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TRANSITION OF BACTERIAL SPORES INTO VEGETATIVE CELLS

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We propose to discuss certain aspects of bacterial spore activation, germination, postgerminative development, and sporulation. Since most of our personal experimental experience has been with *Bacillus megaterium*, our remarks will, in the main, deal with this species. The discussion may appear to be somewhat disjointed, but we hope that some idea of the types of problems with which we have been concerned will emerge. Bacterial spores have a low metabolic activity and are resistant to such adverse environmental influences as heat, desiccation, toxic chemicals, and ionizing radiation. They are not stainable with such simple aqueous dyes as methylene blue; they are refractile; they contain from 2 to 10 times as much calcium as vegetative cells, and from 5 to 15% of their dry weight is present as dipicolinic acid (pyridine-2,6-dicarboxylic acid; DPA) often in combination with calcium. DPA is characteristic of bacterial spores. It is found only in these forms, not in germinated spores or in vegetative cells, and the consensus is that it is somehow concerned with the heat resistance and dormancy of the spore.

The function of the spore is unknown. It is apparently peculiarly adapted to resist environmental influences which are fatal to the homologous vegetative cell. However, since the vast majority of bacterial species does not form spores and has managed to survive eons of such deleterious influences, we can hardly conclude that spore formation is a necessary prerequisite to survival of bacteria. However, the spore form may enable certain bacteria to fill microclimatic ecological niches, where the environment is inhospitable to vegetative forms.

The term dormant commonly refers to the metabolic inactivity of the spore as compared with the vegetative cell; but it has also been applied to the inability of spores to respond to conditions that support rapid germination of spores which have somehow been activated. Activated spores are, then, poised for germination and are no longer dormant. Activation can be accomplished by a number of means and is usually reversible. Germination, the next step in the transition of spores into dividing vegetative cells, is characterized by the irreversible loss of the typical spore properties. The preponderance of evidence is that germination is a degradative process and does not involve protein synthesis. The next step in the sequence leading to a vegetative cell, postgerminative development or outgrowth, involves synthesis of new macromolecules. Under the usual cultural conditions, the vegetative cell divides and may eventually form new spores.

This paper, illustrated with slides, was presented at a meeting of the Division on October 27, 1967.

## Activation

Dormancy may be broken by a treatment, not required for vegetative growth, but which is required for germination or which stimulates germination. A spore which has undergone such a treatment—activation—retains its heat resistance, nonstainability, refractility, and low metabolic activity, but its qualitative and quantitative requirements for the induction of germination have been altered (Keynan & Halvorson, 1965). Activation increases the rate and extent of germination, and this increased competence to germinate is an index of the extent of activation.

Activation treatments have been described for insects, fungi, and higher plants (Sussman & Halvorson, 1966) and include temperature, light, moisture, and chemicals. The best known activation treatment for bacterial spore germination is sublethal heating of aqueous suspensions of spores. Indeed, bacterial spore dormancy is often defined in terms of heat activation requirements—that is, the less the requirement for heat activation, the less the dormancy. The phenomenon of heat activation is reputed to have been discovered by C. Weizmann (1919), who, using the anaerobic sporeformer *Clostridium acetobutylicum* in the fermentation of starch to produce acetone, desperately needed during World War I as a solvent for the explosive cordite, heated his spore suspension at 90–100°C for one to two minutes. The spores were then able to germinate and grow, and an economical production of acetone was achieved. Curran and Evans (1945) first systematically demonstrated that spores which did not germinate, or whose germination was delayed, overcame this dormancy when they were heat activated. We have also found that heating of spores changed their qualitative and quantitative requirements for the induction of germination. Heated *B. megaterium* spores were capable of germinating on lower concentrations and on a greater variety of carbon compounds (TABLE 1) than were unheated spores (Hyatt & Levinson, 1964). Both the rate and the extent of germination were increased as a result of the heat treatment. Similarly, germination of unheated *B. megaterium* spores

TABLE 1

CARBOHYDRATES AND RELATED COMPOUNDS SUPPORTING GERMINATION OF UNHEATED AND HEATED *BACILLUS MEGATERIUM* SPORES

Carbon Source	Spore Germination (%)	
	Unheated	Heated
D-glucose	28	60
D-mannose	30	64
2-deoxy-D-glucose	24	50
D-fructose	3	24
L-sorbose	4	30
$\alpha$ -methyl-D-glucoside	5	27
glucosamine	29	60
N-acetylglucosamine	24	40

Germination determined after 2 hr at 30°C in phosphate buffer (50 mM, pH 7.0) plus carbon source (25 mM for unheated spores, 2.5 mM for spores heated at 60°C for 10 min). No germination in phosphate buffer alone. No germination with any of the 72 additional compounds tested.

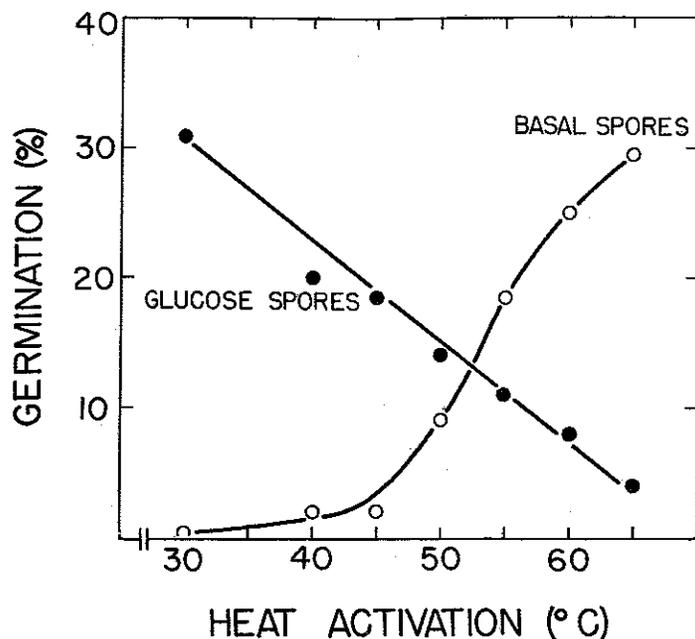


FIGURE 1. L-alanine induced germination of *Bacillus megaterium* spores. Spores were produced either on a "basal" liver medium (○) or on this medium, supplemented with 0.05 M D-glucose (●). Distilled water suspensions of spores were heated for 10 min and were then incubated for 2 hr at 30°C with 0.1 M L-alanine in 0.05 M phosphate buffer (pH 7.0). (Holmes *et al.*, 1965. Reproduced with permission of the American Society for Microbiology.)

was supported by only three of the 48 nitrogenous compounds tested (L-alanine, glucosamine, and N-acetylglucosamine), but the germination of spores, previously heated at 60°C for ten minutes was supported by ten additional nitrogenous compounds (Levinson & Hyatt, 1962), and the concentration of germinant necessary for spore germination was reduced as a consequence of the prior heating.

We hold that dormancy is not an immutable, generalized characteristic of spores, but must be considered relative both to the medium used in producing the spores and to the germinant (Keynan *et al.*, 1961; Levinson & Hyatt, 1964). Perhaps one of our laboratory's more startling pieces of evidence that spore dormancy depends both on sporulation and on germination conditions (Holmes *et al.*, 1965) concerned the production of spores whose germination on L-alanine was suppressed by prior heating at temperatures which accelerated their germination on glucose. *B. megaterium* spores, grown on unsupplemented liver extract medium, required heat activation for germination on L-alanine (FIGURE 1). When spores were grown on the same medium which had been supplemented with glucose, they germinated on L-alanine without heat activation; indeed, their germination on L-alanine actually was suppressed by prior heating. On the other hand (FIGURE 2), spores produced both on the unsupplemented and on the glucose-supplemented medium were activated for germination on glucose as a result of prior

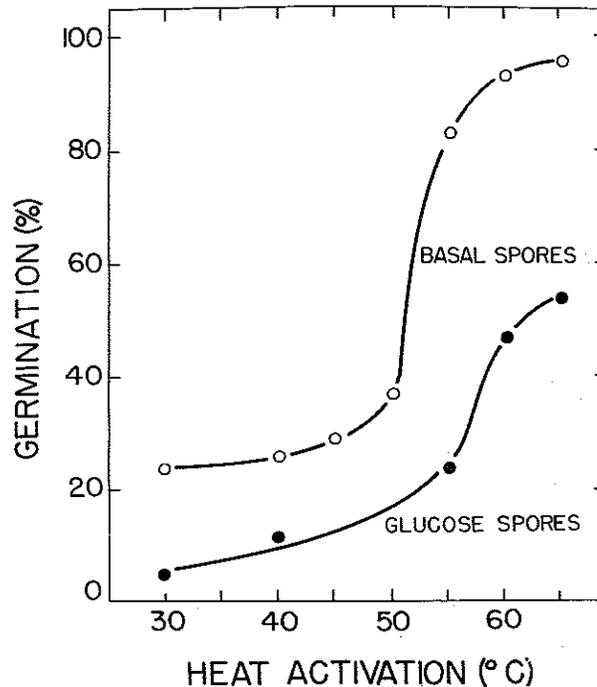


FIGURE 2. D-glucose induced germination of *Bacillus megaterium* spores. Spores were produced on "basal" (○) or 0.05 M D-glucose-supplemented medium (●). Distilled water spore suspensions were heated for 10 min and were then incubated for 2 hr at 30°C with 0.01 M D-glucose in 0.05 M phosphate buffer (pH 7.0). (Holmes *et al.*, 1965. Reproduced with permission of the American Society for Microbiology.)

heating. Here we have an example of a temperature-induced dormancy for germination on a particular substrate—L-alanine. It is possible that a spore component, essential to L-alanine-induced germination, was physiologically heat-sensitized by growing the spores with carbohydrate.

In our work with *B. megaterium*, we have found that untreated spores, and spores which had been heated dry at 65°C for up to 40 hours were dormant. However, spores which had been heated in aqueous suspension at 65°C for ten minutes germinated rapidly and completely in glucose (FIGURE 3), the measure of germination being the decrease in turbidity of spore suspension or the conversion of nonstaining resting spores to staining germinated spores. About 50% of the spores germinated after heating for ten minutes at a lower temperature of 55°C. Treatment of dry spores with water vapor at room temperature, like heat treatment of aqueous spore suspensions, increased both the rate and extent of germination (Hyatt *et al.*, 1966). Water vapor treatment at a relative humidity (RH) of 75% for 18 hours at 30°C resulted in activation approximating that of heat shock for 65°C for 10 minutes; and exposure at 60% RH for one hour at 30°C approximated heat shock at 55°C. The degree of water vapor activation depended on the time and temperature of exposure to water vapor and on the relative humidity. Thus, at

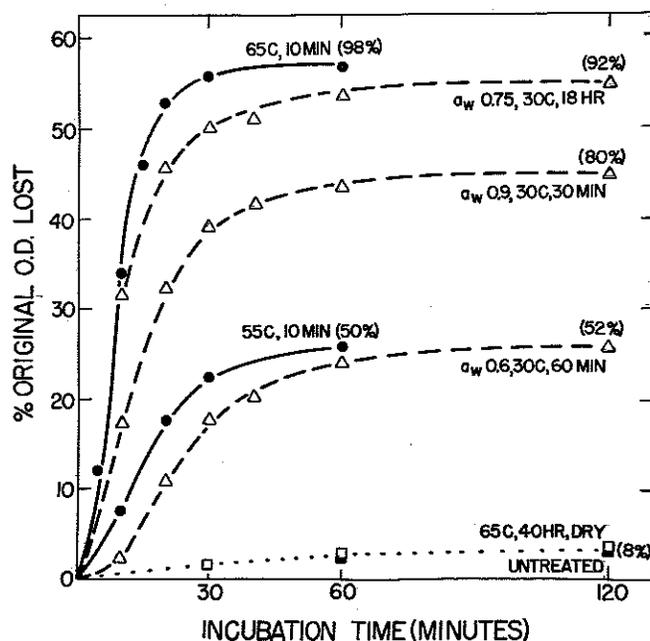


FIGURE 3. Heat and water vapor activation of *Bacillus megaterium* spores. Heat activation: spores (2 mg of spores per ml of distilled water) were heated as indicated. Water vapor activation: dry, lyophilized spores were exposed over water-sulfuric acid mixtures of the indicated water activity levels ( $a_w$  = equilibrium relative humidity). After exposure, spores were suspended in liquid water. Germination: spore suspensions were incubated for 2 hr at 30°C with 0.025 M glucose in 0.05 M phosphate buffer (pH 7.0). Optical density (O.D.) was measured at 560  $m\mu$ ; stainability with 0.5% aqueous methylene blue (2 hr) is indicated in parentheses.

30°C, exposure to water vapor at an RH of 75 or 90% for one to two hours maximally activated spores; lower relative humidities gave less activation, but even at 25% RH, there was some activation after long (one week) exposure. We are currently investigating the basis for activation of spore germination by exposure of spores to water vapor, the failure of spores to be activated in liquid water at room temperatures, and the possible relation of water vapor activation to spore activation by heating in aqueous suspension. Although these studies have not, as yet, been completed, water seems to play a key role in both heat activation and in water vapor activation.

We have also shown (Holmes & Levinson, 1967b) immersion in aqueous ethanol at 30°C, in a broad concentration range, to be an effective activation agent. There was little or no activation at ethanol concentrations below about 20%. Separate treatment with either pure water or pure ethanol at 30°C did not activate spores. Exposure of spores for five minutes at 30°C to 60% ethanol resulted in an activation for glucose-induced germination equivalent to that obtained by heating of spores in aqueous suspension for 10 minutes at 65°C (FIGURE 4). Heating of spores in the absence of water was not effective in activation. Spores, whether activated by heating in aqueous suspension or with aqueous ethanol at room temperature, were deactivated by exposure

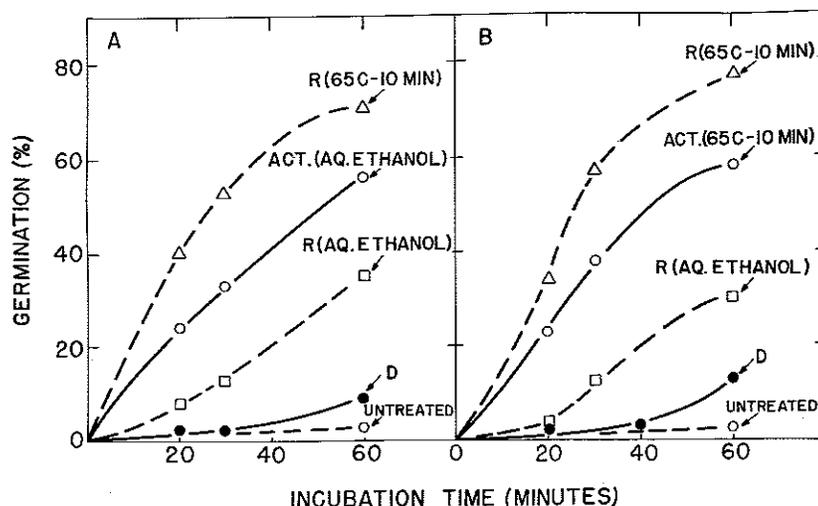


FIGURE 4. Deactivation and reactivation of (A) aqueous ethanol-activated and of (B) heat-activated spores of *Bacillus megaterium*. Spores were activated (ACT) with aqueous ethanol (60%) for 5 min at 30°C, or in water suspension at 65°C for 10 min. Deactivation (D) was with 100% ethanol (18 hr, 66°C). Reactivation (R) conditions were as for activation. Spores were germinated at 30°C with 0.01 M glucose in 0.05 M phosphate buffer (pH 7.0). Germination was determined by stainability with 0.5% aqueous methylene blue. (Holmes & Levinson, 1967. Reproduced with permission of North-Holland Publishing Co.)

to absolute ethanol for 18 hours at 66°C; and the deactivated spores could be fully reactivated by heat shock and at least partially reactivated by aqueous ethanol. Deactivated spores were not, however, reactivable in the absence of water. We suggest that water, at some spore site, allows germinants to function. It is possible that the activation processes we have described permit water to reach this site. Ethanol and heating, by decreasing the surface tension or by altering the polymeric structure of water, may facilitate permeation of water into and hydration of the critical spore site. Water may be removable from the active site, by a liquid desiccant (absolute alcohol), a dehydration treatment which also deactivates spores. It may be (Keynan & Halvorson, 1965) that activation results from protein denaturation, with "unblocking" of some critical enzymes. The function of water at the hypothesized site may be to hydrate protein rendering it more readily subject to denaturation.

#### Germination

Spore germination is, in general, marked by the loss of certain spore characteristics. Spore suspensions become less turbid; the previously non-staining spore becomes stainable with methylene blue; appears dark rather than bright under phase optics; loses dipicolinic acid and much of the calcium associated with heat resistance; becomes heat- and disinfectant-labile; and begins to acquire an increased metabolic activity. All of these parameters

have been used in the quantitation of germination. On the basis of microscopic observations of thousands of individual living *B. cereus* spores under phase optics, Vary and Halvorson (1965) postulated two morphological spore germination endpoints. These were microlag—the average time required for spores to begin darkening—and microgermination—the average time required to complete phase darkening once it had begun. Microgermination time was very short in comparison with microlag. The kinetics of spore germination, based on these microscopic observations were consistent with equations (McCormick, 1965) based on spectrophotometric data (loss of turbidity or optical density of spore suspensions). We were concerned with the apparent interchangeability and synonymy of the turbidimetric and microscopic criteria of germination and with the possible kinetic equivalence of the other common parameters of germination (Levinson & Hyatt, 1966).

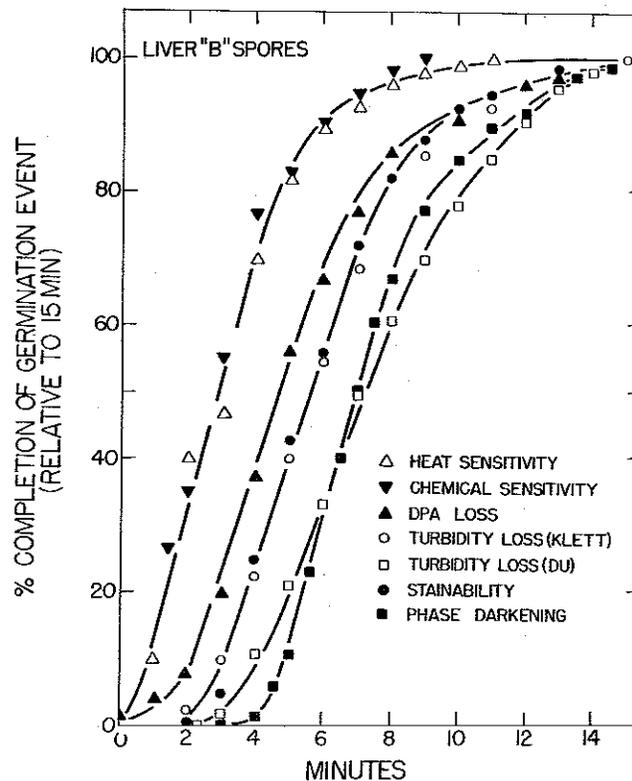


FIGURE 5. Kinetics of *Bacillus megaterium* spore germination events. Spores were produced on liver extract (LB) medium. Heat activated ( $60^{\circ}\text{C}$ , 10 min) spores were germinated by incubating at  $30^{\circ}\text{C}$  with a mixture of glucose (0.025 M) and L-alanine (0.001 M) in 0.05 M phosphate buffer (pH 7.0). In 15 min, when germination had been completed, 88% of the spores were sensitive to heat and to  $\text{HgCl}_2$ ; 82% were stainable and phase-dark; 26% of the original turbidity (DU) had been lost; and 68  $\mu\text{g}$  of the original (85  $\mu\text{g}$  per mg of spores) DPA had been released by the spores. (Levinson & Hyatt, 1966. Reproduced with permission of the American Society for Microbiology.)

Generally, we produced *B. megaterium* spores on a medium containing a liver fraction (LB spores). In some cases, spores (A-K spores) were produced on a peptone medium similar to that devised by Arret and Kirshbaum (1959). Heat activated spores were germinated by incubation at 30°C in a mixture of glucose and L-alanine. Each germination event was plotted as percentage of completion of that event, this normalization permitting comparison of the rate of change among all of the parameters. Fifty percent completion times were noted as reference points. Each germination parameter was referred to stainability with methylene blue, and we were thus able to arrive at an estimate of the sequence of events during germination.

Stainability of LB spores with methylene blue began after about two minutes of incubation (FIGURE 5). By 15 minutes, maximum stainability had been achieved, and this event was 50% completed in 5.5 minutes. Spores in all stages of phase darkening, from those just beginning to lose refractivity to those which had completed darkening, were fully stainable. Inception of stainability was, then, coincident with the beginning of phase darkening, that is, with the end of microlag. Fully phase dark spores (those which had completed microgermination) began to appear at about four minutes, and the time for 50% completion of full phase darkening was seven minutes. The difference between the 50% completion times for stainability and full phase darkening—between beginning and completion of phase darkening—represented microgermination time, and corresponded exactly to the 1.5-minute microgermination time as determined by the more tedious Vary and Halvorson (1965) technique involving continuous microscopic observation of individual living spores.

Optical density losses during germination were plotted relative to completion of this event and were compared with stainability and phase-contrast changes. Measurements with the Beckman DU spectrophotometer were closely related to phase darkening (50% completion at 7.2 minutes for DU; and at 7.0 minutes for phase darkening) and reflected the end of microgermination. The apparent kinetic coincidence of stainability and Klett-Summerson colorimeter readings was an artifactual function of using round rather than plane-sided cuvettes in the Klett instrument.

Dipicolinic acid release paralleled but preceded stainability. The time for 50% completion of the release of DPA was 4.7 minutes in contrast to the 5.5 minutes required for 50% completion of stainability.

Loss of heat resistance, a classical criterion of spore germination, was 50% completed by about 3.0 minutes, a time at which spores had just started to become stainable. Sensitivity to toxic chemicals (exemplified by HgCl<sub>2</sub>) occurred at about the same time as heat sensitivity, and this event was also 50% completed at about 3.0 minutes.

In summary (FIGURE 5), the spores first lost resistance to heat and to toxic chemicals (50% complete in 3.0 minutes). This was followed by DPA loss (4.7 minutes); by acquisition of stainability (5.5 minutes); by phase darkening (7.0 minutes); and by DU-measured loss of turbidity (7.2 minutes). Selection of any one parameter or time would give a distorted picture of germination kinetics. With these *B. megaterium* LB spores, using a 15-minute incubation time, germination determined by all of the parameters would be identical and could be accurately measured by microscopic techniques. However, after five minutes of incubation, depending on the germination criterion, one might conclude that germination had progressed

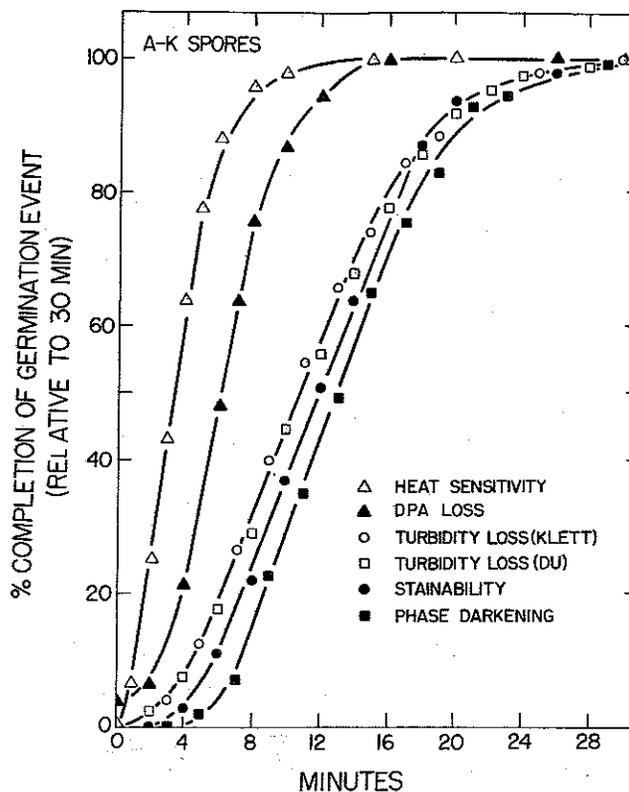


FIGURE 6. Kinetics of *Bacillus megaterium* spore germination events. Spores were produced on Arret-Kirshbaum (A-K) medium. Heat activated (60°C, 10 min) spores were germinated on glucose+ L-alanine (see FIGURE 5). In 30 min, when germination had been completed, 98% of the spores were heat-sensitive; 96% were stainable and 93% were phase-dark; spore suspensions had lost 54% of their original turbidity; and 117  $\mu\text{g}$  of their original (128.5  $\mu\text{g}$  per mg of spores) DPA. (Levinson & Hyatt, 1966. Reproduced with permission of the American Society for Microbiology.)

either to 82% (heat and chemical sensitivity); 56% (DPA loss); 43% (stainability); 21% (DU-measured O.D. loss); or to 11% of completion (phase darkening).

Time course plots for all of the germination parameters of LB spores were approximately parallel (FIGURE 5). The rate of germination in the rectilinear portion of the curves was about 18% of completion per minute. On the other hand (FIGURE 6), with spores produced on the A-K medium, the slopes of the heat sensitivity and DPA curves were steeper than curves for the other parameters, suggesting that this spore population was more homogeneous with regard to loss of heat resistance and DPA than with regard to the other germination criteria. By altering the sporulation medium, we have produced spores whose germination rate, as measured by stainability and by O.D. loss, was decreased from 18 to 7% of completion per minute; but whose rate of acquisition of heat sensitivity remained at about 18% per

minute. It is possible that one mechanism was responsible for stainability, phase darkening, and turbidity loss, but another mechanism, less sensitive to modification through alteration of the sporulation medium, governed loss of heat resistance and release of DPA.

#### *Germination vs. Postgerminative Development*

The activated spores, having germinated, will, on the proper medium, emerge from the spore case, elongate to assume the typical bacillary shape, and divide. We usually refer to stages from emergence through the first cell division as postgerminative development, although others (Campbell, 1957) term them outgrowth. Germination and postgerminative development are separate, though ordered processes, with different physiological requirements, and we shall outline some of these differences.

Resting spores had no measurable oxygen uptake. When they germinated, oxygen consumption on glucose (FIGURE 7) became readily apparent, reflecting an increase in metabolic activity (Levinson & Hyatt, 1956). Little, if any, glucose was consumed in germination, but it was utilized (with concomitant oxygen consumption) by the germinated spores (Levinson & Hyatt, 1963). Inhibitors of respiration, such as cyanide, did not inhibit germination although they were effective inhibitors of postgerminative development (Levinson & Hyatt, 1962; Hyatt & Levinson, 1962). After incubation for about

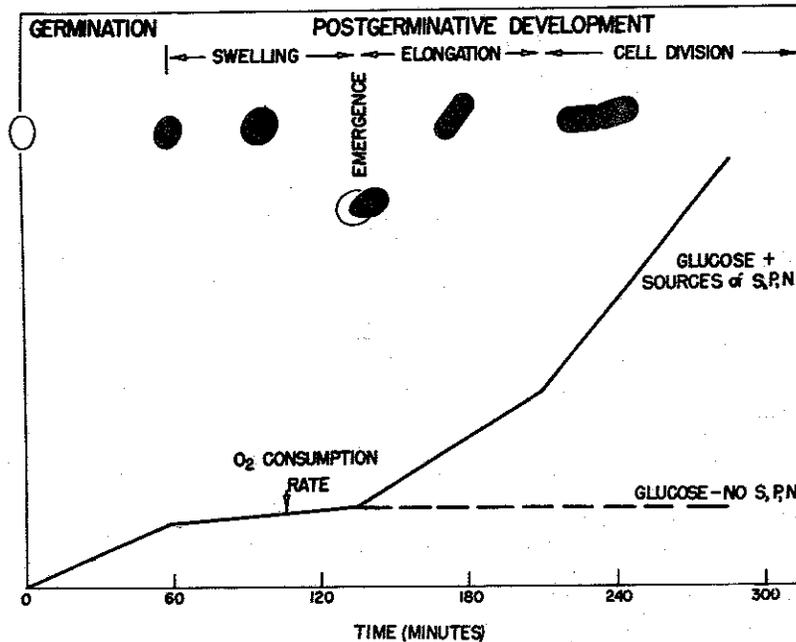


FIGURE 7. Diagrammatic representation of respiratory changes accompanying germination and postgerminative development of *Bacillus megaterium* spores.

60 minutes on a medium containing all the nutrients required for germination and postgerminative development, when germination was completed, the respiratory rate ceased to increase, and remained steady for another 1 to 1.25 hours, during which time the germinated spores swelled. At about 130 minutes, the cells emerged from the spore cases, and emergence was accompanied by a new burst of metabolic activity. The newly emerged cells lengthened to assume the typical bacillary shape. Between 3.5 and 4 hours, with the inception of cell division, there was another increase in the slope of the curve. Thus each morphological stage in the development of the germinated spore—swelling, emergence, and cell division—was marked by a change in the slope of the oxygen consumption rate curve. Such oxygen consumption rate curves have proven useful in investigation of the effects of various substances and conditions on spore germination and postgerminative development.

Sulfur (Hyatt & Levinson, 1957), phosphate (Hyatt & Levinson, 1959), nitrogen (Levinson & Hyatt, 1962) and carbon (Hyatt & Levinson, 1964) sources were necessary for the complete transition of spore to vegetative cell. In the absence of any of these ingredients, spores germinated, but could not complete postgerminative development. For example, spores germinated in the absence of sulfur, but they did not develop further. The addition of sulfur as  $K_2SO_4$  (or even as  $H_2SO_4$ ), in a concentration as low as 0.1 mM (3.2 ppm of S), permitted complete postgerminative development with its associated bursts of respiratory activity.

Carbon sources which support germination of *B. megaterium* spores do not necessarily support postgerminative development (Hyatt & Levinson, 1964). The 80 compounds which we have tested as carbon sources (TABLE 2) manifested all possible combinations of ability and inability to support germination and postgerminative development. As with carbon sources, the ability of a nitrogenous compound (Levinson & Hyatt, 1962) to support germination did not imply that it was utilizable in postgerminative development.

*B. megaterium* spores germinate under anaerobic conditions, but postgerminative development does not occur in the absence of oxygen. The pH optimum for germination differs from that for postgerminative development (Hyatt & Levinson, 1959). Antibiotics which inhibit vegetative development do not affect germination (Stuy, 1956). Spores may be exposed to doses of ionizing radiation which do not impair their ability to germinate, but which destroy their capacity for cell division (Levinson & Hyatt, 1960). Furthermore, extracts of spores contain a  $Mn^{++}$ -stimulated neutral pyrophosphatase, but vegetative cell extracts have diminished  $Mn^{++}$ -activated pyrophosphatase and increased  $Co^{++}$ -activated acid pyrophosphatase activity (Levinson, *et al.*, 1958).

Demonstration of the differences in requirements for germination and postgerminative development lends support to the postulate that metabolic pathways for germination and for stages of postgerminative development are different one from the other.

#### *Sporulation*

Under electron microscopy (FIGURE 8), the spore appears as a body surrounded by a spore case composed of several spore coat layers. Internal to

TABLE 2

CARBON COMPOUNDS IN GERMINATION AND POSTGERMINATIVE DEVELOPMENT OF HEATED *BACILLUS MEGATERIUM* SPORES

Germination	Cell Division	Carbon Compounds	
+	+	D-glucose (60) glucosamine (60) $\alpha$ -methyl-D-glucoside (27), elongation only	D-fructose (24) N-acetylglucosamine (40)
+	-	D-mannose (64) L-sorbose (30)	2-deoxy-D-glucose (50)
-	+	D-ribose D-galactose lactose maltose sucrose D-trehalose	mannitol glycerol L-aspartic acid fumaric acid Ca-gluconate raffinose
-	-	D-arabinose L-rhamnose DL-glyceraldehyde phosphorylated sugars	sorbitol pyruvic acid citric acid

Numbers in parentheses indicate % germination of heated spores (60°C, 10 min) after 2 hr at 30°C in phosphate buffer (50 mM, pH 7.0) + the indicated C compound (2.5 mM). Cell division after 6 hr at 30°C in medium containing phosphate buffer (pH 7.0, 50 mM), L-alanine (25 mM), K<sub>2</sub>SO<sub>4</sub> (1 mM), and C source (25 mM).

the coats, the outer cortical layer, cortex, cell-wall primordium, and plasma membrane envelop the cytoplasmic core of the spore. In the proper medium, the spore case splits open, a new cell emerges from the germinated spore, and, after several divisions, forms spores again.

We have now developed a simple glucose-ammonia-mineral salts medium (Holmes & Levinson, 1967a) in which a new cell (primary cell) emerges from the spore case, but does not divide. Instead the emergent cell forms a spore without having divided (FIGURE 9). This shortened cycle (initial spore → primary cell → second-stage spore without intervening cell division) is called microcycle sporogenesis (Vinter & Slepecky, 1965).

Spores in the glucose-ammonia medium germinated within 15 minutes at 30°C (FIGURE 10). The optical density of the suspension decreased as the spores became less refractile, and the heat sensitivity increased. The optical density increased again as the primary cells emerged and enlarged. At about 10 hours, there was another increase in turbidity associated with the appearance of refractile second-stage spores and heat resistance. There was no increase in numbers of cells in the 18-20 hours required for completion of the cycle.

During the first hours of microcycle sporogenesis, glucose disappeared, and the acids resulting from glucose utilization decreased the pH. The acid produced was then oxidized and the pH rose again. In order for the cycle to go to completion, it was essential that the acids be oxidized. If more glucose were added at any time before second-stage sporulation had occurred, there

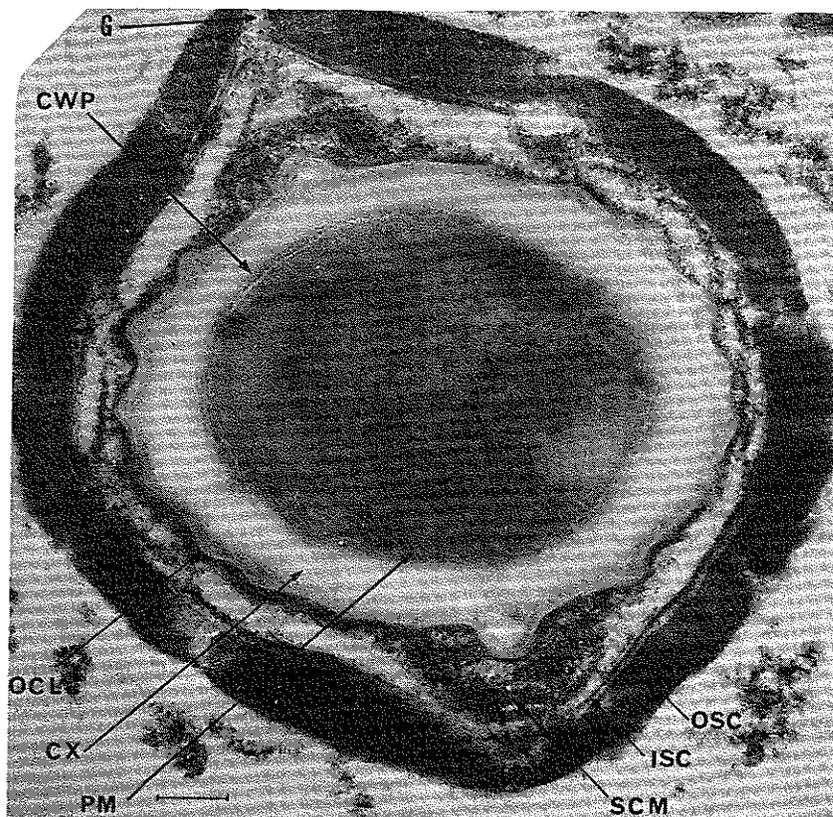


FIGURE 8. The essential structural features of a *Bacillus megaterium* spore. Marker represents  $0.1 \mu$ . CWP, cell-wall primordium; Cx, cortex; G, germinal groove; ISC, inner spore coat; OCL, outer cortical layer; OSC, outer spore coat; PM, plasma membrane; SCM, subcoat material. (Freer & Levinson, 1967. Reproduced with permission of the American Society for Microbiology.)

was renewed production of acid, with complete suppression of second-stage sporulation.

Glucose was, in fact, not an absolute requirement for microcycle sporogenesis. Sodium acetate (40 mM) spared 90% of the glucose requirement, reducing it to 1 mM. Acetate alone was not adequate as the sole carbon source; addition of either glucose or a tricarboxylic acid cycle intermediate, such as succinate, was essential. Spores could be germinated in the absence of a carbon source (in KI, for example) and, if acetate and succinate were added, sporulation occurred.

Utilization of acetate was essential for sporulation. This was examined from two viewpoints: (1) suppressing development of the ability of primary cells to oxidize acetate and (2) allowing this competence to develop, but then rendering the acetate-oxidizing ability inoperative.

Spores germinated, without a carbon source (FIGURE 11), and given acetate and succinate at the beginning of incubation, did not develop the com-

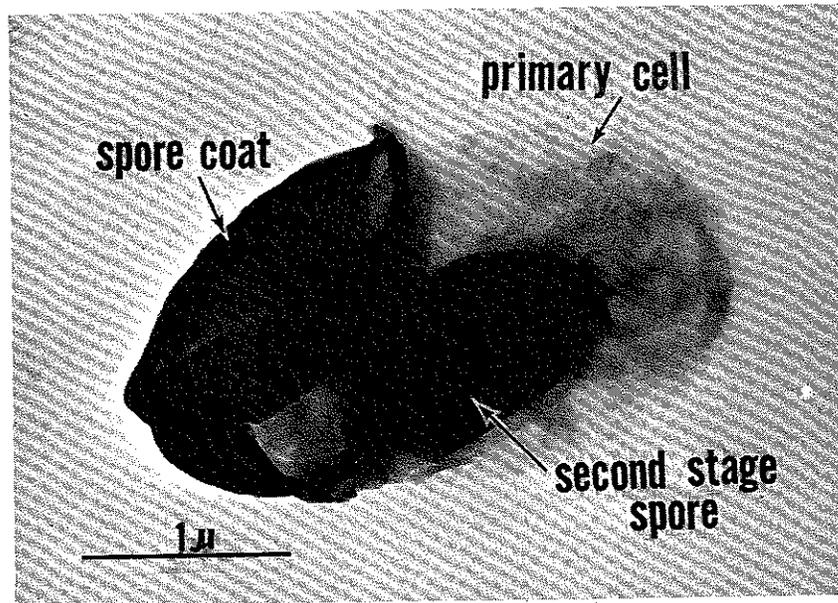


FIGURE 9. An 18-hr primary cell of *Bacillus megaterium* containing a second-stage spore. The primary cell has emerged from the ruptured spore coat of the initial spore and a smaller second-stage spore lies within it. (Holmes & Levinson, 1967. Reproduced with permission of the American Society for Microbiology.)

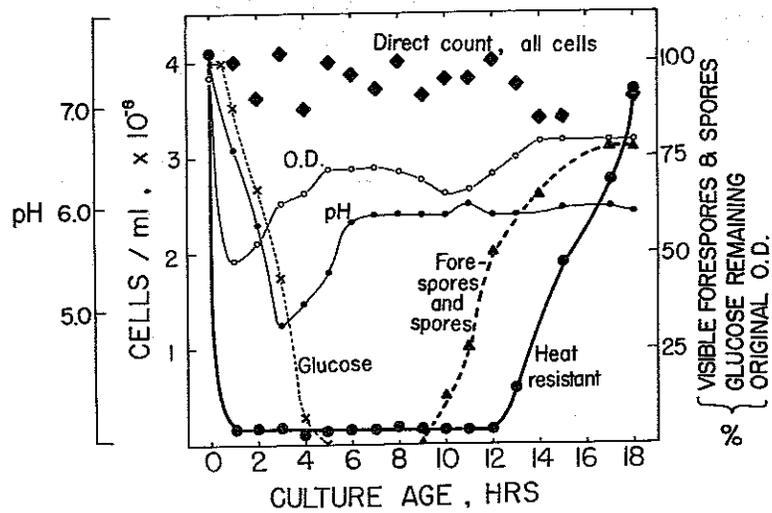


FIGURE 10. Events during microcycle sporogenesis of *Bacillus megaterium* in glucose-ammonia medium. (Holmes & Levinson, 1967. Reproduced with permission of the American Society for Microbiology.)

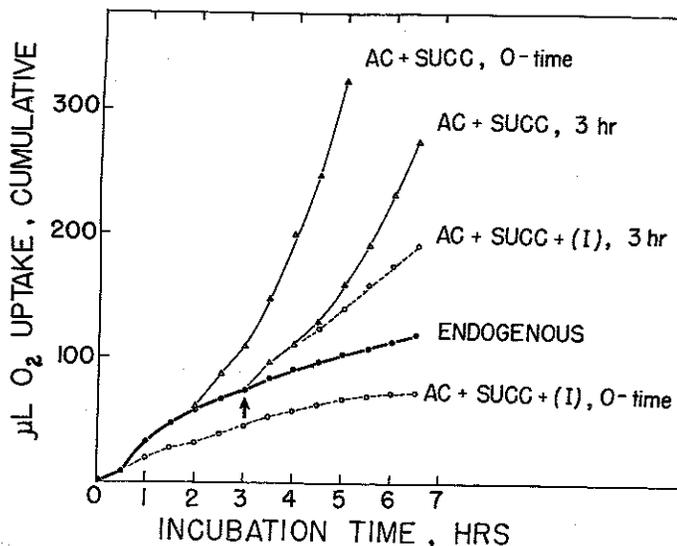


FIGURE 11. Oxygen uptake by *Bacillus megaterium* primary cells under various conditions. Initial spores were germinated with 0.01 M KI, 0.006 M  $K_2HPO_4$ , and 0.0015 M  $K_2SO_4$  (endogenous). Acetate (AC) 0.04 M and succinate (SUCC), 0.002 M, were added with or without inhibitor (I), either at 0-time, or after 3 hr of incubation (arrow) at 30°C. Inhibitors (chloramphenicol, 15  $\mu$ g/ml or actinomycin-D, 50  $\mu$ g/ml) produced the same results whether added at the same time as, or 15 min prior to, the addition of the acids. Sporulation occurred only in the cultures represented by the top two curves (AC + SUCC, 0-time: AC + SUCC, 3 hr). (Holmes & Levinson, 1967. Reproduced with permission of the American Society for Microbiology.)

petence to oxidize these acids for two hours. They developed this competence even in the absence of acetate and succinate and, if presented with these acids at three hours, began to oxidize them without lag. In both cases (acids added either at zero time or after three hours of incubation), the cells proceeded to sporulate without division. The ability to utilize acids did not develop if cells were incubated in the presence of one of the known inhibitors of protein synthesis, inferring that the synthesis of enzymes required for acetate oxidation was inhibited. When these enzymes were not synthesized, because of the presence of inhibitor, there was no sporulation. If acetate and succinate were presented to the primary cells together with inhibitor at three hours, the acids were oxidized by the enzymes synthesized until that time, but no further enzyme production occurred and the cells did not sporulate. A functioning tricarboxylic acid cycle was probably required for microcycle sporogenesis. At least one of the enzymes of the tricarboxylic acid cycle, aconitase, was absent during germination, but was present during acid oxidation.

Fluoroacetate, an inhibitor of acetate oxidation, also inhibited the sporulation of cells which had developed the competence to oxidize this acid. Fluoroacetate inhibited only when added before six hours, i.e., before the pH had risen and substantial acetate oxidation had already occurred. Tricarboxylic acid cycle components, such as succinate, relieved the inhibition of acetate oxidation and also relieved the inhibition of microcycle sporulation.

We consider acetate and a tricarboxylic acid cycle intermediate to be minimum organic metabolic requirements for microcycle sporogenesis. Glucose (in the glucose-ammonia medium) may serve to supply the newly germinated cell with priming amounts of four-carbon compounds for the operation of the tricarboxylic acid cycle.

The microcycle sporulation system offers a unique opportunity for studying structural changes involved in spore germination and formation without the complications introduced by cell division. There is not enough space to show the entire series of electron micrographs obtained with Drs. John Freer and Milton Salton at New York University. Suffice it to say that using ultrathin sectioning techniques, we were able to follow the structural transition from initial spore to second-stage spore in the absence of cell division (Freer & Levinson, 1967). In the final stage of microcycle sporogenesis (FIGURE 12), the second-stage spores had matured and were in the process of being released from the primary cell. Remnants of the initial spore, of the primary cell wall, and of the primary cell plasma membrane could often be seen.

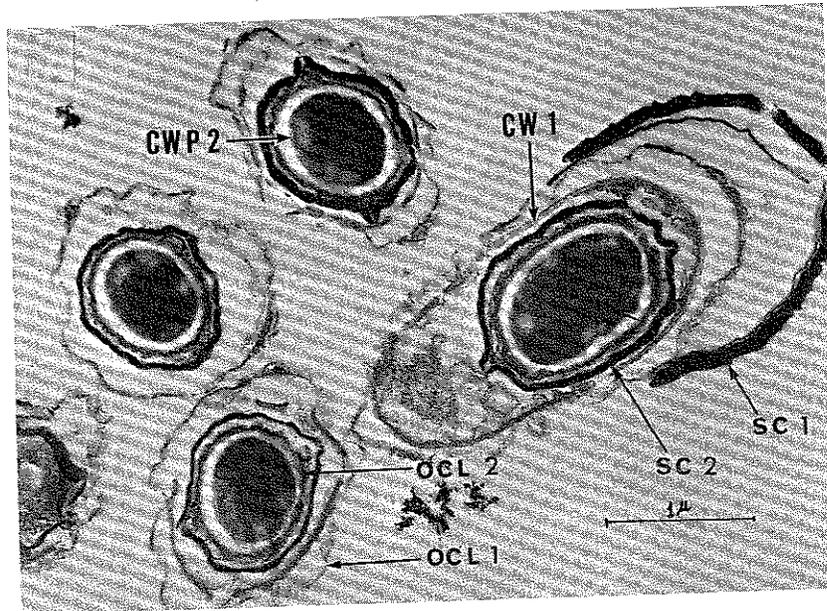


FIGURE 12. Ultrathin sections from a 20-hr culture undergoing microcycle sporulation on glucose-ammonia medium. The numerals 1 and 2 after abbreviations refer to initial and second-stage spores respectively. CW, cell wall; OCL, outer cortical layer; SC, spore coat. Second-stage spores are enveloped by the outer cortical layer of the initial spore, and the sporangium on the right shows, in addition, the persistent spore coat of the initial spore. Remnants of the cell wall, and perhaps of the plasma membrane, of the primary cell are seen. The cytoplasmic cores of the second-stage spores in the upper center and in the lower part of the electron micrograph appear to be surrounded by new cell-wall primordium. (Freer & Levinson, 1967. Reproduced with permission of the American Society for Microbiology.)

## Summary

The spore form then, for whatever purposes, allows the bacterium to take effective "time out" from the competition of making a living. Certain delicate stimuli, such as gaseous water, can, without necessarily endangering this somnolent state, place the spore in a position to sample its chemical environment. If the environment offers one of a limited number of compounds—germinants—the protected state is rapidly and irreversibly abandoned, and the bacterium faces tasks common to all life—growth and reproduction. These latter processes require more complicated nutrition than that necessary for arousing the dormant spore, or for inducing the aroused (activated) spore to germinate. The quality and quantity of the diet will determine whether the germinated spore forms a vegetative cell, and whether this bacillus reproduces and resporulates; reproduces; resporulates directly; or dies. The minimal menu consonant with survival is associated with direct sporulation. A bacillus, activated and germinated by an environment too sparsely furnished to support reproduction but with sufficient nutriment to form a vegetative cell, can still resporulate and retire once more from competition.

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