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Regulation of Development in *Myxococcus xanthus*: Effect of 3′:5′-Cyclic AMP, ADP, and Nutrition

(fruiting body formation/cell–cell interaction/differentiation/assay for cAMP/scanning electron microscopy)

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ABSTRACT **An assay was developed to study the regulation of fruiting in *Myxococcus xanthus*. The nucleotides, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and adenosine diphosphate (ADP), were found to greatly stimulate fruiting under the assay conditions. Very sharp concentration optima were observed. Even under conditions of starvation, these nucleotides greatly increased the number of aggregation sites. Nutrition was found to influence fruiting body morphology.**

The effect of amino acids on the nucleotide stimulation of fruiting was studied under our assay conditions. L-Methionine and L-isoleucine (1 mM) completely blocked the cyclic-AMP- or ADP-stimulated fruiting. In contrast, either L-threonine or D,L-diaminopimelic acid synergistically enhanced the amount of fruiting in the presence of these nucleotides. The data presented suggest the existence of differentiation-related regulatory compounds in *M. xanthus*.

Myxococcus xanthus is a Gram negative, rod-shaped bacterium that grows in nature on decaying organic material and, occasionally, by preying upon other microorganisms (1, 2). Under certain nutritionally limiting conditions, vegetative cells migrate toward specific sites, accumulating in mounds. Individual cells within the mounds undergo morphogenesis to form myxospores. Mounds of mature myxospores are referred to as fruiting bodies. Thus, the *Myxobacteria* represent an exciting bacterial system for the study of problems related to cell–cell interaction and the regulation of differentiation (3).

The effects of nutrition on fruiting body formation have been studied in several laboratories (4–6). Our method in this study was to find a growth medium for *M. xanthus* that would permit vegetative growth under conditions just below the threshold of starvation and fruiting. Presumably, vegetative cells in this physiological state would recognize and respond to added chemical signals that might normally trigger fruiting. Using a semiquantitative assay, we have found evidence for the existence of specific differentiation-related regulatory compounds in *M. xanthus*.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions. *M. xanthus* FB strain DZ2 was obtained from a pure culture maintained by Mrs. Mary Human. Cultures were grown in liquid medium containing 1% Casitone (Difco) and 0.1% $MgSO_4 \cdot 7H_2O$ at 30° with aeration. *Escherichia coli* strain CA8306, deficient in adenyl cyclase, and therefore unable to synthesize cyclic AMP (cAMP), was obtained from J. Beckwith.

Assay for Fruiting Body Formation. The standard assay for fruiting consisted of spreading 25 μl of a washed cell suspension of strain DZ2 on test agar and incubating the plates at 30° for 5–7 days. The plates were examined under the dissecting microscope and the number of fruiting bodies per plate determined. The washed cell suspension was obtained from an exponentially growing culture by centrifugation at 1000 × *g* for 10 min, followed by a single wash and resuspension in 0.1% $MgSO_4 \cdot 7H_2O$ at a cell density of approximately 2×10^8 cells per ml. The test agar contained 0.1% Casitone, 0.1% $MgSO_4 \cdot 7H_2O$, 1.5% Bacto agar (Difco), and the chemical to be assayed. The melted agar was dispensed aseptically into 12 × 50-mm disposable petri dishes with tight lids (Falcon, no. 1006); final volumes were 2 ml. Assays were performed in triplicate.

In some experiments the assay was modified by omitting the Casitone from the test agar. For these experiments, the washed cell inoculum was concentrated to 2×10^{10} cells per ml.

Fruiting Body Formation on E. coli Medium. Standard 15 × 100-mm petri dishes were filled with 25 ml of autoclaved medium containing about 2.5×10^{10} washed stationary phase cells of *E. coli* CA8306, 0.1% $MgSO_4 \cdot 7H_2O$, 1.5% Bacto agar, and supplements as described in the *text*. Fruiting bodies were examined microscopically after 5 days.

Electron Microscopy. Fruiting bodies were prepared for scanning electron microscopy by affixing a thin piece of agar containing the fruiting bodies to a stub using an adhesive made by dissolving Scotch transparent tape in chloroform. Specimens were fixed with 1% osmium vapor for 6–24 hr, placed in a vacuum evaporator, and coated with palladium–gold. Specimens were examined under the Coates–Welter Cwik-scan field emission scanning electron microscope and photographed with Polaroid type 55 P/N film (Polaroid Corp., Cambridge, Mass.).

Chemicals. All compounds used in the fruiting assay medium were purchased from Sigma Chemical Co., St. Louis, Mo. All amino acids used were the L isomer unless otherwise specified.

RESULTS

Stimulation of Fruiting Body Formation in M. xanthus by cAMP. The important role of 3′:5′-cyclic AMP in fruiting body formation in the cellular slime mold *Dictyostelium discoideum* (7, 8) led us to investigate whether a possible role(s) for this compound exists in the developmental cycle of *M. xanthus*. In a preliminary experiment, about 10^8 exponential phase bacteria were spread on petri plates containing 0.1% Casitone and 0.1% $MgSO_4 \cdot 7H_2O$, a medium reported to support some fruiting (6). Crystals of cAMP were deposited in the center of experimental plates; other plates were main-

tained as controls. After 5 days of incubation at 30°, the difference between the experimental and control plates was very striking. Control plates with no added cAMP had no fruiting bodies. The cAMP plates showed three concentric zones: an inner zone of growth inhibition, a zone of very extensive fruiting, and an outer zone of vegetative growth.

A Semiquantitative Assay for Fruiting Body Formation. Modifications in the growth medium and inoculum were investigated to determine the optimal experimental conditions for testing the biological activity of the compounds under study. Fig. 1 shows the effect of starvation on fruiting body formation in the absence or presence of cAMP. In the absence of cAMP, fruiting body formation was stimulated by starvation and inhibited by high Casitone concentrations. Fruiting could be stimulated by cAMP only at levels of nutrition near the threshold of starvation. The results indicated that 0.1% Casitone was a good threshold medium for subsequent experiments.

Cell density was also found to affect fruiting. An increase in the cell density of the inoculum from 2×10^7 cells per ml to 2×10^{10} cells per ml caused a decrease in the number of fruiting bodies per plate, from 237 to 51 in the presence of cAMP and from 88 to 0 in the absence of cAMP. The value chosen for our standard assay conditions was 2×10^8 cells per ml.

Other factors such as freshness of the medium, humidity, temperature, scratches on the agar, and physiology of the inoculum also were found to influence the quantitative results. Since some of these factors were difficult to control, specific numbers of fruiting bodies per plate are less meaningful than relative numbers.

The morphology of the fruiting bodies that formed on the

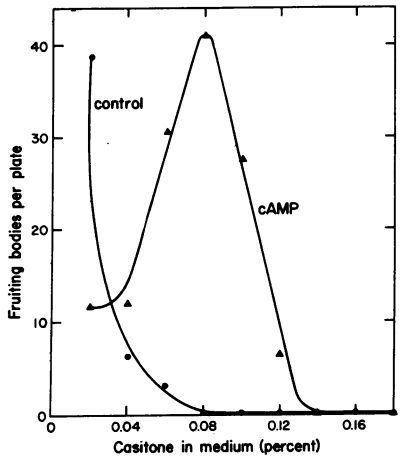

FIG. 1. Effect of nutrition (Casitone concentration) on fruiting in the presence or absence of cAMP. Petri plates (12 × 50 mm) containing 1.5% agar and Casitone, with or without cAMP (0.15 mM), were inoculated with 25 μl of a washed exponential cell suspension (2×10^8 cells per ml). Plates were incubated 5 days at 30°. Fruiting bodies were counted under a dissecting microscope.

Casitone assay medium differs from that of fruiting bodies that formed on *E. coli* agar (Fig. 2). While the most prominent structure on both media was a mound of myxospores, the Casitone fruiting bodies were usually flatter and broader; occasionally they were surrounded by a ring of myxospores.

Effect of cAMP, ADP, and Other Adenylates on Fruiting. Under our standard assay conditions, cAMP stimulated

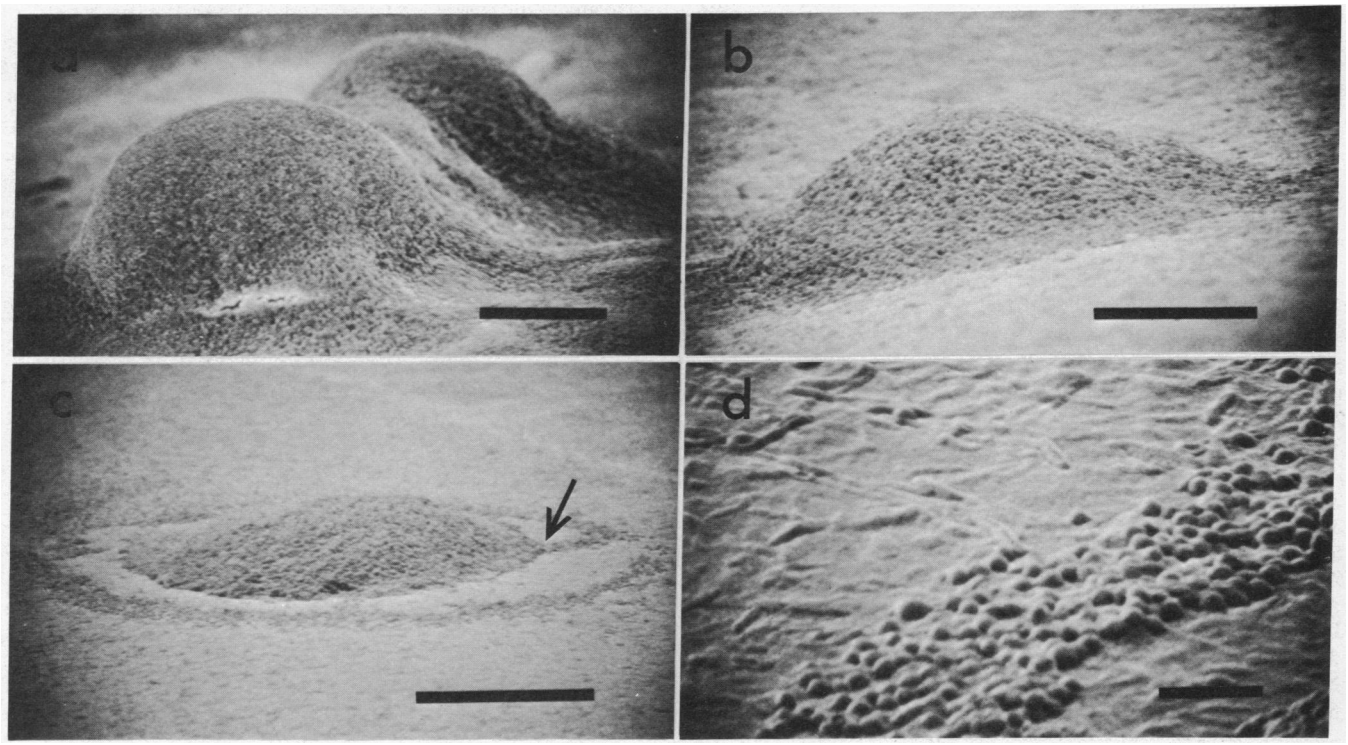

FIG. 2. Scanning electron micrographs of fruiting bodies of *M. xanthus* DZ2. (a) Cells grown on *E. coli* agar. The bar represents 50 μm. (b and c) Cells grown on 0.1% Casitone medium. The *arrow* indicates the focal point for migration towards the fruiting body from surrounding positions. The bar represents 50 μm. (d) Detail of Fig. 2c showing the ring of myxospores surrounding some fruiting bodies. The ring may be the result of cellular morphogenesis within an oriented slime trail. The bar represents 5 μm.

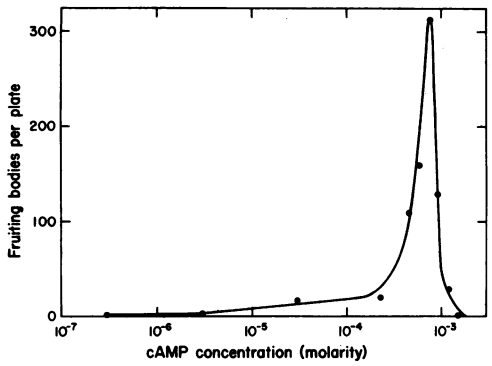

FIG. 3. Effect of cAMP on fruiting body formation. The assay conditions were as described in *Materials and Methods*.

fruiting (Fig. 3). Variation of the cAMP concentration had a great effect upon the number of fruiting bodies per plate. The optimum concentration was about 0.75 mM. Concentrations of 1.5 mM and above inhibited growth and fruiting.

Preliminary experiments show that *M. xanthus* excretes cAMP into the medium while fruiting. Colonies grown on indicator plates show *M. xanthus* feeding cAMP to *E. coli*. The indicator plates contained 5-bromo-4-chloro-3-indolyl β-D-galactoside, isopropyl-β-D-thiogalactoside, arabinose, M-9 salts (modified to contain 0.01 M phosphate), *E. coli* agar, and live *E. coli* CA8306. Under these conditions the *E. coli* lawn produced a blue halo around fruiting colonies of *M. xanthus*.

Other compounds were tested for their ability to stimulate fruiting (Table 1). To our surprise, ADP was the most potent compound tested. Adenosine triphosphate, adenosine 2′-monophosphate, adenosine 3′-monophosphate, and adenosine 5′-monophosphate also showed lower but significant stimulation of fruiting. Phosphate alone had no stimulatory activity at the concentrations tested. The other nucleotides tested had no stimulatory effect on fruiting. These included uridine 5′-monophosphate, guanosine 5′-monophosphate, cytidine 5′-monophosphate, and guanosine 3′:5′-cyclic monophosphate.

TABLE 1. *Stimulation of fruiting body formation in M. xanthus by selected adenine-containing compounds**

Addition to test medium	Concentration range tested (mM)	Fruiting bodies per plate†	Percent of ADP
None	—	0	0
Inorganic PO_4	0.1–1.0	0	0
ADP	0.012–2.3	650	100
3′:5′-cAMP	0.015–1.5	314	48
2′:3′-cAMP	0.014–1.4	65	10
2′-AMP	0.014–1.4	123	19
3′-AMP	0.014–1.4	132	20
5′-AMP	0.014–1.4	131	20
ATP	0.009–0.9	110	17
Adenosine	0.019–1.9	40	6
Deoxyadenosine	0.020–2.0	25	4
Adenine	0.038–3.8	42	6
NAD	0.008–0.8	5	1
S-adenosylmethionine	0.10–1.0	0	0

* The assay conditions are as described in *Materials and Methods*.

† The data presented are the average number of fruiting bodies obtained at the concentration optimum for fruiting.

TABLE 2. *Effect of methionine, isoleucine, and threonine on fruiting body formation in the presence and absence of cAMP and ADP**

Additions:	None	cAMP (0.3 mM)	ADP (0.23 mM)
None	2	63	199
Methionine (2.0 mM)	0	0	0
Isoleucine (6.0 mM)	0	0	0
Threonine (1.0 mM)	93	607	984

* The assay conditions were as described in *Materials and Methods*. The data presented represent the mean number of fruiting bodies per plate.

cAMP and ADP stimulated the fruiting response when added to nonnutritive medium consisting of only agar and 0.1% $MgSO_4 \cdot 7H_2O$ (Fig. 4). Under these conditions, there was no net cell growth. The bacteria aggregated at a few centers, where they subsequently differentiated to form myxospores. The fruiting bodies were planar, as shown in Fig. 5, unlike the mounds observed on either *E. coli* agar or 0.1% Casitone agar (Fig. 2). Increased numbers of planar fruiting bodies were formed in the presence of cAMP and ADP, although they were smaller in size than the controls. Thus, the cAMP and ADP effect on fruiting frequency was separate from the starvation-dependent effect on fruiting body morphology.

Effect of Amino Acids on Fruiting Body Formation. Rosenberg *et al.* reported that methionine and isoleucine inhibited fruiting while threonine reversed that inhibition (6). We tested various concentrations of all the amino acids under our assay conditions. We found that methionine and isoleucine were in fact very potent inhibitors of fruiting and moreover completely reversed the cAMP or ADP stimulation of fruiting (Table 2). In contrast, either threonine (Table 2) or D,L-diaminopimelic acid (Table 3) synergistically enhanced the cAMP or ADP stimulation of fruiting. The inhibitory effect of methionine and isoleucine even prevented fruiting on *E. coli* agar, the most potent fruiting medium available except

TABLE 3. *Effect of diaminopimelic acid plus cAMP on fruiting body formation**

Addition to test medium (mM)		
D,L-Diaminopimelic acid	cAMP	Fruiting bodies per plate
0.0	0.0	10
1.0	0.0	5
2.0	0.0	8
4.0	0.0	23
8.0	0.0	94
0.0	0.15	100
1.0	0.15	125
2.0	0.15	317
4.0	0.15	279
8.0	0.15	527

* The assay conditions are as described in *Materials and Methods*.

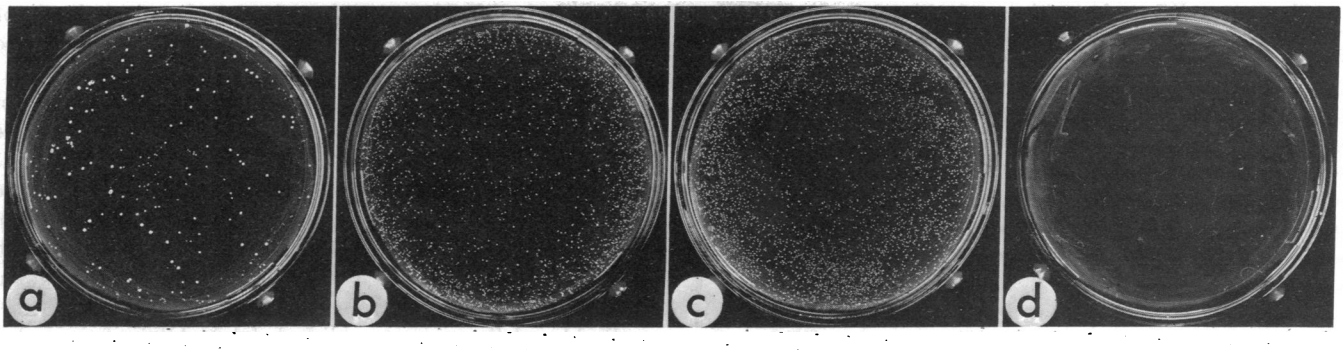

FIG. 4. Effect of cAMP on cellular aggregation and fruiting on nonnutritive medium. The plates were prepared and inoculated as described in *Materials and Methods*. (a) No addition; (b) +cAMP, 0.075 mM; (c) +cAMP, 0.15 mM; (d) +cAMP, 0.6 mM. The petri plates are 50 mm in diameter.

for rabbit dung pellets (Table 4). The methionine analogues, ethionine and norleucine, as well as high concentrations of lysine or Castitone, were also found to inhibit fruiting.

DISCUSSION

The data presented suggest that the triggering of fruiting body formation in *M. xanthus* is subject to multiple controls. One level of regulation appears to be under nutritional control. For example, 0.2% Casitone totally prevented fruiting, even in the presence of *E. coli* agar. This level of regulation is altered in a mutant of *M. xanthus* that forms fruiting bodies on medium containing 1% Casitone (6). The data in Table 4 show that methionine and isoleucine will completely block fruiting at low levels (1 mM), while lysine will block fruiting at higher levels (4 mM). This result suggests that these amino acids are at least some of the nutritional factors regulating fruiting. If the amino-acid composition of Casitone is about the same as casein (9), then 0.2% Casitone contains about 1 mM isoleucine, sufficient amounts to account for the inhibition of fruiting in this medium.

Rosenberg *et al.* (6) observed that methionine, isoleucine, and spermidine, positive effectors for aspartokinase activity in *M. xanthus* (10), inhibited fruiting. In contrast, threonine, an inhibitor of aspartokinase activity, reversed this inhibition of fruiting. To explain this correlation, they hypothesized that a decrease in aspartokinase activity induced starvation for diaminopimelic acid which, in turn, triggered fruiting. The results in Table 3 show that exogenous diaminopimelic acid actually stimulated fruiting. Further, it is not reported to be an inhibitor of aspartokinase activity in *M. xanthus* (10), although it does inhibit aspartokinase I in *Bacillus subtilis* (11). However, the inverse correlation between aspartokinase activity and the induction of fruiting is still very interesting and may account, in part, for the tremendous stimulation of fruiting we obtained in the presence of exogenous ADP, an inhibitor of the activity (6).

Nutrition also affects the morphology of fruiting bodies (Figs. 2 and 5). Under conditions of adequate nutrition, for example, *E. coli* agar, fruiting bodies appeared more highly developed than fruiting bodies on poorer media. Starvation seemed to inhibit the aggregation and culmination process. Perhaps the slime, the substance that holds cells together within the fruiting body, is produced in lower amounts during starvation. The electron micrographs suggest that severe starvation triggers myxospore induction in cells that have finished only the first steps of aggregation (Fig. 5); even tardy cells distant from the fruiting body proper formed myxospores during starvation. Thus, it appears that during strong nutritional stress, cellular morphogenesis in *M. xanthus* is dominant over multicellular development. Nutrition and cellular physiology must, therefore, be considered in taxonomic studies of the Myxobacteria based on fruiting body morphology.

A second level of regulation in the control of fruiting appears to involve cAMP and ADP. Given the proper nutritional conditions, these nucleotides vastly increased the number of fruiting bodies formed. The nucleotides increased the number of nucleation sites for aggregation and fruiting even under conditions of starvation (Figs. 3 and 4). It is not known if

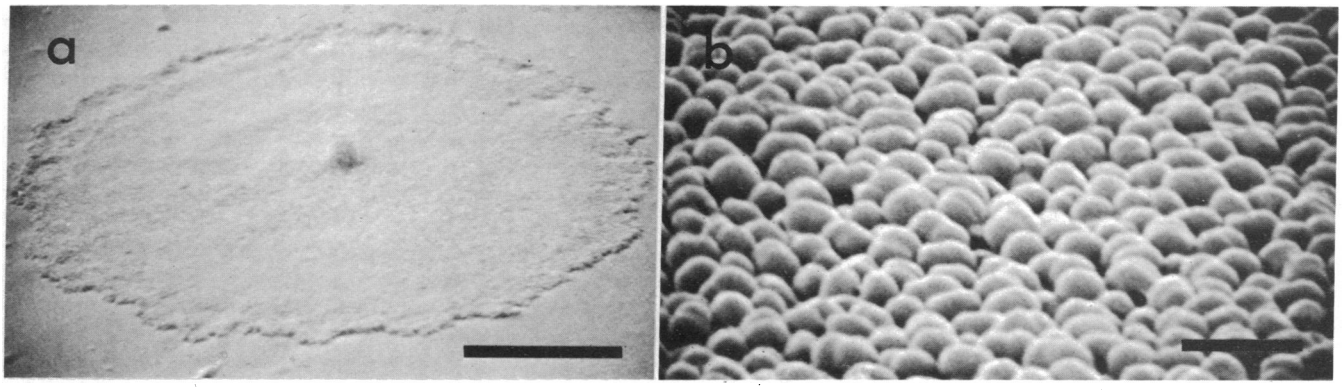

FIG. 5. Planar fruiting body of *M. xanthus*. The structures observed in Fig. 4 were examined by scanning electron microscopy. (a) Planar fruiting body. The bar represents 100 μm. (b) Detail of panel a. The bar represents 2 μm.

TABLE 4. *Effect of nutrition on fruiting body formation on agar containing killed E. coli**

Addition to medium	Concentration	Type of growth after 5 days
None	—	FB
Casitone	0.01%	FB
	0.10%	FB
	0.14%	FB
	0.2%	VEG
	0.5%	VEG
	1.0%	VEG
Methionine	1 mM	VEG
	2 mM	VEG
	4 mM	VEG
Ethionine	1 mM	VEG
Norleucine	1 mM	VEG
Isoleucine	1 mM	FB
	2 mM	FB
	4 mM	VEG
Lysine	1 mM	FB
	2 mM	FB
	4 mM	VEG
DL-Homoserine	4 mM	FB

* The plates were prepared as described in *Materials and Methods*. FB, fruiting bodies; VEG, nonfruiting vegetative growth.

these compounds have any chemotactic activity in *M. xanthus* as they do in some eukaryotic systems (7, 8).

When cAMP or ADP was added together with threonine or diaminopimelic acid to the assay medium (Tables 2 and 3), the number of fruiting bodies obtained was very high. The synergistic increase in fruiting under these circumstances suggests the interaction of at least two regulatory controls. If two such controls exist, one might expect them to be interdependent, since the inhibition of fruiting by methionine or isoleucine cannot be overcome by the addition of cAMP or ADP (Table 2). At this time we do not know if the ADP or cAMP stimulation of fruiting acts directly at specific receptor sites or indirectly. Indirect stimulation could involve alterations in nucleotide levels, inhibition of aspartokinase (6), or both. It is difficult to further characterize these levels of control with feeding experiments; perhaps this could be achieved by the isolation of appropriate mutants.

The data suggest possible mechanisms for the regulation of fruiting body formation. For example, cAMP or a molecule that interacts with cAMP, may trigger transcription at differentiation-specific promoter sites, in a manner similar to the regulation of β-galactosidase (12). Support for the idea of differentiation-specific promoter sites has already been found in *M. xanthus* by the isolation of spontaneous rifampin-resistant mutants that can no longer form fruiting bodies (Rudd and Zusman, unpublished data). Alternative models involving interaction of nucleotides with the cell surface are also possible. The study of the molecular mechanisms controlling differentiation in *M. xanthus* is likely to provide important information on basic biological regulation.

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