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Heat Activation of *Streptomyces viridochromogenes* Spores

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The lag period preceding germination of *Streptomyces viridochromogenes* spores during incubation in a defined germination medium was completely eliminated by a gentle heat shock. The rate of germination was not affected. The optimum pH for activation extended from 6.0 to 9.6. The time of heating required for maximum activation was 1 min at 60 C, 2 to 5 min at 55 C, 20 min at 50 C, and 40 to 50 min at 45 C. Activated spores had the same temperature and pH optima and nutritional requirements for germination as unactivated spores. Activated spores deactivated during incubation for 8 h at 25 C and were activated again by a second heat shock. Spores that had been aged for 4 weeks or longer did not germinate in the defined germination medium unless they were first heat activated.

We reported that spores of *Streptomyces viridochromogenes* germinate when incubated in a defined medium (9, 10). The optimum rate of germination requires the presence of L-alanine, adenosine, L-glutamic acid, *para*-aminobenzoic acid, calcium, and magnesium ions. The spores do not germinate in the absence of CO_2. A lag period of approximately 20 min precedes a rapid decrease in optical density (OD) of spore suspensions accompanied by a loss of refractility of the spores. We now report that a mild heat treatment eliminates the lag period preceding the beginning of germination events and is absolutely required for germination of aged spores in the defined medium. This is the first report of activation of streptomycete spores.

Activation, resulting in increased germinability, is commonly observed for bacterial endospores and fungal spores (12, 16, 17). A comprehensive review of activation of endospores has been published (12) and should be consulted for specific references.

MATERIALS AND METHODS

Production of spores and germination procedure. The methods used to grow *S. viridochromogenes*, harvest spores, and assay for germination have been described (10). Germination was measured by following the decrease in turbidity as measured by the OD of a spore suspension during incubation at 35 C in a defined germination medium (DGM) containing 0.1% L-alanine, 0.1% L-glutamic acid, 0.01% adenosine, 0.01% *para*-aminobenzoic acid, 0.002% $CaCl_2 \cdot 2H_2O$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05 M tris (hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.3), and 0.001% Triton X-100.

Heat activation procedure. A 0.15-ml sample of a concentrated spore suspension (approximately 5.0 mg [dry weight]/ml) was added to 3.0 ml of 50 mM Tris-hydrochloride buffer (pH 8.32), containing 0.001% Triton X-100, which had been prewarmed in a water bath to a temperature of 55 C. The spore suspension was incubated at 55 C for 10 min and then cooled in a 25 C water bath. The spores were sedimented by centrifugation (10,000 rpm for 15 min) at 25 C, and the supernatant fluid was poured off. The spores were suspended in 10 ml of 0.05 M Tris-hydrochloride buffer (pH 7.3) containing 0.001% Triton X-100 (TX buffer) and centrifuged again, and the supernatant fluid was discarded. The spores were then suspended in 0.2 ml of TX buffer and sonically treated for 5 min in a water bath sonicator (Heat Systems Ultrasonics, Inc.) to disperse clumps of spores. Samples of 50 μl were used in the germination assays.

Viability measurements. To determine the effect of the heat activation procedure on the viability of spores, a 0.1-ml sample was removed from an unactivated spore suspension and serially diluted into sterile broth medium consisting of 1.0% glycerol, 0.1% L-asparagine, 0.1% K_2HPO_4, and 0.1% yeast extract (GYA medium). Samples of 0.1 ml were removed from the dilution tubes and spread onto the surface of GYA agar medium (GYA plus 2% agar), using a bent glass rod. The spore suspension was then subjected to the heat activation procedure. A 0.1-ml sample of the activated spore suspension was then removed and treated as just described. The plates were incubated at 30 C for 1 week, after which colonies were counted.

RESULTS

Effect of heat on spore germination. The change in OD of a spore suspension when incubated at 35 C in TX buffer containing 0.002% $CaCl_2 \cdot 2H_2O$ and 0.01% $MgSO_4 \cdot 7H_2O$ (TX salts) is shown in Fig. 1, curve A. A slow, essentially linear decrease in OD was observed. This OD change was accompanied neither by loss of

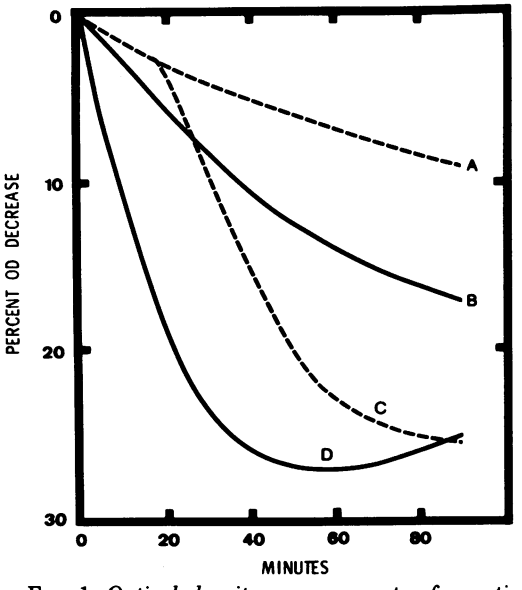

FIG. 1. *Optical density measurements of unactivated and heat-activated spore suspensions. For heat activation, spores harvested after 10 days of growth were heated in 50 mM Tris-hydrochloride buffer (pH 8.32) containing 0.001% Triton X-100 for 10 min at 55 C. The spores were washed and suspended in TX buffer. The unheated or heat-activated spores were inoculated into TX salts or DGM, and the OD was monitored with a recording spectrophotometer during incubation at 35 C. (Curve A) Unactivated spores suspended in TX salts; (curve B) heat-activated spores suspended in TX salts; (curve C) unactivated spores suspended in DGM; (curve D) heat-activated spores suspended in DGM.*

spore refractility nor by any other germination events (9, 10), but was due instead to settling of the spores. We know that this OD decrease involved settling because shaking the spore suspension briefly after the 90-min incubation period returned the OD to the original zero-time value. When the spores were heated in TX buffer (pH 8.32) for 10 min (optimum heat activation conditions, as will be shown later) followed by incubation in TX salts, the rate of OD decrease was more rapid than in the unheated control (Fig. 1, curve B). This decrease in OD did involve a slight loss of spore refractility and was accompanied by a small but detectable increase in the rate of endogenous metabolism and the excretion of carbon-containing spore constituents (C. Hirsch, Ph.D. thesis, Univ. of Wisconsin, Madison, 1975).

When unheated spores were incubated in DGM, a 15- to 20-min lag period preceded a rapid decrease in OD of the spore suspension (Fig. 1, curve C). This OD decrease was accompanied by normal germination events, including the complete loss of spore refractility (9, 10). Significantly, when heated spores were incubated in DGM, germination proceeded without the 15- to 20-min lag period observed for unheated spores (Fig. 1, curve D). The heated spores began to lose refractility almost immediately upon suspension in DGM and appeared completely phase dark by about 40 min. The rate of germination (slope of OD curves) was not appreciably affected by the heat shock, and the maximun OD decrease was nearly the same with heat-treated and untreated spores.

Examination of an unactivated germinating spore suspension with phase-contrast optics revealed that some of the spores remained phase bright, whereas the majority had become phase dark (10). This lack of synchrony, caused by the failure of a minor fraction of the spores to germinate, was not observed with heat-shocked spores. All of the heat-treated spores were phase dark after 40 min of incubation in DGM (not shown). Thus, heat treatment both eliminated the pregermination lag period and synchronized germination of the spores. Heat-treated spores will hereafter be referred to as having been activated.

Effect of calcium ions on germination of activated spores. It was reported above that the OD of an activated spore suspension when incubated in TX salts decreased more rapidly than did that of unactivated spores incubated in TX salts. The slow decrease in OD of the activated spores was accompanied by the occurrence of some of the events involved in germination. The rates at which these events occurred, however, were much slower than those observed when the spores were incubated in DGM. Thus, heat treatment seems to initiate some processes involved in germination that are independent of nutrients in the surrounding medium. We also observed that suspensions of activated spores in TX buffer (no added salts) underwent a decrease in OD (Fig. 2, curve A) that was identical to that of unactivated spores incubated in TX salts (Fig. 1, curve A). The activated spores did not initiate germination events, including the loss of refractility and the excretion of carbon-containing components, as they did when incubated in TX salts. The basis for the different results obtained with TX buffer and TX salts was determined. The only difference between the two suspending media was that TX salts contain $MgSO_4$ and $CaCl_2$ and TX buffer did not. Incubation of activated spores in TX buffer containing 0.01% $MgSO_4 \cdot 7H_2O$ and no $CaCl_2$ resulted in an OD decrease (Fig. 2, curve B) the same as observed for activated spores incubated in TX buffer (which contained

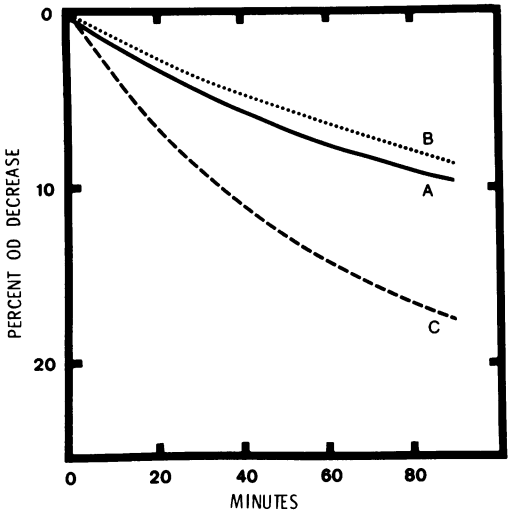

FIG. 2. *Requirement of calcium ions for decrease in optical density of spores during incubation in TX buffer. Activation, germination conditions, and explanation of data presentation are as described in legend to Fig. 1. (Curve A) Activated spores incubated in TX buffer; (curve B) activated spores incubated in TX buffer plus 0.01% $MgSO_4 \cdot 7H_2O$; (curve C) activated spores incubated in TX buffer plus 0.002% $CaCl_2 \cdot 2H_2O$.*

neither Ca nor Mg ions; compare Fig. 1, curve A). Incubation of activated spores in TX buffer containing 0.002% $CaCl_2 \cdot 2H_2O$ and no $MgSO_4$ resulted in a decrease in OD (Fig. 2, curve C) nearly identical to that observed for activated spores in TX salts (Fig. 1, curve B). These data indicate that calcium ions are essential for loss of refractility and presumably also for other germination events that are initiated by heat activation.

Effect of pH on activation. Spores were incubated at 55 C for 10 min in buffers containing 0.001% Triton X-100 at pH values ranging from 3.65 to 10.75. Tris-maleate at 50 mM was used for the pH range 3.65 to 6.52, Tris-hydrochloride was used at 50 mM for the pH range 7.05 to 9.62, and sodium carbonate-bicarbonate was used at 100 mM for the pH range 9.16 to 10.83. The spores were sedimented, washed, and assayed for germinability. The optimal pH range extended from 6.0 to 9.6. Germinability of spores was much reduced when they were heated at lower pH values. The spores germinated very poorly after heating in the alkaline carbonate buffers. This was apparently due to an inhibitory effect and not to the alkaline pH itself since the spores germinated optimally at pH 9.6 (Tris only). A pH value of 8.32 was used in all subsequent heat activation experiments.

Relationship of temperature and incubation time. To determine the optimal temperature and time of heating for activation, spores were incubated in TX buffer at 45, 50, 55, and 60 C. The TX buffers were prepared so that the pH at each test temperature was 7.5. Samples were removed at various times and assayed for germinability (Fig. 3). Spores incubated at 60 C were maximally activated after 1 min. Longer exposure to this temperature caused a rapid decrease in germinability. This was presumably due to killing of the spores. Several instances of the lethality of temperatures in this range to *Streptomyces* spores have been reported (1, 18; M. M. Rice, Ph.D. thesis, Univ. of Wisconsin, Madison, 1965). Spores incubated at 55 C were maximally activated after 2 to 5 min. Longer incubation did not significantly affect subsequent germination. A determination of viability after 0 and 10 min of incubation at 55 C showed that 94% of the spores remained viable. The time for spores to be maximally activated during incubation at 50 C was approximately 5 to 10 min. Spores incubated at 45 C were maximally activated at approximately 15 min and did not germinate as well (maximum OD decrease) as those activated at the higher temperatures. Lower temperatures were not tested, but spores incubated in TX buffer at 25 and 4 C germinated with the same kinetics even after standing for extended periods of time (9). For all subsequent experiments, spores were activated by heating at 55 C for 10 min. This provides a normal germination

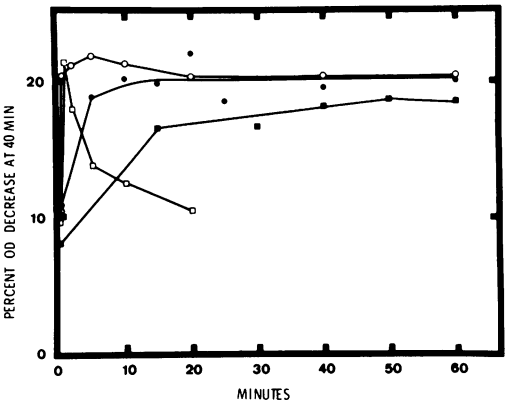

FIG. 3. *Effect of time of exposure to different temperatures on activation of spores. Spores were incubated in TX buffer at the temperatures tested. At different times, samples were removed and the rate of germination of the spores in DGM was determined. The results are expressed as the percent decrease in OD at 40 min. Symbols: (■) 45 C; (●) 50 C; (○) 55 C; (□) 60 C.*

curve (OD decrease) without significantly affecting viability of the spores.

Optimum conditions for germination of activated spores. We previously established that germination of unactivated spores occurred most rapidly at a temperature of 35 to 40 C (10). An experiment was designed to determine whether this also was true for activated spores. After activation, spores were incubated in DGM at temperatures ranging from 23 to 50 C. The pattern for the effect of incubation temperature on germination rate was virtually the same as for the unactivated spores. The maximal rate was at 35 to 40 C and decreased at higher and lower temperatures.

To determine whether activation altered the pH optimum for germination, heat-activated spores were incubated in DGM containing 50 mM Tris-hydrochloride or Tris-maleate buffers at pH values ranging from 4.87 to 8.49. The maximum rate of germination occurred over the pH range of 5.8 to 7.3. Germinability decreased sharply at more acid or alkaline pH values. This pattern of germination at different pH values is essentially the same as we previously reported for unactivated spores (10).

It is known that heat activation of *Bacillus* endospores results in simplification of the nutritional requirements for germination (15). We designed an experiment to determine whether this was true for activated spores of *S. viridochromogenes*. Spores were heat activated and their germination was followed during incubation in DGM or DGM lacking each of the four germinants: L-alanine, L-glutamate, adenosine, or *p*-aminobenzoic acid. The optimum rate of germination required all components of DGM (Table 1). The rates of germination in the absence of individual germinants were essentially the same as found previously for germination of unactivated spores (10).

Reversibility of heat activation. A suspension of spores was heat activated, washed, and suspended in TX buffer, pH 7.3. A sample of spores when added to DGM germinated as expected for activated spores (Fig. 4, curve A). The spores were allowed to stand at 25 C for 17 h and then were tested again for germinability. The spores germinated only after a lag period and with practically the same kinetics as spores that had not been heat activated (Fig. 4, curves B and D). When the activated spores that had stood for 17 h in TX buffer and had become deactivated were again heat shocked, they were activated as evidenced by germination in DGM at practically the same rate as obtained with the original activated spore suspension (Fig. 4, curve C).

An experiment was designed to determine the effect of time of incubation at 25 C on deactivation of heat-activated spores. The activated spores were incubated without agitation in TX buffer at 25 C for 67 h. At various times samples were removed and inoculated into DGM, and germinability of the spores was assayed. The rate of germination of the activated spores decreased during the first 8 h of incubation in TX buffer (signifying deactivation) and then

TABLE 1. *Nutritional requirements for germination of heat-activated spores*

Germination medium	% Decrease in OD[a]	% of DGM
DGM	22.1	100.0
DGM – alanine	15.9	72.0
DGM – glutamate	16.1	72.9
DGM – adenosine	17.1	77.4
DGM – PABA[b]	18.7	84.6

[a] Percent decrease in OD after 40 min of incubation.

[b] PABA, *para*-Aminobenzoic acid.

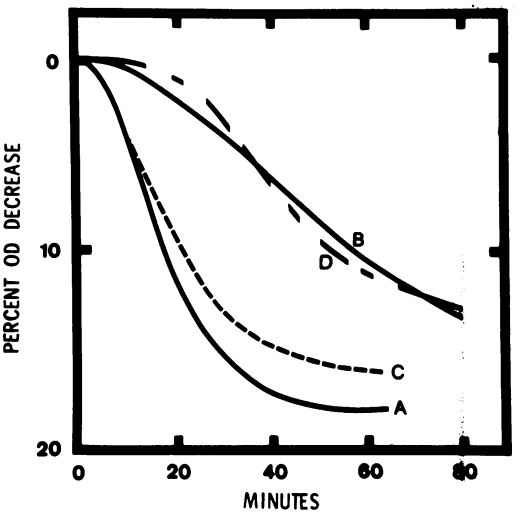

FIG. 4. *Deactivation and reactivation of heat activated spores. Spores were heat activated as described in the legend to Fig. 1 and suspended in TX buffer. A sample was tested for germinability in DGM. The remaining spores were incubated at 25 C for 17 h. One sample was assayed for germinability and another sample was heat activated and then tested for germinability. (Curve A) Heat-activated spores, zero time; (curve B) heat-activated spores after 17 h of incubation (deactivated); (curve C) deactivated spores subjected to a second heat activation treatment; (curve D) spores that had not been heat activated.*

remained approximately constant (Fig. 5). A control suspension of spores that had not been activated germinated in nearly identical fashion before and after the 67-h incubation period.

Effect of activation on aged spores. We have reported that spores harvested from plates after 21 days of incubation germinated less readily in DGM than 10- or 14-day spores and that heat activation restored germinability of the aged spores (10). A more careful analysis was made of this latter phenomenon. Spores were harvested at weekly intervals from plates that had been incubated up to 7 weeks. The ability of the spores to germinate in DGM was tested before and after activation. The unactivated spores did not germinate in DGM when they were harvested from plates incubated for 7 days (Fig. 6). The 14-day spores did germinate rapidly. The ability to germinate in DGM then decreased rapidly so that 4-week and older unactivated spores did not germinate. The spores harvested after incubation for each of the time periods germinated equally well after they were subjected to the heat activation procedure. Spores harvested after 10, 14, and 21 days of growth germinated equally well in yeast extract (10). Spores incubated for 7 weeks, which did not germinate in DGM unless activated, did germinate in yeast extract as well as do younger spores (data not shown).

DISCUSSION

A concise definition of activation is found in the glossary section of the comprehensive review of bacterial spores edited by Gould and Hurst (7). This definition states that activation "results from some treatment which does not itself initiate germination, but afterwards allows spores to germinate more rapidly or more completely or both." Spores of *S. viridochromogenes* that have been heat shocked germinate more rapidly than spores that have not been heated. We feel that it is justifiable to use the connotation of activation as applied to endospores of bacilli to spores of *Streptomyces*.

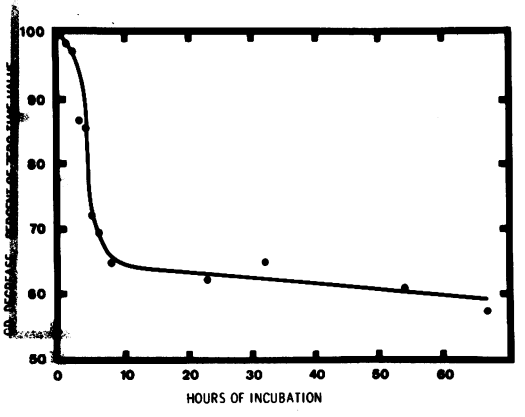

FIG. 5. *Rate of deactivation of heat-activated spores during incubation at 25 C. Spores activated as described in the legend to Fig. 1 were incubated in TX buffer at 25 C. At the times tested, samples were removed and assayed for germinability in DGM. The amount of OD decrease after 40 min of incubation in DGM of spores at each sampling time is expressed as the percentage of the OD decrease obtained with freshly activated spores (zero-time value) after 40 min of incubation in DGM.*

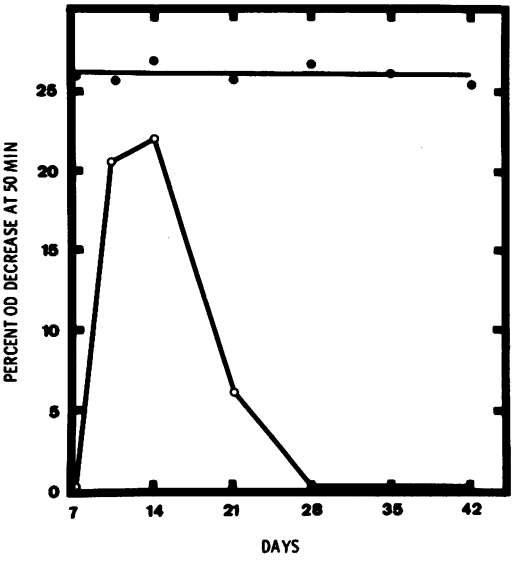

FIG. 6. *Germinability before and after heat activation of spores harvested from growth medium after various periods of growth. Spores harvested from plates after periods of growth ranging from 7 to 42 days were tested for their ability to germinate in DGM before and after being heat activated. The results are expressed as the percent OD decrease obtained after 50 min of incubation in DGM. Symbols: (○) Unactivated spores; (●) activated spores.*

The requirement of activation for germination of dormant cell forms occurs in a wide variety of organisms including bacteria, fungi, protozoa, and seeds of plants (17). A variety of treatments, such as exposure to heat, cold, chemical agents, mechanical abrasion, and light, have been shown to serve as activators.

Temperature has an important effect on germination of endospores of two thermophilic actinomycetes, *Thermoactinomyces vulgaris* and *Actinobifida dichotomica*. The exact nature of the temperature effect is controversial. An optical density assay shows a dramatic activation of spores of *T. vulgaris* strain 136 when the spores are heated at 100 C for 10 min (11). Heating spores of *T. vulgaris* CUB 76 and *A. dichotomica* CUB 339 at 100 C for up to 30 min increased

the number of colonies subsequently formed on a nutrient medium over the numbers obtained with unheated spores (5). These observations were reported to be evidence for heat activation of the spores. Eight of ten strains of *T. vulgaris* (including one designated CUB 76H) germinated less well in peptone broth after being heated at 90 C for 15 min (3). The other two strains germinated better in the peptone broth, but germinated less well in an L-alanine medium, after the heat shock. Kirillova et al. (13) state that spores of *T. vulgaris* strain 136, when heated at 100 C for 10 or 20 min, did not germinate at all in three incubation media during the time that unheated spores were 100% germinated in the media. Foerster (6) reported that spores of several strains of *T. vulgaris* and one of *A. dichotomica* germinated less readily after heating for 30 min at 70, 80, 90, or 100 C. The reason for the contradictory results for the effect of heat on germination of these spores, activation versus inhibition, is unclear. The effect of low temperature on the spores is not so confusing. Incubation of *T. vulgaris* spores at temperatures below their optimal growth temperature of 55 C activates the spores (2, 11, 13). The most effective temperature was 20 C (13).

Most studies of the mechanism of the activation process have been made with endospores of bacilli (see reference 12).

Some interesting similarities and differences are noted when comparing activation of *S. viridochromogenes* spores to endospores of bacilli. Newly formed endospores will often not germinate in a nutrient medium unless they are first activated. The streptomycete spores require activation for germination only when incubated in DGM, and even then the requirement is dependent on spore age. The spores, when harvested after 7 days or after 28 to 42 days of growth on solid media, will not germinate in DGM. Spores harvested at 10, 14, and 21 days of growth do germinate in DGM after a preliminary lag period. The spores at each of these ages germinate equally well in DGM after heat activation. Spores at the different ages germinate equally well in yeast extract medium and do not require activation.

The relationship between the time of exposure at different temperatures required to give maximum activation of *S. viridochromogenes* spores is not linear. The time required to reach the maximum OD decrease becomes progressively longer as the temperature is lowered. This same pattern is observed during heat activation of spores of bacilli (15). The temperatures that result in rapid activation are lower for spores of the streptomycete than for the bacilli.

Activation decreases or eliminates the lag period preceding germination of *S. viridochromogenes* spores but does not affect the rate of germination (rate of OD decrease of spore suspension). Activation of endospores of bacilli results in a decreased pregermination lag and an increased rate of germination (14).

Activation of endospores of bacilli and *S. viridochromogenes* spores is a reversible process. Both lose the capacity for rapid germination if incubated in a nongermination medium after activation. The time required for this deactivation is much less for the streptomycete spores, 8 h at 25 C as compared with 48 h or longer for endospores at this temperature.

Activation of endospores is accompanied by an increase in the rate of endogenous metabolism, acquisition of the ability to oxidize glucose, excretion of dipicolinic acid (8), and an increase in the activity of intracellular proteases (4). Spores of *S. viridochromogenes* exhibit an increase in their endogenous metabolism rate and excrete small amounts of cell carbon after they have been activated (C. Hirsch, Ph.D thesis). Neither of these two processes will occur if the activated spores are incubated in the absence of calcium ions.

It is known that the nutritional requirements for germination of endospores of bacilli are less complex after they have been activated. The requirements for components of DGM for germination of *S. viridochromogenes* spores are exactly the same before and after activation.

Keynan and Evenchik (12) evaluated various theories and possible explanations for the mechanism of activation of *Bacillus* endospores. Based on analyses of accumulated data, they proposed that activation may involve reversible changes in the tertiary structure of some spore macromolecule. A likely possibility would be reduction of disulfide bonds in the spore coat resulting in increased permeability of the spores. Deactivation, the reversal of activation, would involve oxidation of the sulfhydryl groups to reestablish the disulfide bonds. As Keynan and Evenchik point out, this hypothesis is compatible with most of the observations that have been made dealing with endospore activation.

There is no reason to suggest that the mechanism involved in endospore activation would apply likewise to the streptomycete spores. If activation of the streptomycete spores does involve breaking chemical bonds, the types of bonds would be weaker than those that might be involved in endospore activation since the energy (as heat) required is much less for the former. Also, the time for deactivation of the streptomycete spores is considerably shorter,

again suggesting the possibility of weaker bonds being involved.

ACKNOWLEDGMENTS

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