JOURNAL OF BACTERIOLOGY, Sept. 1972, p. 674-681
Vol. 111, No. 3

Interference-Contrast and Phase-Contrast Microscopy of Sporulation in *Clostridium thermosaccharolyticum* Grown Under Strict Anaerobiosis

CHARLES ELLER AND Z. JOHN ORDAL

Department of Food Science, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Received for publication 25 May 1972

Cells of *Clostridium thermosaccharolyticum* grown under strict anaerobiosis (modified Hungate technique) were examined during growth and sporulation by employing Nomarski interference-contrast and Zernike phase-contrast optics to delineate the sequence of morphological changes leading to the formation of free, mature spores. A 0.5% L-arabinose, liquid, complex medium was used to obtain a yield of 30 to 40% free, refractile spores (ca. 10^8/ml) by 48 hr of incubation. The mean doubling time for the glucose culture (vegetative cells) was found to be 80 min, and that for the L-arabinose culture (sporulating cells), 498 min. By 8 hr of incubation, beginning spore formation became evident in the arabinose culture by the development of a distinct arrowhead-shaped terminal swelling. By 32 hr of incubation or shortly thereafter, Nomarski optics showed the mature spore to be uniformly spherical, whereas the enlarged terminal swelling containing it was not. The use of phase-contrast and interference-contrast optics permitted the characterization of the distinctive morphological changes occurring during sporulation of *C. thermosaccharolyticum*.

Clostridium thermosaccharolyticum is a thermophilic, saccharolytic, nonproteolytic, spore-forming anaerobe (13) producing acetate, butyrate, CO_2, and H_2 as fermentation end products; in addition, cells committed to sporulation produce ethanol (9). This bacterium is of economic importance as it produces spores of high heat resistance and is responsible for the spoilage of canned goods known as "hard swells" (12, 18). The growth and sporulation of this microbe has been the subject of several recent investigations from this laboratory. Carbohydrates (such as arabinose) which have the common effect of reducing the growth rate stimulate sporulation; those (such as glucose) which allow unrestricted growth repress sporulation (7).

McClung (13) noted that the morphology of sporulation was a most distinctive feature of this bacterium; however, no report has dealt intensively with the rather remarkable morphological changes occurring during sporogenesis. It is important to note that completely free spores apparently have not often been observed for this bacterium; the spore remains attached to the sporangium even after extended incubation (13, 17). In this investigation, we examined cells of *C. thermosaccharolyticum* during growth and sporulation under strict anaerobiosis by using Nomarski interference-contrast and Zernike phase-contrast optics to follow the sequence of morphological changes leading to the production of free, refractile spores.

MATERIALS AND METHODS

Microorganism. *C. thermosaccharolyticum*, National Canners Association strain 3814, originally obtained from George York, University of California, Davis, was used in this study.

Anaerobic culture technique. The method used for maintenance of strict anaerobiosis was similar to that originally described by Hungate (10) and modified from that of Eller, Crabill, and Bryant (2) principally in that round-bottom flasks were used as culture vessels instead of roll tubes. Anaerobic conditions were continuously preserved during preparation, sterilization, inoculation, and sampling of the medium. Gas (100% CO_2) was passed into round-bottom flasks (either 200- or 500-ml capacity) through the barrel of a 2-ml Luer-Lock syringe con-

taining sterile cotton, fitted with a bent 3-inch (ca. 7.6 cm), 16-gauge needle. Whenever a flask was opened, a gassing needle was immediately inserted, and the flask was closed with a no. 5 solid rubber stopper as the needle was withdrawn. The stopper was then wired on tightly. Gas from the cylinder was passed through a column of hot (300 to 400 C) reduced copper wire to remove traces of oxygen. The copper oxide produced was reduced by periodic passage of hydrogen gas (100% H_2) through the column. Descriptions of a similar copper column setup are given by Moore (14) and by Hungate (11).

Culture sampling. Periodic sampling of a culture was facilitated by the transfer of the flask culture incubated in a 56 C incubator (Lab-Line Imperial II Radiant Heat Incubator, Lab-Line Instruments, Inc., Melrose Park, Ill.), to a water bath at 56 C. The stopper was then removed with concomitant insertion of a gassing syringe needle. A sample (10 ml) was then taken with a sterile pipette after first sucking up headspace CO_2 through the pipette. After sampling, the rubber stopper was placed at the top of the neck of the flask, and the gassing needle was brought halfway out of the neck; after about 15 sec the needle was swiftly withdrawn as the stopper was plunged into the neck of the flask. After fully seating the stopper, it was rewired on, and the culture flask was placed back into the incubator for continued incubation. These steps did not disturb the oxidation-reduction potential of the culture as shown by the indicator, rezasurin, nor was the temperature of the culture disturbed.

Media. Stock cultures were made in pea broth (*p*H 6.8) in screw-capped tubes (12% Alaskan whole seed peas, 2% peptone [Difco], and 10 μg of manganese [$MnSO_4 \cdot H_2O$] per ml). Seed peas were obtained from W. A. Burpee Co., Clinton, Iowa. The stock cultures were overlaid with sterile nutrient agar (Difco) as a seal immediately after inoculation. No strict anaerobic procedures were used for these stock cultures since good growth and some sporulation is readily obtained in freshly prepared pea broth. After the cultures had been incubated for 8 hr at 56 C (*p*H 5.8), they were placed and stored at 4 C. The stock cultures were transferred at approximately 6-month intervals. For each experiment, a stock culture tube or tubes, depending upon the amount of inoculum needed, was removed from storage at 4 C and incubated at 56 C overnight (usually for 24 hr; final *p*H, 5.1). A 5% pea broth inoculum (ca. 10^9 cells) was inoculated directly into the test medium without any intervening series of transfers. A 5% inoculum was found by Hoeniger, Stuart, and Holt (6) to be more sporogenic for *Clostridium perfringens* than were other inocula between 1 and 10%. It was found in the present study that an active culture method was unnecessary to obtain good growth and sporulation with a minimum lag period when strict anaerobic techniques were utilized throughout preparation, inoculation, and incubation of the test media. The test media employed were slightly modified from that of Pheil and Ordal (17) and Hsu and Ordal (8). The medium used to obtain sporulating cells of *C. thermosaccharolyticum* had the following composition (with the constituents being expressed as final percentages in the medium): peptone (Difco), 0.5%; yeast extract (Difco), 0.5; L-arabinose, 0.5; $CaCl_2 \cdot 2H_2O$, 0.01; $(NH_4)_2SO_4$, 0.1; $MgSO_4$, 0.01; $MnSO_4 \cdot H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.0005; $CuSO_4 \cdot 5H_2O$, 0.0005; $MoSO_4$, 0.0001; $FeSO_4 \cdot 7H_2O$, 0.00005; thiamine hydrochloride, 0.0001; *p*-aminobenzoic acid, 0.000002; biotin, 0.0000001; resazurin, 0.0001; Na_2CO_3, 0.16; cysteine $HCl \cdot H_2O$, 0.05; and CO_2 gas phase, 100.00. A solution of the mineral salts was prepared in a concentration 20 times that required in the medium; a stock solution of the vitamins was also prepared in a concentration 20 times that required and stored at 4 C. In preparing the medium, all constituents, except Na_2CO_3 and cysteine·HCl were added to the required amount of double-distilled water (Corning Distilling Apparatus, model AG-1b, Corning Glass Works, Corning, N.Y.) contained in a round-bottom flask and boiled under CO_2. After stoppering (the stopper was wired on), the medium was autoclaved at 121 C for 15 min. After autoclaving and cooling to 56 C (the resazurin in the medium was colorless, indictating a redox potential below −0.042 v), the sterile CO_2-equilibrated Na_2CO_3 and cysteine·HCl were anaerobically added to the medium. The *p*H of the complete medium was 6.5. The cysteine reducing agent was made up as a 2.5% solution and tubed anaerobically under CO_2 (sterile, rubber-stoppered tubes, 18 by 150 mm, flint glass, Bellco-Glass Inc.). The Na_2CO_3 was made up as an 8% solution, tubed anaerobically under CO_2, and equilibrated with CO_2 for 30 min immediately before addition to the medium. After the addition of the Na_2CO_3 and the cysteine·HCl, the medium was inoculated while it was maintained at 56 C; the *p*H of the inoculated medium remained at 6.5. The medium for obtaining only vegetative cells of *C. thermosaccharolyticum* was identical to that just described with the exception that the carbohydrate used was 0.5% glucose.

Growth determination of bacteria. Growth of cultures was estimated by following the changes in optical density (OD) at 600 nm with a Bausch and Lomb Spectronic 20 colorimeter. Uninoculated, oxidized (pink) medium was used as a reference, and dilutions of cultures with the medium were made as necessary (above 0.60 OD unit). The exponential growth rate constant, called k, expressed as the number of generations (doublings) per hour, was calculated from the formula $k = (\log_{10}N_t - \log_{10}N_0)/0.301t$, where N_0 is the total cell count at a certain time, N_t is the count at a subsequent time, and t is the time elapsed; 1/k, the time required for the population to double (mean doubling time), was also calculated.

Spore and vegetative cell counts. A Petroff-Hausser bacteria-counting chamber was used for direct phase-microscope counts to determine the total number of vegetative cells, sporulating cells, and free refractile spores. Culture samples were diluted with 50% glycerol as necessary to obtain the proper number of bacteria per counting square and to retard cell movement.

Microscope observations and photography.

Warm liquid agar (nutrient agar, Difco) was allowed to flow freely over the surface of a clean glass slide held at a steep angle. After almost instantaneous cooling, a very thin film of solidified agar coated the slide. A small drop of culture suspension was placed on a glass cover slip; the cover slip was then gently inverted and placed onto the film of agar. The sides of the coverslip were sealed with candle wax. Observations were made with a Zeiss photomicroscope equipped with Zernike phase-contrast and Nomarski interference-contrast optics. All photographs were taken with an electronic flash device through oil-immersion objectives (phase-contrast, Ph3, neofluor, numerical aperture 1.3; interference-contrast, planachromat, numerical aperture 1.25) and ×8 ocular with an achromatic, aplanatic phase-contrast and Inco condenser, numerical aperture 1.4. Kodak high-contrast copy 35-mm film was chosen as the film of choice with H & W control film developer (The H & W Co., St. Johnsbury, Vt.) used for maximum contrast and resolution. Contrast and resolution were enhanced by placing Zeiss immersion oil between the condenser and the slide. The finished prints had a magnification of approximately ×4,000.

RESULTS AND DISCUSSION

Growth studies. The time course of unrestricted growth of *C. thermosaccharolyticum* in a 0.5% glucose batch culture under strict anaerobiosis is shown in Fig. 1. Interestingly, the absorbance of the culture fell rapidly after reaching a maximum at 9 hr, whereas the total cell count remained unchanged after 9 hr of incubation. Microscopic observation of the cells after 9 hr showed that they lost cellular density and appeared as phase-pale cells; hence, the cells seemed to maintain their cellular integrity while leaking much of their internal contents into the medium. This microscopic appearance is typical for glucose-grown cells after the cells have passed the log phase of growth. The *p*H of the culture dropped markedly between 7 and 9 hr of log-phase growth and became stabilized thereafter. The final *p*H, 5.6, was higher than usually occurs during unrestricted growth on glucose (can be as low as *p*H 4) due to the presence of the CO_2-bicarbonate buffer system in the medium. Hence, restricting a sharp drop in *p*H alone did not result in stimulation of sporulation. One of the inhibitory effects on sporulation to be considered would be the lowering of *p*H to an unfavorable level; however, Pheil and Ordal (17) also showed that *p*H alone was not of primary concern in inducing sporulation. What is of primary concern apparently is whether or not the carbon source substantially reduces the growth rate of the bacterium (7).

The time course of growth of *C. thermosaccharolyticum* in a 0.5% L-arabinose batch culture under strict anaerobiosis is given in Fig. 2. Here, the total cell counts represented not only vegetative cells, but also sporangia (cells in various stages of terminal swelling, i.e., spore formation) and free refractile spores. It can be seen in Fig. 2, in comparison to the glucose growth curves in Fig. 1, that cells in the arabinose medium grew at a much slower rate, were not produced in as high numbers, did not yield as high an absorbance at maximum log

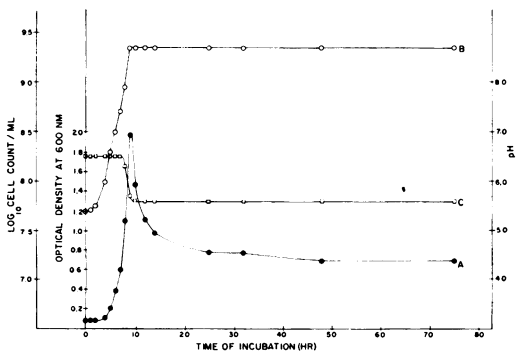

FIG. 1. *Unrestricted growth of C. thermosaccharolyticum in batch culture in a 0.5% glucose medium under strict anaerobic conditions. Curve A (●), change in optical density (600 nm) during the entire growth period; curve B (○), corresponding change in the total number of bacterial cells; and curve C (□), change in pH. Note that only vegetative cells make up the total cell counts; no spores are produced by the bacterium growing unrestrictedly on glucose.*

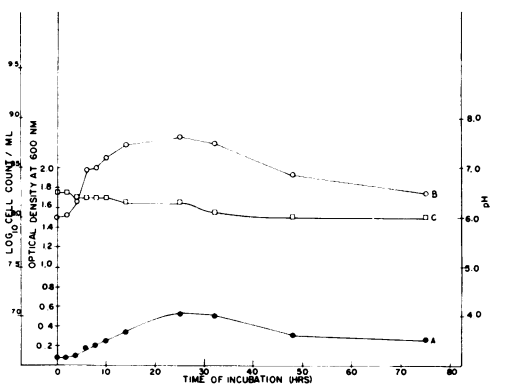

FIG. 2. *Growth of C. thermosaccharolyticum in batch culture in a 0.5% L-arabinose medium under strict anaerobiosis. Curve A (●), change in optical density (600 nm) during the entire growth period; curve B (○), corresponding change in the total number of bacteria; and curve C (□), change in pH. Note that both vegetative cells and sporangia, as well as free, mature spores, make up the total cell numbers at certain times, since growth on L-arabinose effects sporulation in this bacterium.*

phase, and only increased the acidity of the medium to *p*H 6. It is believed that in sporulating cultures, such as those growing at the expense of arabinose, some of the acetate formed during the initial period of batch growth is reduced to ethanol because of the greater reducing capabilities of sporulating cells (9).

The exponential growth rate constant, k, for the glucose culture (vegetative) was calculated to be 0.75 generation/hr, for a mean doubling time of 80 min. The L-arabinose culture (sporulating) had a k value of 0.12 generation/hr, for a mean doubling time of 498 min. Total cell count data between 2 and 9 hr of log growth in glucose and 2 and 25 hr of growth in arabinose were utilized in these calculations.

Sporulation studies. The time course of sporulation (both sporangia and free spore formation) in *C. thermosaccharolyticum* in a 0.5% L-arabinose batch culture is presented in Fig. 3. Initially about 8% sporangia were introduced with the pea broth inoculum. By 6 hr of incubation, the per cent sporangia dropped to 3% because of cell division occurring without concomitant spore development. By 8 hr of incubation, spore formation became distinctly evident by the development of "arrowheads" by some of the elongated cells (Plate I, Fig. 5 and 6). Arrowhead formation rather than cell elongation was taken as the first overt sign of sporulation by phase-contrast microscopy, since elongated cells can be found as well in glucose-grown vegetative cultures, although the predominant cell type in vegetative cultures at mid to maximum log phase was a short rod. Fitz-James (3) observed a marked increase in cell size (elongation) during spore formation in *Clostridium pectinovorum*. By arrowhead formation, we refer to the distinct arrowhead shape of the terminal phase-dark swelling of the sporulating cell, the swelling being somewhat pointed at its apex; this can be considered the forespore stage. By 10 hr of incubation, most of the sporangia, which then numbered about 17% of the cells, exhibited the arrowhead appearance. By 14 hr of incubation sporangia represented about 33% of the culture cells; rods with phase-dark, swollen, slightly pointed to completely spherical ends comprised the majority of the sporangia (Plate I, Fig. 7 and 8). By 25 hr of incubation the numbers of sporangia reached a peak at 2.85×10^8/ml (44%); most of the sporangia exhibited slightly to moderately refractile terminal spore ends with little cellular material enveloped around the developing refractile spore (Plate I, Fig. 9 and 10). After 25 hr the number of sporangia began to decrease rapidly. Concomitant

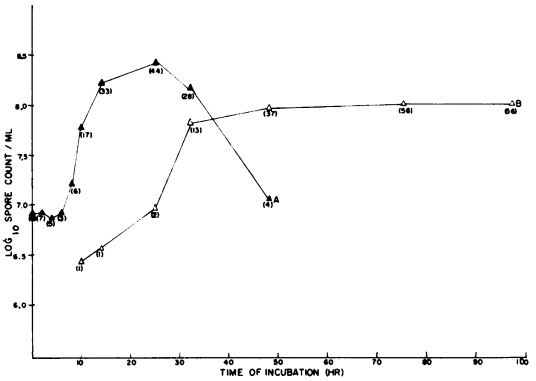

FIG. 3. *Sporulation of C. thermosaccharolyticum in batch culture in a 0.5% L-arabinose medium under strict anaerobiosis. Curve A (▲), change in number of sporangia (cells in various stages of terminal swelling, i.e., spore formation) and curve B (△), change in number of free, mature spores. The percentage of sporulation is shown by numbers in parentheses for both sporangia and free spores. The percentage of free spores in reality becomes somewhat fictitious after 48 hr of growth due to lysis of vegetative cells and sporangia which do not fully mature.*

with this decrease in sporangia, the level of free, spherical, refractile spores (released from sporangia) rose significantly. After 32 hr of incubation the sporangia (28%) were essentially all fully phase-bright terminal spores (Plate I, Fig. 11–14). At this stage, many of the vegetative cells, both short and long rods, were phase-pale, indicating leakage of their cellular contents into the medium. This fact was also indicated by a decrease in absorbance as shown in Fig. 2. Also at this incubation period, the level of free, spherical, phase-bright spores (Plate I, Fig. 17) had risen to 13% of the bacterial cells. By 48 hr of incubation, sporangia and vegetative cells were lysing, few intact sporangia were evident, and the percentage of free spores reached 37%. In reality, it was about at this point that a stated percentage of free spores present became somewhat fictitious because of the occurrence of cell lysis. Here, it is more meaningful to quantitate in terms of spore numbers rather than as percentages. By 48 hr of incubation, the total number of free, refractile spores was 10^8/ml, nearly the maximum free spore crop per milliliter. By 75 hr of incubation, the maximum free, refractile spore number per milliliter was attained: 1.1×10^8/ml. No sporangia and few intact vegetative cells were present at this incubation period. Perkins (16) pointed out that the finding of a medium which stimulates spore production by the thermophilic anaerobes would represent a

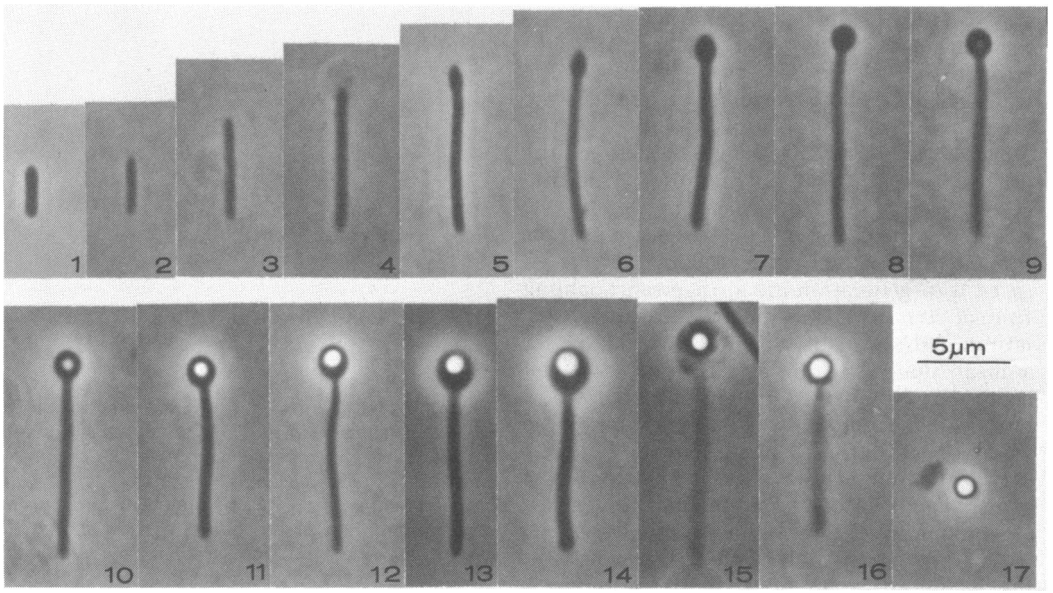

PLATE I. *Phase-contrast microscopy of the sequential morphological changes during the sporulation of C. thermosaccharolyticum. The cells were grown in a 0.5% L-arabinose medium under strict anaerobiosis.* FIG. 1. *Short, stout vegetative cell (glucose-grown).* FIG. 2-4. *Thin sporulating cell undergoing cell elongation.* FIG. 5 and 6. *Initial terminal swelling of the sporangium (forespore) giving an "arrowhead" appearance.* FIG. 7. *Enlarged arrowhead swelling becoming more round at the tip.* FIG. 8. *Completed spherical phase-dark terminal swelling.* FIG. 9 and 10. *Developing refractility at the center of the spherical end.* FIG. 11. *Fully refractile spore in the terminal swelling.* FIG. 12-14. *Further engulfment of the refractile spore by cellular material producing a much enlarged swollen end.* FIG. 15 and 16. *Lysis of the sporangial contents around the spore and in the rod occurs as a prelude to release of the spore.* FIG. 17. *A free, mature, spherical, refractile spore. Scale marker = 5 μm.*

major contribution to the study of bacterial spores because spores of the thermophilic anaerobes are the most heat resistant known, and information about spore resistance might be revealed if sufficient quantities could be produced for analyses. The procedure used in this study regularly produced 10^8 free spores/ml. Preliminary heat resistance studies demonstrated that these spores possessed a moderately high heat resistance (surviving for 3 min at 121 C). It was interesting to note that somewhat less than half of the sporangia produced in the medium proceeded to form free, refractile spores. It is not known why so many of the sporangia which appear to be committed to sporulation do not go on to maturity. In summary, the sporulation process of this bacterium as evidenced by phase-contrast microscopy commenced at about 7 to 8 hr after inoculation and was completed by about 48 hr.

Sequential development during sporogenesis as viewed under phase-contrast. The sequential course of morphological events occurring during sporulation of *C. thermosaccharolyticum* is detailed in Plate I, Fig. 2 through 17. It is important to note, first of all, that these elongated rod-shaped bacteria as seen in Plates I and II must be very flexible since they bend quite readily when immobilized on the agar-coated slides; they are normally straight rods and would appear so if viewed in a hanging drop preparation. A vegetative, glucose-grown cell is shown in Plate I, Fig. 1. Pheil and Ordal (17) measured the size of the cells and found that the sporangia were long (8.9 to 10 μm) and thin (0.35 μm), whereas the vegetative (nonsporulating) cells were shorter (2.0 to 3.5 μm) and thicker (0.5 μm); this size difference is readily apparent in Plate I, Fig. 1 in comparison to Fig. 2-4. We observed a general nongranular appearance to both "healthy" vegetative cells and sporangia; both were homogeneously phase-dark. Granulation or a mottled appearance was pronounced in old and lysing (dying) cells and sporangia; this was probably due to sites of coagulation of cellular materials along with leakage of these materials and not due to actual inclusion granule formation. When cells were stained with Sudan black B, occasional sudanophilic granules were seen in young vegetative cells and sporangia; however, these

granules occurred quite sparsely, as many cells were without them. Both glycogen and volutin stains failed to demonstrate such materials in our preparations.

After cell elongation occurred, a swelling formed at one end of the rod giving the appearance of an arrowhead rather than a club (Plate I, Fig. 5 and 6); the apex of the terminal swelling having a definite pointed shape. Clubbing or a characteristic drumstick appearance is seen in some of the other members of the clostridia (3, 19, 20). According to Fitz-James and Young (4), the formation of the forespore septum is the first distinct sign that the cell is commencing sporulation. As shown by Hitchins, Kahn, and Slepecky (5), these septa are not easily discerned in *Bacillus* by phase-contrast microscopy, but can be depicted with interference-contrast optics. We were unable to detect the forespore septum with either of the two optical techniques. The arrowhead end of the cell continued to enlarge; it became more round (Plate I, Fig. 7) and eventually became a completely spherical, phase-dark terminal swelling (Plate I, Fig. 8). Next, refractility began to develop in the center of the spherical, swollen end (Plate I, Fig. 9 and 10) until a fully phase-bright spore was formed (Plate I, Fig. 11); this was followed by an enlargement of the swollen end of the cell (Plate I, Fig. 12–14). In many instances, the refractile spore appeared off-center, being situated closer to one side of the swollen end. The spore and the sporangium at this stage are considered uniquely characteristic and dramatic of the species. Next, the cellular sporangial mass, both in the rod and surrounding the spore, lost density as evidenced by a decrease in phase darkness (Plate I, Fig. 15 and 16); after completion of lysis the mature, refractile, spherical spore was released (Plate I, Fig. 17).

Comparison of sporulation as seen with phase-contrast and interference-contrast. Plate II, Fig. 1 through 12, depicts the gross morphological changes which occur during sporogenesis of *C. thermosaccharolyticum* when identical cells are viewed both by phase-contrast and by interference-contrast microscopy. In general agreement with Hitchins, Kahn, and Slepecky (5), the bacteria viewed using the Nomarski system (i) showed a strong "shadow cast effect" or brilliant relief image, almost as if side lighting was used, (ii) exhibited an optically flat appearance, (iii) lacked the halo, particularly evident around the spore swelling, characteristic of phase-contrast optics, and (iv) appeared as slightly larger cells than if viewed under phase-contrast. Hitchins, Kahn, and Slepecky (5) found that the Nomarski system also gave more detail, particularly showing the presence of well-defined forespore septa and granules; however, in the present investigation, little advantage was seen in this regard. Although more detail was generally lacking, we did find that sporangia with fully refractile spores showed some additional detail under the Nomarski system. As depicted in Plate II, Fig. 8 through 11, the spore appears to be bulging out of the terminal swelling giving a "fried egg" appearance. Hence, the entire swelling itself does not appear round like a ball, the impression one might obtain from phase-contrast microscopy, but rather is optically flatter than the spore it contains. It was also observed that the decreased cell density as seen with phase-contrast when a cell is lysing appeared as a more optically flat cell with Nomarski optics (compare Plate II, Fig. 8 and 9 with Fig. 10 and 11). In general, in agreement with Hitchins, Kahn, and Slepecky (5), the development of refractility as seen with phase-contrast was paralleled by an enhancement of the "shadowing effect" (three-dimensional image) of spores with interference-contrast optics. However, phase-contrast microscopy showed more readily the development of spore refractility. Observation of Plate II, Fig. 6 and 7 shows that under Nomarski optics no difference is apparent between the sporangia in the two figures, whereas under phase-contrast, the initial development of spore refractility is obvious in Fig. 7. It is concluded that for this bacterium there are advantages for using both light microscopic techniques for following the sequence of morphological changes occurring during sporogenesis.

We have deliberately avoided discussing the morphology of sporulation of *C. thermosaccharolyticum* in terms of the seven stages as currently defined for sporulation in both *Bacillus* and *Clostridium* (1, 4, 5, 15), since the two optical techniques employed in this study did not adequately reveal details other than gross changes occurring during the sequence of sporulation. Nuclear stains were done, but we were not able to detect stage 1 or the axial chromatin stage. Stages 2 and 3, forespore septation and development, were evidenced only by arrowhead formation and development, and no septa were seen. Stage 4, cortex formation, and Stage 5, coat formation, were only indicated by changes in spore refractility as viewed by phase-contrast microscopy. Maturation, or stage 6, was evidenced by an increase in the size of the terminal swelling after full spore refractility had developed and by decreased cell density preceding lysis of the sporangium. Stage 7, or the free refractile spore

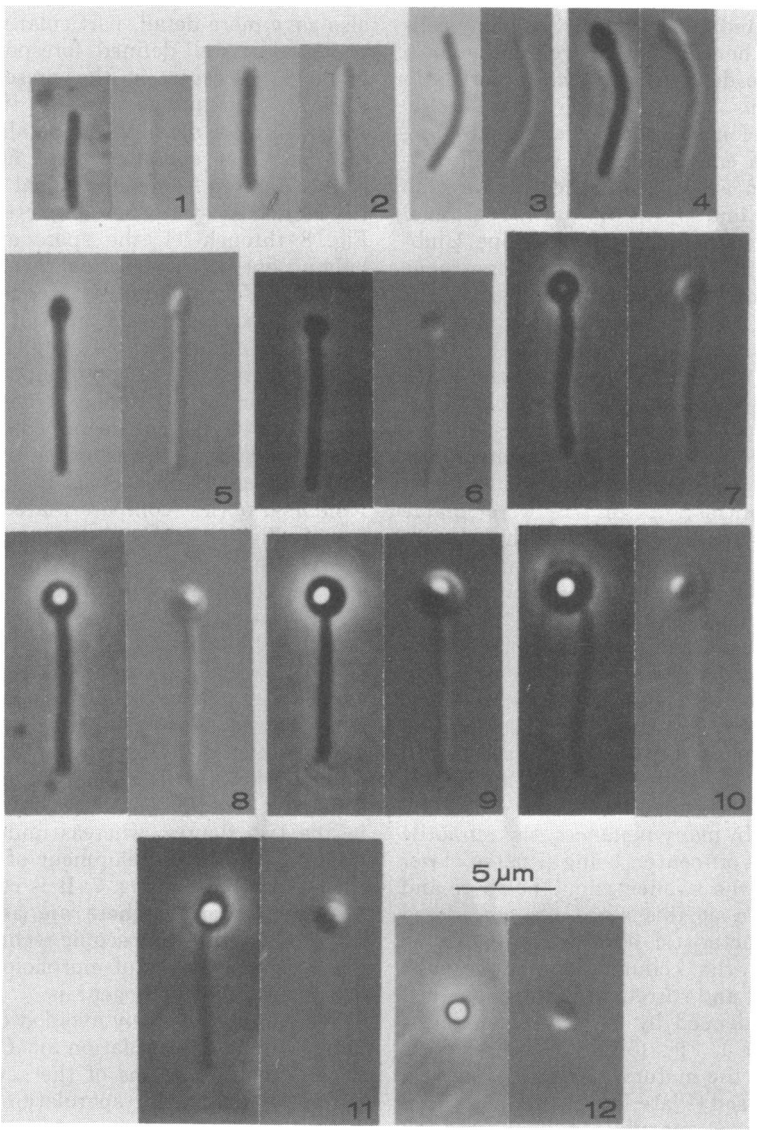

PLATE II. *Gross morphological changes occurring during sporulation of C. thermosaccharolyticum as revealed by both phase-contrast and interference-contrast microscopy. Cells were produced in a 0.5% L-arabinose medium under strict anaerobiosis. The right side of each figure represents interference-contrast optics; the left side, phase-contrast optics of the identical cell.* FIG. 1–3. *Thin, sporulating cell undergoing elongation.* FIG. 4. *Terminal swelling of the sporangium (forespore) exhibiting an "arrowhead" shape.* FIG. 5. *Arrowhead swelling becoming rounder.* FIG. 6. *Completed spherical terminal swelling.* FIG. 7. *Initial development of spore refractility in the center of the swelling.* FIG. 8. *Fully refractile spore in the spherical swelling.* FIG. 9. *Further envelopment of the spore by cellular material forming an enlarged swollen end.* FIG. 10 and 11. *Decrease in cellular density and lysis of the sporangial material before the release of the spore.* FIG. 12. *A free, mature, spherical, refractile spore. Scale marker = 5 µm.*

stage, was readily depicted. Furthermore, these developmental stages in sporulation up to and including cortex formation have proven to be nearly identical in all species of the *Bacillaceae* thus far studied (15); we have as yet no reason to believe that they are any different in this thermophilic anaerobe. It is the spore coat stage which occurs differently in various sporeformers; the coats being single, double, laminated, or ridged, depending on the species

(15). Through the use of thin-sectioning and electron microscopy, we are looking further into the sporulation process of *C. thermosaccharolyticum*; however, the utilization of phase-contrast and Nomarski interference-contrast microscopy has allowed delineation of the interesting gross changes during the sporulation sequence of this thermophilic anaerobe.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service training grant FD 00004 from the Food and Drug Administration.

LITERATURE CITED

1. Balassa, G. 1971. The genetic control of spore formation in bacilli. Curr. Top. Microbiol. Immunol. **56**:99-192.
2. Eller, C., M. R. Crabill, and M. P. Bryant. 1971. Anaerobic roll tube media for nonselective enumeration and isolation of bacteria in human feces. Appl. Microbiol. **22**:522-529.
3. Fitz-James, P. C. 1962. Morphology of spore development in *Clostridium pectinovorum*. J. Bacteriol. **84**: 104-114.
4. Fitz-James, P. C., and E. Young. 1969. Morphology of sporulation, p. 39-72. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press Inc., London.
5. Hitchins, A. D., A. J. Kahn, and R. A. Slepecky. 1968. Interference contrast and phase contrast microscopy of sporulation and germination of *Bacillus megaterium*. J. Bacteriol. **96**:1811-1817.
6. Hoeniger, J. F. M., P. F. Stuart, and S. C. Holt. 1968. Cytology of spore formation in *Clostridium perfringens*. J. Bacteriol. **96**:1818-1834.
7. Hsu, E. J., and Z. J. Ordal. 1969. Sporulation of *Clostridium thermosaccharolyticum*. Appl. Microbiol. **18**:958-960.
8. Hsu, E. J., and Z. J. Ordal. 1969. Sporulation of *Clostridium thermosaccharolyticum* under conditions of restricted growth. J. Bacteriol. **97**:1511-1512.
9. Hsu, E. J., and Z. J. Ordal. 1970. Comparative metabolism of vegetative and sporulating cultures of *Clostridium thermosaccharolyticum*. J. Bacteriol. **102**:369-376.
10. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1-49.
11. Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3B. Academic Press Inc., New York.
12. McClung, L. S. 1935. Studies on anaerobic bacteria. III. Historical review and technique of culture of certain thermophilic anaerobes. J. Bacteriol. **29**:173-187.
13. McClung, L. S. 1935. Studies on anaerobic bacteria. IV. Taxonomy of cultures of a thermophilic species causing "swells" of canned foods. J. Bacteriol. **29**:189-203.
14. Moore, W. E. C. 1966. Techniques for routine culture of fastidious anaerobes. Int. J. Syst. Bacteriol. **16**:173-190.
15. Murrell, W. G. 1967. The biochemistry of the bacterial endospore, p. 133-251. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in Microbial Physiology, vol. 1. Academic Press Inc., New York.
16. Perkins, W. E. 1965. Production of clostridial spores. J. Appl. Bacteriol. **28**:1-16.
17. Pheil, C. G., and Z. J. Ordal. 1967. Sporulation of the "thermophilic anaerobes." Appl. Microbiol. **15**:893-898.
18. Reed, J. M., C. W. Bohrer, and E. J. Cameron. 1951. Spore destruction rate studies on organisms of significance in the processing of canned foods. Food Res. **16**: 383-408.
19. Santo, L. M., H. R. Hohl, and H. A. Frank. 1969. Ultrastructure of putrefactive anaerobe 3679h during sporulation. J. Bacteriol. **99**:824-833.
20. Takagi, A., T. Kawata, S. Yamamoto, T. Kubo, and S. Okita. 1960. Electron microscopic studies on ultrathin sections of spores of *Clostridium tetani* and *Clostridium histolyticum*, with special reference to sporulation and spore germination process. Jap. J. Microbiol. **4**:137-155.