JOURNAL OF BACTERIOLOGY, Apr. 1976, p. 13-23

Vol. 126, No. 1
Printed in U.S.A.

Nutritionally Defined Conditions for Germination of *Streptomyces viridochromogenes* Spores

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Received for publication 17 December 1975

Spores of *Streptomyces viridochromogenes* were removed from the surface of solid media with glass beads and suspended in a buffer-detergent solution. Addition of yeast extract and glucose resulted in rapid loss of refractility of the spores. Appearance of germ tubes followed. Germination was accompanied by a decrease in the optical density (OD) of the suspension. The OD decrease was used as an assay for germination. A defined germination medium (DGM) comprised of L-alanine, L-glutamic acid, adenosine, *para*-aminobenzoic acid, and calcium and magnesium ions provided a germination rate nearly equal to that of complex media. The germination rate was essentially the same if D-alanine and D-glutamate replaced the L-isomers. The optimum pH and temperature for germination were 7.0 and 35 C. Germination was absolutely dependent on the presence of CO_2. Spores harvested after growth for longer periods than the usual time (10 days) became less germinable in DGM. The same was observed for spores grown at 37 C as compared with 30 C. Spores incubated in DGM for various time periods before being transferred to a buffer solution did not continue to germinate. Spores harvested after growth of eight species of *Streptomyces* did not show a decrease in OD when incubated in yeast extract medium. Another strain of *S. viridochromogenes* did exhibit an OD decrease in the medium. Comparative properties of spores of streptomycetes, fungi, and bacilli are discussed.

One of the most intriguing life cycles exhibited by prokaryotic organisms is that of bacteria belonging to the genus *Streptomyces*. Part of the life cycle of these bacteria involves formation of spores that are dormant (13, 20), hydrophobic, resistant to desiccation, but only slightly resistant to heat or chemicals (20). Upon encountering a favorable environment the spores germinate. During germination the spores swell and lose refractility (1, 12, 14), and one or more germ tubes emerge. On a medium solidified with agar, the spore grows into a multiply branched coenocytic mycelium. Some of these mycelia differentiate and form aerial hyphae (28). Within these aerial hyphae the reproductive processes occur. The hyphae fragment by an apparent ingrowth of an inner wall layer (2, 29, 30) to form single nucleated (16) spores within a fibrous hydrophobic sheath (2, 29). The vegetative mycelia, not involved in reproduction, ultimately lyse (28).

Mycelia growing in liquid shaken culture do not seem to sporulate (4). Claims of sporulation in liquid culture (3, 6) are not well documented. We know of no report of spores having been isolated from liquid cultures of *Streptomyces*.

When considering the vast amount of research directed to other developmental systems of bacteria, such as spores of *Bacillus*, cysts of *Azotobacter*, myxospores of *Myxobacterium*, and stalks of prosthecate bacteria, it is curious that the developmental biology of *Streptomyces* has received so little attention. This neglect cannot be due to unimportance of these bacteria. Actinomycetes, mainly *Streptomyces*, produce the great bulk of the available arsenal of antibiotics and play an important role in the microbial activities occurring in soil (8).

The only concerted effort directed to defining the properties of *Streptomyces* spores and physiological processes accompanying their germination has been done in Russia, mostly in the laboratory of Kalakoutskii. This work, as well as the work of others, was recently summarized in an excellent review (20).

As an extension of our previous work with the fragmentation division bacteria (7, 11, 22), we decided to undertake a comprehensive study of the developmental biology of streptomycetes. The most fruitful approach seemed to be to begin with the spore. The logical starting point was to choose a manageable species, develop

procedures for obtaining reasonable crops of spores in the native (dry) state that were free of contamination of vegetative hyphae, and develop defined conditions for rapid germination of the spores. This paper reports the accomplishment of these goals. Brief reports directed to the properties and events accompanying germination (13) and heat activation (14) of spores of *S. viridochromogenes* have been published.

MATERIALS AND METHODS

Description of organisms and growth conditions. The organism used in most of the experiments was an unidentified strain obtained from K. B. Raper. We tentatively identified the culture as *Streptomyces viridochromogenes*. This was verified by Alma Dietz, based on patterns of pigment production on several media and scanning electron microscopy of the spore surface. The culture appears to be similar to *S. viridochromogenes* strain NRRL B-1511, obtained from T. G. Pridham. Other strains of *Streptomyces* used in some experiments were *S. griseus* 1947, obtained from Elizabeth McCoy, *S. griseus* NRRL B-1965, *S. venezueleae* NRRL B-2247, and *S. albo-niger* NRRL B-2403, obtained from T. G. Pridham, *S. coelicolor* 3740 and *S. erythrous* Lily F43, obtained from K. B. Raper, and *S. venezueleae* 3534, obtained from H. A. Lechevalier. A strain isolated locally from soil, which produces a soluble blue pigment and appears to be a strain of *S. coelicolor,* was also studied.

For most experiments, cells were grown on a defined medium (SN) containing 1.0% sucrose, 0.1% $NaNO_3$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.02 M potassium phosphate buffer (pH 7.0), 2.0% agar, and 0.5% of a trace salts mixture (TS). The TS consisted of 0.4% $CaCl_2 \cdot 2H_2O$, 0.2% $MnSO_4 \cdot 4H_2O$, 0.1% $FeSO_4 \cdot 7H_2O$, and 0.005% $ZnSO_4 \cdot 7H_2O$ in 0.1 N HCl. Sucrose, as a 50% solution, and trace salts were sterilized separately. Some experiments involved growth on a complex medium (GYA) containing 1.0% glycerol, 0.05% yeast extract (YE), 0.1% L-asparagine, 0.1% K_2HPO_4, 2.0% agar, and 1.0% TS. Glycerol as a 25% solution and TS were sterilized separately.

Preparation of spores. Plates of SN medium were inoculated with spores obtained from GYA slants that had been incubated for 10 days. The spores were washed from the slant surfaces, and 0.2-ml samples were transferred to the surface of each plate and spread evenly with a bent glass rod. After incubation at 30 C for 10 days, the spores were harvested by using a procedure adapted from the method of DeJong and McCoy (5). Approximately 200 glass beads (4 mm in diameter) that had been acid cleaned, dried, and sterilized were placed on the surface of each plate. The beads were rolled around on the agar surface by gently tipping the plates. The spore-coated beads were poured into a sterile bottle and stored at −20 C.

Suspensions of spores were obtained by vigorously shaking the coated beads in cold 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.3) containing 0.001% Triton X-100 (TX buffer). The Triton X-100 is necessary to wet the otherwise hydrophobic spores. The beads were removed by decanting and the spores were sedimented by centrifugation in the cold, washed twice by suspension in TX buffer, centrifuged, and again suspended in TX buffer at a density of 10^{10} spores/ml (Petroff-Hausser counts). Clumps of spores were dispersed by 15 min of sonic treatment in the cold in an ultrasonic cleaning bath (Heat Systems Ultrasonics, Inc.). The spore suspensions were stored at 2 C. No germination, as evidenced by loss of refractility or emergence of germ tubes, occurred during storage for up to 6 months. Also, no change in the rate or extent of germination or loss of viability occurred during this time.

Germination assay. Germination of spores was assayed by measuring the decrease in turbidity as measured by optical density (OD) at 600 nm of spores suspended in a defined germination medium (DGM) at 35 C, using a Cary 15 or Beckman DBG spectrophotometer. DGM consists of TX buffer, 0.01% $MgSO_4 \cdot 7H_2O$, 0.002% $CaCl_2 \cdot 2H_2O$, 0.1% L-alanine 0.1% L-glutamic acid, 0.01% adenosine, and 0.01% *p*-aminobenzoic acid (PABA). On some occasions spores were germinated in a 0.1% YE solution. The OD of the spore suspensions was monitored continuously during incubation without agitation in the cuvettes. In some preliminary exeriments the spore suspensions were agitated by a tiny magnetic bar revolving in the cuvettes. We observed that this practice led to clumping of spores and a decreased OD reading. This procedure was later abandoned since the germination assay is not complicated by clumping of spores if the suspensions are not agitated.

Photomicrographs were made by using a Zeiss phase-contrast microscope and Kodak HC film. Samples of spore suspensions were placed on a slide coated with 1% agar and covered with a number 0 cover slip.

YE and agar were purchased from Difco Laboratories, Inc., HyCAS from Sheffield Chemical Co., Triton X-100 from Ruger Chemical Co., Inc., and Tris buffer from Sigma Chemical Co.

RESULTS

Spore harvest. Fifteen cultures of *Streptomyces* were screened for production of spores when grown on plates of SN medium and for purity of spore preparations obtained by the glass bead procedure. Microscopic examinations were made of the organisms and of spores washed from beads by TX buffer after growth for various periods up to 2 weeks. It was determined that a strain of *S. viridochromogenes* gave more reproducible and cleaner spore crops than the others. When spores of this organism were spread over the surface of plates of SN medium, confluent mycelial growth was evident after 24 h of incubation. Spores were observed in microscope preparations of about 72 h. The growth medium became greenish-brown and then progressively more gray as spores were formed. The maximum level of spores was

reached after 7 to 10 days of growth. Spores were routinely harvested at 10 days.

The spores suspended in TX buffer were greenish-gray in color, phase bright, and virtually free of contamination by mycelial fragments. The spores tended to remain in chains, which were easily broken by gentle sonic treatment. By weighing dried beads before and after rolling them over plates of media, we determined that approximately 3 mg (dry weight) of spores was obtained per plate. This represents approximately 10^{10} spores per plate.

The amount and purity of spores obtained on SN medium was essentially the same as during growth on several other media tested. The maximal amount of spores was obtained a few days earlier, and the total yield was a little more on GYA medium. On some complex media droplets of liquid formed on the surface of the mycelial growth. When this occurred, it was impossible to harvest spores with the glass beads.

In liquid shaken cultures of SN or GYA medium the organism grew as balls composed entirely of multiply branched mycelia. Spores were not observed in the growth liquor or mycelial growth even after several weeks of incubation. The growth media turned blue-green after 2 to 3 days of growth and then became deep brown. The mycelial growth became black in color after 4 to 5 days of incubation. Varying the concentration of sucrose or nitrate in SN medium to give C:N ratios from 10:1 to 0.1:1 did not result in formation of spores in liquid shaken cultures.

Germination in complex media. The turbidity changes shown in Fig. 1A were the result of incubating spores static and shaken in TX buffer containing 0.01% $MgSO_4 \cdot 7H_2O$ and 0.002% $CaCl_2 \cdot 2H_2O$ (TX salts) and in 0.1% YE-1.0% glucose (YEG) medium. The static experiments involved incubating the spore suspensions in cuvettes in the spectrophotometer without agitation. The shaken experiments involved shaking spore suspensions in a water bath and, at 10-min intervals, removing samples for determination of the OD. The static spore suspension in TX salts exhibited a linear decrease in OD during the 2-h period. The OD of spores shaken in TX salts remained constant for 1 h and then decreased slowly. The significant decrease in the OD of the static suspension and slight decrease in the shaken suspension was not due to germination of the spores, but was apparently the result of their clumping and settling. This important point was verified by the observations that the spores remained refractile (Fig. 2A, B) and did not show the increase in endogenous respiration or excretion of spore components that occurred during germination (13; unpublished data). When a suspension of spores that had been incubated statically for 2 h was shaken briefly, the OD of the suspension returned to essentially the same value as was observed initially.

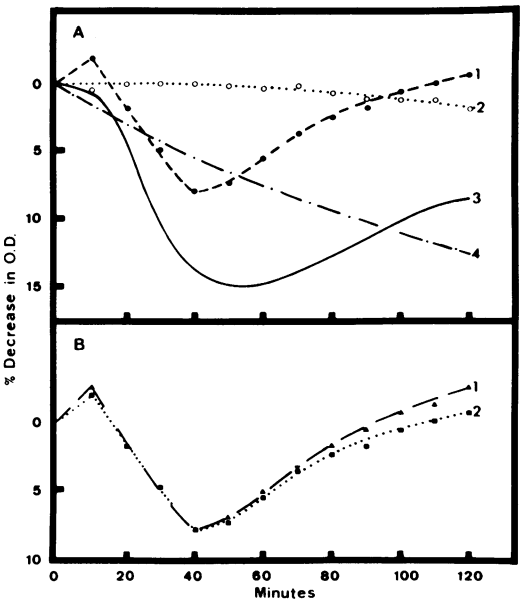

FIG. 1. *Turbidity of spore suspensions incubated static and shaken in TX salts and YEG medium. (A) Actual turbidity changes accompanying incubation of spores. (Curve 1) YEG medium, shaken; (curve 2) TX salts, shaken; (curve 3) YEG medium, static; (curve 4) TX salts, static. (B) Turbidity in YEG medium corrected for changes in turbidity due to clumping and settling. (Curve 1) YEG medium, static; (curve 2) YEG medium, shaken. Spores were incubated in TX salts or YEG medium without agitation in cuvettes in the spectrophotometer, and the OD was measured continually. The agitated suspensions involved incubation of spores in TX salts or YEG medium in a water bath shaker. Samples were removed at 10-min intervals for OD determinations.*

When the OD curves obtained during germination in YEG medium (Fig. 1A) were corrected for the OD decrease in TX salts, the results for shaken and static cultures were nearly the same (Fig. 1B). The OD of the suspension increased slightly for 10 min. During this time the spores remained refractile (Fig. 2C). The linear decrease in OD that followed was accompanied by loss of refractility of the spores (Fig. 2D). The OD reached a minimum at 40 to 45 min, when the spores were phase dark, and then began to rise. The increase in OD was accompanied by emergence of germ tubes (Fig. 2E).

These experiments establish some important characteristics of *S. viridochromogenes* spore germination: spores incubated in TX salts at

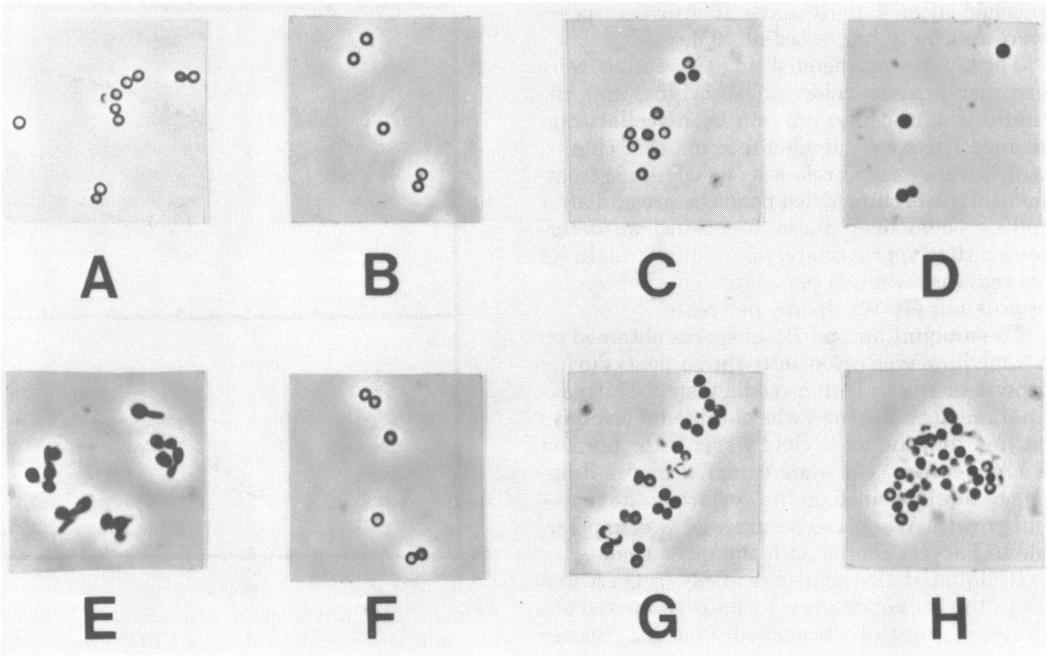

FIG. 2. *Photomicrographs of spores incubated in different media. (A) Incubated in TX-buffer, 120 min, shaken; (B) incubated in TX-buffer, 120 min; (C) incubated in YEG medium, 10 min; (D) incubated in YEG medium, 50 min; (E) incubated in YEG medium, 150 min; (F) incubated in DGM, 20 min; (G) incubated in DGM, 90 min; (H) incubated in DGM, 150 min. Spores were incubated without agitation (except A) in the different media at 35 C. At various times, samples were spread on an agar layer and photomicrographs made with a phase-contrast microscope.*

35 C for 2 h did not germinate; germination is accompanied by loss of spore refractility and can be measured by the decrease in OD of spore suspensions; germination is separable from and followed by outgrowth; and germination of static or shaken spores occurs at essentially the same rate. The last two points are pertinent to this and our subsequent investigations because germination is measured by following the decrease in OD of static spore suspensions.

Germination in defined medium. Early experiments designed to develop a defined germination medium revealed that the rate of germination was stimulated by calcium and magnesium ions. The effects of metal ions on germination will be dealt with later. For the experiments leading to defined germination conditions, the spores were suspended in TX salts. Addition of HyCAS (1.0% final concentration) to a spore suspension resulted in germination of the spores at nearly the same rate (linear OD decrease) as was obtained with YEG.

The effect on germination of adding 20 individual L-amino acids at 0.1% final concentration to spore suspensions was tested. Most of the amino acids had little or no effect, but seven of them (alanine, histidine, tyrosine, aspartic acid, glutamic acid, arginine, and asparagine) did cause the spores to germinate, although at a significantly slower rate than with HyCAS. A combination of all seven amino acids did produce germination rates comparable to HyCAS. The seven amino acids were next tested in all possible combinations of pairs. Two pairs, tyrosine-glutamate and alanine-glutamate, caused spores to germinate significantly more rapidly than any of the other pairs. The tyrosine-glutamate pair caused slightly more rapid germination than alanine-glutamate. Because tyrosine is poorly soluble and unstable in solution, we chose the alanine-glutamate pair for all subsequent work. The rate of germination of spores suspended in TX salts containing alanine and glutamate is shown in Fig. 3, curve B. Stimulation of the germination rate was slight, but a definite increase over TX salts only (curve A).

A mixture of adenosine, uridine, cytidine, and guanosine, each at 100 μg/ml, was tested for its effect on germination of spores in TX salts containing alanine and glutamate. The rate of germination more than doubled as compared with alanine and glutamate alone. Adenosine and guanosine, each alone, gave the same result as the mixture of nucleosides. The result

obtained with adenosine is shown in Fig. 3, curve C.

The rate of germination of spores suspended in TX salts plus alanine, glutamate, and a mixture of vitamins was nearly double the rate obtained with the two amino acids plus adenosine. Testing individual vitamins resulted in the discovery that PABA at 100 μg/ml gave the same amount of stimulation of germination as the complete vitamin mixture. The stimulation of germination by PABA is shown in Fig. 3, curve D.

The combination of L-alanine, L-glutamate, adenosine, and PABA, when added to spores suspended in TX salts, produced a rate of germination approaching that of YEG or HyCAS and greater than that of any combinations of the chemicals (Fig. 3, curve E). The decrease in OD after a 20-min lag was accompanied by a loss in refractility and slight swelling of the spores (Fig. 2F-H). This combination comprises the DGM used in all our subsequent experiments.

The effect on germination of the D-isomers of alanine and glutamate was tested. Addition of either D-alanine or D-glutamate at 0.1% final concentration to DGM had absolutely no effect on the rate of germination. Replacing the L-glutamate in DGM with D-glutamate resulted in a slight decrease in the rate of germination (data not shown). This rate was identical to that obtained when both L- and D-glutamate were omitted from DGM. Replacing L-alanine with D-alanine resulted in a slight lengthening of the lag period and a slight decrease in the germination rate. Germination was nearly completely suppressed if both D- and L-alanine were removed from DGM. Germination of *S. viridochromogenes* spores in DGM is, therefore, almost completely dependent on alanine, and both the D- and L-isomers serve equally well as germinants.

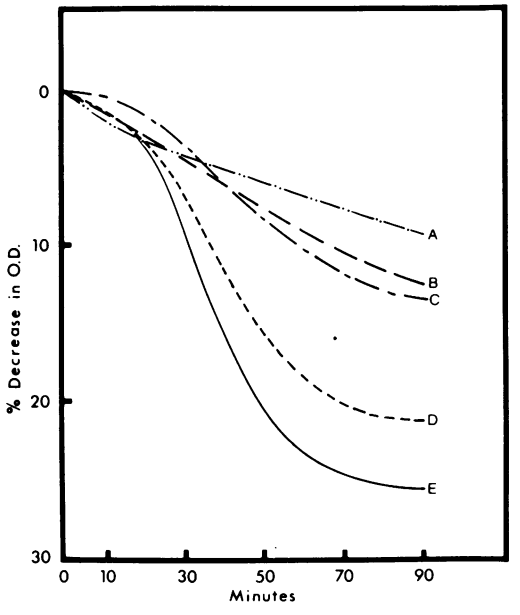

FIG. 3. *Optical density changes accompanying germination of spores in nutritionally defined media. (Curve A) TX-salts buffer; (curve B) TX-salts buffer plus 0.1% L-alanine and L-glutamic acid; (curve C) same as curve B except 100 μg of adenosine per ml was added; (curve D) same as curve B except 100 μg of para-aminobenzoic acid per ml was added; (curve E) same as curve D except 100 μg of adenosine per ml was added (the final defined germination medium). Spores suspended in the different media at an initial OD of approximately 0.5 were incubated at 35 C without agitation, and the OD was monitored.*

Effect of metal ions, pH, and temperature. The germination rate of spores was determined in DGM amended by various combinations of divalent cations and the chelating agent ethylenediaminetetraacetic acid (EDTA). The rate of spore germination was increased by nearly 40%, compared with the rate without metals added, by a mixture of Ca, Mg, Mn, and Zn ions (Table 1). Germination was stimulated slightly when Mn or Zn was removed from the mixture and was decreased by approximately 50% when either Ca or Mg was omitted. A low concentration of Fe inhibited the rate of germination by over 50%. Phosphate had little effect on germination; this was also true when the amount of phosphate was doubled to 20 mM. Germination was nearly completely blocked by 0.5 mM EDTA in the presence of metal ions and 0.1 mM EDTA in the absence of metal ions. These results clearly indicate a requirement for spore germination in DGM of Ca and Mg ions.

TABLE 1. *Effect of divalent metal ions on germination of spores of S. viridochromogenes*

Metal ions present[a]	% OD decrease, 60 min
None	12.2
Ca, Mg, Mn, Zn (complete)	19.1
Complete − Ca	10.5
Complete − Mg	11.5
Complete − Mn	21.4
Complete − Zn	20.3
Complete + Fe	8.0
Complete + PO_4	17.3
None + EDTA (0.1 mM)	3.3
Complete + EDTA (0.5 mM)	2.7

[a] The final concentrations (millimolar) of metals tested were: 0.14 Ca (as $CaCl_2 \cdot 2H_2O$); 0.06 Mn (as $MnSO_4 \cdot H_2O$); 0.02 Fe (as $FeSO_4 \cdot 7H_2O$); 0.001 Zn (as $ZnSO_4 \cdot 7H_2O$); 0.41 Mg (as $MgSO_4 \cdot 7H_2O$); and 10.0 PO_4 (as $NaH_2PO_4 \cdot H_2O$).

To determine the effect of pH on the rate of germination, DGM buffered with Tris-maleate buffer at pH values of 6.5 to 8.5 was inoculated with spores, and the changes in OD were monitored for 1 h. The spores germinated at all pH values tested, but the rate was maximum at pH 7.0.

Spores were incubated in DGM at temperatures ranging from 25 to 45 C. The spores germinated at temperatures between 25 and 40 C but not at 45 C. The maximal rate of germination was at 35 C.

Demonstration of CO_2 requirement for germination. The stimulation of germination by PABA suggested that one-carbon metabolism may be essential for spore germination. An experiment was designed to determine whether CO_2 is required for germination. Air was scrubbed free of CO_2 by passage through spargers immersed in two bottles containing 20% KOH followed by a column (1 by 2 inches; about 2.5 by 5 cm) of ascarite. The CO_2-free air was then bubbled through a small culture tube containing 0.1% YE and another tube containing DGM. The tubes were covered with serum caps and vented with a syringe needle. The YE and DGM tubes were flushed with CO_2-free air for 30 min before 0.05 ml of a concentrated spore suspension was added to each. Two more tubes, one containing YE and the other DGM, were inoculated with spores and were bubbled with normal air. A loopful of culture was removed from each tube at various times during incubation at 35 C, and the morphology of spores was recorded by photomicrographs. Spores incubated in YE and exposed to the air became phase dark at 50 min and germ tubes were evident at 110 min (Fig. 4). The spores incubated in DGM and exposed to air were phase dark at 50 to 70 min and exhibited germ tubes after 150 min of incubation. The spores sparged with CO_2-free air in YE or DGM remained phase bright for 150 min. At that time, normal air was bubbled through these cultures. The spores in YE and DGM were phase dark 20 and 40 min later, respectively.

Stoppered cuvettes containing YE or DGM were sparged with CO_2-free air for 30 min. Spores were then added with a syringe, and the OD of the suspension was monitored. In both media the lag period preceding the decrease in OD was 10 min longer than for spores incubated in air. A decrease in OD at the normal rate followed. Spores suspended in TX salts excrete a small amount of respiratory CO_2 (13). Presumably, enough CO_2 accumulates in the sealed cuvettes to allow germination to begin. It was not possible to follow the OD of continuously sparged spore suspensions because of clumping of the spores, which resulted in a decrease in OD not related to germination.

Effect of spore age, growth media, growth temperature, and heat shock on germination. Spores were harvested from plates of GYA and SN media after incubation at 30 and 37 C for 10, 14, and 21 days. Ability of the spores to germinate (germinability) was tested in DGM and YE and in DGM after the spores had been heat shocked at 55 C for 10 min. We have described in detail the heat shock activation (more rapid and synchronous germination) of *S. viridochromogenes* spores (14).

Germinability of spores was essentially the same when they were harvested after growth on SN or GYA medium (Table 2). Spores produced at 37 C were less germinable than spores produced at 30 C when incubated in DGM. The 30 and 37 C spores were equally germinable, however, when incubated in YE. Spores that had been grown for 10 and 14 days germinated equally well when incubated in DGM. The 21-day spores were significantly less germinable in DMG. The same pattern of germination was observed for 10-, 14-, and 21-day-old spores incubated in YE except that the decrease in germinability at 21 days was less pronounced. Germinability of spores incubated in DGM after the heat shock treatment was significantly increased. The 10-, 14-, and 21-day spores germinated in DGM equally well after heat activation.

Commitment to germination. An experiment was designed to determine whether spores incubated in DGM are committed to complete the process of germination if they are removed from DGM and incubated in TX salts. Spores were inoculated into tubes each containing 4.0 ml of DGM and were incubated at 35 C. At various times the spores were removed from DGM by filtering the contents of a tube through a membrane filter (Nucleopore, 0.4-μm pore size). The spores were washed on the filter with 5.0 ml of TX salts. The filter was then placed in a beaker containing 2.0 ml of TX salts and agitated for 1 min in a water bath sonicator to resuspend the spores. The spore suspensions were then incubated at 35 C, and the change in OD was monitored (Fig. 5). Spores incubated in DGM for 5 and 10 min, within the time period when the OD of spores suspended continuously in DGM has not yet begun to decrease, showed essentially the same amount of decrease in OD when transferred to TX salts as spores incubated in TX salts from time zero. Spores preincubated in DGM for 20 min (not shown), when the OD lag period for spores continuously incu-

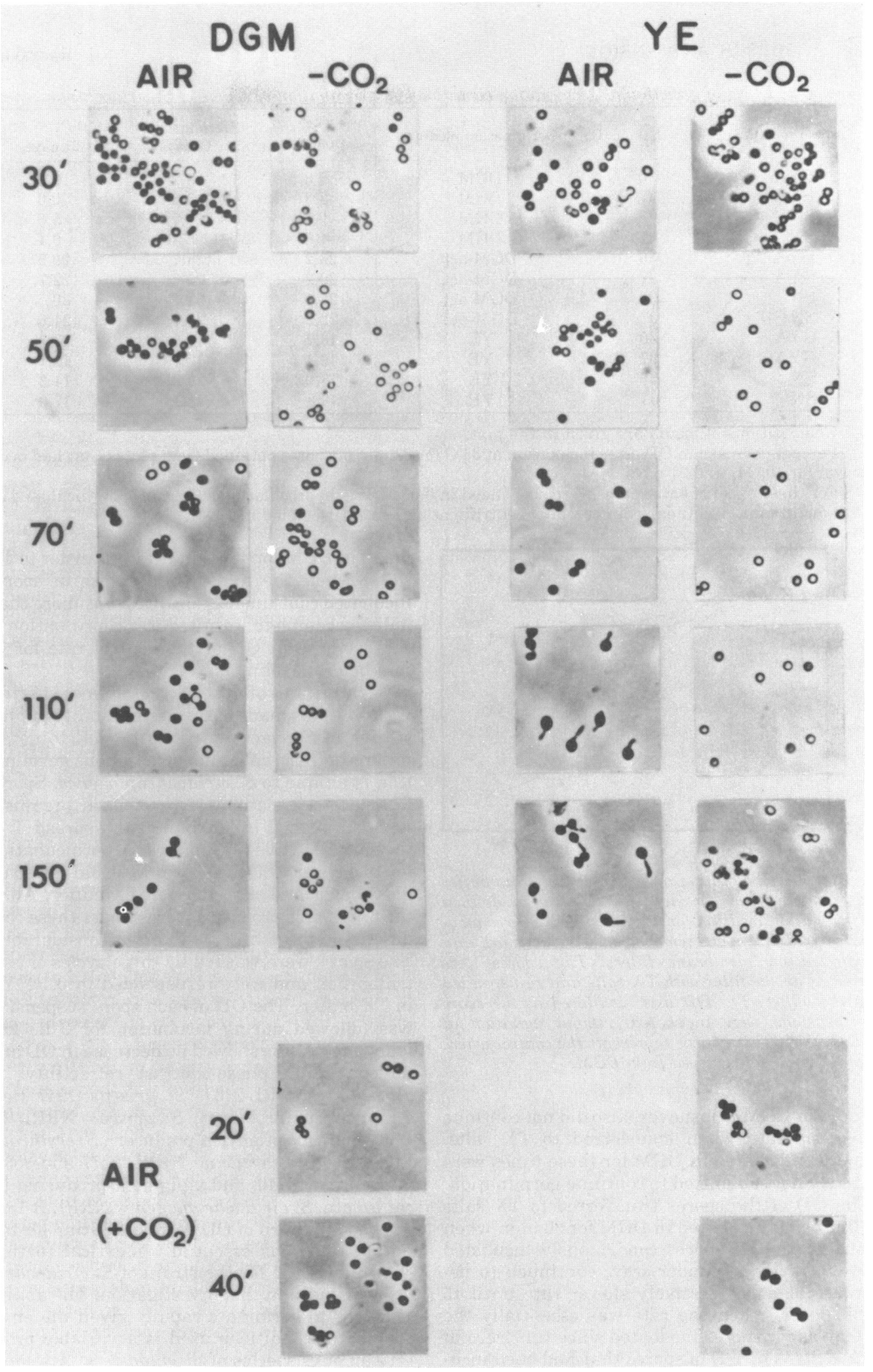

FIG. 4. *Demonstration of requirement of CO_2 for germination of spores in complex and defined media. Spores incubated at 35 C in either DGM or 0.1% YE medium were flushed continuously with either normal air or air that had the CO_2 removed by alkali and ascarite. Samples removed at various times were spread on an agar layer, and photomicrographs were made with a phase-contrast microscope.*

TABLE 2. *Effect of growth and germination conditions on germination of spores of S. viridochromogenes*

Growth medium[a]	Growth temp (C)	Germination medium	Germination[b] 10 days	14 days	21 days
GYA	30	DGM	8.8	8.2	2.6
GYA	37	DGM	3.3	3.9	0.6
SN	30	DGM	9.1	11.7	5.6
SN	37	DGM	4.6	6.4	0.4
GYA	30	DGM-act[c]	23.2	28.7	29.5
GYA	37	DGM-act	20.2	25.0	22.7
SN	30	DGM-act	22.1	32.7	30.7
SN	37	DGM-act	22.2	28.4	24.6
GYA	30	YE	16.7	17.8	13.7
GYA	37	YE	19.1	17.7	12.7
SN	30	YE	18.9	20.2	14.2
SN	37	YE	18.8	22.1	17.0

[a] Compositions of media are given in the text.

[b] Percent decrease in OD after incubation at 35 C for 50 min of spores obtained from plates that had been grown for 10, 14, and 21 days.

[c] For these experiments spores were suspended in 0.05 M Tris-hydrochloride buffer, pH 8.3, incubated at 55 C for 10 min, and then sedimented by centrifugation and resuspended in DGM.

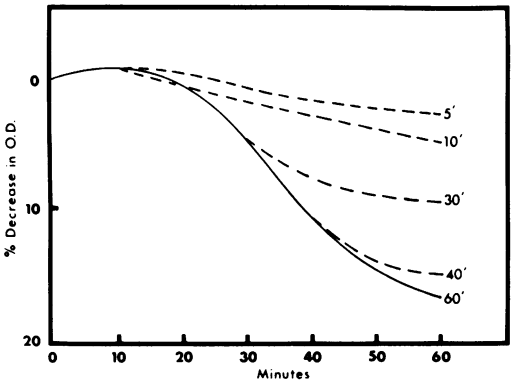

FIG. 5. *Germination of spores in TX salts buffer after having been previously incubated in defined germination medium for various times. Spores incubated in DGM were removed at various times and collected on membrane filters. The spores were washed on the filter with TX salts and resuspended in the buffer. The OD was monitored as the spore suspensions were incubated without agitation at 35 C. The 60-min curve represents the control where spores were not removed from DGM.*

bated in DGM is just over, also did not continue to germinate when transferred to TX salts. Spores incubated in DGM for these times were clearly not committed to continue germination. The OD of the spores transferred to TX salts after being incubated in DGM for 30 min, when germination of spores continuously incubated in DGM is well under way, continued to decrease at a progressively slower rate until 15 min later, when the rate was essentially the same as for spores incubated since time zero in TX salts. The OD of spores that had been incubated in DGM for 450 min before transfer to TX salts, when the total OD decrease of spores incubated continuously in DGM was more than halfway complete, resulted in a continuation of the decrease in OD at the control rate for 10 min before leveling off.

Germination of other *Streptomyces* species. A survey was made of the germinability of nine strains of *Streptomyces* in 0.1% YE to determine whether rapid and synchronous germination is unique to *S. viridochromogenes*. Spores from a stock slant culture of each organism were suspended in TX buffer and spread over the surface of five GYA plates. After incubation for 10 days at 30 C the spores were harvested on glass beads and suspended in TX buffer. All of the organisms produced spores under these conditions, but the amounts varied considerably. The spores were washed by three cycles of centrifugation and were resuspended in 0.1% YE in TX buffer. The OD of each spore suspension was followed during incubation for 2 h. The following strains showed no decrease in OD and no change in phase-contrast refractility: *S. albo-niger* NRRL-2403, *S. griseus* 1947 (low streptomycin producer), *S. griseus* NRRL B-1965 (high streptomycin producer), *S. erythrous* Lily F-43, *S. venezuelae* NRRL 2277 and 3534, *S. coelicolor* 3740, and a pigment-producing local isolate. *S. viridochromogenes* NRRL B-1511 showed a pattern of OD decrease during germination that was essentially identical to that shown in Fig. 1 for the strain of *S. viridochromogenes* used in this investigation. The ability of spores to germinate rapidly given the environmental conditions used is a variable property amongs species of *Streptomyces*. Attempts

are being made to determine the optimum germination conditions for some of the recalcitrant majority.

DISCUSSION

An important point of terminology must be considered. The term "germination" has come to have a different connotation for investigators of bacterial endospores and fungal spores. Germination of bacterial endospores generally means conversion from a dormant to a metabolically active state and is separate from the biosynthetic events of outgrowth resulting in a vegetative cell (9, 26). Germination of fungal spores does not distinguish between breaking of the dormant state and the biosynthetic processes involved in outgrowth of germ tubes (24, 26, 27).

Previous references in the literature to germination of streptomyces spores have used the criterion of discernible emergence of germ tubes (15, 19, 20). Our studies with *S. viridochromogenes* spores, as described in this and our other papers (13, 14), clearly show three distinct stages in the transition from the dormant to vegetative state. We have chosen to name these stages using terms generally employed for bacterial endospores, i.e., activation, germination, and outgrowth. This similarity in terminology should not be taken to imply that the physiological events characterizing these three stages are the same in bacterial endospores and streptomyces spores.

Germination of endospores has been referred to as a trigger reaction (21) because once begun, germination continues in the presence of inhibitors or adverse pH (10). Germination of *S. viridochromogenes* spores does not seem to involve a trigger. Transferring spores of *S. viridochromogenes* from DGM to TX salts after the first 10 min of incubation completely arrested germination. Once germination had started, the shift to TX salts resulted in a continual decrease in the rate of germination. It would be interesting to test the effect of inhibitors on initiation of germination of *S. viridochromogenes* spores, as did Halman and Keynan (10), for endospores. We have shown germination to be inhibited by rifampin and chloramphenicol (13), but have not added the inhibitors to spores already germinating. It is not too surprising that germination of endospores, being essentially a degradative process, involves a trigger reaction. The dependence of spores of *S. viridochromogenes* for a continued supply of germinants to completely germinate fits with our observations that this is a biosynthetic process (13; unpublished data).

Spores of *S. viridochromogenes* do not germinate when suspended in TX buffer, even when shaken at 25 C for several months (unpublished data). Optimum conditions for germination are 35 C, neutral to slightly alkaline pH, and the presence of L-alanine, L-glutamic acid, adenosine, PABA, magnesium, calcium, and CO_2. L-Tyrosine effectively replaces L-alanine. Germination is not absolutely dependent on any of these chemical germinants except for CO_2, which is required for any decrease in OD or phase darkening. The rate of germination is very slow when L-alanine is omitted from the germination medium. Germination requirements for endospores of bacilli are analogous in that germination is usually more rapid and complete in a combination of germinants, but will occur at least partially with any one component only (9). It has been suggested for bacilli that this may be due to heterogeneity of the spore population with respect to status of dormancy (17). This could also be true for spores of *S. viridochromogenes*. Microscopic examination of spores germinated in DGM or various combinations of the components shows that not all the spores are equally phase dark at the time the maximal OD decrease is reached (spores germinating in air in DGM for 70 min, Fig. 4).

Kalakoutskii and Bobkova (18) reported that L-valine stimulated germination of spores of *Actinomyces streptomycini* B-6 (Russian literature uses *Actinomyces* synonymously with *Streptomyces*; 20), whereas glutamic acid stimulated slightly and alanine had no effect. Shigaeva and Romankulov (25) tested the effect of 10 L-amino acids on germination of five *Actinomyces (Streptomyces)* species. Valine stimulated germination of all, alanine slightly stimulated some, and glutamate inhibited germination of all. It must be stressed that both of these reports designated germination as the percentage of spores with visible germ tubes after 6 or 8 h of incubation. Also, it is important to note that in one of the reports (25) spores were washed from agar surfaces. The spores might have been germinated during isolation, by the definition of germination we are using, by nutrients washed from the growth media. This possibility is reinforced by the statement in the review of *Streptomyces* spores by Kalakoutskii and Pouzharitskaya (20) that germinating *A. streptomycini* spores do not show a decrease in OD or refractive index. The contention that an OD decrease does accompany spore germination is supported by Attwell and Cross (1), who showed this to occur during germination of *S. griseus* spores in peptone-yeast extract broth.

S. viridochromogenes, when grown at 37 C, forms spores that are considerably less germinable in DGM than when grown at 30 C. Growth is noticeably more rapid at 37 C, as judged by visual estimation. The spores become progressively less germinable in DGM as the time they are harvested from plates increases from 10 to 21 days. Unfortunately, when this experiment was done, we did not anticipate the decrease in germinability with age and so did not incubate enough plates to provide spores that were older than 21 days. In a later experiment, we observed that spores harvested after growth for longer than 21 days did not germinate in DGM unless heat activated (14). These observations could be explained by the spores becoming more dormant or developing more complex nutritional requirements for germination during aging. In YE medium, the spores germinated equally well whether they had been grown at 30 or 37 C, but they did become slightly less germinable after 21 days of growth. Thus, a mixture of nutrients does reverse significantly the effects of aging on germination in DGM. Whatever does occur to cause the spores to become less germinable in DGM is completely neutralized by a gentle heat shock of 55 C for 10 min. At present, we have no explanation for this apparent aging effect or reversibility by heat shock. We do know some of the physiological reactions occurring in heat-shocked (activated) and germinating spores that were harvested after 10 days of growth (14). Clues to the aging effects might result from a similar study of spores harvested after 21 days of incubation or longer. Whatever occurs to make spores obtained from growth for longer periods less germinable in DGM does not occur when the spores are removed from the growth medium. Spores shaken in TX buffer at 25 C or stored on glass beads in a desiccator for up to 3 months do not become more or less germinable in DGM than the freshly harvested spores (13; unpublished data).

It is apparent from a study of the photomicrographs of germinating spores, particularly in DGM, that the population does not germinate synchronously. Some phase-bright spores remain when the majority of spores are phase dark (note Fig. 2G, Fig. 4; spores aerated in DGM for 50, 70, and 110 min). This lack of synchrony of germination threatened to make results of studies of germination difficult to interpret. Fortunately, we discovered that heat activation of the spores produces good synchrony of germination (14).

In retrospect, we were indeed fortunate in choosing *S. viridochromogenes* for our studies. It was not until we were well into the investigation that we tested germinability of some other streptomycetes in YE medium. None of the nine strains tested germinated during 2 h of incubation. The only published curve that we could find showing a decrease in OD of germinating streptomycete spores was for *S. griseus* CUB 340 spores suspended in peptone-yeast extract broth (1). The maximum decrease in OD occurred at 5 h, which compares with 40 to 50 min for *S. viridochromogenes* spores germinating in YE medium. Our choice was all the more fortuitous because, as shown by Lindenfelser and Pridham (23), *S. viridochromogenes* is a very stable organism with respect to physiology, pigmentation, and sporulation. *S. viridochromogenes* is not such an ideal organism for study in one respect, however: it is a weak antibiotic producer (23). When we began this investigation we had hoped to find a relationship between antibiotics and formation, dormancy, and germination of streptomycete spores. Perhaps the ease of germination of spores of the weakly antibiotic-producing *S. viridochromogenes* and difficulty of germination of other streptomycetes, some of which are good producers, will ultimately reveal such a relationship. This will involve discovering how to germinate spores of a good antibiotic-producing strain rapidly, synchronously, and with defined conditions.

ACKNOWLEDGMENTS

This investigation was partially supported by National Science Foundation grant GB 41716. C.F.H. was supported by Public Health Service training grant 5T01-GM00686-10-MIC from the National Institute of General Medical Sciences.

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