

Microcysts of the Cellular Slime Mold *Polysphondylium pallidum*¹

I. Factors Influencing Microcyst Formation

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Microcyst formation can be induced by increasing the osmotic pressure of the surrounding medium. Certain ions such as K^+ , Ca^{++} , or Mg^{++} may be needed in the encystment process, and the presence of divalent cations increases the rate of encystment and cyst maturation. Chloride of potassium is optimal for encystment, but other anions of potassium are either less effective or toxic. The optimal pH for encystment was found to be pH 6.0. The use of agar plates containing KCl revealed the importance to the encystment process of inhibiting cell aggregation. When myxamoebae of *Polysphondylium pallidum* strain Pan-17 are deposited on KCl-agar plates, approximately 20% of the population proceeds through aggregation to sorocarp formation at the concentration of KCl optimal for microcyst formation. However, the same proportion of myxamoebae remains unaligned, or forms defective aggregation centers, if synergistic inhibitors (such as incubation in darkness or at low temperature) are employed in addition to KCl. The possibility that this is due to heterocytosis has been excluded. Accordingly, it is suggested that during the stationary phase approximately 20% of the population becomes committed to forming component cells of fruiting bodies, and that these myxamoebae cannot be induced to form microcysts by exposure to KCl. In *P. pallidum* strains WS-320 on the other hand, the imposition of synergistic inhibitors leads to the total encystment of the cell population. This suggests that, in contrast to Pan-17, the myxamoebae of the latter strain remain potentially equal and exhibit minimal presumptive specialization.

After a period of growth, myxamoebae of the cellular slime molds that were formerly free-living, undergo a process of aggregation and form multicellular organizations in which the constituent cells progressively acquire functional specialization and subsequently differentiate as either stalk cells or spores in the communal fructifications, or sorocarps. Under certain conditions, not known prior to this study, some myxamoebae fail to enter the aggregation stage, but instead form microcysts; i.e., the myxamoebae encyst as individuals.

The microcysts of *Polysphondylium pallidum* are typically spherical, range from 4 to 6 μ in diameter, and appear finely granular when viewed with light or phase-contrast microscopy. The cyst walls are highly refractile, and, when stained with appropriate reagents (1), show reactions

suggesting the presence of cellulose. When microcysts germinate on 0.1 L-P agar (lactose, 1 g; peptone, 1 g; Difco agar, 15 g; distilled water to 1 liter), the emergent myxamoebae leave behind empty, thin, hyaline, and seemingly homogeneous cyst walls. The life cycles of this and other cellular slime molds are described and illustrated by Bonner (2) and Raper (15).

Microcyst formation has been observed to occur in several species of slime molds (1), but they have not been carefully investigated as to origin or structure. The purpose of this study was to characterize the environmental factors that lead to microcyst formation in selected strains of *P. pallidum*. To do this, the myxamoebae were first grown in two-membered cultures with bacteria on a nutrient-poor agar and then placed in or upon precisely defined, cyst-inducing media under controlled conditions.

MATERIALS AND METHODS

Slime molds. Differences in handling the two strains employed by us are based upon experimental findings.

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The two strains differ somewhat in the pattern of their fructifications and in their nutritional requirements, as reported by Hohl and Raper (10).

Stock cultures of *P. pallidum* Pan-17 were prepared by inoculating plates of 0.1 L-P agar with a suspension of *Escherichia coli* strain B/r and slime mold spores. The mixed inoculum was spread with a bent-glass rod and the cultures were incubated in continuous fluorescent light at 25 ± 1 C.

Stock cultures of *P. pallidum* WS-320 were prepared by first inoculating plates of 0.1 L-P agar with *E. coli* B/r as wide, crossed bands, after which spores of the slime mold were implanted at the middle of the cross. The plates were incubated at room temperature (24 to 26 C) under normal laboratory conditions of alternate day and night.

Growth medium. Growth medium was 0.1 L-P agar [lactose, 1 g; peptone, 1 g; agar (Difco), 15 g; and distilled water to 1 liter].

Growth and preparation of the test myxamoebae. Plates of 0.1 L-P agar medium were poured at 60 C, cooled immediately, and then kept in a refrigerator for 0 to 6 hr prior to being used for the cultivation of *P. pallidum* Pan-17, and for 24 hr in the case of *P. pallidum* WS-320. The plates were inoculated with 0.05 ml of a mixed suspension of *E. coli* B/r and slime mold spores (4×10^6 to 6×10^6 spores/ml). The age of the spores was 1 to 5 days. The suspension was spread over the agar surface with a sterile, bent-glass rod, and the plates were placed in metal cans for dark incubation at 25 C. Under these conditions, the myxamoebae reached the stationary phase but did not aggregate. Myxamoebae of 34 ± 1 hr in the case of *P. pallidum* Pan-17 and 41 ± 1 hr in the case of *P. pallidum* WS-320 were washed from the surface of the plates with double-distilled deionized water, and were centrifuged 3 times at $175 \times g$ for 5 min to free them of bacteria. In each experiment, it was required that a sample of myxamoebae, tested as a control, must undergo aggregation and form normal fruiting bodies when deposited on non-nutrient agar (1% Difco Purified Agar in double-distilled water).

Induction of encystment in shaken flasks. The myxamoeba suspensions were adjusted to a concentration of 5×10^7 to 7×10^7 cells/ml, and 0.1-ml samples of these suspensions were transferred to the replacement medium. Flasks containing the replacement medium (5 ml of the test solution in 50-ml Erlenmeyer flasks) with added myxamoebae were placed in a Gyrotory shaker (320 rev/min) at 25 C for 24 hr.

Counting procedure. After the flasks were seeded with myxamoebae, their density was determined immediately by hemacytometer counts. After 24 hr, the flasks were swirled on a Vortex Jr. stirrer to break up any clumps of microcysts, and the percentage of microcysts was determined. Such computations were based upon the examination of from 600 to 1,000 cells per count.

Iso-osmotic concentrations. The calculation of iso-osmotic concentrations was based upon available data on the depression of the freezing point of solutions of known compounds at different molar concentrations (8, 13).

Preparation of the test agar plates. The agar plates

for microcyst induction were prepared by heating 1.5% Difco Purified Agar in double-distilled deionized water in the steamer for about 1 hr. Ten ml of the agar was added to 5 ml of the test solution, thus yielding a final agar concentration of 1%. Plates were then poured and left for 24 hr at room temperature before myxamoebae were deposited on them.

Light versus dark incubation. For tests involving the influence of light, a cool-white 15-w fluorescent lamp was placed at a distance of about 20 cm from the plates; the intensity at this level was about 170 ft-c. The plates were placed in metal cans when cultures were to be incubated in the dark. Such dark incubation also necessitated prior processing of the myxamoebae in darkness (washing, centrifuging, and depositing the myxamoebae on the plates).

RESULTS

Effect of osmotic pressure and of different ions. Myxamoebae of strain Pan-17 were inoculated into KCl solutions of different molarity to determine the effect of different salt concentrations on the production of microcysts. The results, presented in Fig. 1, indicated that the optimal concentration for microcyst formation was 0.08 M.

Iso-osmotic solutions of different compounds were then tested, including: NaCl (0.08 M), CaCl₂ (0.056 M), MgCl₂ (0.056 M), K₂HPO₄ (0.062 M), K₂SO₄ (0.062 M), KHCO₃ (0.08 M), KI (0.079 M), KNO₃ (0.082 M), K₃ citrate (0.055 M), K acetate (0.08 M), sucrose (0.144 M), and glucose (0.144 M). The results obtained with some of these are shown in Fig. 2. Glucose and sucrose gave percentages of microcysts approximately equal to KCl. MgCl₂ and CaCl₂ yielded smaller percentages of microcysts when used as single salts, but NaCl alone failed to induce any measurable encystment. When, however, a low level of Ca⁺⁺, Mg⁺⁺, or K⁺ (2 mM) was added in an iso-osmotic solution of NaCl, microcyst formation was increased dramatically, as shown in Fig. 3.

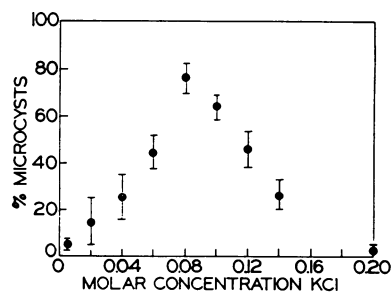


FIG. 1. Percentage of microcysts formed from populations of pregrown myxamoebae of *Polysphondylium pallidum* Pan 17 after incubation in solutions containing different molar concentrations of KCl. Incubation, 24 hr in shaken culture.

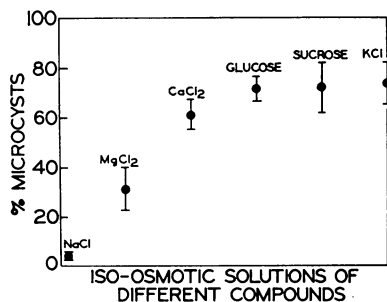


FIG. 2. Comparative effects of iso-osmotic solutions of six different compounds on percentage of microcysts formed from pregrown myxamoebae of *Polysphondylium pallidum* Pan-17. All compounds employed have the same osmolarity. Incubation, 24 hr in shaken culture.

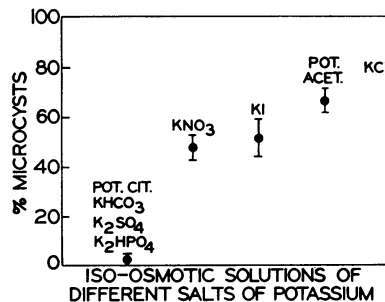


FIG. 4. Effect of different anions, as supplied in iso-osmotic solutions of several salts of potassium, on percentages of microcysts formed by myxamoebae of *Polysphondylium pallidum* Pan-17. A solution of KCl (0.08 M) serves as the control.

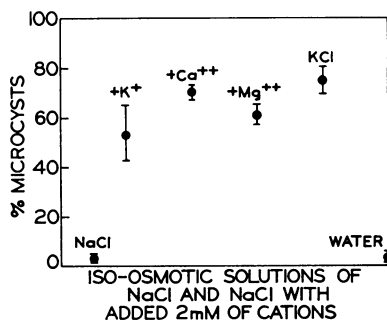


FIG. 3. Stimulatory effect of cations (K^+ , Ca^{++} , or Mg^{++}) upon percentages of microcysts formed by myxamoebae of *Polysphondylium pallidum* Pan-17 when these cations were incorporated at low concentration in an iso-osmotic solution of NaCl. Water and KCl solution (0.08 M) serve as controls.

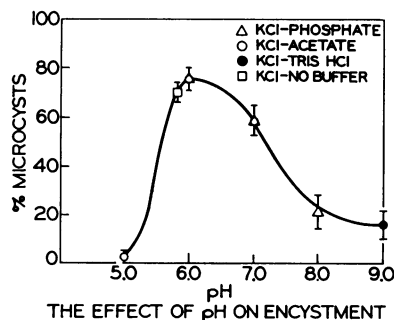


FIG. 5. Effect of pH on microcyst formation by pregrown myxamoebae of *Polysphondylium pallidum* Pan-17 when tested in an iso-osmotic solution of KCl and KCl with different buffers.

Heavy metals in a trace-element solution (10) were toxic and led to the rupturing of the myxamoebae. Anions other than chloride were either less effective or completely destructive of the myxamoebae, as indicated in Fig. 4.

Effect of pH. As seen in Fig. 5, the optimal pH for encystment was found to be pH 6.0; pH 5.0 was toxic to the myxamoebae, and a higher pH either destroyed the myxamoebae or inhibited encystment.

Effect of some other compounds on cyst formation. The addition of chloramphenicol (25 μ g/ml), streptomycin (25 μ g/ml), or glucose (5 mM) to an iso-osmotic solution of KCl did not alter the percentage of microcyst formation. In contrast, ethylenediaminetetraacetic acid (EDTA) at 2 mM concentration reduced the percentage of microcysts to $18 \pm 8\%$.

Rate of encystment. There was usually a 4-hr lag period before myxamoebae started to encyst after they were transferred to the KCl solution

and were further incubated in shaken flasks. The rates of encystment in 0.08 M KCl solution, and in iso-osmotic solutions of KCl with added 2 mM Ca^{++} or Mg^{++} , or both, are shown in Fig. 6. When divalent cations were added, there was an increase in the rate of encystment, and up to 90% of the final yield occurred within 4 hr. The results depicted in Fig. 6 for different time intervals are presented as the percentages of the final yield of microcysts. This was done because the addition of divalent cations usually led to bubbling of some myxamoebae; i.e., part of the cell was pinched off and subsequently encysted. Accordingly, the microcysts were not uniform in size. The presence of divalent cations also increased the rate of cyst maturation. A mature microcyst has a comparatively thick wall, but an immature cyst has a much thinner wall, a difference that is easily detected via phase microscopy. A rounded but unencysted cell appears dark with little or no halo apparent at the cell margin.

Formation of microcysts on agar plates. The results reported with different KCl solutions in

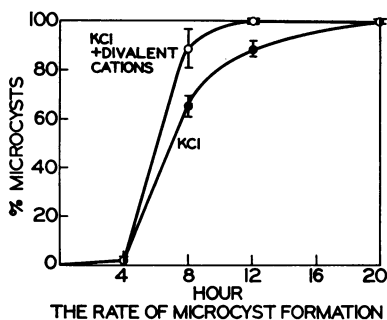


FIG. 6. Rate of microcyst formation by pregrown myxamoebae of *Polysphondylium pallidum* Pan-17 as influenced by the addition to an iso-osmotic solution of KCl of divalent cations (2 mM Ca^{++} and/or Mg^{++}). Rate is expressed as percentage increase with time. Each point represents an average of 10 determinations.

shaken flasks were retested on agar plates. Myxamoebae were prepared as previously described to yield a concentration of 5×10^6 to 7×10^6 myxamoebae/ml; then small drops of the cell suspensions were deposited on agar plates. The population in each drop, which covered an area of 2- to 3-mm diameter, yielded a density of 400 to 600 myxamoebae/mm² on the agar plate. Each plate was covered with a glass lid after excess water had been adsorbed, and the results were recorded after 24 hr. These revealed an interesting situation. As shown in Table 1, the optimal concentration for cyst formation on agar was 0.12 M KCl instead of 0.08, and some small fruiting bodies were still observed.

One important difference between the shaken cultures and the agar plates was that in the former we were mechanically preventing the myxamoebae from aggregating. Because the myxamoebae deposited on agar plates were not so inhibited, some of them might have initiated aggregation before the KCl could become effective, and these myxamoebae might have proceeded to form fruiting bodies while the remainder formed microcysts. If we could inhibit aggregation by imposing factors other than those contributed by KCl, this might lead to encystment of the whole population.

This possibility was tested by examining the effect of several factors which are known to influence the aggregation process, such as dark incubation (3, 11, 14), the addition of certain chemical compounds (4, 9), and incubation at low temperature (12, 14). The effects of different conditions of incubation on cell aggregation and microcyst formation by myxamoebae deposited on agar are shown in Table 2. Note that abundant microcysts were formed on KCl agar independent of light, dark, or the presence of charcoal. In contrast, on KCl-free non-nutrient agar, incuba-

TABLE 1. Determination of the optimal KCl concentration for microcyst formation by myxamoebae of *Polysphondylium pallidum* Pan-17 on agar plates^a

Molar concn of KCl	Amoebae	Aggregates	Fruiting bodies	Cysts
0.0	0 ^b	0	++++	0
0.04	0	0	++++±	±
0.08	±	0	+++	+
0.12	±	0	+	++++
0.16	+	++	0	+
0.20	++++	0	0	0

^a The experiment was carried out in fluorescent light (170 ft-c), and the plates were examined 24 hr after the myxamoebae were deposited. The addition of CaCl_2 to the agar did not change the results.

^b Code: ± to +++++, relative abundance of the developmental stage indicated.

TABLE 2. Comparison between dark and light incubation, and the effect of added charcoal on the production of microcysts vs. sorocarps by myxamoebae of *Polysphondylium pallidum* Pan-17^a

Plate	Incubation	Charcoal	Amoebae	Non-branched sorocarp	Branched sorocarp	Cysts
KCl agar	Light	0	± ^b	±	0	+++
	Dark	0	+	0	0	+++
	Light	+	±	±	0	+++
	Dark	+	±	±	0	+++
NNA (non-nutrient agar)	Light	0	0	++++	0	0
	Dark	0	±	0	+++	±
	Light	+	0	++++	0	0
	Dark	+	±	0	+++	±

^a Possible differences in relative humidity between dark- and light-incubated cultures have been excluded. Plates were examined 24 hr after the myxamoebae were deposited.

^b Code: ± to +++++, relative abundance of the developmental stage indicated.

tion in light wholly precluded microcyst formation and all myxamoebae formed small, non-branched sorocarps, whereas some microcysts developed in dark-incubated cultures along with fewer and somewhat larger (branched) sorocarps. In other experiments, myxamoebae were deposited on KCl agar and incubated at different temperatures from 15 to 35 C. Microcysts were produced at a level of approximately 75% at 15 to 30 C, but sparsely at 35 C; sorocarps developed only (in limited numbers) at 25 and 30 C, whereas fructification occurred from 15 to 30 C on KCl-free agar. In contrast to their behavior on

KCl agar, only 54% \pm of comparable cells formed microcysts when they were placed in KCl solution and incubated in shaken flasks at 15 C. The reason for this disparity is not immediately apparent but may reflect, in large measure, cell response to a liquid environment versus a solid substrate. Several chemical compounds (M. A. Toama, Ph.D. thesis, University of Wisconsin, 1967) were also tested. It was found that most of the compounds did not inhibit aggregation, and when this did occur, it may have resulted from the inhibition of the overall activity of the cells so that no cysts were formed.

From the previous results we can conclude: (i) that approximately 20% of the population is capable of aggregating and forming fruiting bodies on agar in the presence of a concentration of KCl that is optimal for microcyst formation, and (ii) that physical conditions which influence aggregation, such as low temperature and dark incubation, synergistically and completely inhibited aggregation in the presence of KCl. However, these conditions did not lead to an increase in the percentage of microcysts. The same proportion of cells (ca. 20%) remained either as unaligned myxamoebae or formed defective centers.

Heterocytosis. The previous results led us to consider the possibility that the wild type of *P. pallidum* Pan-17 might contain two types of cells (heterocytosis): a cyst former and a noncyst former. This possibility was tested by a method used previously in the cellular slime molds (6), and heterocytosis was not found to occur in *P. pallidum* Pan-17.

Microcyst formation in P. pallidum WS-320. The optimal concentration of KCl for microcyst formation in shaken cultures was found to be 0.06 M, and the percentage encystment was $96 \pm 5\%$. However, when the myxamoebae were deposited on agar plates containing KCl, the optimal concentration of this salt was found to be 0.12 M. When the plates were incubated in light, more than 50% of the cells proceeded to aggregate at the optimal KCl concentration. When the plates were incubated in darkness, a dramatic difference was observed. Practically all of the myxamoebae formed microcysts. This was not observed in similar cultures of *P. pallidum* Pan-17. The rate of encystment in *P. pallidum* WS-300 followed a course similar to that of *P. pallidum* Pan-17, except that the lag period was about 8 hr instead of 4 hr.

When the myxamoebae were washed with 0.02 M KCl solution instead of water, practically all of the myxamoebae proceeded to aggregate on KCl plates in the presence of either light or charcoal, and more than 50% aggregated in the dark. Such

washing did not produce a comparable effect in *P. pallidum* Pan-17.

The synergistic effect of low temperature, in the presence of KCl as an inhibitor of aggregation in WS-320, was observed at 15 C; the percentage of aggregates was dramatically decreased, whereas the percentage of cysts showed a corresponding increase. Thus, synergistic inhibition of aggregation in this strain is linked with an increase in the percentage of microcysts, a situation quite different from that found in *P. pallidum* Pan-17.

DISCUSSION

The encystment of unaggregated myxamoebae of *P. pallidum* was due primarily to increased osmotic pressure. This was shown by using different molar concentrations of KCl and iso-osmotic solutions of different compounds such as glucose and sucrose. It is known that slime mold myxamoebae are not permeable, or have low permeability, to glucose and sucrose during the stationary phase (5, 17). The toxicity of some compounds, which have the same osmolarity as KCl, led to the production of low percentages of encystment, or in some cases destroyed the myxamoebae. Such toxicity could have been due to the cation, as in the case of higher concentration of Mg^{++} , or to the anion, as in the case of potassium salts of SO_4^{++} , HPO_4^{++} , HCO_4^+ , and of citrate. The toxicity of the anion could have stemmed from its high pH, because the optimal pH for encystment was pH 6.0, or it could have resulted from the formation of insoluble complexes with divalent cations.

The absence of encystment in an iso-osmotic solution of NaCl indicated that certain cations, such as K^+ , Ca^{++} , or Mg^{++} , at a concentration of 2 mM may be needed in the encystment process. The importance of divalent cations has been shown by the increased rate of encystment and cyst maturation in their presence and by the inhibiting effect of the chelating agent ethylenediaminetetraacetic acid.

Tests on agar plates, rather than in shaken flasks, revealed two important points in the case of *P. pallidum* Pan-17. (i) Aggregation was inhibited mechanically in shaken cultures, and, as a consequence, the optimal KCl concentration for encystment was less than that on agar plates where the salt is believed to act as an inhibitor for aggregation as well as to provide the optimal tonicity needed for encystment. (ii) Approximately 20% of the population always proceeded through aggregation and sorocarp formation at the optimal KCl concentration; and, even in the presence of synergistic inhibitors of aggregation, such as low temperature or dark incubation, this percentage of myxamoebae remained either un-

aligned or formed only defective aggregation centers.

The possibility that this was due to heterocytosis was excluded. Accordingly, we set forth a hypothesis to explain this behavior. At the stationary phase, a certain percentage (ca. 20%) of the population undergoes an irreversible commitment as "potential center-former," and, following deposition on agar, these myxamoebae will proceed through aggregation and sorocarp formation on KCl plates, unless they are synergistically inhibited from aggregating. The rest of the population (ca. 80%) consists of uncommitted cells or "responders," and these, when triggered by an optimal KCl concentration, will form microcysts. If not so triggered, these cells will join the aggregates previously established by the potential center-forming myxamoebae.

Concepts similar to that of "potential center-formers" have been previously reported for populations of myxamoebae in *Polysphondylium* (7, 16). In cloned populations of *P. violaceum*, Shaffer (16) has clearly shown that an aggregation center is initiated by the differentiation of a single cell, "the founder," and that such cells attract the remainder of the population. The percentage of myxamoebae capable of functioning as potential founders is ca. 10%. Two types of cells have also been previously reported in *P. pallidum* Pan-17 (7); one is the acrasin-producing cell which forms the center, and the other the acrasin-sensitive cell which responds to the attraction of the former, but none of which produces significant acrasin per se. Under the conditions of his tests, there was one center for every 32 cells.

Whether the center-formers (7), the founders (16), and the potential center-formers of strain Pan-17 are the same, and whether the percentage is 3 or 10 or 20%, cannot be ascertained at the present. Variation in the percentage could be due to differences in the methods of growth and testing. Nevertheless, the data strongly suggest that there are two types of cells, one of which achieves at an early stage a certain state of differentiation not possessed by the other.

It is not known why some of the cells become "potential center-formers"; neither is it known what determines the percentage of these, or what is their ultimate fate in the normal developmental process. The ability of these cells to form fruiting bodies consisting of spores and stalk cells, in the presence of optimal KCl, seems to indicate, however, that they are committed to sorocarp formation at an early stage.

The myxamoebae of *P. pallidum* WS-320 did not show the same type of behavior as Pan-17.

Apparently, all the cells of this strain remain potentially equal for a longer period of time, and whether they form aggregates or cysts will depend upon the environmental conditions that surround the cells. Thus, when the cells are deposited on KCl plates, which inhibits aggregation, and in the presence of light, which stimulates aggregation, the percentage of myxamoebae forming microcysts will be about 50%. However, when synergistic inhibitors such as dark incubation combined with low temperature were employed, cyst formation was favored and high percentages of microcysts were obtained (about 100%). If synergistic stimulants to aggregation, such as washing the cells with 0.02 M KCl instead of water were used, aggregation was favored and involved percentages of myxamoebae greater than 50%. Thus, the conversion of a whole population to microcysts depended primarily on the complete inhibition of aggregation (KCl, washing with distilled water, and incubation in darkness and at low temperature), coupled with the presence of microcyst-inducing agents and conditions (optimal toxicity and the presence of certain ions: Ca^{++} , Mg^{++} , K^+).

The results obtained with *P. pallidum* ES-320, although different from those obtained with Pan-17, tend to support the view that the aggregates formed by the latter strain, under conditions optimal for microcyst formation, were due to the presence of "potential center formers." This does not appear to be the case with WS-320. Once more it is emphasized that what is true for one organism is not necessarily true for another strain, even within the same species.

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