JOURNAL OF BACTERIOLOGY, July 1980, p. 377-382
0021-9193/80/07-0377/06$02.00/0

Vol. 143, No. 1

Streptomyces viridochromogenes Spore Germination Initiated by Calcium Ions

DAVID EATON† AND JERALD C. ENSIGN*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Initiation of germination of heat-activated *Streptomyces viridochromogenes* spores occurs in media containing only calcium ions and organic buffer. The calcium-induced initiation of germination was accompanied by a decrease in absorbance of the spore suspension, an increased rate of endogenous metabolism, the loss of spore carbon, and the loss of heat resistance. Calcium amounts to 0.28% of the dry weight of freshly harvested spores. The amount of calcium remained the same after incubation of spores in water and after heat activation. The spore content of calcium doubled after incubation in 0.5 mM $CaCl_2$ for 5 min at 4°C and during calcium-induced germination. Nearly all of the calcium appears to be bound to sites external to the spore membrane, since the chelating agents (ethylenedinitrilo)tetraacetic acid and arsenazo III removed virtually all of the calcium ions. The calcium ions must be present during the entire initiation of germination period. Germination ceases after an (ethylenedinitrilo)tetraacetic acid wash and begins again immediately after addition of calcium ions.

Spores of *Streptomyces viridochromogenes* germinate when incubated in a defined medium (DGM) comprised of alanine, glutamic acid, adenosine, $MgSO_4$, and $CaCl_2$ (4). The early events in germination, to be referred to as initiation of germination in this paper, involve loss of refractility of the spores accompanied by a decrease in optical density, excretion of spore carbon, increased respiratory activity and ATP content, and release of a germination inhibitor compound (4, 6). Germination is dependent on a continual supply of CO_2 which serves to replenish tricarboxylic acid intermediates through anaplerotic fixation reactions (2).

The data to be presented establish that the physiological events associated with germination of the spores can be initiated by calcium ions alone. Initiation of germination is dependent on the capacity for production of energy. As mentioned above, germination is accompanied by increased respiratory activity and ATP production. In addition, chemicals that affect energy production, such as cyanide, carbonyl cyanide-*m*-chlorophenylhydrazone, arsenate, dicumarol, and phenazine methosulfate, completely inhibit the initiation process, and the germination inhibitor molecule (6) was recently found to be a specific sodium-potassium ATPase inhibitor (unpublished observations). Germination of *S. antibioticus* spores is also dependent on energy production (3). Initiation of germination of streptomyces spores appears to be quite a different process than that of *Bacillus megaterium* endospores, where production of energy is not involved (1, 8). Germination of fungal spores requires a continuous supply of energy (9, 10).

We report in this paper the results of an investigation of the mechanism by which calcium ions initiate germination of *S. viridochromogenes* spores.

MATERIALS AND METHODS

Organisms and growth conditions. The organism used in these experiments was *S. viridochromogenes* NRRL B-1551.

Spores obtained from growth on sucrose nitrate agar and prepared as previously described (4) were stored at 4°C as an aqueous suspension. The germination properties of stored suspensions were stable for as long as 1 year. Before each germination experiment, the spores were suspended in 0.5 M of the tetrasodium salt of EDTA and incubated at 4°C for 5 min to remove divalent cations. The spores were then sedimented by centrifuging and washed three times with distilled water.

Germination conditions. The various germination media were buffered at pH 7.4 with TX-buffer, which consisted of 0.05 M Tris buffer (Tris-hydrochloride) and 0.001% (wt/vol) Triton X-100. Triton X-100 was added to prevent clumping of the spores.

When present in the various germination media, the concentrations of $CaCl_2$, KCl, and glucose were 0.54, 7.0, and 13.9 mM, respectively. A previously described defined germination medium (DGM) was used in some experiments (4).

Heat-activated spores (5) were used in all germination experiments. An aqueous spore suspension was diluted 1:20 into TX-buffer prewarmed to 55°C (pH 7.3) in a water bath. After 10 min at 55°C, the spore

† Present address: U. S. Department of Agriculture, Forest Service, Forest Products Laboratory, Madison, WI 53705.

suspension was rapidly cooled, and the spores were sedimented by centrifugation at 10,000 × *g* for 5 min at 4°C. The spores were resuspended in the germination media to be tested.

Germination assay. Germination was assayed as the decrease in absorbance at 600 nm of spore suspensions incubated at 37°C in a Beckman model 25 spectrophotometer (Beckman Instruments, Inc.). The density of the spores was adjusted to initial absorbance values of 0.5 to 0.6 (0.9 to 1.1 mg/ml, dry weight). The data are corrected for a small amount of absorbance decrease caused by settling of the spores and are expressed as the percentage of the initial absorbance. The observed absorbance decreases were due to a loss of spore refractility (6). Settling or clumping of spores was not a significant factor since the same results were obtained when the spore suspensions were shaken vigorously for 2 to 3 s or subjected to a 2-s sonic shock with a microprobe at 5-min intervals during a 60-min incubation period.

Heat resistance. Heat-activated spores were incubated in various media at 37°C. Samples were removed at various times and diluted 1:100 into TX-buffer at 60°C. After a 10-min period, the spore suspensions were cooled rapidly by diluting 1:10 in water at 0°C. After a 1-min sonication treatment in a Heat Systems water bath sonicator, which dispersed clumps of spores, samples were serially diluted in sterile water and spread on a medium containing 1.0% glycerol, 0.1% yeast extract, 0.1% asparagine, 0.1% K_2HPO_4, and 2.0% agar (Difco). Colonies were counted after 2 days of incubation at 30°C.

Measurement of metal ions. The calcium, magnesium, and potassium concentrations of spores were determined using a Perkin Elmer model 303 atomic absorption spectrophotometer (Perkin Elmer Corp.). Harleco (Hartman Leddon Co.) standards were used for calibration. All glass and plastic containers and pipettes were prewashed with 1% HCl. The spores were washed four times by centrifuging at 10,000 × *g* for 10 min at 4°C and resuspending in double-distilled water. For analyses they were suspended in a solution of 1% lanthanum oxide dissolved in 5% HCl.

Radiorespirometry. Endogenous metabolism of spores was quantitated by radiorespirometry. Spores uniformly labeled with ^{14}C were obtained after growth of the organism on sucrose nitrate agar containing 2 μCi of [*U*-^{14}C]sucrose per ml (6). After harvesting, washing, and heat activation, the spores were incubated in various media at 37°C. A stream of sterile humidified air was bubbled through the spore suspension. The $^{14}CO_2$ evolved was collected by bubbling of the effluent air through Carbo-Sorb (Packard Instrument Co.) or a 1:1 mixture of phenethylamine-methanol. The solution absorbed virtually all of the $^{14}CO_2$ in the air. The ^{14}C content of the samples was quantitated with liquid scintillation spectrometry by using Aquasol scintillation fluid (Packard Instrument Co.).

Glucose oxidation was determined by incubating spores at 37°C in various media containing 20 μCi of [*U*-^{14}C]glucose per ml. The $^{14}CO_2$ was determined as described above.

Release of ^{14}C-labeled spore carbon. Spores were labeled with ^{14}C and harvested as described above. After heat activation, the spores were incubated at 37°C in various media through which streams of sterile air were bubbled. Samples were removed at various times and filtered through a 0.20-μm pore size membrane filter (Metricel Ga-8, Gelman Instrument Co.). The ^{14}C content of the filtrate was quantitated as above. The ^{14}C content of the filtrate did not change when acidified with H_2SO_4. Thus, none of the label in the samples came from $^{14}CO_2$.

Chemicals. [*U*-^{14}C]sucrose was purchased from Schwarz-Mann. Lanthanum oxide, Tris buffer, and arsenazo III {2,2'-[1,8-dihydroxy-3,6-bisulfo-2,7-naphthalene-bis(azo)]dibenzenearsonic acid} were purchased from Sigma Chemical Co. Triton X-100 was purchased from Rugor Chemical Co.

RESULTS

Requirement of calcium for germination. Previous work established that initiation of germination of *S. viridochromogenes* spores begins after a 10- to 20-min lag period when they are incubated in a medium containing alanine, glutamic acid, adenosine, *p*-aminobenzoic acid, $CaCl_2$, $MgCl_2$, and KCl. The pregermination lag is greatly reduced after activation by a heat shock of 55°C for 10 min (5). Initiation of germination of the heat-activated spores was found to occur in the presence of Ca ions only (Fig. 1). After a 5-min lag period, the absorbance of the

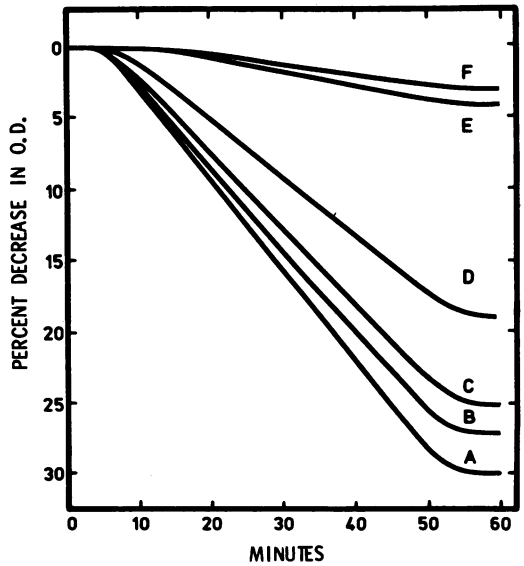

FIG. 1. *Effect of calcium and potassium ions and glucose on germination of S. viridochromogenes spores. Heat-activated spores were incubated in media containing: (A) calcium, potassium, and glucose: (B) calcium and potassium; (C) calcium and glucose; (D) calcium; (E) potassium; and (F) glucose. Germination was estimated as the percent decrease in optical density of the spore suspension. The curves shown represent the average recorder tracings from at least three repetitive experiments.*

spore suspension declined linearly to a maximum of 18% reached at 55 min (curve D). Both the rate and amount of absorbance decrease were increased by addition of glucose, KCl, or both (curves A, B, and C). However, the spores did not germinate when incubated with glucose or KCl in the absence of $CaCl_2$ (curves E and F). The amount of absorbance decrease observed with calcium-glucose, calcium-potassium, and calcium-potassium-glucose media was approximately the same as occurs during germination in the previously used defined germination medium (4). Both Sr and Ba ions (0.54 mM) were as effective as Ca ions in initiating germination (data not shown). Germination did not occur in the presence of 0.5 to 1.0 mM Zn, Fe, Mg, La, Mn, Co, and various monovalent cations. Spores which were not heat activated did not initiate germination in any combination of calcium, potassium, or glucose.

The optimal Ca ion concentration for initiating germination in DGM was 0.5 mM (Fig. 2). Higher concentrations inhibited germination. Microscopic examinations revealed that the spores incubated for 50 min with 0.5 mM Ca were phase dark, whereas those incubated with 1.7 and 2.5 mM Ca were, respectively, partially and completely phase bright. An increased rate of respiration during spore germination was reported previously (6), and Ca ions stimulate the rate of endogenous metabolism (see Fig. 4). The possibility exists that the observed inhibition of germination by greater than optimal Ca ion concentrations was caused by oxygen depletion during the static incubation. To test this, a fine stream of air was bubbled through the spore suspension for 30 s of each minute. The maximum amount of absorbance decrease of the germinating spores was the same at Ca ion concentrations greater than 0.5 mM (Fig. 2). Microscope examinations revealed the spores in each instance to be phase dark. Germination did not occur when the spore suspensions were purged continuously with a fine stream of oxygen-free nitrogen gas for the 50-min period (data not shown).

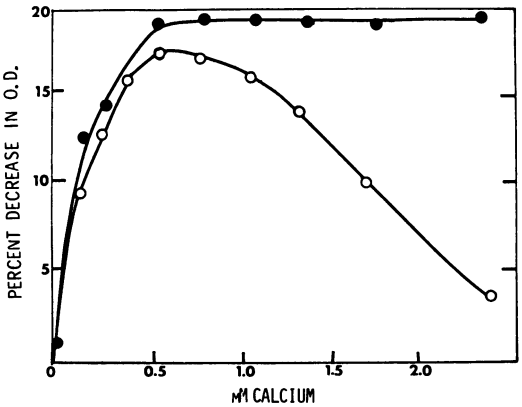

FIG. 2. *Effect of calcium ion concentration on germination of S. viridochromogenes spores. Heat-activated spores were incubated in DGM containing the amounts of calcium shown. The experimental points illustrate the percent decrease in optical density of the spore suspension after 50 min of incubation. Symbols: ○, incubated statically; ●, incubated with aeration 30 s of each min.*

Measurement of calcium levels in spores. The calcium content of the spores was measured, and the results are shown in Table 1. Fresh spores suspended in TX buffer contain 0.28% of their dry weight as calcium. This value did not change after incubation in water for 7 days or after heat activation. Incubation of spores with 0.5 mM Ca for 5 min or longer at 4°C resulted in a near doubling of their calcium content. Spores which had been germinated by incubation in calcium-potassium-glucose medium for 60 min also contain the same increased level of calcium.

Treatment of spores in 0.01 N HCl for 5 min at 4°C removed virtually all of the calcium. The acid caused a 26% decrease in viability of the spores and removed 25% of their spore carbon (data not shown). Treatment of the spores with 0.5 M EDTA at pH 10.0 or 0.02% arsenazo III for 5 min also resulted in removal of virtually all of the calcium from the spores. The spores maintained complete viability and lost only 0.7% of their spore carbon after the EDTA extraction (data not shown). Because the EDTA caused no apparent damage to the spore membrane and arsenazo III does not pass permeability barriers

TABLE 1. *Calcium ion content of S. viridochromogenes spores*[a]

Treatment	% dry wt as Ca ions
None (control)	0.28 (0.26–0.30)[b]
Incubated 7 days in water at 4°C, then washed	0.28 (0.26–0.30)
Incubated with:	
0.5 mM $CaCl_2$ at 4°C	0.55 (0.50–0.60)
0.01 N HCl washed	0.02 (0.00–0.04)
0.5 M EDTA washed	0.02 (0.01–0.03)
0.02% Arsenazo III washed	0.02 (0.01–0.04)
Germinated for 60 min in Ca-K-glucose medium	0.55 (0.50–0.60)
0.5 M EDTA or 0.01 N HCl washed then incubated with 0.5 mM Ca at 4°C	0.55 (0.50–0.60)

[a] The amount of calcium in the samples of spores was determined by atomic absorption spectrophotometry as described in the text. The spores were washed four times with double-distilled water before analyses.

[b] Ranges for replicate determinations.

or affect cell properties (7, 11), it seems reasonable to conclude that virtually all of the calcium content of the spores is located exterior to their permeability barrier.

When spores which had been depleted of calcium by HCl or EDTA extraction were incubated for 5 min in 0.5 mM Ca ions at 4°C, they accumulated the same amount of the cation as untreated spores (0.55 to 0.56% dry weight).

The spore contents of Mg and K ions were measured and found to be 0.17 and 2.0% of the spore dry weight, respectively (data not shown). These values did not change appreciably after incubation in water, heat activation, or germination in calcium-potassium-glucose medium. Treatment of spores with EDTA removed only approximately 50% of the Mg ions.

Requirement for continuous presence of calcium during germination. An experiment was designed to determine whether calcium acts as a trigger in initiation of spore germination. Heat-activated spores were preincubated for 10 min in the presence of glucose or calcium and glucose. The spores were then washed with 0.5 M EDTA followed by water and then suspended in fresh media and incubated for an additional 50 min. The results (Fig. 3) show that spores preincubated for 10 min in calcium-glucose medium had begun to germinate after a lag period of a few minutes and continued to germinate when transferred to medium containing only Ca ions. When the calcium-glucose-preincubated spores were transferred to medium containing only glucose, germination ceased. Addition of Ca ions to these spores 30 min later resulted in immediate resumption of germination. Spores preincubated in glucose only did not initiate germination. This was true if incubation was prolonged another 50 min. The glucose-preincubated spores germinated without a lag when transferred to a medium containing only Ca ions. These data clearly establish that Ca ions were required continuously during germination and did not serve only to trigger germination events.

Endogenous respiration and spore carbon release studies. Previous studies established that two early events associated with germination of *S. viridochromogenes* spores are excretion of spore carbon and an increased endogenous respiration rate (6). A study was made of the effect of calcium ions on these processes. The heat-activated spores incubated in buffer respired endogenously at a constant rate such that at 120 min approximately 0.4% of their spore carbon was converted to $^{14}CO_2$ (Fig. 4). The addition of Ca ions resulted in a rapid increase in the respiration rate for the first 60 to 75 min followed by a less rapid rate. At 120 min, 1.05% of the spore carbon had been converted to

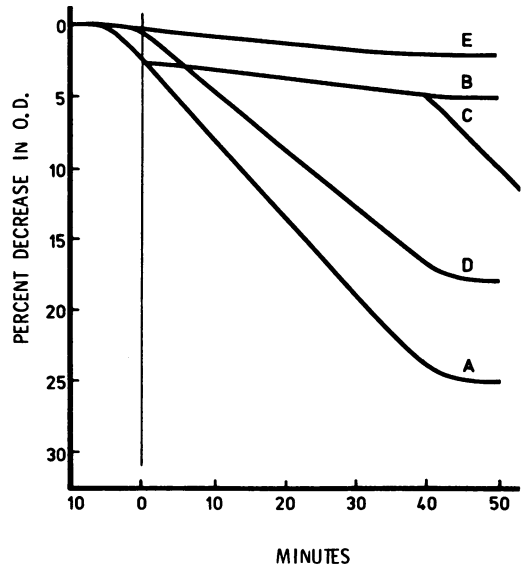

FIG. 3. *Continuous requirement for calcium ions for germination of S. viridochromogenes spores. Heat-activated spores were incubated for 10 min in media containing glucose only or calcium ion plus glucose. The spores were then sedimented by centrifugation, washed with 0.5 M EDTA followed by water, and then incubated in a fresh medium. (A) Preincubated in calcium-glucose, shifted to calcium only; (B) preincubated in calcium-glucose, and then shifted to glucose only; (C) as in B except calcium was added at 40 min; (D) preincubated with glucose, shifted to calcium only; (E) preincubated with glucose only, shifted to glucose only. The curves shown represent the average recorder tracings from at least three repetitive experiments.*

$^{14}CO_2$. The respiration rate in medium containing glucose and no Ca ions was only slightly greater than that in buffer. In the presence of glucose and Ca ions, the respiration rate increased to a rapid constant rate, and nearly 1.7% of the spore carbon had been converted to $^{14}CO_2$ at 120 min.

The results for release of spore carbon are shown in Fig. 5. Approximately 3% of the spore carbon was excreted by spores incubated for 120 min in the buffer containing glucose. Addition of Ca ions to this medium resulted in a release of 5% of the carbon after 60 min of incubation, after which no more carbon was released during another hour of incubation. The amount of carbon released in the presence of both Ca ions and glucose was much greater, a maximum amount of 15% being released after 60 min of incubation.

Heat resistance studies. The relationship of germination to heat resistance was tested. Heat-activated spores were incubated in the following media: TX-buffer, TX-buffer plus potassium, TX-buffer plus calcium, TX-buffer plus

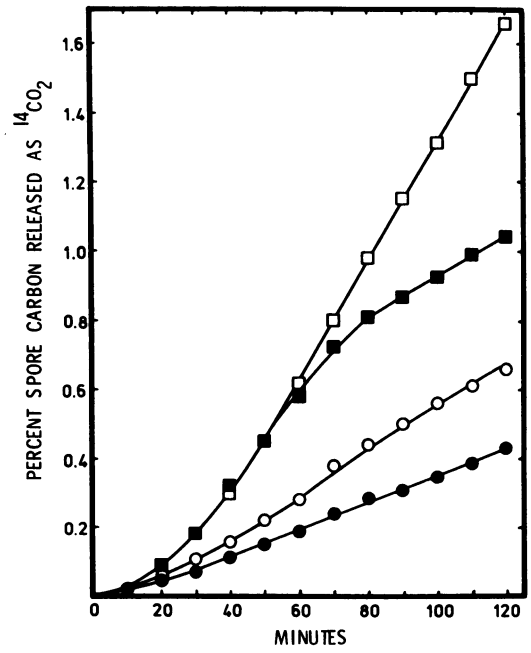

FIG. 4. *Rate of endogenous metabolism of spores of S. viridochromogenes. Spores labeled with ^{14}carbon were heat activated and incubated in various media. The media were aerated and the $^{14}CO_2$ was collected and measured. Symbols: □, calcium-glucose medium; ■, TX-buffer plus calcium ions; ○, TX-buffer plus glucose; ●, TX-buffer.*

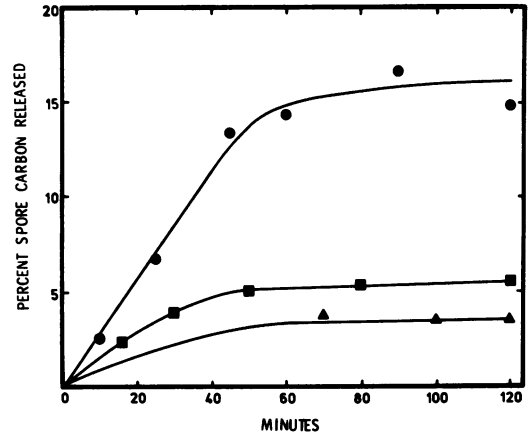

FIG. 5. *Rate of release of spore carbon from spores of S. viridochromogenes. Spores labeled with ^{14}carbon were heat activated and incubated in various media. Samples were removed at different times, the spores were removed by filtration, and the amount of label in the supernatant fluid was measured. Symbols: ●, calcium-glucose medium; ■, TX-buffer plus calcium ions; ▲, TX-buffer plus glucose.*

glucose, and TX-buffer plus calcium and glucose. At various times, samples were removed and heated at 60°C for 10 min and then cooled rapidly, and viable cell counts were made. As shown in Table 2, the nongerminating spores (buffer, potassium, or glucose only; see Fig. 1) remained almost completely heat resistant. The germinating spores became progressively less heat resistant. The rate of loss of heat resistance parallels the rate of germination (see Fig. 1). This decrease in heat resistance of the germinating spores may be a result of either a mixture of heat-susceptible germinated and resistant dormant spores or of a gradual loss of resistance by a synchronously germinating population. To test this, a sample of spores at the mid-germination point (22 min of germination in calcium-glucose medium) was heated at 60°C, and viability was tested at various times. The loss of viability was exponential with time (Fig. 6), indicating that the spore population was germinating synchronously and that the spores were equally susceptible to heat.

DISCUSSION

Hardisson et al. (3) defined three stages of germination of *S. antibioticus* spores. The first stage, which involves phase darkening accompanied by a 20% decrease in absorbance of a spore suspension, is apparently equivalent to what we refer to in this paper as initiation of germination. This first stage of germination of *S. antibioticus* spores requires only the presence of either calcium, magnesium, or ferrous ions and is completely blocked by inhibitors of ATP synthesis. *S. antibioticus* spores do not require heat activation for germination, and the dry weight of the spores does not change during the first stage of germination (3). Spores of *S. viridochromogenes* will not initiate germination with only magnesium or ferrous ions present, and calcium-initiated germination occurs only with heat activated spores. Also, initiation of germination is accompanied by a loss of spore carbon (6; this paper).

TABLE 2. *Relationship of heat resistance to germination of S. viridochromogenes spores*[a]

Additions to TX-buffer	% viability	
	30 min	60 min
None (control)	100	100
Potassium ions	97	95
Calcium ions	62	47
Calcium plus potassium ions	62	38
Calcium ions plus glucose	53	19
Glucose	93	88

[a] Heat-activated spores were incubated in the test media at 37°C. Samples removed at 30 and 60 min were subjected to a heat shock of 60°C for 10 min and viable counts determined as described in the text.

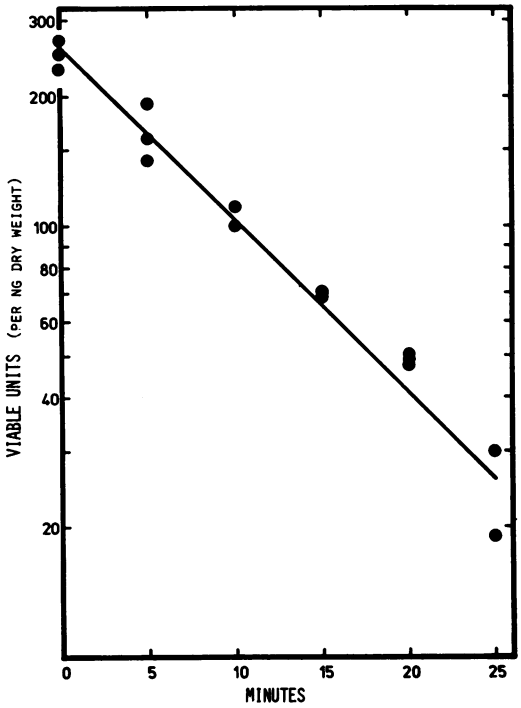

FIG. 6. *Thermal resistance of partially germinated spores of S. viridochromogenes. Heat-activated spores were germinated for 22 min by incubation in calcium-glucose medium at 37°C. They were then diluted into TX-buffer at 60°C and incubated at this temperature. At the times indicated, samples were serially diluted and plated onto solid media, and colonies were counted after 48 h of incubation. Data are plotted by using the method of least squares (r^2 = 0.96).*

Our work does not yet answer the question of the mechanism of calcium-induced initiation of germination of *S. viridochromogenes* spores. Events known to be associated with germination, such as increased respiratory activity, release of carbon-containing materials, loss of heat resistance, and macromolecule biosynthesis, are dependent on the continuous presence of calcium ions. The observation that the chelating agents EDTA and arsenazo III remove virtually all of the calcium from spores and at the same time arrest germination events suggests that the site of calcium activity is external to the cytoplasmic membrane.

ACKNOWLEDGMENTS

This investigation was supported by the College of Agricultural and Life Sciences, University of Wisconsin, and by Public Health Service grant 13948 from the National Institute of Allergy and Infectious Disease.

We thank Carl Bowser, Department of Geology, University of Wisconsin, for assistance and use of the atomic absorption spectrophotometer.

LITERATURE CITED

1. **Dills, S. S., and J. C. Vary.** 1978. An evaluation of respiration chain-associated functions during initiation of germination of *Bacillus megaterium* spores. Biochim. Biophys. Acta **541**:301–311.
2. **Grund, A. D., and J. C. Ensign.** 1978. Role of carbon dioxide in germination of spores of *Streptomyces viridochromogenes*. Arch. Microbiol. **118**:279–288.
3. **Hardisson, C., M. B. Manzanal, J. A. Salas, and J. E. Suarez.** 1978. Fine structure, physiology and biochemistry of arthrospore germination in *Streptomyces antibioticus*. J. Gen. Microbiol. **105**:203–214.
4. **Hirsch, C. F., and J. C. Ensign.** 1976. Nutritionally defined conditions for germination of *Streptomyces viridochromogenes* spores. J. Bacteriol. **126**:13–23.
5. **Hirsch, C. F., and J. C. Ensign.** 1976. Heat activation of *Streptomyces viridochromogenes* spores. J. Bacteriol. **126**:24–30.
6. **Hirsch, C. F., and J. C. Ensign.** 1978. Some properties of *Streptomyces viridochromogenes* spores. J. Bacteriol. **134**:1056–1063.
7. **Scarpa, A., F. J. Brinley, Jr., and G. Dubyak.** 1978. Antipyrylazo III, a "middle range" Ca^{2+} metallochromic indicator. Biochemistry **17**:1378–1386.
8. **Scott, I. R., and D. J. Ellar.** 1978. Metabolism and the triggering of germination of *Bacillus* megaterium. Use of L-(^{3}H)alanine and tritiated water to detect metabolism. Biochem. J. **174**:635–740.
9. **Smith, J. E., and D. R. Berry.** 1974. The fungal spore, p. 80–105. *In* An introduction to biochemistry of fungal development. Academic Press, Inc., New York.
10. **Sussman, A. S.** 1969. The dormancy and germination of fungus spores. Symposia Soc. Exp. Biol. **23**:99–121.
11. **Weissman, G., T. Collins, A. Evers, and P. Dunham.** 1976. Membrane perturbation: studies employing a calcium-sensitive dye, arsenazo III, in liposomes. Proc. Natl. Acad. Sci. U. S. A. **73**:510–514.