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Induction of Cellular Morphogenesis in *Myxococcus xanthus*

I. General Description

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ABSTRACT

DWORKIN, MARTIN (University of Minnesota, Minneapolis), AND WILLIAM SADLER. Induction of cellular morphogenesis in *Myxococcus xanthus*. I. General description. J. Bacteriol. **91**:1516–1519. 1966.—The details of a process for converting vegetative rods of *Myxococcus xanthus* to microcysts rapidly (120 min), quantitatively, and synchronously are presented. The conversion is induced by 0.5 M glycerol. On the basis of a number of morphological and physiological parameters, the microcysts thus produced are identical with those produced within fruiting bodies. Microcyst formation requires Mg^{++} or Ca^{++} and aeration, and is considerably stimulated by the presence of the growth medium. The process is reversible, by the removal of the inducer, and will take place only with exponentially growing cells. Mutants unable to form microcysts have been isolated. These are also deficient in their ability to form fruiting bodies.

"It is perhaps remarkable that biology, which was for so long a morphological science has made so little attempt, now that it has passed into the experimental phase, at a causal analysis of the mechanisms that give use to the shapes which have been so extensively studied" (8).

Undoubtedly, part of the difficulty can be attributed to the relative structural complexity of the systems traditionally studied. The success of Cantino (2), working with *Blastocladiella*, an aquatic Phycomycete, and of Wright (9), working with the slime mold *Dictyostelium*, suggests that the task of obtaining biochemical correlates of morphogenesis may be considerably simplified by using a microbial system. A further simplification should result from using a procaryotic rather than a eucaryotic organism as the experimental system.

Myxococcus xanthus is a fruiting myxobacterium which demonstrates colonial morphogenesis (chemotactic aggregation of vegetative rods to form fruiting bodies) as well as cellular morphogenesis (conversion of vegetative rods to round, refractile microcysts). In addition to undergoing a well-defined life cycle, the organism possesses most of the convenient attributes of the more conventional bacteria, and is thus amenable to the techniques which have made bacteria an effective tool for biochemical and genetic investigations.

We previously described a method for inducing the conversion of vegetative rods to microcysts quantitatively, relatively synchronously, and rapidly (about 120 min; 5). The purpose of the present report is to describe the conversion process in some detail.

MATERIALS AND METHODS

Organism. M. xanthus strain FB (4) was used in all experiments. This strain is morphogenetically competent, forming fruiting bodies and microcysts, and will grow in a dispersed state in liquid media.

Cultivation. The details of growth and cultivation of *M. xanthus* strain FB have been presented elsewhere (3). Inocula for experiments were prepared from cells growing in liquid CT medium [2% Casitone (Difco), 0.01 M K_2HPO_4-KH_2PO_4 (*p*H 7.5), 0.008 M $MgSO_4$].

Induction of microcyst formation. A typical microcyst induction experiment was performed as follows. An exponentially growing culture of *M. xanthus* FB grown in liquid medium (40 ml in a 300-ml Erlenmeyer flask, incubated with shaking at 30 C) was chilled, centrifuged at 0 C, washed once with 40 ml of cold distilled water, and inoculated into 1 or 2% Casitone with 8×10^{-3} M $MgSO_4$. The initial cell density was usually about 5×10^8 cells per milliliter (about 200 Klett units with a no. 66 filter). The culture

was incubated with shaking at 30 C, and microcyst formation was initiated by the addition of glycerol to a final concentration of 0.5 M. Alternatively, the washed cells were concentrated to about 10^{10} cells per milliliter (about 4,000 Klett units with a no. 66 filter), 5 ml was placed in a test tube (17 by 150 mm), Dow-Corning antifoam was added, and the culture was vigorously bubbled with air through a Pasteur pipette. Morphological changes were observed by means of phase-contrast microscopy.

RESULTS

Description of conversion. A figure describing morphological and turbidity changes in a culture of *M. xanthus* undergoing glycerol-induced microcyst formation has already been published (5). The optical density of the culture rises immediately after the addition of glycerol and within a minute or so drops somewhat below the original value. There is then a period of 10 to 15 min during which there is no change in the turbidity. This is followed by a linear decrease which levels off at about 60 min. There is then a stable period of about 15 min, followed by a slow linear increase.

Morphologically, no change is seen until 30 to 40 min have elapsed. Then the rods begin to shorten, and at about 60 min are clearly ovoid shaped. Within a 5- to 10-min period, the ovoids convert to nonrefractile spheres which then slowly acquire refractility over a 50- to 60-min period. During the ovoid-to-sphere conversion the cells begin to form clumps.

The process is quite synchronous, and almost 100% of the cells are converted to microcysts.

A comparison of glycerol-induced microcysts with fruiting body microcysts revealed the following similarities.

(i) *Morphological change.* When vegetative rods of *M. xanthus* are placed on an agar medium consisting of autoclaved cells of *Escherichia coli* or 0.05% tryptone, they will convert to microcysts as a part of the normal developmental cycle. The sequence of morphological changes involved in the conversion has been described (6), and is identical with that occurring during the glycerol-induced conversion of rods to microcysts.

(ii) *Effect of sonic treatment.* Suspensions of vegetative cells, spheroplasts (produced by incubation of vegetative cells in the presence of 4.0 M glycerol), fruiting body microcysts (vegetative cells incubated for 9 days on 0.05% tryptone-agar plates), and glycerol-induced microcysts were subjected to sonic treatment for 1 min in an MSE sonic oscillator. Microcysts produced within fruiting bodies have a tendency to form clumps and are therefore difficult to count. In this case the suspension of 9-day-old microcysts was dispersed with a glass Potter-Elvehjem type tissue homogenizer. The remaining clumps were then allowed to settle out. There were 1.1 and 0.6% survivors of vegetative cells and spheroplasts, respectively, whereas the numbers of both glycerol microcysts and plate microcysts increased slightly (to 112%). This increase probably reflected a disruption of some remaining clumps of microcysts by the sonic treatment.

(iii) *Germination.* Fruiting body microcysts and glycerol microcysts were compared in terms of their ability to germinate. The suspensions were treated in an MSE sonic oscillator for 30 sec at 4 C (to remove remaining vegetative cells), counted in a Petroff-Hausser counter, and then plated on CT agar. In both cases, there was close to 100% germination efficiency.

(iv) *Susceptibility to bacteriophage infection.* A bacteriophage for *M. xanthus* (MX-1) will not adsorb to either plate microcysts or glycerol microcysts (1).

Nutritional requirements for microcyst formation. Glycerol-induced microcyst formation proceeds optimally in the presence of CT medium containing from 0.3 to 2% Casitone. A defined amino acid mixture (3) which will support growth of *M. xanthus* (generation time of about 10 hr compared with 4 hr on CT medium) will also support microcyst formation in the presence of glycerol. Under these conditions, however, the process is asynchronous and requires about 5 hr for complete conversion.

Inorganic requirements. Two different basal media were used for determining the inorganic requirements for microcyst induction by glycerol —either the defined amino acid growth medium or 1% Casitone which had been partially deionized by passage through an AG11A8 ion retardation (Bio-Rad Laboratories, Richmond, Calif.) column. With the defined medium, Ca^{++} was absolutely required. A suitable concentration was found to be 1.5×10^{-2} M. In the deionized Casitone, either Mg^{++} or Ca^{++} at 5×10^{-3} M was required. In neither case did microcyst formation take place without the addition of the divalent cation.

We have no information regarding the amount of Ca^{++} or Mg^{++} remaining in the partially deionized Casitone.

Effect of temperature. Two parameters were used for evaluating the effect of temperature on microcyst formation. These were the length of time before visible morphogenetic change and the time between the appearance of visible morphogenetic change and the appearance of refractility. The results listed in Table 1 indicate an optimal temperature at about 35 C.

Effect of glycerol concentration. Between con-

TABLE 1. *Effect of temperature on microcyst formation*

Temp	Effect	
	Length of lag	Additional min for appearance of refractile spheres
	min	
10	—*	—*
20	160	200
30	35	60
35	30	60
40	40	70

* No microcysts formed after 21 hr.

centrations of glycerol of 0.4 and 1.0 M, there is no marked difference in the rate or extent of microcyst formation. At concentrations higher than 1.0 M, the rate of formation decreases, and at concentrations greater than 3.0 M spheroplasts rather than microcysts are formed. Below 0.4 M there is a sharp decrease in the percentage of the population which is converted to microcysts and at concentrations of 0.1 M and lower no changes at all are induced (Table 2).

Reversibility. If, at any stage prior to the appearance of nonrefractile spheres, the cells are centrifuged, washed with cold distilled water, and resuspended in CT medium in the absence of glycerol, the sequence of morphological changes leading to that stage is reversed and the cells revert to vegetative rods.

Effect of growth phase of the culture. A stationary-phase culture was used to inoculate fresh CT medium. Cells were then removed after 2, 12, 23, 35, and 73 hr of incubation. These represented lag, middle-log, late-log, early-stationary, and late-stationary phase cells. The cells were then tested for their response to 0.5 M glycerol. Cells from middle-log phase of growth behaved in a typical fashion, undergoing a 40-min lag, with refractile spheres appearing at 100 min. With late-log phase cells, the lag was 50 min, with refractile spheres beginning to appear at 110 min. Cells from the early portion of the stationary phase required a 70-min lag before beginning the conversion. Refractile spheres then began to appear at 140 min. Cells from late stationary phase failed to undergo any significant amount of conversion. Finally, lag-phase cells required a 170-min lag with refractile spheres beginning to appear at 250 min (Table 3).

TABLE 2. *Effect of concentration of glycerol on microcyst formation*

Concn of glycerol	Effect		
	Length of lag	Additional min for appearance of refractile spheres	Per cent microcyst formation
M	*min*		
5.0	—*	—*	
3.0	75	305	>99
2.0	40	100	>99
1.0	35	65	>99
0.5	35	55	>99
0.4	35	60	>99
0.3	50	60	90–95
0.2	55	60	<10
0.1	—†	—†	

* Spheroplasts formed in 30 min.
† No microcysts formed.

Effect of aeration. Erlenmeyer flasks (250-ml volume) containing 5, 20, 40, and 100 ml of CT medium plus 0.5 M glycerol were inoculated and incubated with shaking. A fifth flask containing 40 ml of the glycerol-CT medium was inoculated and incubated without shaking. Flasks with 5 and 20 ml of medium formed microcysts rapidly and typically. The others formed microcysts much less rapidly. The stationary flask required the longest period of time both for the initiation (2 hr) and completion (4 hr) of the morphological change.

Effect of glycerol on other myxobacteria. M. xanthus strain VC will form neither fruiting bodies nor microcysts on any of the media which normally permit the expression of the developmental cycle. When *M. xanthus* VC is exposed to 0.5 M glycerol in CT medium, the cells convert to microcysts in a manner identical with strain FB.

A species of *Cytophaga* and *Sporocytophaga myxococcoides* (ATCC 10010) are not induced to form microcysts by the glycerol technique.

Isolation of microcyst-nonforming mutants. A small percentage of the population (about 1%) consistently failed to form microcysts. These residual vegetative cells were separated from the microcysts by centrifugation at about $10^3 \times g$ for

TABLE 3. *Effect of age of culture on microcyst formation*

Growth phase of cells	Microcyst formation	
	Length of lag	Additional min for appearance of refractile spheres
	min	
Lag	170	80
Middle log	40	60
Late log	50	60
Early stationary	70	70
Late stationary	—*	—*

* No microcysts formed.

5 min. Under these conditions, most of the vegetative cells remained in the supernatant fluid. These were cultivated and induced, and the cycle was repeated five times. There was an ever-increasing percentage of microcyst-nonforming cells in the culture, culminating in a population which demonstrated no conversion whatever to microcysts. Two such variants, designated FB-s3 and FB-s4, were isolated and checked for their ability to form fruiting bodies. FB-s3 formed rudimentary fruiting bodies containing no microcysts while FB-s4 formed none whatever.

DISCUSSION

The data presented permit the conclusion that glycerol microcysts and plate microcysts have fundamentally the same properties. Further, on the basis of morphological events leading to their formation, germination ability, and response to sonic vibration, we may clearly distinguish between glycerol microcysts and spheroplasts.

We have no direct information which would explain the absorbancy changes in the culture during microcyst formation. The fact, however, that absorbancy begins to decrease at a linear rate at least 15 min prior to any visible morphological change suggests that the decrease reflects a change in the optical properties of the cell rather than an alteration in its shape.

The Ca^{++} requirement for microcyst formation in the defined medium is reminiscent of the report by Fluegel (Bacteriol. Proc., p. 46, 1962), which demonstrated that $CaCl_2$ was required for fruiting body formation by *M. fulvus*. Since we have no data on the inorganic composition of the so-called deionized Casitone, it is difficult to interpret the observation that, with Casitone instead of the defined amino acid mixture as the medium for microcyst formation, Mg^{++} replaces Ca^{++}.

Humphrey and Vincent (7) demonstrated that walls of *Rhizobium* contain most of the cellular Ca^{++} and that Ca^{++} deficiencies induce morphological aberrations. It is quite possible that in *M. xanthus* the reorganization of the cell wall (which is most likely a part of microcyst formation) requires the structural participation of Ca^{++}.

The reversibility of microcyst formation when glycerol is removed is most interesting. It should be emphasized that this does not represent germination but rather an exact reversal of the stages leading to microcyst formation.

For optimal microcyst formation to occur, the cells must be taken from the exponential phase of growth, must be well aerated in a growth medium, and must be kept at a temperature between 30 and 35 C. In other words, the requirements for optimal growth must be satisfied for glycerol induction of microcyst formation to take place optimally. It is not yet clear what relation, if any, this process has to microcyst formation within the fruiting body, where availability of oxygen is clearly limited and where the cells may no longer be in the exponential phase of growth.

It is of considerable interest that variants selected on the basis of their inability to form microcysts were also found to be deficient in fruiting body formation. This suggests either that microcyst formation is somehow causally related to fruiting body formation, or that the genetic loci for the two properties are so closely linked that a deletion has resulted in a loss of both of them.

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