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# Some Properties of *Streptomyces viridochromogenes* Spores

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Dry spores of *Streptomyces viridochromogenes* exhibited no endogenous metabolism when tested by the sensitive radiorespirometric technique. Wetting the spores resulted in a sharp increase in endogenous respiration followed by a gradual decrease to a constant level, whereby 0.02% of the spore carbon was respired to $CO_2$ per h. The rate of endogenous metabolism increased slowly as unactivated and heat-activated spores germinated in a defined germination medium, reaching rapid rates only after germination was completed. Components of the defined germination medium, adenosine, L-alanine, and L-glutamic acid, were oxidized to $CO_2$ at appreciable rates only after germination was complete. The $QO_2$ (microliters of $O_2$ uptake per hour per milligram [dry weight] of spores) values for endogenously respiring spores were 3.9 for unactivated and 7.8 for activated spores. Various sugars, amino acids, and organic acids were oxidized only slowly or not at all by unactivated and activated spores. The dry spores contained $5.2 \times 10^{-2}$ μmol of ATP per g (dry weight). The ATP content of spores increased approximately 4-fold after suspension in buffer and approximately 11-fold after heat activation. During germination, the ATP level increased to a level of 1 μmol of ATP per g (dry weight) and remained constant. Germination was accompanied by excretion from the spores of approximately 8 and 12% of the total spore carbon from unactivated and activated spores, respectively. A potent germination inhibitor was released from the germinated spores. The germination inhibitor had no effect on heat-activated spores or spores which had begun germination for as short a time as 5 min.

Bacteria belonging to the genus *Streptomyces* are unique in their mode of growth and in their capacity to produce a vast assay of antibiotic substances. Growth on a solid substrate occurs as a highly branched mycelial mat from which arise specialized aerial hyphae. Multiple septation of the aerial hyphae forms conidial spores, which are the reproductive units of the organism. The spores germinate in a suitable environment and form germ tubes which grow into the characteristic multiply branched mycelia. Much of what is known of the life cycle of these bacteria is described in the excellent review by Kalakoutskii and Agre (12).

Several years ago, we began a comprehensive study of the developmental biology of *Streptomyces*. A general overview of part of the work was published (9). We developed procedures for procuring dry spores of *Streptomyces viridochromogenes* and for germinating the spores in a nutritionally defined minimal germination medium (10). Germination is accompanied by loss of spore refractility and a decrease in the optical density of a spore suspension. In the defined medium, germination begins after an approximately 20-min lag period and is complete 20 to 25 min later. A heat shock of 10 min at 55°C eliminates the lag period but has no effect on the subsequent rate of germination (11). Heat activation is reversible; heat-shocked spores incubated for 8 h at 25°C require another shock for germination without lag. Aged spores will not germinate in the defined medium unless activated.

In this communcation, we report the results of a study of some properties of *S. viridochromogenes* spores and of some of the events which accompany their germination.

## MATERIALS AND METHODS

**Culture conditions and germination of spores.** The organism used, growth conditions, procedures for obtaining spores, heat activation of spores, and germination assays were previously described (10, 11).

**Radiorespirometry studies.** Rates of endogenous metabolism of spores were determined using radiorespirometry. Spores uniformly labeled with $^{14}C$ were obtained by growing the organism for 10 days at 30°C on a sucrose-nitrate-salts agar medium (10) containing 10 μCi of [*U*-$^{14}C$]sucrose per ml. Spores were harvested in the dry state using sterile glass beads (10). The

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weight of spores was determined by weighing the beads before and after harvesting the spores. The endogenous metabolism of dry spores was measured by placing 200 of the spore-coated glass beads (approximately 3 mg [dry weight] of spores) into a sealed sterile glass chamber 4.5 cm wide by 1.0 cm deep. The chamber was incubated at 35°C while being flushed continuously with filter-sterilized air, which entered through an inlet tube at one end of the chamber and then passed through an outlet tube at the opposite end. In some experiments the air used to flush the chamber was dried by passage through two columns (2.5 by 30 cm) of anhydrous $CaSO_4$ (Drierite). In other experiments, the air was saturated with water by sequential passage through spargers located at the bottoms of two bottles, each containing 500 ml of sterile water. Any $^{14}CO_2$ evolved by the labeled spores was collected by passing the effluent air from the chamber through a sparger located at the bottom of a tube containing 3.0 ml of a 1:1 mixture of phenethylamine-methanol. The solution absorbed all of the $^{14}CO_2$ in the air. At various times during the course of incubation, the phenethylamine-methanol mixture was replaced with fresh solution. The $^{14}C$ content of samples was quantitated by liquid scintillation spectrometry using Aquasol scintillation fluid (Packard Instrument Co., Chicago, Ill.).

The effect of wetting on release of $^{14}CO_2$ from dry spores was tested by incubating 200 spore-coated glass beads for 8.0 h in a second chamber which was continually flushed with desiccated air. After 8.0 h, 50 ml of sterile 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.3) plus 10 mg of Triton X-100 per liter (TX buffer) was added aseptically to the chamber. The spores were suspended in the buffer and stirred by means of a 5-mm-long magnetic bar in the chamber. The air used to flush the chamber was changed from dry to humid, the $^{14}CO_2$ in the effluent air was trapped, and the radioactivity was counted.

The total initial $^{14}C$ content of the harvested spores was determined by suspending the spores from 25 beads in cold TX buffer and washing them several times with TX buffer. The radioactivity present in samples of the washed spores was then determined by liquid scintillation spectrometry.

To measure the endogenous metabolism of germinating spores, the spores were removed from the glass beads with cold TX buffer and then washed by centrifuging three times with 200 ml of TX buffer. The spores were finally suspended in TX salts (TX buffer containing 100 mg of $MgSO_4 \cdot 7H_2O$ and 20 mg of $CaCl_2 \cdot 2H_2O$ per liter). A 40-ml sample (0.25 mg [dry weight] of spores per ml) was placed in a sterile 125-ml flask and incubated at 35°C while shaking at 120 rpm. Filter-sterilized, humidified air was passed through the suspension, and any $^{14}CO_2$ evolved by the labeled spores was collected from the effluent air and measured as described. Germination was effected by adding (final concentrations, wt/vol) 0.1% yeast extract plus 1.0% glucose or a defined germination medium (DGM) containing 0.1% L-alanine, 0.1% L-glutamic acid, 0.01% adenosine, and 0.01% *p*-aminobenzoic acid.

A similar experimental design was used to measure the oxidation by spores of the components of DGM. Unlabeled spores were incubated in DGM containing $^{14}C$-labeled L-alanine, L-glutamic acid, adenosine, or *p*-aminobenzoic acid, each used at a concentration of 0.5 μCi/ml. Oxidation of the DGM components was then followed by monitoring the $^{14}CO_2$ produced.

**Oxygen uptake studies.** The consumption of oxygen by spores was measured using a Yellow Springs Instruments Co. model 53 polarographic oxygen analyzer coupled to a Sargent recorder. The reaction chambers contained 0.5 ml of unactivated or activated spore suspension, 0.1 ml of a 1.0% solution of test substrate (except L-tyrosine, for which 0.5 ml of a 0.01% solution was tested), and TX buffer to a total volume of 3.0 ml. The reactions were maintained at 35°C and mixed continuously with a magnetic bar. The rate of endogenous oxygen consumption was determined without added substrates.

**Release of spore carbon.** The release of carbon-containing substances from washed $^{14}C$-labeled spores was determined. Tubes containing 10 ml of labeled spores suspended in TX salts were incubated at 35°C while being aerated by bubbling with humidified air at a rate of 400 ml/min. Samples of 0.5 ml were removed at various times and filtered through 0.45-μm-pore size membrane filters (type HA, Millipore Corp., New Bedford, Mass.). The radioactivity of the filtrates was quantitated by liquid scintillation spectrometry using Aquasol scintillation fluid. A test showed that the radioactive content of the filtrate samples did not change when acidified with HCl. Thus, none of the label in the samples came from $^{14}CO_2$.

**ATP measurements.** ATP was extracted from spores with 90% (vol/vol) dimethyl sulfoxide ($Me_2SO$). A 0.1-ml sample of spore suspension was added to 2.5 ml of $Me_2SO$, and the spores were then extracted for 4 min at ambient temperature. The mixture was then diluted by adding 5 volumes of 0.01 M morpholinopropane sulfonic acid buffer, pH 7.4. The spores were sedimented by centrifugation (10,000 × *g* for 10 min), and a sample of the supernatant fluid was assayed for ATP content. This procedure extracted almost 98% of the ATP in the spores, as determined by first extracting a sample of spores and then repeating the extraction until no further ATP was obtained. A second procedure involving extraction of the spores with 5.0 N formic acid yielded results identical to those obtained with the $Me_2SO$ extraction procedure.

The ATP concentration of the $Me_2SO$ extracts was determined using the luciferin-luciferase assay with a DuPont 760 luminescence biometer. The instrument was calibrated with an ATP solution of known concentration. For each experiment, the ATP calibration standard was prepared in the same medium in which the spores were suspended and then processed by using the same procedure used to extract ATP from the spores.

The ATP content of dry spores was determined by extracting spores still coating the glass beads used in the harvesting procedure. The ATP content of wetted spores was determined by washing dry spores from spore-coated glass beads with TX buffer at ambient temperature and then immediately extracting a sample of the spore suspension.

**Chemicals.** [*U*-$^{14}C$]sucrose (434 mCi/mmol), L-[*U*-$^{14}C$]alanine (173 mCi/mmol), L-[*U*-$^{14}C$]glutamic acid

(260 mCi/mmol), and [*U*-$^{14}$C]adenosine (570 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. *p*-[*U*-$^{14}$C]aminobenzoic acid (1.41 mCi/mmol) was purchased from Mallinckrodt Chemical Works. The luciferin-luciferase preparations used for ATP analyses were purchased from E. I. duPont de Nemours & Co., Inc., Wilmington, Del. ATP came from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were reagent grade of the highest purity available.

## RESULTS

**Endogenous respiration of dry and wet spores.** Spores of *Streptomyces* are produced on aerial hyphae and are surrounded by a hydrophobic outer sheath (23). It is likely that the sphores exist in nature in a nearly dry condition and as such may be completely dormant. An experiment was designed to test this idea by measuring the rate of endogenous respiration of dry spores using the sensitive technique of radiorespirometry. Dry air was passed over $^{14}$C-labeled spores which coated glass beads. The beads had been rolled over the surface of confluent growth on agar medium so that the spores had not been wetted. The $^{14}CO_2$ content of the effluent air was monitored as an index of endogenous respiration. A small amount of label was detected during the first hour (Fig. 1). Thereafter, no $^{14}CO_2$ was released from the spores. The spores in this dry state are absolutely dormant with respect to endogenous respiration. After 15 h of incubation, the dried air passing over the spores was changed to water-saturated air. An immediate increase in the rate of a small but detectable level of formation of $^{14}CO_2$ followed (Fig. 1, inset). The rate of endogenous metabolism increased over a 5-h interval to a level representing $1.5 \times 10^{-3}$% of the spore carbon being converted to $^{14}CO_2$ per h. This rate was maintained for an additional 10 h.

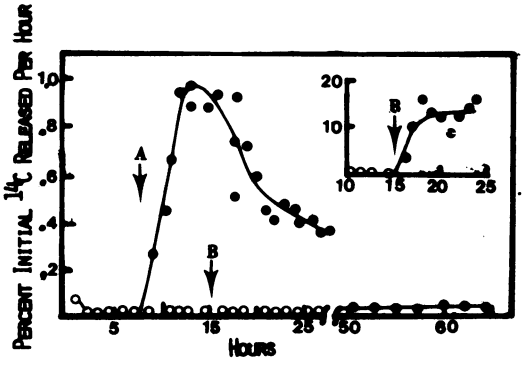


FIG. 1. *Release of $^{14}CO_2$ by dry and wet spores. Spores uniformly labeled with $^{14}$C during growth on [U-$^{14}$C]sucrose were harvested dry from agar medium. The release of $^{14}CO_2$ from the spores incubated in a dry or humid air atmosphere, or wetted with TX buffer, was monitored. The results are presented as the percentage of the initial spore $^{14}$C released as $^{14}CO_2$ per 1-h intervals. The inset shows the results obtained when dry spores were incubated in a humid atmosphere. The ordinate of the inset represents the percentage ($\times 10^{-4}$) of the initial spore carbon released as $^{14}CO_2$, and the abscissa represents time in hours. Symbols: (○) dry spores; (●) wet spores. Inset: (○) spores incubated in desiccated air; (●) spores incubated in water-saturated air. Arrow A indicates the time when TX buffer was added. Arrow B indicates times when desiccated air was switched to water-saturated air.*

To test the rate of endogenous metabolism of wet spores, TX buffer was injected into the chamber containing the spore-coated beads. This resulted in a rapid increase in the rate of endogenous metabolism over a 4-h period to a maximum of nearly 1.0% of the spore carbon being converted to $^{14}CO_2$ per h. The rate then decreased rapidly until at 50 h a constant level of endogenous respiration equal to approximately 0.02% of the spore carbon converted to $^{14}CO_2$ per h was maintained.

A microscopic examination of the spores at 27 and 65 h revealed no evidence that the spores had germinated, i.e., no loss of refractility, swelling of the spores, or germ tube production.

**Endogenous respiration of germinating spores.** Spores of *S. viridochromogenes* germinate during a 30- to 40-min period of incubation in various complex media or a defined germination medium (10). Germination events begin after a 10- to 20-min lag period. A gentle heat shock (55°C for 10 min) eliminates the lag period but does not affect the subsequent rate of germination (10). The results of measuring, by radiorespirometry, the rates of endogenous metabolism of unactivated and activated spores during germination in DGM are shown in Fig. 2.

Unactivated and activated spores possessed barely detectable levels of endogenous respiration during the first 20 min of incubation. During this time, the unactivated spores were not germinating but the activated spores were germinating (11). The rate of endogenous respiration then increased as the activated and unactivated spores completed germination and entered the outgrowth stage. At 150 min, the activated spores had respired 4.3% and the unactivated spores had respired 2.5% of their respective contents of $^{14}$C to $^{14}CO_2$. Unactivated spores incubated in TX salts did not germinate but showed a slow, constant rate of endogenous respiration. Activated spores incubated in TX salts, after an initial 20-min lag period, respired at a constant rate six times greater than did the similarly incubated unactivated spores. At 150 min, the activated spores had respired 1.7% and the unactivated spores had respired 0.3% of their re-

spective contents of $^{14}C$ to $^{14}CO_2$.

**Oxidation of substrates by germinating spores.** The ability of activated spores to oxidize the components of DGM was studied by measuring the $^{14}CO_2$ produced from $^{14}C$-labeled DGM substrates (Fig. 3). During the first 30 min, when the spores were actively germinating (11), the oxidation of each of the DGM components was barely detectable. Between 30 and 50 min, when germination was nearly complete, the rate of adenosine oxidation began to slowly increase and continued to increase after 75 min, when outgrowth was occurring. The pattern of L-alanine oxidation was similar, but the rate was less than half that of adenosine oxidation. L-Glutamic acid was oxidized at a very slow but constant rate for the first 100 min, after which time the rate of oxidation began to increase slowly. No oxidation of *p*-aminobenzoic acid was detected (data not shown).

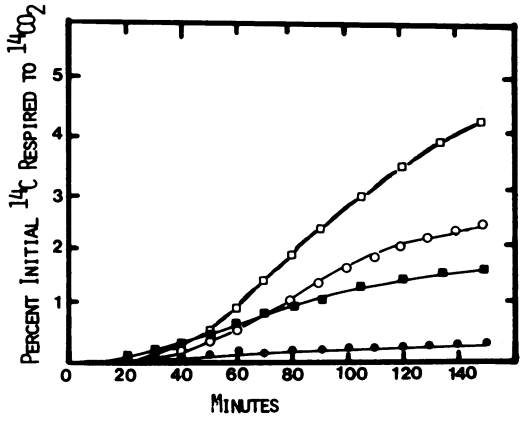


FIG. 2. *Endogenous respiration of spores. Unactivated or heat-activated spores uniformly labeled with $^{14}C$ were incubated in DGM or TX salts, and the $^{14}CO_2$ evolved from the spores was measured. The results are presented as the percentage of the initial spore $^{14}C$ respired to $^{14}CO_2$. Symbols: (□) activated spores in DGM; (○) unactivated spores in DGM; (■) activated spores in TX salts; (●) unactivated spores in TX salts.*

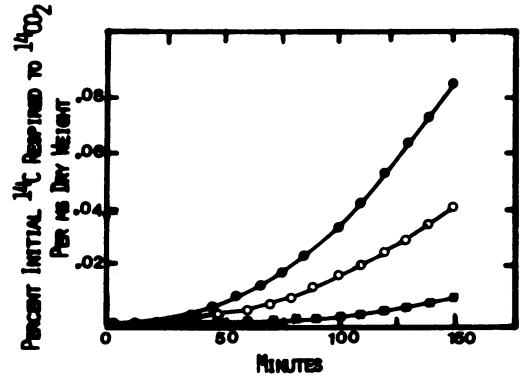


FIG. 3. *Oxidation of DGM components by activated spores germinating in DGM. Activated spores were incubated in DGM containing one of the $^{14}C$-labeled DGM components, and the $^{14}CO_2$ evolved was measured. The results are presented as the percentage of the initial substrate $^{14}C$ respired to $^{14}CO_2$ per milligram (dry weight) of spores. Symbols: (●) adenosine; (○) L-alanine; (■) L-glutamic acid.*

After 150 min of incubation, the total amounts of $^{14}C$-labeled adenosine, alanine, and glutamic acid which had been oxidized to $^{14}CO_2$ per milligram (dry weight) of spores were 0.085, 0.041, and 0.010%, respectively.

A similar experiment using unactivated spores showed that the general pattern of substrate oxidation was the same as observed for activated spores. However, the lag period preceding germination of unactivated spores delayed by 20 min the time at which the onset of oxidation of the DGM components began.

**Oxidation of various compounds.** The ability of unactivated and activated spores to oxidize a variety of compounds was tested using a dissolved-oxygen monitor. The rates of endogenous $O_2$ consumption by spores germinating in yeast extract and DGM were also determined. The spores incubated in the presence of the other test substrates showed no signs of germination (phase darkening or germ tubes) during the 1-h incubation periods. The results were corrected for endogenous metabolism by subtraction. The $QO_2$ value (Table 1) for endogenous metabolism of unactivated spores was half that of activated spores. Of the substrates tested

TABLE 1. *Oxygen consumption by unactivated spores*

| Substrate[a] | μl of $O_2$ uptake/h per mg (dry wt)[b] | |
|---|---|---|
| | Unactivated spores | Activated spores |
| None (endogenous) | 3.9 | 7.8 |
| Yeast extract[c] | 11.0 | 10.4 |
| DGM[c] | 5.3 | 9.6 |
| Glucose | 3.4 | 11.8 |
| Glycerol | 3.4 | 4.8 |
| Trehalose | 2.8 | 0 |
| L-Alanine | 1.6 | 3.3 |
| L-Tyrosine | 1.3 | 2.9 |
| Succinate | 1.2 | 2.9 |
| Adenosine | 0 | 3.6 |

[a] All substrates were tested at 33 μg/ml except for L-tyrosine, which was tested at 16.6 μg/ml, and DGM, where 0.3 ml of a 10×-concentrated solution was added.

[b] All values (except endogenous) were corrected for endogenous metabolism by substraction. Averages of triplicate determinations.

[c] These spores were germinating; for all other values the spores showed no detectable signs of germination.

which did not effect detectable germination, only glucose and glycerol were oxidized rapidly. With the exception of trehalose, which was not oxidized by activated spores, the compounds were oxidized more rapidly by activated spores. The following substrates were oxidized at $QO_2$ values less than 2.0: fructose, L-glutamate, L-serine, α-ketoglutarate, oxaloacetate, pyruvate, and fumarate.

Activated and unactivated spores oxidized components of yeast extract without a lag at rapid and nearly equal rates. Unactivated spores germinate in yeast extract after a 15- to 20-min lag period (10), whereas activated spores germinate in yeast extract without lag (11). Unactivated spores began rapid oxidation of DGM components only after a 10- to 15-min lag period. Consequently, the $QO_2$ values for the first hour were nearly half that of activated spores where oxidation with DGM began rapidly. The $QO_2$ values with DGM as substrate from 60 to 120 min were 10.1 for both activated and unactivated spores (data not shown).

**ATP concentration of spores.** The results of measuring the ATP contents of spores stored dry and wet and during germination are shown in Table 2 and Fig. 4. Spores stored on glass beads at −20°C for 1 month and spores stored on glass beads in a desiccator at room temperature for nearly 2 years had essentially the same ATP contents. The viability of the long-term stored spores was the same as for the fresh spores (data not shown).

A 4.2-fold increase in ATP occurred when spores were washed from glass beads with TX buffer. This increase occurred within 1 min. The ATP content of the wetted spores was the same after the spores had been stored at 4°C for 1 month. Heat activation of the spores by incubation at 50°C for 10 min more than doubled the ATP content over the level in the wetted spores.

Addition of DGM to the activated spores resulted in an increase in ATP content (Fig. 4). There was a slow increase in ATP during the first 8 to 10 min followed by a more rapid increase reaching a maximum at 30 to 40 min, which corresponds to the time when germination was complete as indicated by the optical density curve. The ATP level of germinated spores was 19.4-fold higher than that of dry spores.

TABLE 2. *ATP content of spores*

| Spores | ATP content (μmol/g, dry wt) | Relative amt |
|---|---|---|
| Dry, stored at −2°C, 1 month | $5.2 \times 10^{-2}$ | 1.0 |
| Dry, stored at room temperature, 22 months | $6.0 \times 10^{-2}$ | 1.1 |
| TX buffer suspension, zero time | $2.2 \times 10^{-1}$ | 4.2 |
| TX buffer suspension, 1 month at 4°C | $2.4 \times 10^{-1}$ | 4.5 |
| Heat activated | $6.0 \times 10^{-1}$ | 11.3 |
| Germinated | $10.1 \times 10^{-1}$ | 19.4 |

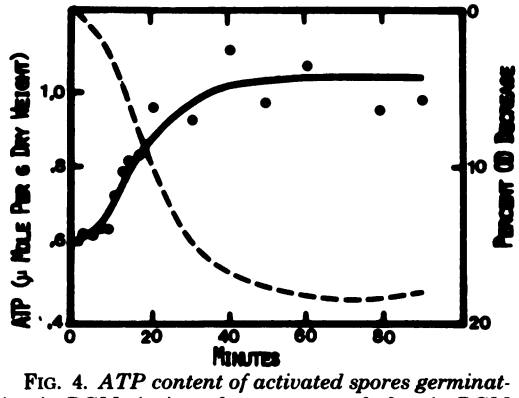


FIG. 4. *ATP content of activated spores germinating in DGM. Activated spores were shaken in DGM at 35°C. Samples of 1.0 ml were removed at different times; ATP was extracted with $Me_2SO$ and measured with the luciferin-luciferase assay. The dashed line shows the optical density changes of the spore suspension.*

**Excretion of spore carbon.** The decrease in optical density of spore suspensions during germination might be accompanied by a loss of spore constituents. This was tested with $^{14}C$-labeled spores. During germination, samples were filtered through membrane filters, and the amount of $^{14}C$ released by the spores was determined (Fig. 5). Unactivated spores germinating in DGM began to slowly excrete $^{14}C$-containing material into the medium after a 10-min lag period (curve B). After 20 min, the rate increased to a rapid, constant value until 40 to 50 min, when it became progressively slower. The 10- to 20-min period when little $^{14}C$-labeled material was released corresponds to the lag period preceding germination of the unactivated spores (10). Most of the spore carbon was released between 20 and 60 min, corresponding to the time when germination occurred. By 90 min, these spores had released approximately 9.5% of their initial $^{14}C$ content. Activated spores incubated in DGM (curve A) excreted $^{14}C$-carbon at essentially the same rate as unactivated spores but without the initial lag. By 90 min, the activates spores had released nearly 12% of their initial $^{14}C$ content.

Unactivated spores incubated in TX salts excreted very little spore carbon (curve D). Activated spores incubated in TX salts excreted labeled material at a slow, constant rate (curve C) and had released slightly more than 1% of their initial $^{14}C$ content at 90 min.

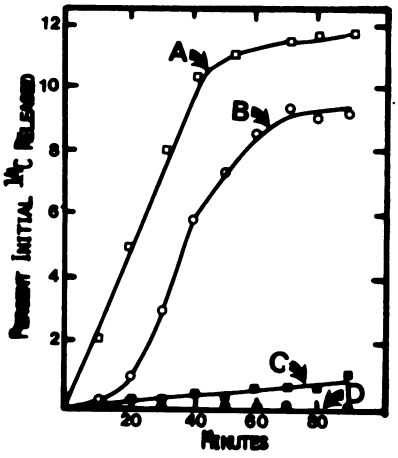


FIG. 5. *Release of carbon-containing material. Unactivated or activated spores uniformly labeled with $^{14}C$ were incubated in DGM or TX salts. The release of carbon-containing material from the spores was followed by monitoring the amounts of filterable $^{14}C$. The results are presented as the percentage of the initial spore $^{14}C$. Symbols: (□) activated spores in DGM; (○) unactivated spores in DGM; (■) activated spores in TX salts; (●) unactivated spores in TX salts.*

The possibility that spore carbon was excreted during the heat activation procedure was tested. The spores released 0.92% of the $^{14}C$ originally present during 10 min of incubation at 50°C. Spores incubated at room temperature for the same time lost only 0.07% of their total label.

**Effect of excreted materials on germination.** The possibility that the materials excreted from germinated spores might either stimulate or inhibit germination of fresh spores was tested. Spores were germinated in DGM for 60 min and then were removed by centrifugation. A 0.1-ml sample of the supernatant fluid was added to unactivated spores suspended in DGM. These spores did not germinate (Fig. 6, curve A). When the supernatant fluid (0.1 ml) was added after 10, 20, or 30 min of incubation, the spores germinated normally (curve C). Spores heat activated and incubated in DGM plus 0.1 ml of the supernatant fluid germinated normally (curve B). These results show that germinating spores release something that is inhibitory to germination of unactivated spores and seems to affect only the initiation of germination.

The germination inhibitor also blocked the excretion of spore carbon and the increased endogenous respiration which normally accompanies germination (unpublished observations). The inhibitor was released from spores only during germination. It could not be removed from spores by extraction with various organic solvents, hot or cold water, or dilute acids or bases.

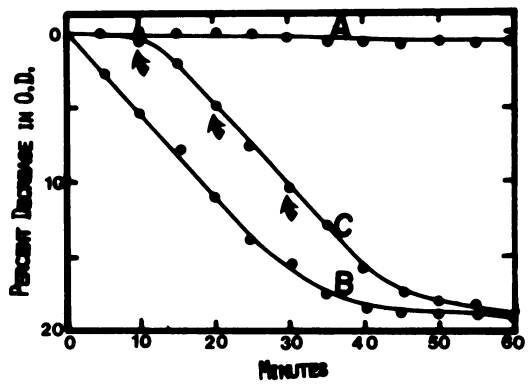


FIG. 6. *Effect of supernatant from germinated spores on germination. Activated and unactivated spores were incubated in DGM. A 0.1-ml sample of the supernatant fluid obtained after removing spores which had germinated in DGM over a 60-min period was added. (Curve A) Sample added at zero time to unactivated spores; (curve B) sample added at zero time to activated spores; (curve C) samples added to unactivated at times indicated by arrows.*

As little as 0.01 ml of the supernatant fluid from a suspension of germinated spores, when added to a fresh suspension, completely blocked all germination events. Attempts to purify and identify the germination inhibitor are now in progress. The materials released during germination exhibit weak antibiotic activity against *Staphylococcus aureus* and *Arthrobacter crystallopoietes*.

The germination inhibitor activity was extracted from germination supernatant fluids with chloroform and more polar organic solvents. The inhibitor activity was adsorbed to diethylaminoethyl-cellulose, anion-exchange resin (Biorex 5, Bio-Rad Laboratories, Richmond, Calif.), activated charcoal, and silicic acid. The activity was dialyzable and was not excluded during gel filtration on Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) in methanol (exclusion limit, approximately 4,000 molecular weight). When germination supernatant fluids were heated at 85°C for 10 min, virtually no loss of inhibitory activity occurred; however, heating in a boiling-water bath for 5 min resulted in a complete loss of activity. The inhibitory activity was not destroyed by a 30-min exposure to cold 5% trichloroacetic acid or 0.5 N NaOH at 37°C. These observations suggest that the germination inhibitor is a low-molecular-weight, acidic compound, which is relatively heat stable and probably not a protein or RNA.

## DISCUSSION

The extreme condition of dormancy where an organism displays absolutely no metabolic activ-

ity is termed cryptobiosis (15, 21). By using endogenous respiration as an index of relative dormancy, dry spores of *S. viridochromogenes* are cryptobiotic. This complete dormancy is attributable to the desiccated state of the spores, since mere exposure to humid air is sufficient to begin endogenous metabolic processes (Fig. 1). The amount of metabolism following exposure to water-saturated air, about $10^{-3}$% of the spore carbon respired per h, is still 10- to 100-fold lower than the lowest known respiration rate of starving bacterial cells (1). The rate of endogenous metabolism of the spores when incubated in buffer is close to that of starving cells of *A. crystallopoietes* (0.02 and 0.03% of cell carbon respired to $CO_2$ per h, respectively) (2). *A. crystallopoietes* is unusual in remaining 100% viable while being shaken at 30°C in buffer for 1 month or longer. A suspension of *S. viridochromogenes* spores incubated while being shaken at 25°C in TX buffer remained completely viable for 6 months (unpublished observations).

A curious characteristic of unactivated *S. viridochromogenes* spores is their slow oxidation of some potential substrates and failure to oxidize others. DeJong and Olszowy (Bacteriol. Proc., p. 77, 1972), using Warburg respirometry, reported the endogneous $QO_2$ for *S. viridochromogenes* spores to be 2.5. They found the $QO_2$ values to double with carbohydrates but to be the same as the endogenous rate when amino acids or organic acids were present.

The ability of *S. viridochromogenes* spores to oxidize various substrates shows that the spores are permeable and possess complete enzyme systems. Spores of *Actinomyces streptomycini* (apparently *S. griseus*, 19) contain functional enzymes involved in carbon, nitrogen, and phosphorous metabolism (13), the tricarboxylic acid cycle (13), and electron transport (22). Thus, with respect to the rate of metabolism of both exogenous and endogenous substrates, streptomycete spores are similar to fungal spores (6) and unlike spores of bacilli (4).

The rate of endogenous metabolism of both unactivated and activated spores increases only slightly during initiation of germination (Fig. 2). The rapid endogenous metabolism rate of activated spores incubated in TX salts, which is nearly the same as for activated and unactivated spores in DGM, is explained by the observation that activated spores do in fact slowly germinate in TX salts as evidenced by loss of spore carbon and refractility (11). This endogenous germination is absolutely dependent upon the calcium ions in the TX salts (D. Eaton and J. C. Ensign, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I128, p. 176).

Substrates contained in DGM are not oxidized extensively until germination of activated spores is nearly complete (Fig. 3). The spores must be dependent upon endogenous metabolism for their energy requirements during that time. The spores contain a large supply of trehalose which is at least partially depleted during germination (A. White, unpublished observations). Spores of *S. hygroscopicus* utilize both trehalose and glycogen during germination (7).

The rate of endogenous metabolism of the spores begins to decline (Fig. 2) as the rate of oxidation of DGM components increases rapidly (Fig. 3). This shift in oxidation from endogenous to exogenous carbon sources coincides with outgrowth of germ tubes and onset of vegetative growth.

The ATP content of dry spores of *S. viridochromogenes* (Table 2) is nearly twice that of bacterial endospores (17). The ATP level in fully germinated spores, 10 μmol/g (dry weight), is within the range reported for vegetative cells of most bacteria (for compilation, see Table 1 of reference 14).

Spores of *Bacillus* lose a considerable part of their constituents during the early stages of germination (see review, reference 7). This loss is accompanied by a decrease in refractility of the spores. Spores of *S. viridochromogenes* lose approximately 10 to 20% of their spore carbon during the early stages of germination. This excretion of spore carbon is definitely associated with germination since it occurs immediately in activated spores and only after a lag period in unactivated spores. Spores incubated in DGM do not germinate or lose spore carbon in the absence of calcium ions or $CO_2$ (unpublished observations).

The material released from *Bacillus* spores originates primarily from a cortex region and is comprised of calcium ions, dipicolinic acid, and peptidoglycan (7). The origin and identity of the carbon-containing material excreted from germinating spores of *S. viridochromogenes* is not known. Some preliminary work directed to identification of this material indicates a heterogeneous mixture of anthrone- and ninhydrin-positive substances. Some amino sugars are present. Spores of streptomycetes do not contain dipicolinic acid (12) or unusually high levels of calcium ions (18; D. Eaton, unpublished observations).

An important component of the material excreted during spore germination is one or more substances which potently inhibit germination of other spores of the organism. The germination inhibitor affects only initiation of germination, since heat-activated spores and unactivated spores which have begun germination become

completely resistant (Fig. 6). Partially purified preparations of the germination inhibitor show weak antibiotic activity. This suggests the possibility that the germination inhibitor may be an antibiotic. The possibility that antibiotics play a role in development and maintenance of dormancy of spores was discussed by Demain (3).

The presence of germination inhibitors in fungal spores is well documented (16). D-Alanine, an inhibitor of germination of some *Bacillus* spores, is produced and excreted during germination (5).

We can only speculate on the advantages of organisms like streptomycetes including germination inhibitors in their spores. The inhibitor might well act as a timing mechanism to insure that spores do not germinate in the vicinity of the mother colony which has depleted the environment of its nutrients. The first spore to receive a signal to germinate will certainly gain a competitive edge against other spores if it excretes an inhibitor to keep them in a dormant state for a longer period. If the germination inhibitor is also an antibiotic inhibiting other potential competitors, so much the better. If the first early germinating spore finds its environment adverse and dies, there is little consequence to survival of the species. The other spores produced by the parent will ultimately overcome the inhibitor and germinate. The end result would be a succession of germinating spores, thus increasing the chances that some of the spores would ultimately find the environment suitable for growth and reproduction. A somewhat similar idea for the role of alanine racemase in *Bacillus* spores was presented by Stewart and Halvorson (20). They speculated that the conversion of L-alanine to D-alanine, which is a potent germination inhibitor, ensures that the entire population of spores will not germinate at the same time.

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## LITERATURE CITED

1. **Altman, D. L., and D. S. Dittmer.** 1974. Biology data book, vol. 3. Federation of American Societies for Experimental Biology, Bethesda, Md.
2. **Boylen, C. B., and J. C. Ensign.** 1970. Long-term starvation survival of rod and spherical cells of *Arthrobacter crystallopoietes*. J. Bacteriol. **103**:569-577.
3. **Demain, A. L.** 1974. How do antibiotic-producing microorganisms avoid suicide? Ann. N.Y. Acad. Sci. **235**:601-612.
4. **Desser, H., and E. Broda.** 1969. Radiochemical investigations of the respiration of spores of *Bacillus cereus*. Arch. Mikrobiol. **65**:76-86.
5. **Fey, G., G. W. Gould, and A. D. Hitchins.** 1964. Identification of D-alanine as the auto-inhibitor of germination of *Bacillus globigii* spores. J. Gen. Microbiol. **35**:229-236.
6. **Gottlieb, D.** 1976. Carbohydrate metabolism and spore germination, p. 141-163. *In* D. J. Weber and W. M. Hess (ed.), The fungal spore: form and function. John Wiley & Sons, Inc., New York.
7. **Gould, G. W.** 1969. Germination, p. 397-444. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press Inc., New York.
8. **Hey-Ferguson, A., M. Mitchell, and A. D. Elbein.** 1973. Trehalose metabolism in germinating spores of *Streptomyces hygroscopicus*. J. Bacteriol. **116**:1084-1085.
9. **Hirsch, C. F., and J. C. Ensign.** 1975. Germination of *Streptomyces viridochromogenes* spores, p. 28-35. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
10. **Hirsch, C. F., and J. C. Ensign.** 1976. Nutritionally defined conditions for germination of *Streptomyces viridochromogenes* spores. J. Bacteriol. **126**:13-23.
11. **Hirsch, C. F., and J. C. Ensign.** 1976. Heat activation of *Streptomyces viridochromogenes* spores. J. Bacteriol. **126**:24-30.
12. **Kalakoutskii, L. V., and N. S. Agre.** 1976. Comparative aspects of development and differentiation in actinomycetes. Bacteriol. Rev. **40**:469-524.
13. **Kalakoutskii, L. V., and M. I. Douzha.** 1967. Enzymatic activity of intact conidia of actinomycetes. Mikrobiologiya **36**:279-283.
14. **Knowles, C. J.** 1977. Microbial metabolic regulation by adenine nucleotide pools. Symp. Soc. Gen. Microbiol. **27**:241-283.
15. **Lewis, J. C.** 1969. Dormancy, p. 301-358. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press Inc., New York.
16. **Macko, V., R. C. Staples, Z. Yaniv, and R. R. Granados.** 1976. Self-inhibitors of fungal spore germination, p. 73-100. *In* D. J. Weber and W. M. Hess (ed.), The fungal spore: form and function. John Wiley & Sons, Inc., New York.
17. **Nelson, D. L., and A. Kornberg.** 1970. Biochemical studies of bacterial sporulation and germination. XX. Phosphate metabolism during germination. J. Biol. Chem. **245**:1146-1155.
18. **Pozharitskaja, L., R. Aslanjan, V. Lebedev, and L. V. Kalakoutskii.** 1973. Content of metals in resting and germinating spores of *Actinomyces streptomycini* B-6. Mikrobiologiya **42**:313-314.
19. **Shirling, E. B., and D. Gottlieb.** 1968. Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. **18**:279-392.
20. **Stewart, B. T., and H. O. Halvorson.** 1953. Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. J. Bacteriol. **65**:160-166.
21. **Sussman, A. S.** 1969. The prevalence and role of dormancy, p. 1-38. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press Inc., New York.
22. **Taptykova, S. D., L. V. Kalakoutskii, and N. S. Agre.** 1969. Cytochromes in spores of actinomycetes. J. Gen. Appl. Microbiol. **15**:383-386.
23. **Wildermuth, H.** 1970. Development and organization of the aerial mycelium in *Streptomyces coelicolor*. J. Gen. Microbiol. **60**:43-50.