sporulation process in Clostridium botulinum. I. Correlation of morphological changes with catabolic activities, synthesis of dipicolinic acid, and development of heat resistance. J. Bacteriol. 88:690-694. 1964.—A reasonable degree of synchrony in the sporulation of Clostridium botulinum 62-A was attained by using a large inoculum of a young culture into a medium containing 4% Trypticase and 1 ppm of thiamine. Sporulation was complete within 24 to 36 hr. Cells harvested at various intervals were studied for their fermentative activity with L-alanine and L-proline as substrates. The Q values (microliters of gas per hour per milligram of dry cells) were maximal at the time a large percentage of the cells initiated sporulation as indicated by swelling. They declined to a plateau at about the same level as found in vegetative cells by the time 10% of the cells had completed sporulation, and finally to a much lower level when sporulation was completed. The rates of accumulation of volatile acids (acetic, valeric, and propionic acids) corresponded closely with the catabolic potential observed. However, in the case of acetic acid, there was a significant decrease in the total acid present as the number of mature spores increased to over 50% of the final number. The total acetic acid then increased at a slow rate. The production of basic compounds during growth and sporulation more than balanced the rate of acid production, because the hydrogen ion concentration decreased exponentially throughout the period as indicated by the steady increase in pH. The synthesis of dipicolinic acid coincided closely with the development of heat resistance. Refractility developed 3 to 5 hr in advance of heat resistance.

The physiology of the sporulation process in anaerobic bacteria has received little attention. A prime deterrent has been the difficulty of synchronizing the sporulation process to a degree at which it is possible to correlate functional and morphological changes. Halvorson (1957) obtained very rapid sporulation of Clostridium roseum by using the activation procedure of Collier (1956), and Zola and Sadoff (1958) applied this process to a putrefactive anaerobe and achieved an exponential rate of sporulation which was complete in about 2 days. Thus, it appears that the inoculation of a sporogenic medium with a large number of exponentially growing cells may bring the sporulation of anaerobic bacteria into reasonable synchrony.

Synchronous cultures of Bacillus cereus show maximal oxygen uptake rates after sporulation has been initiated; acetate and pyruvate acids accumulate during vegetative growth and are utilized during sporulation (Nakata and Halvorson, 1960). The enzymes required for acetate oxidation are synthesized during the transition from vegetative growth to the sporulation process, but are apparently active only during the early phases of sporulation (Hanson, Srinivasan, and Halvorson, 1963). Acetic and other acids accumulate during vegetative growth of C. botulinum as a result of the oxidative and reduc-

1 Journal article no. 3370, Michigan Agricultural Experiment Station. This investigation was carried out during the tenure of a Predoctoral Fellowship from the Division of General Medical Sciences, U.S. Public Health Service, and is part of a dissertation submitted to the School for Advanced Graduate Studies, Michigan State University, by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

2 Present address: Pfizer Medical Research Laboratories, Chas. Pfizer & Co., Inc., Groton, Conn.
tive deamination of amino acids (Clifton, 1940; Costilow, 1962). The cells are capable of acetate activation because they have acetokinase (Simmons and Costilow, 1962), and thus are able to utilize free acetate. However, since C. botulinum cannot be expected to oxidize acetate, acetate uptake during sporulation would indicate some role other than that of an energy source.

The objective of this study was to devise a medium and a procedure for the rapid sporulation of C. botulinum which would provide the necessary degree of synchrony for physiological studies. Morphological changes were correlated with the catabolic activity of cells, as indicated by the specific fermentative activity and the rate of accumulation of various types of volatile acids. Also, the synthesis of dipicolinic acid (DPA) was studied in relation to the formation of refractile spores and heat-resistant spores.

**Materials and Methods**

C. botulinum 62-A, obtained from the American Type Culture Collection (ATCC 7948), was used for all studies. The stock culture was maintained as a spore suspension (about 10⁷ per ml) in 0.067 M phosphate buffer (pH 7.0) stored in a refrigerator.

Initial sporulation trials were conducted with the Trypticase-salts-peptone medium (TSP) of Zoha and Sadoff (1968), which was replaced with a medium containing 4% Trypticase (BBL) supplemented with 1 ppm of thiamine·HCl. This medium was used for sporulation in all instances unless otherwise stated. The Trypticase used during this study was from a single lot.

Rapid sporulation was achieved by inoculating the sporulation medium with about 10⁴ heat-shocked (80°C for 10 min) spores per ml, incubating at 37°C for 10 hr, and using this culture to inoculate the medium for sporulation. The volumes were adjusted so that the inoculum constituted 25% of the sporulation culture. Sodium thioglycolate (0.1%) was added to the medium into which the spores were inoculated but not to the sporulation medium. Natural gas was bubbled constantly through the latter culture to provide anaerobic conditions and to prevent settling.

Total counts of cells and spores were made with a Petroff-Hauser counting chamber.

Cells for metabolic studies were harvested by centrifugation and resuspended in 0.067 M phosphate buffer (pH 7.0). The fermentative activity of these cells was tested immediately after harvest in a Warburg respirometer. Hydrogen evolution was differentiated from CO₂ by absorbing the latter in 20% KOH. Dry weights were determined by evaporating 1-ml samples of the suspension to constant weight at 110°C.

The Celite (Johns-Manville Products, Detroit, Mich.) column described by Wiseman and Irvin (1957) was used for the isolation of organic acids from culture media; 3-(4-Aminilino, 1-naphthlazo) 2,7-naphthylene disulfonic acid monoammonium salt was used as the internal indicator in the column. The paper chromatographic method of Kennedy and Barker (1951) was used to identify the volatile acids isolated from the Celite column. The fraction eluted from the column was evaporated on a steam bath after the addition of 1.0 ml of concentrated NH₄OH. A 0.01-ml spot of this sample was utilized for the paper chromatogram. Known samples were prepared in a similar manner.

The DPA content of cells and spores harvested from samples taken at intervals during sporulation was determined by the procedure of Janssen, Lund, and Anderson (1958).

**Results**

To observe metabolic changes associated with sporulation, it is necessary to have the culture in reasonable synchrony. The TSP medium developed by Zoha and Sadoff (1958) for a putrefactive anaerobe was the only one found in the literature which appeared promising in this respect, and it was not successful for C. botulinum. Incubation in TSP for 5 to 7 days was required for the number of spores to reach 80-90% of the total count. In contrast, cultures in a broth containing 4% Trypticase and 1 ppm of thiamine·HCl were sporulated within 24 to 36 hr of incubation, depending on the culture. The addition of thiamine as suggested by Lund (1956) proved to be exceedingly important, because both the rate and extent of sporulation were improved on its addition to Trypticase (Fig. 1). Gas evolution was evident in the thiamine-containing medium but not in the control.

Subsequent studies demonstrated much variation in different lots of Trypticase. Some lots were not nearly so sporogenic as others. This variation was independent of thiamine.

The cultures were sufficiently well synchronized
Log of No.  

FIG. 1. Effect of added thiamine-HCl (1 ppm) on the sporulation of Clostridium botulinum in a 4% Trypticase medium. Subsamples were taken at indicated intervals from 200-ml cultures incubating at 37 C. Natural gas was bubbled through the cultures constantly. Counts were made with a Petroff-Hauser counting chamber.

to find a high percentage of the cells in each of the following morphological stages by microscopic examination: (i) vegetative, (ii) swollen cells, (iii) forespores (swollen cell containing a refractile body with a diffuse edge), and (iv) highly refractile spores. Continued incubation of the culture for 5 to 7 days resulted in lysis of the sporangia and liberation of the endospores. The most difficult observation was the differentiation of the immature forespores and mature spores, but the trained observer can do this with reasonable accuracy. This degree of synchrony was believed to be adequate for studies of some of the basic metabolic activities occurring during sporulation.

Catabolic rates. The Stickland reaction system was selected as an indicator of the catabolic functioning of cells during sporulation, because it is one of the principal energy-gaining pathways utilized by C. botulinum (Clifton, 1940; Costilow, 1962). Cells harvested from cultures after various periods of incubation were used to ferment a mixture of L-alanine and L-proline, and the $Q_{\text{H}_2}$ (microliters of CO$_2$ evolved per hour per milligram of dry cells in a helium atmosphere) and $Q_{\text{H}_4}$ (microliters of H$_2$ evolved per hour per milligram of dry cells in a helium atmosphere) values were determined.

Cells from 14- to 16-hr cultures were found to have the highest fermentative capacity for this system (Fig. 2). At this time, over 90% of the cells were obviously swollen and, thus, had initiated sporulation. As immature forespores appeared, the Q values declined to about the level found in the vegetative cells, where they remained relatively constant during most of the spore
maturation period. The harvest from the sporulated culture had the lowest fermentative activity on L-alanine and L-proline.

Acid production. The volatile acids produced during the growth and sporulation of *C. botulinum* were separated on a Celite column (Wiseman and Irvin, 1957), and were identified as acetic, propionic, and valeric acids. The valeric acid was probably the optically active form which is obtained on the oxidative deamination and subsequent decarboxylation of isoleucine. Concentrations of the individual acids in the culture at various periods were determined by titration of the appropriate fractions from the Celite column.

Both acetic and valeric acid were produced at increasing rates until over 90% of the cells contained forespores (Fig. 3). Valeric acid was formed thereafter at a much slower rate; there was a significant decrease in the acetic acid concentration during the early part of the period of rapid increase in the number of refractile spores. Only a relatively small amount of propionic acid was formed.

Even though there was over 0.05 meq/ml of volatile acid produced in the culture during sporulation, the pH of the culture increased steadily until sporulation was practically complete. This undoubtedly resulted from the production of NH₃ and basic amino compounds. Direct nesslerization of samples demonstrated that the free amino nitrogen increased greatly during the entire period.

**Synthesis of poly-β-hydroxybutyrate and DPA.** Granules of poly-β-hydroxybutyrate accumulate in cells of aerobic bacilli prior to sporulation (Law and Slepecky, 1961), and the cells become very granular in appearance. No such granulation is observable in cells of *C. botulinum*, and assays conducted for this compound by the procedure of Law and Slepecky (1961) were negative.
Preliminary data (Day and Costilow, 1961) from less highly synchronized cultures indicated that DPA synthesis occurred prior to the appearance of refractile spores. However, this did not prove to be true. Refractile spores are formed in advance of DPA synthesis, and the latter coincides closely with the development of heat resistance (Fig. 4). The total DPA level attained was about 28 μg/ml of culture. Clean spores were found to contain DPA at concentrations from 3 to 4% of their dry weight.

Effect of added volatile acids and pH adjustment on sporulation. The addition of acetic, valeric, and propionic acids to the sporulation medium initially and the adjustment of the pH to 7.5 had no effect on the time of onset nor on the extent of sporulation of C. botulinum.

DISCUSSION

The sporulation process in C. botulinum has not been brought into ideal synchrony, but the rate of sporulation attained was sufficient to reflect functional changes which could be correlated with morphological changes. Thus, the swollen cells recognizable at the initiation of sporulation have the highest fermentative activities with a mixture of L-alanine and L-proline as substrates, and the rate of volatile acid accumulation increases up to this point. The intermediate stages in development from the swollen cell to the mature spores are not so well defined, but it is possible to correlate the presence of increasing numbers of forespores with a specific fermentative capacity not significantly different from that observed with vegetative cells and with a net decrease in the acetic acid concentration in the culture. Also, correlations of the development of refractility with DPA synthesis and the development of heat resistance are quite similar to those reported for B. cereus strain terminalis (Hashimoto, Black, and Gerhardt, 1960). These correlations indicate that the system is adequate for use in conducting more detailed studies of the physiology of this process.

The increased catabolic rate after the onset of sporulation corresponds to the increased oxygen demand observed by Halvorson (1957) in B. cereus var. terminalis, indicating an increase in the energy available to the cell. Also, the picture of acetate utilization indicated by the determination of total acetic acid free in the medium is similar to that reported by Nakata and Halvorson (1960).

LITERATURE CITED


