Microcyst Germination in *Myxococcus xanthus*

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Germination of glycerol-prepared microcysts of *Myxococcus xanthus* was studied. The sequence of morphological events during germination resembled that of germinating fruiting body-microcysts. The turbidity drop of a culture of germinating microcysts could be described by McCormick's formula derived for germinating *Bacillus* spores. The rate of uptake of labeled glycine and acetate did not change during germination. Temperature, aeration, and pH optima for germination were the same as for vegetative cell growth. Germination was induced by protein hydrolysates and the individual amino acids glycine, alanine, valine, aspartic acid, and glutamic acid. A number of organic compounds, including sugars, alcohols, aldehydes, ketones, organic acids, and chelating agents, did not induce germination. The inorganic ions HPO$_4^{2-}$, Mg$^{++}$, Ca$^{++}$, and NH$_4^+$ induced germination, although ionic strength was not a factor. Microcysts incubated in distilled water at concentrations greater than about 10$^9$ cells/ml germinated; supernatant fluid from such suspensions (germination factor) induced germination of less concentrated suspensions. The activity of germination factor was resistant to boiling, but was lost on charring and dialysis. Germination of microcysts and growth of vegetative cells was equally sensitive to a variety of metabolic inhibitors, including penicillin and chloramphenicol. Germination was more resistant than vegetative growth to inhibition by antibiotics of the streptomycin family and by actinomycin D.

Many myxobacteria undergo morphogenesis at both the colonial and cellular levels, with formation of fruiting bodies from large numbers of vegetative cells, and subsequent conversion of these vegetative cells into microcysts. Such microcysts resemble bacterial endospores, in that both are resting forms which exhibit enhanced resistance to deleterious conditions. A better comparison (8), however, might be with *Azotobacter* cysts, which, like microcysts, are formed by a shortening of the entire vegetative cell. Dworkin and Voelz (8) described germination of microcysts of *Myxococcus xanthus* on a solid nutrient medium. About 5 hr after plating on germination medium, the spherical microcysts lost phase refractivity, and rod-shaped vegetative cells began to emerge. Remnants of microcyst walls were left behind as the newly emerged cells resumed vegetative growth. Electron micrographs (18, 19) confirmed this description.

Dworkin and Gibson (5) described a method for inducing synchronous, complete conversion of vegetative cells into microcysts by incubation in liquid medium containing glycerol. These microcysts, unlike those obtained from fruiting bodies, readily germinated in liquid media (W. S. Ramsey and M. Dworkin, Bacteriol. Proc., p. 17, 1966). This facilitated a physiological examination of microcyst germination, the results of which are reported in this paper.

**MATERIALS AND METHODS**

*Organism.* The organism used was *M. xanthus* strain FB, a fruiting myxobacterium which grows dispersed in liquid culture (3).

*Cultivation of vegetative cells.* Cells were grown and maintained by daily transfer in 40 ml of CT medium (2% Casitone in 0.01 M potassium phosphate buffer [pH 7.6] + 0.008 M MgSO$_4$). Cultures were incubated in 250-ml Erlenmeyer flasks at 30 C with shaking.

*Production of microcysts.* Log-phase cultures at a density of 6 X 10$^8$ to 12 X 10$^8$ cells/ml were chilled on ice, harvested by centrifugation at 4 C, washed once with ice-cold distilled water, and suspended in microcyst induction medium consisting of 1% Casitone, 0.008 M MgSO$_4$, and 0.5 M glycerol. Cultures of 40 ml in 250-ml flasks or 200 ml in 2-liter flasks were incubated at 30 C with shaking for 6 hr. Although the primary morphological change of rod-shaped vegetative cells to approximately spherical microcysts took place by 2 hr, incubation was continued for a total of 4 days.
6 hr. During this period, the microcysts became more phase-refractile. Microcysts were harvested by centrifugation at 4 C, washed three times with ice-cold distilled water, and stored at 0 C.

Germination of microcysts. Microcysts were routinely suspended in CT medium at 1.3 X 10^6 to 1.5 X 10^6 cells/ml and were incubated at 30 C with shaking. A Petroff-Hausser counter was used to determine cell concentration. Following the convention of endospore workers (11), the complete loss of phase refractivity was used as a parameter of germination. In every case, except when noted otherwise, germination was followed by outgrowth. Estimates of the percentage of microcysts which germinated were made when less than the entire culture germinated. When testing solutions of individual compounds in water for their ability to induce germination, incubation was continued for at least 12 hr. In inhibitor experiments, CT was used as the basal medium to which inhibitors were added. In all experiments, suspensions of microcysts in CT and water were included as controls.

Production of germination factor. Microcysts were suspended at 1.3 X 10^6 cells/ml in distilled water. A portion of this suspension (10 ml) was incubated for 1 hr. The microcysts, unchanged at this point in number and microscopic appearance, were removed by centrifugation, and the supernatant fluid was used as germination factor.

Assay of germination factor. Microcysts were suspended at 1.3 X 10^6 cells/ml in 10 ml of the liquid to be tested. The suspensions were incubated; germination within 2 hr indicated the presence of active germination factor.

Inhibition of vegetative growth. Vegetative cells were incubated in microcyst induction medium minus glycerol. After 6 hr of incubation, the vegetative cells were harvested and suspended in CT medium at 1.5 X 10^6 cells/ml. A portion of this suspension (9 ml) was added to 1 ml of concentrated inhibitor solution and incubated in a 250-ml Erlenmeyer flask with attached Klett tube. Growth was monitored with a Klett colorimeter and was reported as turbidity increase after 12 to 20 hr incubation relative to that of a control flask without inhibitor. At this time, control cultures were in late log or early stationary phase (one Klett unit, using the 54 filter, represents about 3 X 10^6 exponentially growing cells/ml). Growth curves of cultures containing inhibitors indicated that these compounds acted by increasing the generation time of the cells.

Uptake of 14C-labeled compounds. 14C-labeled compounds were added to suspensions of microcysts in CT medium. Samples of 0.3 ml were taken at intervals and filtered through 25-mm diameter, 0.45-μ pore size Millipore or Gelman membrane filters. The filters were washed with 5 ml of ice-cold medium without labeled compounds, dried, and placed in bottles containing 10 ml of scintillation fluid consisting of 70% toluene, 30% absolute ethyl alcohol, 4.0 mg/liter of 2,5-diphenyloxazole and 100 mg/liter of 1,4-bis-2-(5-phenyloxazolyl)-benzene. Radioactivity was determined by use of a Packard Tri-Carb liquid scintillation system.

Chemical analysis. Inorganic phosphate was determined by the method of Sumner (16).

Photomicrographs. Photomicrographs were made with a Zeiss microscope with transmitted light phase-contrast optics at a magnification of 640 times.

RESULTS

Description of germination. Figure 1 depicts phase-contrast photomicrographs of individual cells and turbidity changes of a culture of microcysts undergoing germination in CT medium. Of the cells, 10% were found to have germinated when the culture was examined after 40 min of incubation. Some loss of phase refractivity was observed in most of the microcysts at this time. In the following 20 min, additional microcysts completely lost refractility, to raise the total to about 90%. Almost all of the remaining microcysts subsequently germinated. The newly germinated cells elongated and became crooked and lumpy until division occurred. Such cells tended to collect in large clumps. During the period of germination, there was a reproducible pattern of turbidity changes.

McCormick (12) derived a formula which described the turbidity drop of a suspension of germinating Bacillus cereus spores. This involved a plot of log log 1/Y versus log time when Y represented the fraction of total turbidity change at any time. Such a plot gave a straight line, which led to the formula Y = e^{-kt^2} when K = ln 1/Y, Y = Y at time t = 1, and c was a constant. Vary and Halvorson (17), using a similar formula, studied germination of individual cells and suggested that k might correspond to the microlag, the time interval between introduction of spores into germination medium and beginning of germination of the individual spore; and c might correspond to microgermination time, the time required for complete loss of phase refractivity of the individual spore. A similar plot of our data on microcyst germination resulted in a straight line (Fig. 2). Thus, the turbidity drop of cultures of germinating M. xanthus microcysts can also be described by McCormick's formula.

Temperature. J. G. Holt (Ph.D. Thesis, Purdue Univ., Lafayette, Ind., 1960) reported the optimal growth temperature of M. xanthus to be 30 to 33 C. We examined germination in CT medium at 15, 30, and 37 C. Germination was fastest at 30 C, with 50% germination by 45 min of incubation and 100% by 60 min. Germination was much slower at 15 C, and was absent at 37 C, even after 14 hr of incubation.

pH. The optimal initial pH for vegetative growth of M. xanthus FB is in the range 7.2 to 8.2 (3). Germination was determined in CT
medium which had been adjusted to pH values of 2.0, 4.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.6, 9.0, 11.0, and 12.3. Germination occurred within 3 hr of incubation at an initial pH of 6.5 to 8.5; optimal germination was in the range of 7.0 to 8.0. Loss of phase refractility also took place at pH 12.3, although no outgrowth occurred at this pH. No germination was observed after incubation in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer alone at pH 4.0 to 11.0. This concentration of buffer did not inhibit germination when added to CT medium. Thus, pH changes alone did not induce germination.

Oxygen. The effects on germination of aerobic and anaerobic atmospheres were determined. (Vegetative growth of M. xanthus is strictly aerobic.) Suspensions of microcysts at 1.3 × 10^9 cells/ml in water and CT were incubated in Warburg flasks under N₂ (<7 ppm of O₂) or air. Normal germination occurred in the aerobic flasks, but no germination was observed in the anaerobic flasks.

Heat shock. Some bacterial and fungal spores will not germinate unless activated by sublethal exposure to heat. Accordingly, microcysts were heated in CT medium for various times at 40°C and examined for germination at 30°C. Heating at 40°C did not hasten germination; rather, if continued for more than 10 min, it acted to retard germination. Heating for as long as 120 min did not diminish the ultimate ability of microcysts to germinate. Thus, heat activation was neither required for nor stimulated germination.

Complex media. The following enzymatic hydrolysates of protein at concentrations of 0.1 and 1.0% induced germination within 3 hr of incubation: Trypticase Soy Broth (BBL), vitamin-free Casitone (Difco), enzymatic casein hydrolysate (BBL), Antibiotic Medium No. 3 (Difco), tryptose broth (Difco), Trypticase (BBL), and Proteose Peptone No. 3 (Difco). Yeast extract (Difco) and Casamino Acids (Difco, an acid hydrolysate), which were originally acidic, induced germination at these concentrations only after the pH was adjusted to 7.0. N-Z Amine type A (Sheffield Chemical, Norwich, N.Y.) induced germination only at 1%. The proteins lactalbumin (Nutritional Biochemicals Corp., Cleveland, Ohio) and gelatin (Eastman Organics, Rochester, N.Y.) did not induce germination.

Amino acids. In view of the ability of protein hydrolysates to induce germination, a number of individual L-amino acids were tested. All solutions were in distilled water and were tested at 0.01, 0.05, and 0.1 M at pH 7.0 (except cystine, which was tested at pH 10). Germination was not induced by arginine, cysteine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, theonine, tryptophan, or tyrosine. The following amino acids induced germination: glycine at 0.01 and 0.05 M, alanine at 0.01, 0.05, and 0.1 M, valine at 0.05 and 0.1 M,

![Fig. 1A. Turbidity of a culture of microcysts germinating in CT medium at 5 × 10⁶ cells/ml. One Klett unit, with a 54 filter, represents about 3 × 10⁶ exponentially growing cells/ml.](image)

![Fig. 1B. Phase-contrast photomicrographs of microcysts during germination. Photographs were made after the indicated minutes of incubation in CT medium: (a) 0; (b) 40; (c) 60; (d) 90; (e) 120. Marker represents 5.0 μ.](image)
aspartic and glutamic acids at 0.01 M. In addition, two synthetic amino acid media, S and SF (4), were tested. S medium consists of 17 amino acids, MgSO₄, and potassium phosphate buffer, and supports growth of M. xanthus vegetative cells. SF medium, when solidified with agar, supports microcyst formation from previously starved vegetative cells. Both S and SF media prepared without phosphate buffer also induced germination.

**Other organic compounds.** The following solutions of organic compounds in distilled water were tested and found inactive in inducing germination: sucrose from 10⁻⁴ to 1 M; α-D-glucose, D-fructose, D-xylose, and maltose hydrate from 10⁻⁴ to 5 × 10⁻¹ M; starch from 0.05 to 5.0%; ethyl alcohol from 10⁻⁴ to 1 M; 2-propanol, (t) amyl alcohol, and phenol from 10⁻⁴ to 10⁻¹ M; acetone, formaldehyde, and acetaldehyde from 10⁻⁴ to 1 M; vanillin from 10⁻⁴ to 10⁻³ M; sodium acetate from 10⁻³ to 1 M; sodium bicarbonate, sodium formate, and sodium citrate from 10⁻² to 1 M.

**Chelating agents.** Germination of Bacillus and Clostridium spores (14) and rupture of Azotobacter cysts (10, 13) have been induced by chelating agents. Water solutions of trisodium citrate from 10⁻² to 1 M (pH 6.9 to 7.4) and of ethylenediaminetetraacetic acid from 10⁻³ to 10⁻¹ M (pH 7.0 to 7.2) were tested with M. xanthus microcysts. These chelating agents neither induced germination nor disrupted the microcysts.

**Inorganic compounds.** We observed that potassium phosphate buffer, 0.01 M, pH 7.6 (phosphate buffer), a component of CT medium, induced germination within 2 hr. Phosphate buffer tested between pH 4.0 and 11.0 was found to induce germination at pH 7.0 to 9.5. In this pH range, the predominant anion is HPO₄²⁻. The activity in inducing germination was shown to be related to pH rather than to K⁺ concentration by the inability of 0.01 to 0.1 M solutions of KH₂PO₄ to induce germination. A number of other salts was tested (Table 1), and the anion HPO₄²⁻, as well as the cations Mg⁺⁺, Ca⁺⁺, and NH₄⁺, induced germination. Phosphate buffer reliably induced synchronous germination within 2 hr; induction of germination with Mg⁺⁺, Ca⁺⁺, and NH₄⁺ was not as dependable and required longer incubation.

**Ionic strength.** The ability of salt solutions to induce germination might have been an effect of ionic strength. Accordingly, solutions of KCl and NaCl of the same ionic strength as phosphate buffer were tested and were found to be without ability to induce germination.

**Water germination.** We observed early in this study that microcysts suspended in distilled water occasionally germinated. The concentration of microcysts was found to be a factor in water germination (Fig. 3). All microcysts germinated within 2 hr at concentrations at or above 1.3 × 10⁸ cells/ml. Lower concentrations germinated slower, and at 0.33 × 10⁹ cells/ml and lower no germination occurred, even after extended incubation. Microcysts which germinated in water grew into normal appearing cells which grew and

**Fig. 2.** McCormick plot of turbidity drop of culture of germinating microcysts using data from Fig. 1. Y indicates the fraction of the ultimate turbidity drop occurring at any specific time.
divided when transferred to CT medium. The newly emerged cells did not appear as lumpy and crooked as did cells germinated in CT medium. Germination factor. J. C. Vary (Bacteriol. Proc., p. 37, 1965) found that concentrated water extracts of dormant B. cereus spores induced germination of heat-activated spores. With this in mind, we prepared germination factor by taking the supernatant fluid from microcyst suspensions which had been incubated in water for 1 hr at 1.3 \times 10^9 \text{ cells/ml} \text{ (see Materials and Methods). This }
\text{supernatant fluid induced germination of microcysts at } 1.3 \times 10^9 \text{ cells/ml (Fig. 4). Active preparations could be obtained with as little as 15}\ \text{min of incubation. The pH of the germination factor was the same as that of distilled water. The activity of germination factor was stable to boiling for 5 min but was lost by dialysis or by heating at 900 C for 1 hr. Determinations of the dry weight of microcysts germinating in water (Fig. 5) indicated a loss of about 10\% at the time of germination. We conclude that the active principle in the germination factor was released from the microcysts. Phosphate buffer and germination factor. Since both phosphate buffer and germination factor induced rapid and synchronous germination, it seemed possible that phosphate was the germination factor. Accordingly, germination factor was analyzed for inorganic phosphate (Table 2). Known concentrations of phosphate were also tested. The activity of germination factor seemed related to its phosphate content, with the critical concentration between 0.018 and 0.057 \mu \text{ mole/ml of inorganic phosphate. In order further to test this relationship, germination factor was prepared, the amount of phosphate in the sample was determined, and phosphate buffer containing the same concentration of phosphate was prepared. Parallel dilutions were made of each solution, and all were tested for ability to induce germination (Table 3). The ability to induce germination did not strictly follow inorganic phosphate concentration, which indicated that germination factor contained something which induced germination in addition to simple inorganic phosphate. Uptake of \(^{14}\text{C-labeled compounds. Dworkin and Niederpruem (6) suggested that the inability of microcysts to respire might be a reflection of their decreased permeability to substrates. If this were the case, a general change in permeability of
was determined (Fig. 6). In neither case were large changes in rate of uptake observed during germination or subsequent outgrowth, although a slight decrease in the rates occurred at 60 min. 

Inhibitors. Inhibitor studies were undertaken to learn about physiological requirements for germination from the known modes of action of some inhibitors. The effects of several inhibitors on germination and growth of vegetative cells are shown in Table 4. In most cases, germination was inhibited by about the same concentrations which produced substantial inhibition of growth of vegetative cells, that is, more than 50% inhibition of turbidity increase. Phenethyl alcohol at 0.012 M induced microcyst formation as well as inhibited cell growth (7). Sodium lauryl sulfate caused lysis of vegetative cells; thus, the 81% inhibition of turbidity increase by 0.001% sodium lauryl sulfate probably exaggerates the true inhibition of growth. These results indicated that the processes of germination of microcysts and growth of vegetative cells were both inhibited at about the same order of magnitude of concentrations of a variety of inhibitors.

The effects of the inhibitors novobiocin, gramicidin, chlortetracycline, and sulfanilamide are listed in Table 5. Gramicidin in the concentrations tested was without effect on germination and growth. Again, the compounds which affected germination and growth did so at about the same order of magnitude of concentrations.
TABLE 4. Effect of inhibitors in CT medium on germination of microcysts and growth of vegetative cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc</th>
<th>Microcysts germinated after incubation for</th>
<th>Percentage inhibition of growth of vegetative cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hr</td>
<td>15 hr</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>All</td>
<td>All</td>
<td></td>
</tr>
<tr>
<td>Phenethyl alcohol</td>
<td>0.004 M</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>0.012 M</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>0.024 M</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Sodium lauryl sulfate</td>
<td>0.001%</td>
<td>50%</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>0.01%</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>10^-4 M</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>10^-4 M</td>
<td>None</td>
<td>None</td>
<td>83 %</td>
</tr>
<tr>
<td>Potassium fluoride</td>
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<td>All</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>10^-4 M</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>10^-5 M</td>
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</tr>
<tr>
<td>Mercuric chloride</td>
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</tr>
<tr>
<td></td>
<td>10^-5 M</td>
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<td>None</td>
</tr>
<tr>
<td>Sodium azide</td>
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<td>All</td>
</tr>
<tr>
<td>10^-3 M</td>
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</tr>
<tr>
<td>10^-2 M</td>
<td>None</td>
<td>None</td>
<td>86 %</td>
</tr>
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</table>

* Causes microcyst formation.

W was inhibited at levels substantially below those required to inhibit germination.

DISCUSSION

The course of germination of glycerol-induced microcysts corresponded well with descriptions of germination on agar of fruiting body microcysts (8). The conversion of a bacterial spore to a vegetative cell has been thought of as proceeding in three steps, activation, germination, and outgrowth (11). Microcysts differed in their conversion to vegetative cells, primarily in that activation was not required and germination as well as outgrowth required macromolecular synthesis. Changes in dry weight, phase refractility of individual cells, and the kinetics of the turbidity change of cultures of germinating microcysts resembled those in germinating Bacillus spores, even though electron micrographs of thin sections of both spores and microcysts indicated few structures in common (8, 15).

The lack of change in the rate of uptake of acetate and glycine by microcysts during germination and outgrowth suggests that microcysts and vegetative cells do not differ greatly in permeability to nutrients. However, these experiments do not exclude the possibility of such differences, and the rates of uptake of labeled amino acids may reflect the rates of exchange of internal amino acids rather than net uptake.

Germination of fungal (1) and B. globigii spores (9) has been shown to be inhibited by high spore concentrations. On the other hand, the requirement for high concentrations of microcysts

TABLE 5. Effect of novobiocin, gramicidin, chlorotetracline, and sulfanilamide in CT medium on germination of microcysts and growth of vegetative cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc (µg/ml)</th>
<th>Germination of microcysts after incubation for</th>
<th>Percentage inhibition of growth of vegetative cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hr</td>
<td>15 hr</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>All</td>
<td>All</td>
<td></td>
</tr>
<tr>
<td>Novobiocin</td>
<td>1.0</td>
<td>All</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>All</td>
<td>50%</td>
</tr>
<tr>
<td>Gramicidin</td>
<td>10.0</td>
<td>All</td>
<td>50%</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>0.1</td>
<td>All</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>None</td>
<td>50%</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>1.0</td>
<td>All</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>None</td>
<td>50%</td>
</tr>
</tbody>
</table>
of *M. xanthus*, in order for germination in water to take place, appears to be unique. A reasonable interpretation of water germination in *M. xanthus* would include the release from microcysts of an active substance, with low concentrations of microcysts unable to produce enough of this substance to induce germination. The biological significance of the germination factor is not clear. Germination factor would allow germination of high concentrations of microcysts under poor nutrient conditions, whereas lower concentrations of cells would not germinate. Subsequent lysis of most of the newly germinated cells could provide sufficient nutrients to allow a few of the surviving cells to move about until more suitable conditions were found. In addition, the requirement for high concentrations of cells for germination to take place under conditions of low nutrient suggests a role for fruiting bodies. Rather than acting to aid spore dispersal, a role postulated for other fruiting bodies, they may instead create a cell concentration sufficiently high to insure germination under conditions of low nutrient concentration.

The inhibition of germination and growth by similar concentrations of penicillin and D-cycloserine suggests that loss of phase refractility, i.e., germination, is dependent on the formation of cross-linked peptidoglycan. A requirement for protein synthesis for germination was suggested by the inhibition of germination by chloramphenicol. The protein(s) synthesized might include enzymes which would degrade the microcyst wall-capsule complex. Germination was relatively insensitive to antibiotics of the streptomycin family. These antibiotics are believed to cause misreading of the genetic code by interfering with proper attachment of messenger ribonucleic acid to ribosomes (2). Although it is possible that these antibiotics fail to penetrate the microcyst, the data suggest the interesting possibility that, while protein synthesis is required for germination, the germination messenger ribonucleic acid is polynucleotid (and stabilized) before germination is initiated. The inability of growth-inhibiting concentrations of actinomycin D to interfere with germination is consistent with this hypothesis.

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MICROCYST GERMINATION IN M. XANTHUS

LITERATURE CITED