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Direct Transition of Outgrowing Bacterial Spores to New Sporangia Without Intermediate Cell Division

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ABSTRACT

VINTER, VLADIMIR (Syracuse University, Syracuse, N.Y.), AND RALPH A. SLEPECKY. Direct transition of outgrowing bacterial spores to new sporangia without intermediate cell division. J. Bacteriol. **90**:803-807. 1965.—A direct transition was observed of the primary cell developed after germination of *Bacillus cereus* spores into new sporangia without intermediate division stages. Two simple methods were used for replacement of outgrowing spores into diluted medium or saline. Elongated primary cells prevented from division by limitation of nutrients in the suspending medium were able to form new forespores in 8 hr and sporangia in 12 hr. These new sporangia were still marked by attached envelopes of the original spore. Under the same conditions, cells replaced during the first divisions quickly lysed. Spores formed in the elongated primary cell during "microcycle sporogenesis" possessed normal heat resistance and refractility and were later released from sporangia.

Sporogenesis in bacteria under conventional cultivation procedures is preceded by a number of cell divisions. Different methods are used to define the regulation mechanisms controlling sporulation, including induction of sporogenesis by starvation and determination of sequence of cytological, biochemical, and genetic events participating in this process. The whole population, more or less heterogenous, has been used for all these studies, and the term "ontogenesis of the culture" can be used more properly than the "ontogenesis of a single cell." The dependence of the ability to form spores on the age of the culture was mainly studied by use of replacement techniques with transfer of the culture into nongrowth-supporting medium. In all these studies, log- or early stationary-phase cultures were used. A study was undertaken to define the sporeforming ability of the first cell which developed immediately after germination and which was prevented from the first division(s) by transfer into a nongrowth-supporting medium.

MATERIALS AND METHODS

Bacillus cereus (NCIB 8122) spores were incubated in peptone (Difco)-glucose medium (Vinter, 1956) at 30 C. After emergence of the germ cell following spore germination, the spore envelopes remain attached to the cell in this strain (Vinter, 1960). This first cell is marked by these envelopes even during the elongation and division period. A cultivation vessel equipped with an ultrafine fritted-glass filter was developed which facilitated the semicontinuous exchange of nutrient medium for saline (Fig. 1). A Klett tube was attached to the vessel for the determination of turbidity of the culture in a Klett-Summerson photoelectric colorimeter with a no. 66 filter. The second type of cultivation used was the transfer of the cells at different ages after centrifugation from the nutrient medium into saline. Well-washed, heat-shocked (65 C for 15 min) spores were used as inoculum. The percentage of elongated primary cells (longer than 4 μ), slightly refractile prespores, sporangia, and pairs of cells was determined by direct microscopic observation and plate counting (Vinter, 1955). Pairs of cells were considered as one sporangium. Total viable count and spore count (survivors after heating at 65 C for 10 min) were determined by use of the method described by Vinter and Vechet (1964); the total cell number was measured in a Petroff-Hausser chamber. The germinated spores were stained with methylene blue.

RESULTS

A decrease in turbidity of the germinating culture cultivated in the semicontinuous vessel was paralleled by an increase in the number of stainable spores accompanied by swelling of the spores (Fig. 2). After 15 min of cultivation, the

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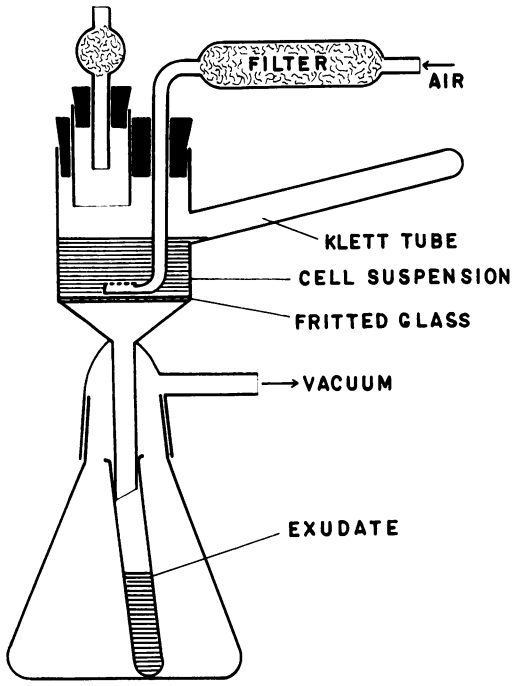

FIG. 1. *Cultivation equipment for semicontinuous exchange of medium.*

germination medium was replaced with the fresh medium to remove the inhibitory components of the spore exudate. The medium was exchanged within 5 min; cells were detached from the surface of the filter by reverse air flow. This treatment increased slightly the number of germinating spores up to a total amount of 90%. Tween 80 (final concentration, 0.01%) was present in the nutrient medium. At 40 min, when the majority of spores swelled to "exosporium size," the medium was semicontinuously filtered off and replaced in less than 5 min with the same volume of saline to roughly a 100-fold dilution of the original nutrient medium. Removal of cells from the filter surface and mixing of the suspension was accomplished by reversing the air flow. Traces of remaining nutrients were sufficient only to allow the slow completion of the elongation stage. About 83% of germinated spores formed normal elongated cells under these conditions within 5 hr of cultivation. Less than 10% of cells were already committed to the first division, but the number of pairs did not increase significantly during further cultivation in saline. The total viable count in the culture was much lower than the total cell direct count, and showed a significant decrease at 7 hr of cultivation and a continuous increase during later stages of culture development. No obvious clumping of cells was observed during cultivation. The differences in values of viable count and total cell count indicated a decreased ability of cells elongated in dilute medium to form colonies. Similarly, according to previous experiments on the effect of penicillin on elongated cells of *B. cereus*, the cells in control nontreated cultures in this stage seem to be more vulnerable to subsequent dilution and plating procedure (Vinter, 1965). No obvious lysis of cells was observed during further cultivation.

The partial terminal detachment of pre-existing spore envelopes (exosporium plus coats) was obvious in almost all fully elongated cells. These envelopes, although sometimes very loose, remained attached to one end, marking very clearly the primary cell developed from the spore. After 5 hr of cultivation, determinations of total, viable, and spore counts were initiated, and the suspending medium was enriched with calcium chloride (final concentration, 3×10^{-4} M). In a later period, a slight granulation of elongated cells was observed. From the seventh to ninth hour of cultivation, the gradual formation of new prespores inside the emerged primary cells was noted, followed by a very slow increase of their refractility. Somewhat after the appearance of fully refractile spores in the later stage, the number of heat-resistant cells increased to more

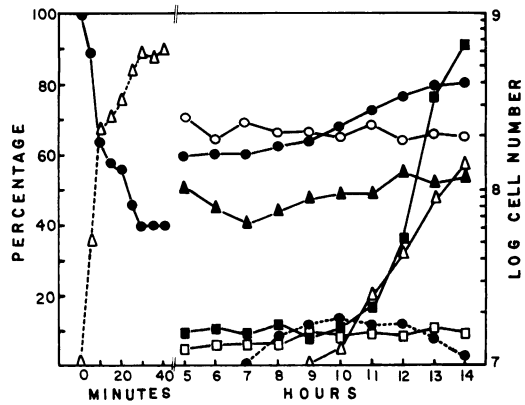

FIG. 2. *Germination of Bacillus cereus spores and the development of new sporangia after replacement of medium. Symbols (connected by solid lines): ●, percentage of turbidity of the culture (after inoculation = 100%); ○, total cell count; ▲, viable count; ■, spore count (per cent of survivors, after heating, from all colony-forming cells); △, percentage of sporangia from all elongated cells; □, percentage of pairs from all elongated cells. Symbols (connected by broken lines); △, percentage of spores stainable with methylene blue; ●, percentage of prespores in elongated cells.*

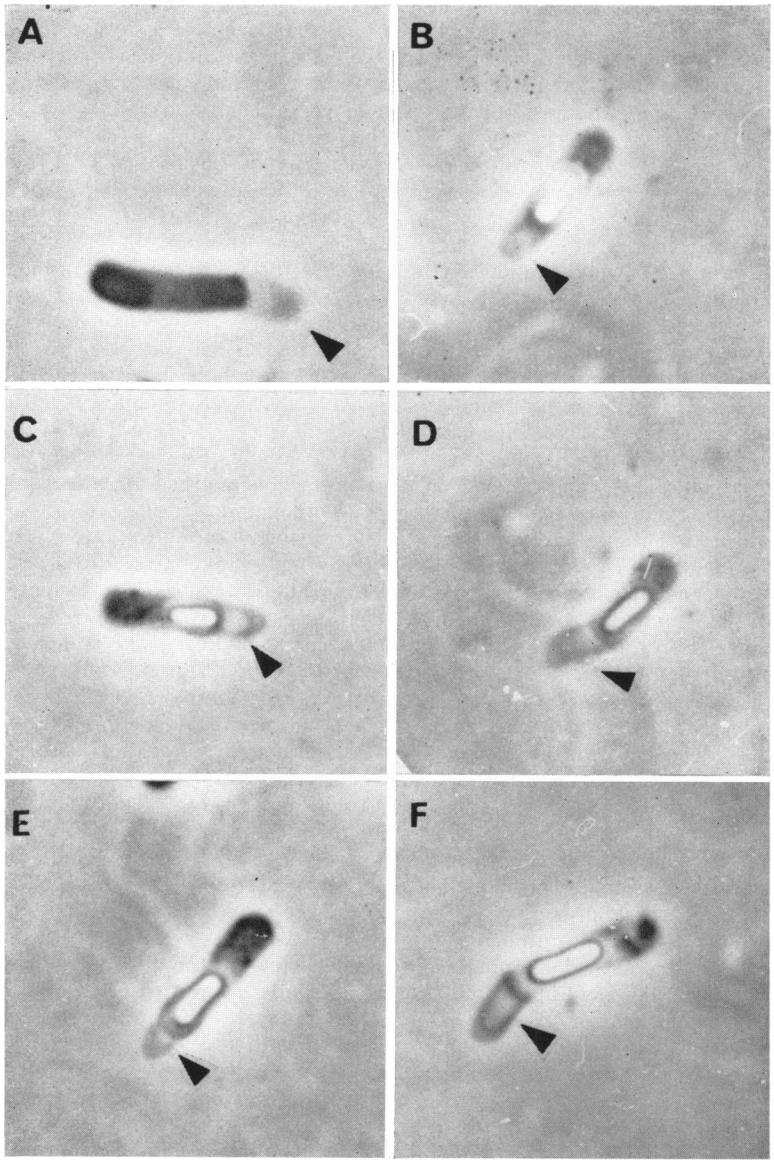

FIG. 3. *Sporulating primary cells of Bacillus cereus. Polar terminal caps consisting of envelopes of original spore are marked by arrows. (A) Prespore; (B–F) mature sporangia.* × *3,500, phase contrast.*

than 90% of the viable count. A slow increase in turbidity paralleled the formation of sporangia.

A low background (about 10%) of survivors detected throughout the whole period of cultivation was due to ungerminated spores. Only the cells which completed the elongation were able to form new spores. From the values obtained, we may postulate that probably only ungerminated spores and cells capable of completing elongation and spore formation could be detected as viable in later periods of cultivation. The time sequence of individual morphological stages was easily reproducible in repeated experiments under the same experimental conditions. The different steps of prespore and spore formation in the primary cells are shown in Fig. 3. These are representative cell types; attempts to follow continuous development of a single spore in a microchamber were unsuccessful. The attached envelopes of original spores mark practically all new sporangia. The new spores are more often formed closer to the marked end of the cell. The

normal spores were formed also in pairs of cells, without noticeable difference between the primary cell marked by spore envelopes and the new daughter cell. After a few more hours of further cultivation in saline, the spores were continuously released from the sporangia. The liberation of spores from sporangia seemed to be faster than in normal cultures. This quick release of spores lasted only a few seconds in many cases and may support previous results showing the lack of typical cell envelopes in swollen and elongated cells after spore germination (Vinter, 1965).

When swollen or elongated spores were transferred to saline after centrifugation, no obvious lysis of cells occurred. However, young pairs or chains of vegetative cells, developing in the early log phase, were extremely sensitive to the transfer. They underwent quick lysis and, after 2 or 3 hr of further cultivation, primarily empty "ghost" cells were present in the culture. Only a negligible fraction of cells in this culture were able to form spores after a longer period of cultivation. The restored ability of the growing cells of *B. cereus* to produce spores in nongrowth-supporting medium is found only during the later log phase of growth.

The ability of outgrowing spores to convert to new sporangia also depended on the stage of development of cells at the moment of replacement. By use of sequential transferring of germinating and outgrowing spores into saline after centrifugation, it was found that outgrowing spores later arrested by early replacement in the stages of nonrefractility, swelling, or only slight elongation were not capable of forming new sporangia. These results confirmed our findings on the necessity of cell development up to normal elongation for conversion of the primary cell into new sporangium in diluted medium. A high degree of synchrony of cells during germination, as well as outgrowth, was necessary for the homogenous response of cells to replacement and, therefore, also for an optimal yield of new sporangia. Rather high asynchrony of the cells during "microcycle sporogenesis" probably reflects the rapid changes in sensitivity of outgrowing spores to replacement procedures.

This same transition from spore to cell to spore has been observed in growth-limiting studies with germinating spores of *B. megaterium;* however, that organism does not possess the spore envelope marker found in *B. cereus*, assuring that the cells observed corresponded to the primary cells as with *B. cereus*.

DISCUSSION

Young, fully growing cells are not able to form spores when the nutrient supply is artificially limited, and, subsequently, these cells die. The outgrowing elongating spores and also the granulated cells in the stationary phase, however, are capable of forming new resistant spores under these conditions. The results on the different sporeforming capabilities of young and old populations of bacilli are in agreement with findings of many authors. The ability of the first cell developed after germination and outgrowth to form a spore again, however, is not known. This direct transition of the primary cell after spore outgrowth to sporangium represents the complete developmental "microcycle." It has no relation to the reversal of spores activated by different methods back to the stage of refractile dormant spores (Riemann, 1961; Riemann and Ordal, 1961; Lee and Ordal, 1963; Keynan et al., 1964). Actually, the normal elongation of the primary cell is required for later conversion to a new sporangium.

Deoxyribonucleic acid (DNA) synthesis in outgrowing spores starts only after the ribonucleic acid content is approximately doubled (Fitz-James, 1955; Woese and Forro, 1960). This stage, corresponding to the later period of swelling in *B. cereus* (Vinter, 1965), was found to be most favorable for maximal conversion of primary cells to new sporangia after replacement.

Sporulation is known to be a sequence of biochemical and morphological events determined by distinct genetic loci. The induction of these processes, more or less specific for sporogenesis, is probably repressed under conditions permitting fast growth. Although the primary cell developed during the outgrowth of the spore under "shift-down" conditions is still capable of relatively fast recycling to the dormant spore, the young cells, already committed to the first division, are not able to realize this process. The rate of metabolism in both types of cells before replacement may be the main factor in different resistance of cells to "shift-down" treatment, controlling the function of genetic determinants for sporogenesis. The explanation of these phenomena on a genetic basis should be taken into consideration. For example, the possibility of involvement of episomic elements switching on the processes typical for sporogenesis (Jacob, Schaeffer, and Wollman, 1960; Rogolsky and Slepecky, 1964) may contribute to this explanation.

Every specific genetic determinant of this process, autonomous or integrated in the chromosome, may exhibit the repression after the stage of first division(s) or after DNA replication is achieved, being still derepressed or activated by "shift-down" treatment in the outgrowing spore. The necessity of partial development of the outgrowing spore after replacement may reflect the need for completion of other biochemical mechanisms involved in sporogenesis. The rapid

lysis of the young growing cells after dilution of the nutrient medium testified to the extremely high overall sensitivity of cells during the first division(s) to this treatment, the inability to develop new sporangia being one among other cell alterations.

The phenomenon of "microcycle sporogenesis" eliminates the necessity of divisions prior to spore formation. The method itself may simplify the studies of regulatory mechanisms determining sporogenesis and of the processes preceding or accompanying spore formation.

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