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CHARACTERISTICS OF AN ABORTIVELY DISPORIC VARIANT OF *BACILLUS CEREUS*

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ABSTRACT

YOUNG, I. ELIZABETH (University of Alberta, Edmonton, Alberta, Canada). Characteristics of an abortively disporic variant of *Bacillus cereus*. J. Bacteriol. **88**:242–254. 1964.—A variant [A(−)3] of the *Bacillus cereus* group has been isolated which appears to begin the formation of a spore at each pole of the cell. These pseudo-forespores, like conventional forespores, are initially formed by invagination of the plasma membrane. However, their maturation does not continue the course of normal spore development. There is no further proliferation of the membrane and no development of the peripheral layers, the cortex, spore coat, or exosporium; there is deposition of apparent cell-wall material between the layers of the septa. Each pseudo-forespore receives approximately one-half of the cell's chromatin. With a supply of fresh nutrients, each is able to resume division after elongation to the bacillary form. Furthermore, the portion of the cell lying between the two pseudo-forespores, containing at most a fragment of the total chromatin, is able to resume growth and division. This growth potential has been interpreted as evidence that the chromatin body of the variant contains more than one set of the genetic instructions characteristic of the organism. A single growth cycle in the presence of dipicolinic acid (thought to cure some *Bacillus* species of phage) results in approximately 40% of the cells producing a single spore. These spores resemble those formed by the organism from which A(−)3 was isolated. The possibility that the abortively disporic state is associated with the presence of an infecting phage is discussed.

Those bacteria which form spores as a part of their natural life cycle generally do so by the production of a single endospore within each cell. Among the variants which have been isolated from *Bacillus cereus* and are altered in their ability to initiate or complete spore formation is one which appears to begin the formation of two spores, one at each pole of the cell. In this paper, the morphology and growth potential of this abortively disporic variant are described, and some evidence is presented that the morphological variation is associated with the presence of an infecting virus.

MATERIALS AND METHODS

Organisms. The strain of *B. cereus* var. *alesti* described previously (Young and Fitz-James, 1959*b*) was the parent organism used in these studies. Subsequently referred to as A(+), this organism produces both a spore and parasporal protein inclusion, or crystal, during the sporulation process (Fig. 1). A common stable variant of A(+) forms a spore but no crystal (Fig. 2). Variants with this characteristic are called A(−) and have been isolated from untreated cultures of A(+), from spores of A(+) which have been irradiated with ultraviolet light, and from spores of A(+) which have been stored in 5% formaldehyde. The culture of A(−) used in these and previous studies of spore development (Young and Fitz-James, 1962) was derived from spores stored in formaldehyde.

Among the colonies derived from ultraviolet-irradiated spores of A(−) was a variant, A(−)1, which forms spores which are unable to achieve full refractility owing to the absence of a morphologically complete cortex (Fitz-James, 1962) (Fig. 3). Since such spores are partially digested during the terminal lysis of spore formation (Fig. 3b), the normally creamy-white colony changes by 36 hr to one which is clear and colorless. The culture of A(−)1 has shown no tendency to revert to A(−) but does spontaneously produce asporogenous variants. Thus, it is maintained by subculture every four days from selected single colonies.

The variant A(−)3 (Fig. 4), which is the subject of this paper, arose spontaneously in a plating of A(−)1. It was recognized by the failure of the colony to become clear at 36 hr.

Media. All cultures were maintained at 30 C on plates of nutrient agar (Difco). For culture

in fluid, the techniques and medium described previously (Young and Fitz-James, 1959*b*) were used.

Microscopy. Living cells were observed in dark phase contrast with a Zeiss VZ condenser (N.A. 1.4) and Neofluar ×100 objective in conjunction with a Kpl ocular.

The growth of individual cells was observed in slide culture with dark phase contrast. For this purpose, cells were transferred directly to a small cone of agar built to the top of the well of a depression slide (1.4 mm in thickness with a well of 0.7 mm thickness) with loopsful of the molten medium. A clean cover slip was placed on the cone and over the well; a drop of water sealed the perimeter of the cover slip to the slide and maintained a moist environment within the well. The outer edges of the cover slip were sealed to the slide with molten wax. The top of the cone was small enough (less than 2 mm in diameter) to allow adequate diffusion of gases to and from the cells to support normal growth.

The chromatin in fixed cells was stained after hydrolysis by the Giemsa method as developed by Robinow (1944).

Photographs in phase contrast were taken on Kodak Royal Pan cut film and those in bright field on either Kodak Panatomic-X or Contrast Process Ortho by use of a Zeiss ×100 planoapochromat objective N.A. 1.3 and ×12.5 Kpl ocular. Phase-contrast and bright-field photographs were taken at initial magnifications of 1,400 and 1,800 times, respectively, and were magnified twice in printing.

Electron microscopy was carried out by Philip Fitz-James of the University of Western Ontario following methods detailed elsewhere (Fitz-James, 1960).

RESULTS

General observations. During vegetative growth of the variant A(−)3, no morphological or cultural characteristic distinguished it from the ancestral strains A(+), A(−), or A(−)1. After the 11 hr of growth typical of fluid cultures at 30 C, there was a period of some 4 hr in which essentially no increase in the number of rods occurred; there was, however, a remarkable division and differentiation within each cell.

Thus, during the first hour after the cessation of division, narrow phase-transparent zones developed at the two ends of each cell (Fig. 8a). These increased in density and extended into the cell as round to ovoid structures (Fig. 8a and 14a). In time of development and in phase-contrast appearance, they were identical to the forespores which develop in normal *Bacillus* species when cultured under similar conditions (Young and Fitz-James, 1959*b*). However, maturation of these bodies into refractile spores did not occur; rather these "pseudo-forespores" remained phase-dense and only on occasion showed bands of increased refractility (Fig. 4).

Lysis of the cytoplasm remaining between the two pseudo-forespores began soon after their development. However, the wall of the mother cell did not disintegrate as in normal spore formation and its persistence prevented the liberation of the pseudo-forespores (Fig. 4).

The degree of synchrony associated with the formation of normal forespores (Young and Fitz-James, 1959*a*, *b*) was not achieved during the formation of pseudo-forespores, nor was there the uniformity from cell to cell which is a characteristic of normal spore formation. Thus, although many cells contained two phase-dense pseudo-forespores, some contained only one with an extracted membraned area at the other pole (Fig. 4). Cells in the same chain were sometimes completely extracted, although the membranes which had surrounded the pseudo-forespores were usually still apparent. In other cells, the central cytoplasm remained intact (Fig. 4 and 14a). These differences were probably reflections of the degree of synchrony and the level of stability developed in the pseudo-forespores before the onset of lytic activity.

Patterns of chromatin. The configurations characteristic of stained bacterial chromatin in division were observed in A(−)3 throughout vegetative growth. With the cessation of division, each cell contained two distinct, somewhat contracted chromatin bodies which extended and fused into a single axial filament (Fig. 7). This sequence of changes in chromatin patterns has been observed to precede the appearance of the forespore during normal spore formation (Young and Fitz-James, 1959*a*). However, with the apparent fragmentation of the axial filament, two brightly staining bands of chromatin appeared at opposite ends of each cell (Fig. 8b). These bands were located in the phase-transparent zones described above. Eventually, as the pseudo-forespores enlarged and protruded

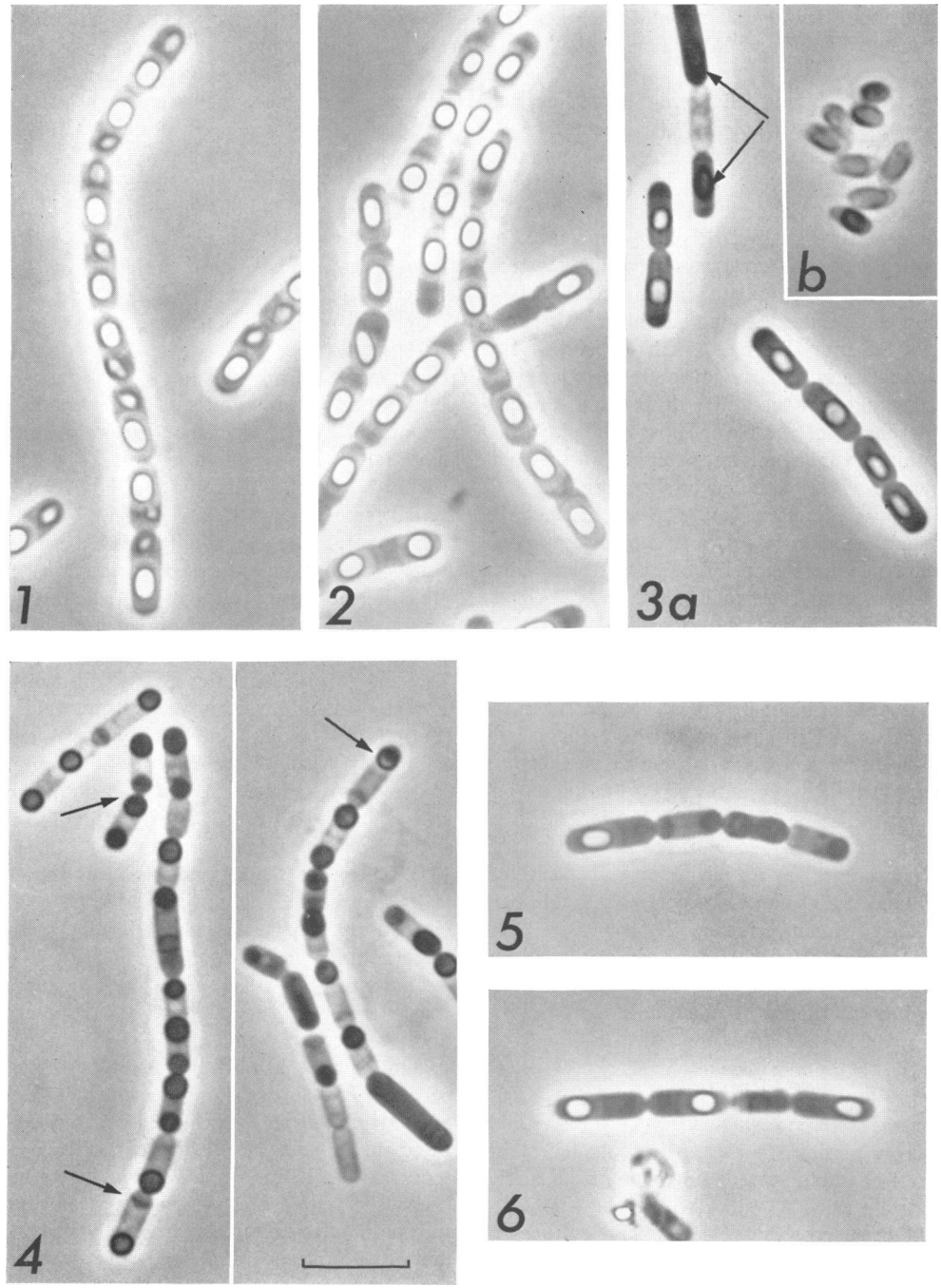


FIG. 1-6

back into the cell, the enclosed chromatin remained as a bar or dot (Fig. 8b). Any chromatin remaining in the cytoplasm of the mother cell could not be demonstrated convincingly by staining techniques (Fig. 8b). However, by analogy to normal spore formation, in which one-half of the cellular chromatin is enclosed in the spore while the other half remains in the mother cell, it could be assumed that each of the pseudo-forespores acquired one-half of the chromatin present in the cell. Direct analyses for deoxyribonucleic acid (DNA) by standard methods (Young and Fitz-James, 1959*a*) supported this assumption; cells which during pseudo-forespore formation contained 35.8×10^{-15} g of DNA per cell produced pseudo-forespores which contained, also on an average, 16.3×10^{-15} g of DNA. However, in electron micrographs of thin sections, there were wisps of material in the central cytoplasm which were of the texture and density expected of bacterial chromatin (Fig. 9). This suggests that the partitioning of the chromatin in individual cells was not so precise as could be assumed from the above chemical and cytological data.

Electron microscopy. Electron micrographs of thin sections revealed that each of the pseudo-forespores was initially separated from the cytoplasm of the mother cell by the invagination of the cytoplasmic membrane in a manner identical to that already described during normal spore formation (Young and Fitz-James, 1959*a*; Fitz-James, 1960). Such transverse growth of the two septa within a cell proceeded almost simultaneously, although on occasion the membrane at one end was more advanced than at the other (Fig. 10).

The continued proliferation of membrane, which in normal spore formation leads to the development of a forespore completely surrounded by a double-layered membrane (Fitz-James, 1960), did not occur. Also, there was no subsequent development of a recognizable cortex, spore coat, or exosporium. There was, however, deposition of material between the layers of the septa which resembled in density and was continuous with the outer cell wall (Fig. 11).

In addition to their failure to develop peripheral integuments, the pseudo-forespores were devoid of dipicolinic acid and possessed no remarkable resistance to heat.

Growth potential of pseudo-forespores. A culture of A(−)3 which has gone to completion on solid medium is comprised of chains of cells which are extracted but for the pseudo-forespores (Fig. 4). To follow the growth potential of these forms, cells from a completed culture were transferred to fresh medium in a growth chamber, and the response of the pseudo-forespores was followed in phase contrast. In Fig. 12, it is apparent that both the pseudo-forespores in a cell increase in size and elongate to the normal bacillary form and that each subsequently divides transversely in the customary fashion. It is characteristic of this variant that a relatively long but predictable lag occurs after transfer before there is any marked change in the size or form of the pseudo-

FIG. 1. *Dark phase-contrast photomicrograph of Bacillus cereus var. alesti (A(+)) during final stages of sporulation. Each cell contains a refractile spore and bipyramidal protein inclusion.*

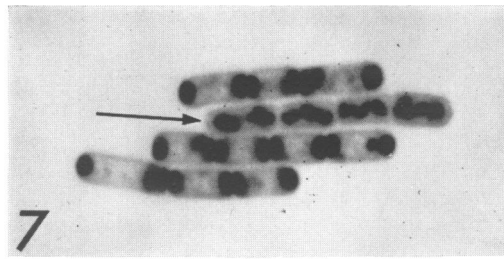
FIG. 2. *Dark phase-contrast photomicrograph of Bacillus cereus var. alesti (A(−)) during final stages of sporulation. Each cell contains a refractile spore but no parasporal body.*

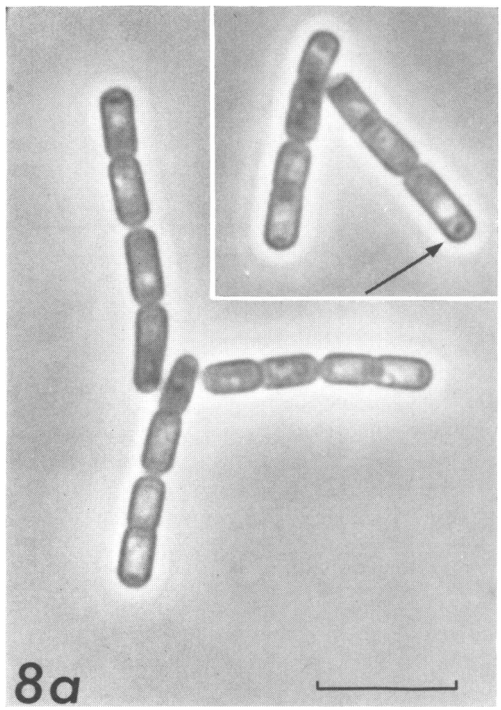
FIG. 3. *Dark phase-contrast photomicrograph of Bacillus cereus var. alesti (A(−)1) during abortive sporulation. (a) Each cell contains a spore which does not achieve full refractility. The spores in the upper cells (arrows) have already lost the degree of refractility achieved. (b) The extracted hulls of the spores which are all that survive the terminal lysis of spore formation.*

FIG. 4. *Dark phase-contrast photomicrograph of Bacillus cereus var. alesti (A(−)3) during the final stages of abortive sporulation. A round to ovoid phase-dark structure, pseudo-forespore, is formed at both poles of the cell (arrow, upper left). Note that during the terminal lysis the cytoplasm between the two pseudo-forespores disappears, but the cell wall of the mother cell remains intact. Many of the pseudo-forespores also lyse, leaving an extracted membraned area (lower arrow). Some pseudo-forespores have bands of increased refractility (upper arrow).*

FIG. 5. *Dark phase-contrast photomicrograph of cells of Bacillus cereus var. alesti (A(−)3) cultured in fluid medium. Notice the cell containing the single subrefractile spore of the A(−)1 type.*

FIG. 6. *Dark phase-contrast photomicrograph of cells of Bacillus cereus var. alesti (A(−)3) cultured in fluid medium, to which had been added dipicolinic acid (0.3 mg/ml). Notice the cells containing single subrefractile spores of the A(−)1 type. Such cells comprise 40% of the population. The magnification of Fig. 1–6 is indicated by the 5-μ marker on Fig. 4.*





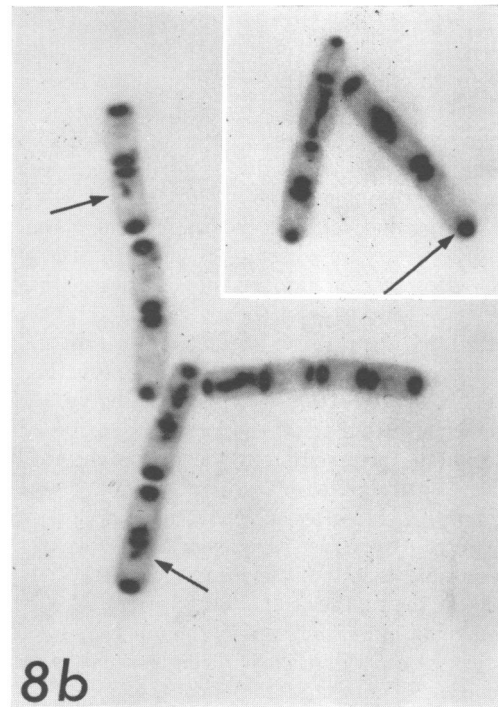


FIG. 7. *Cells of variant A(−)3 grown in fluid culture for 11 hr, and then fixed in the vapors of osmium tetroxide, hydrolyzed, and the chromatin stained with Giemsa. Cells in the marked chain fortuitously show from left to right the sequential changes in the chromatin pattern which immediately precede the appearance of the bars of chromatin at the sites of the pseudo-forespores. In the other cells, the chromatin is seen as bars at each pole.*

FIG. 8. *Another group of cells from the same preparation described in Fig. 7. (a) Appearance of cells in dark phase contrast prior to hydrolysis and staining. Note that there is no extraction of the central cytoplasm and that the pseudo-forespores are differentiated at the poles. The marked cell contains pseudo-forespores which have become round to ovoid. (b) The same cells shown in (a) after hydrolysis and staining of the chromatin with Giemsa. The two arrows on the left point to small fragments of chromatin which might not become included in the pseudo-forespores. Note that cells in which the pseudo-forespores are approaching maturity have no detectable chromatin in the central cytoplasm (right arrow). The magnification of Fig. 7 and 8 is indicated by the 5-μ marker on Fig. 8a.*

FIG. 9. *Electron micrograph of a thin section of the variant A(−)3 from a 10-hr fluid culture. Within the cell, the profiles of completed septa of two pseudo-forespores each enclose a mesosome and a chromatin mass. Some chromatin material is also visible (arrow) in the central compartment of the cell. ×53,000.*

FIG. 10. *Electron micrograph of a thin section of the variant A(−)3 from a 10-hr fluid culture. The profile of one pseudo-forespore appears complete, whereas the other is in a stage of formation (arrows). ×68,000.*

FIG. 11. *Electron micrograph of a thin section of the variant A(−)3 from an 11-hr fluid culture. Further development of the pseudo-forespores has stopped. Cell-wall material (cw) has developed between the two layers of the septa of the pseudo-forespores. ×83,000. The magnification of Fig. 9 to 11 is indicated by the 0.1-μ marker on each figure.*

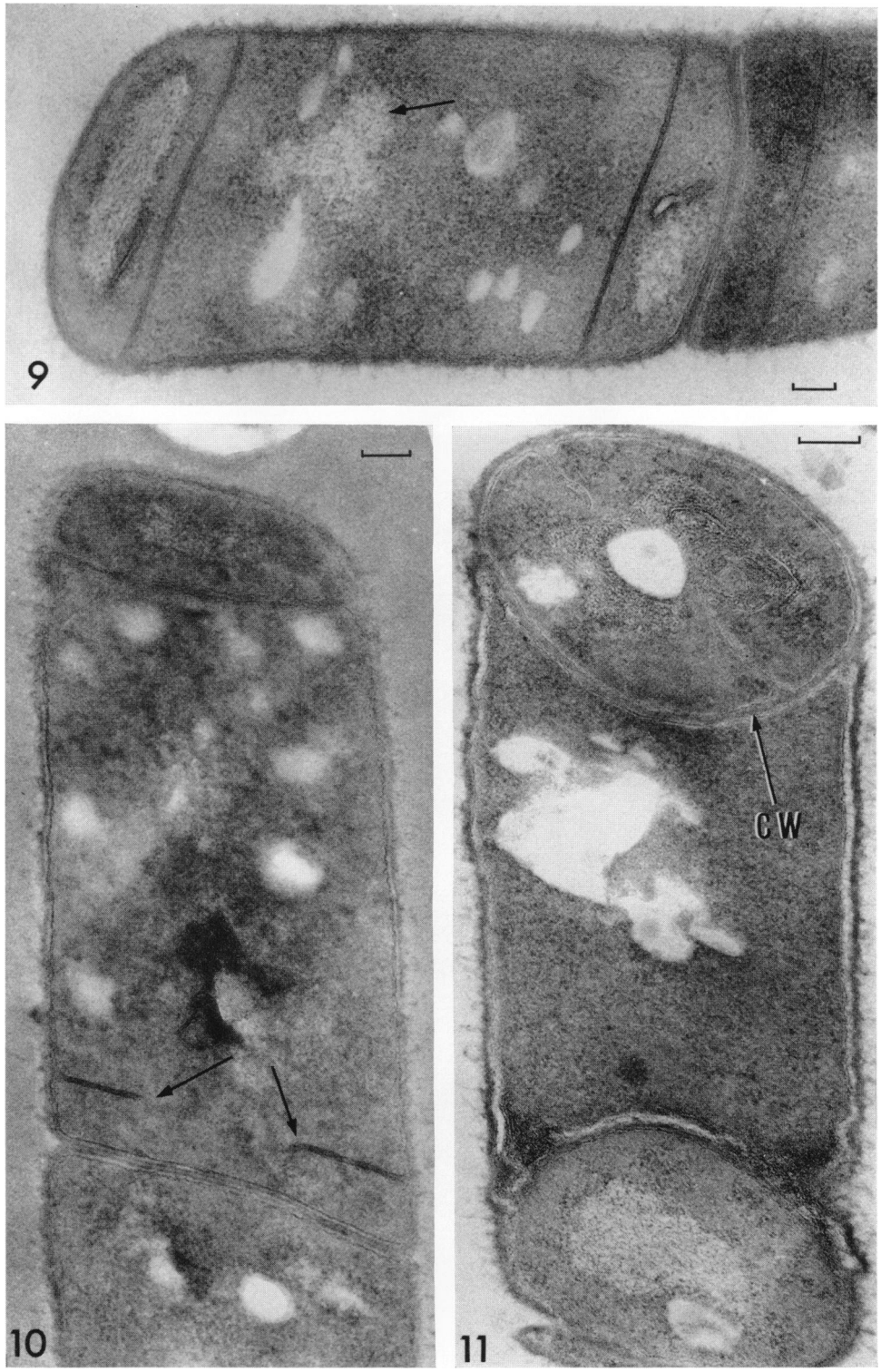


FIG. 9-11

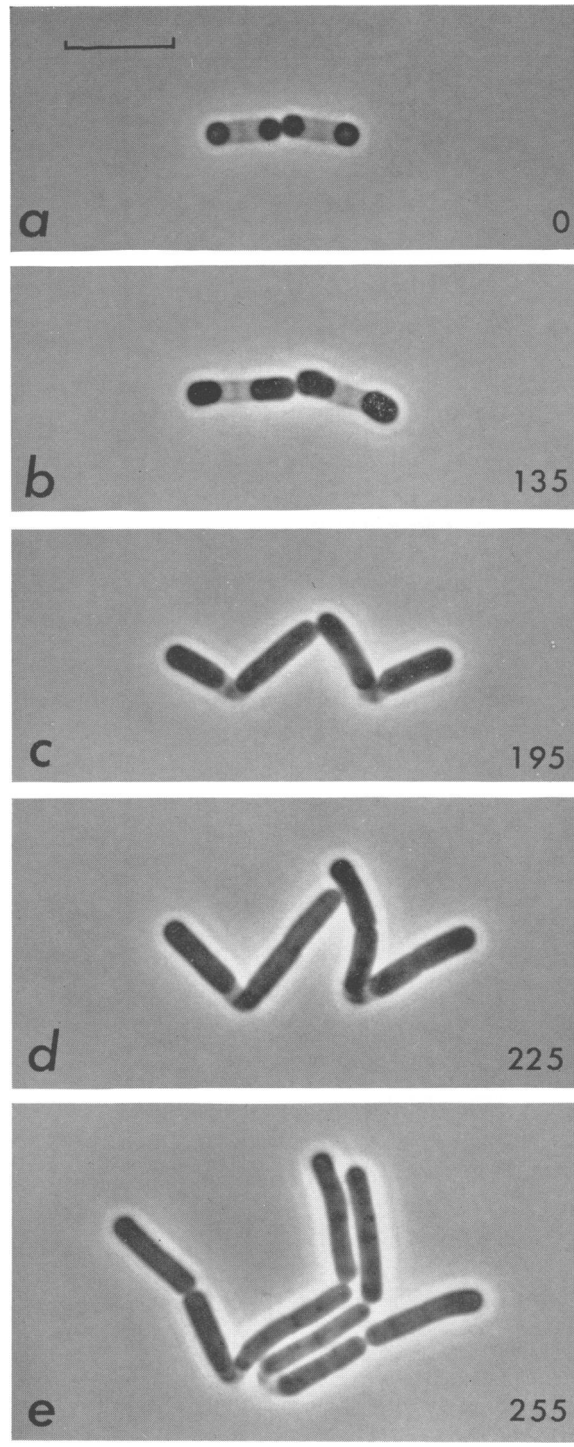


FIG. 12. *Five successive phase-contrast photomicrographs of the same cells of the variant A(−)3 growing on nutrient agar. Numbers indicate the number of minutes since the cells were transferred to the medium. In (a) the two phase-dark pseudoforespores in each cell are round. By 135 min (b), they have increased in size and become more oblong. By 195 min (c), they are rodlike; the second cell from the left is about to divide. In (d) three of the cells have divided once and in (e) all have divided once and some have proceeded through a second division. The magnification is indicated by the 5-μ marker on Fig. 12a.*

forespores. This is in contrast to the trigger-like fashion in which normal refractile spores germinate and proceed to their first division (Fitz-James, 1955). This stationary period was reflected also in the chromatin of the pseudo-forespores which remained in a compact ball or rod until approximately 3 hr after transfer to fresh medium. Then, before the cell proceeded to the first division, the chromatin expanded to an open ring and duplicated (Fig. 15a, b; 16a, b).

The walls of the mother cells lysed when fluid cultures were allowed to stand for 3 to 4 days at 30 C. This lysis released the pseudo-forespores as free phase-dark bodies which also responded to transfer to fresh medium by enlarging to the bacillary form (Fig. 13). There was an extended period over which these free forms showed evidence of their return to growth; few showed any enlargement before 3 hr, whereas others were just beginning to enlarge 6 hr after transfer to fresh medium.

Growth potential of "sporulating" cells. Cells which already possessed two pseudo-forespores but in which the central cytoplasm was not yet lysed responded differently after transfer to fresh medium. Such a cell is shown to the left of Fig. 14. It is apparent that within 60 min of transfer the cell had increased in length, although the two pseudo-forespores remained unchanged in size. By 105 min, a transverse septum had formed in the center of the cell; 30 min later, the division was complete. The product of this division was two cells, each bearing a terminal pseudo-forespore. Growth and division of the bacilli continued while the two pseudo-forespores gradually became pinched off from the ends of the growing chain (Fig. 14f). Eventually, they too elongated to the bacillary form and commenced division. Owing to rapid overgrowth, it has been impossible to continue photography of the progeny of such cells until all three components have reverted to the vegetative form, but it has been possible to recognize pseudo-forespores, carried on the tip of growing chains, which are in a stage of enlargement prior to their first division (Fig. 17 to 19).

In the interval between the transfer of the cells to nutrient medium and the appearance of the first septum, faintly stainable divisional figures of chromatin emerged in the cytoplasm between the two pseudo-forespores (Fig. 20). In stained preparations, these prolific cells were distinguished from the normal bacillary form by the condensed packet of chromatin at each pole.

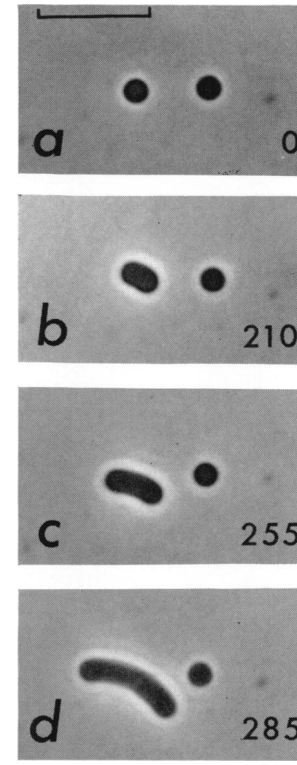


FIG. 13. *Four successive phase-contrast photomicrographs of the same pseudo-forespores of the variant A(−)3 growing on nutrient agar. The free pseudo-forespores were derived from a fluid culture allowed to stand at 30 C for 4 days. Numbers indicate the number of minutes since the cells were transferred to the medium. The pseudo-forespore to the left elongated to a rod (b, c) and began to divide at 285 min. The one to the right did not commence division until 90 min later. The magnification is indicated by the 5-μ marker on Fig. 13a.*

When the pseudo-forespores were no longer ovoid but rod-shaped, that is, just prior to the first division, the chromatin was open and in a stage of duplication (Fig. 18b); at or after the first division, they could no longer be distinguished from their relatives derived from the central portion of the original cell.

Possible basis for the variation. In a search for earlier stages of development, late vegetative forms were examined in an electron microscope. Within a small but definite number of these cells, an array of inclusions was observed which

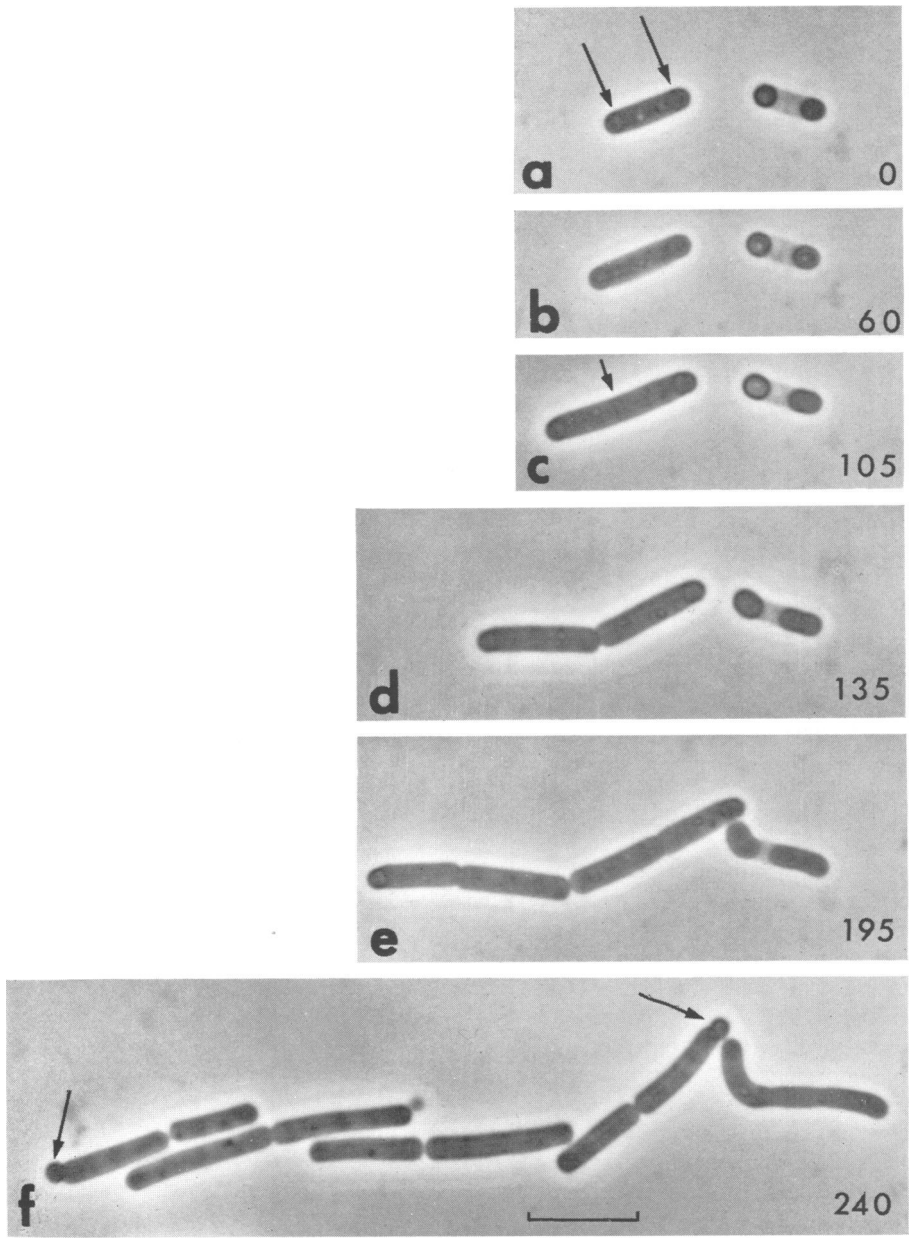


FIG. 14. *Six successive phase-contrast photomicrographs of the same cells of the variant A(−)3 growing on nutrient agar. Numbers indicate the number of minutes since the cells were transferred to the medium. The cell on the left in (a) possesses two terminal pseudo-forespores (arrows) and a central cytoplasm which is not yet extracted. In (b) this cell has increased in length by growth of the central area. In (c) a septum (arrow) has transected the central cell, the division of which is complete in (d). The pseudo-forespores to the right have begun to increase in size, although those on the cell to the left have not. In (e) a further division of the rods has occurred; in (f) the progeny from the central portion of the original cell in (a) numbers 10. The pseudo-forespores are now being pinched off from the two ends (arrows). One of the pseudo-forespores on the right has divided. The magnification is indicated by the 5-μ marker on Fig. 14f.*

was suggestive of viral particles in stages of development. The possibility that the variation to A(−)3 was associated with the presence of a virus was therefore investigated.

Goldberg and Gollakota (1961) showed that repeated treatment of cultures of phage-carrying *B. cereus* T with dipicolinic acid (pyridine-2:6-dicarboxylic acid) cures the cells of the infecting virus. Addition of 0.4 mg/ml of dipicolinic acid to a growing culture of A(−)3 produced no apparent effect on its further growth or the commencement of forespore formation. However, after a single addition of dipicolinic acid, approximately 40% of the cells produced only one forespore per cell. Furthermore, these forespores proceeded to develop into spores which were indistinguishable from those of the cortexless variant A(−)1 (Fig. 3a). Thus, they were enveloped by coats and exosporia and achieved the degree of refractility indicated in Fig. 6. Similarly, in fluid cultures of A(−)3 to which no dipicolinic acid had been added, a few cells (less than 5% of the population) could be found which also produced only a single spore of the A(−)1 type (Fig. 5). In both treated and untreated cultures, such cells arose randomly in chains otherwise comprised of typical A(−)3 cells (Fig. 5). Thus, both the A(−)1 and A(−)3 type of cell derived from a common parent; the cells of A(−)1 were not selected for by the dipicolinic acid treatment, nor were they present in the control cultures of A(−)3 as a result of contamination.

Other evidence which suggests that the variation of A(−)1 to A(−)3 is associated with the presence of a lysogenic phage, along with a description of the structure and composition of the phage, will be presented in a separate report (Fitz-James, *in preparation*).

DISCUSSION

Like all vegetative cells of the *Bacillus* group, those of the variant A(−)3 possess two nuclear bodies in some stage of duplication and division. Thus, each cell has at least two representations of the set of genetic instructions characteristic of the organism. It is perhaps to be expected then that each of the pseudo-forespores, containing approximately one-half of the cell's chromatin should also each possess a set of these instructions. That they do can reasonably be inferred from the observation that both pseudo-forespores are capable of growth and perpetuation of the variant.

Not so predictable was the observation that the "cell" remaining between the two pseudo-forespores was also capable of the resumption of growth and division. This is especially remarkable as this portion of the cell was, by chemical and cytological assessment, devoid of DNA. However, electron microscopy indicated that fragments of nuclear material did, in fact, remain trapped in this area. Certainly, this portion was able to respond quickly to a supply of nutrients, as it resumed vegetative growth and division some hours before the pseudo-forespores. Indeed, stainable divisional figures of chromatin could be demonstrated in this portion within 1 hr of transfer to fresh medium, at which time the "cell" had already increased in length.

Certain proposals may be made from these observations: (i) the vegetative cell carries more than two copies of the set of genetic instructions; (ii) the amount of DNA required to house the set of instructions is less than the amount of DNA in a single chromatin body; (iii) each set of instructions may be capable of directing cell syntheses.

There is no direct evidence, other than that reported here, to support the first two proposals. However, indirect support is found in the following observations from normal *Bacillus* species: spores of closely related species contain amounts of DNA which are multiples of the amount found in the smallest spore (Fitz-James and Young, 1959); spores may exhibit "single hit" or "multiple hit" inactivation parameters on exposure to X rays (Woese, 1958); spores of the same species of *B. megaterium* which are known to vary in shape and size also have variable amounts of chromatin as judged from stained preparations (Robinow, 1960).

If the third proposal is relevant to *Bacillus* species in general, then one could expect the chromatin body which is always discarded during normal sporulation to be genetically competent. However, it does fail to direct the formation of a spore about itself, and there is reason to believe it incapable of continuing vegetative functions, for return to vegetative growth does not occur once spore formation has begun even if fresh nutrients are supplied to the cell (Bayne-Jones and Petrilli, 1933). This has now been reinvestigated in view of the remarkable growth potential of A(−)3.

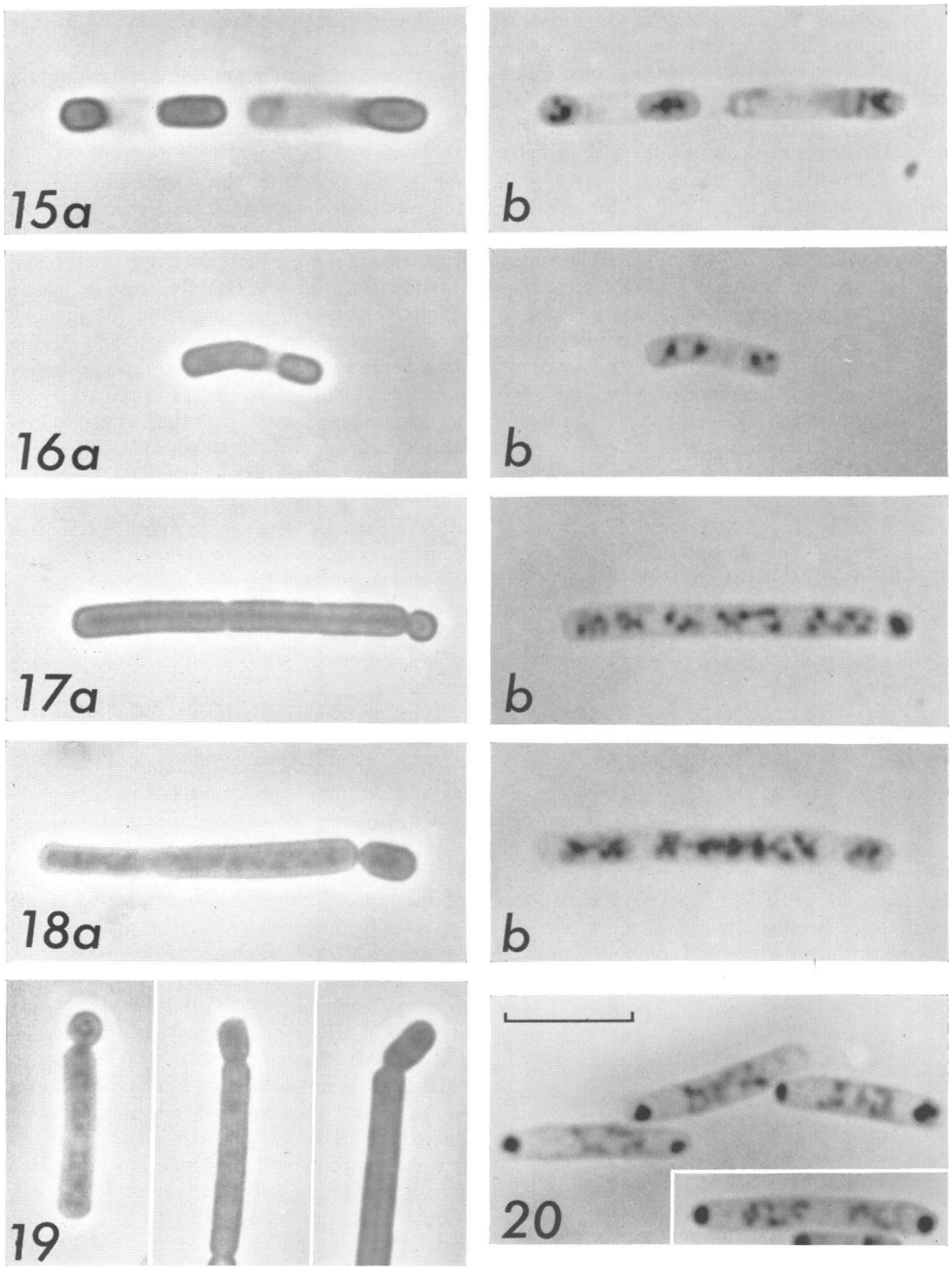


FIG. 15-20

Contrary to the previous reports, it has been found that the vegetative portion of a sporulating cell, containing one-half of the cell's chromatin, is indeed capable of resuming growth and division (Young, *in preparation*).

Thus, it may be found that a chromatin body, although cytologically a continuum of DNA, actually contains several sets of the same information, each of which may find expression under appropriate conditions.

This type of variant may not be unique to *B. cereus*. Electron micrographs of a variant of *B. subtilis* of remarkable similarity to A(−)3 were published by Ryter, Ionesco, and Schaeffer (1961). As they state, "il ne sporule pas à proprement parler, mais présente des segmentations anarchiques." Unfortunately, the organism was not studied genetically or biochemically; no information is available as to whether it too carries an infecting virus.

ACKNOWLEDGMENTS

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FIG. 15. *(a) Dark phase-contrast photomicrograph of pseudo-forespores of the variant A(−)3 fixed in osmium 3 hr after transfer to fresh medium. Note stages of elongation to the bacillary form. (b) The same cells in (a) after hydrolysis and staining of the chromatin with Giemsa. Note that the chromatin in the pseudo-forespore on the left is no longer a compact bar. In the other two pseudo-forespores, the chromatin is in stages of replication and division.*

FIG. 16. *Another pair of cells from the same preparation described in Fig. 15. (a) Note the pseudo-forespore on the left which has completed reversion to the bacillary form and contains two distinct chromatin bodies (b).*

FIG. 17. *(a) Dark phase-contrast photomicrograph of cells of the variant A(−)3 fixed in osmium 4 hr after transfer to fresh medium. Note the pseudo-forespore on the tip of the growing chain. Such cells derive from the type described in Fig. 14. (b) The same cell shown in (a) after hydrolysis and staining of the chromatin with Giemsa. Note the compact ball of chromatin in the expanding pseudo-forespore.*

FIG. 18. *(a) Dark phase-contrast photomicrograph of cells of the variant A(−)3 fixed in osmium 4 hr after transfer to fresh medium. Note the more advanced stage of reversion of the pseudo-forespore to the bacillary form. Such cells also derive from the type of cell described in Fig. 14. (b) The same cell after hydrolysis and staining of the chromatin with Giemsa. Note the duplicating chromatin body in the pseudo-forespore.*

FIG. 19. *Dark phase-contrast photomicrographs of cells of the variant A(−)3 fixed in osmium 4.5 hr after transfer to fresh medium and arranged to show the stages of reversion of the pseudo-forespores to the bacillary form.*

FIG. 20. *Cells of the variant A(−)3 which were transferred to fresh medium when at the stage of development shown in Fig. 14a and then fixed in osmium 90 min later. The chromatin was stained with Giemsa after hydrolysis. Note the elongated cells with brightly stained bars of chromatin at each pole. Note the faintly stained chromatin emerging in the cytoplasm between the two pseudo-forespores. Such cells are at similar stages of development as shown in Fig. 14. The magnification of Fig. 15 to 20 is indicated by the 5-μ marker on Fig. 20.*

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