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Developmentally Induced Autolysis During Fruiting Body Formation by *Myxococcus xanthus*

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The developmental events during fruiting body construction by the myxobacterium *M. xanthus* is an orderly process characterized by several sequential stages: growth → aggregation → formation of raised, darkened mounds of cells → autolysis → myxospore induction. The temporal sequence of autolysis followed by myxospore induction is consistent with the interpretation that developmental autolysis provides essential requirements for the surviving cells to induce to myxospores. At intermediate developmental times on agar plates a fraction of the cell population is irreversibly committed to lyse; i.e., lysis continues in liquid growth medium or in magnesium-phosphate buffer. Lysis is cell concentration independent and is therefore likely to be by an autolytic mechanism. The lysis sequence can be preliminarily characterized as having an early stage during which deoxyribonucleic acid synthesis continues and a later irreversible stage during which deoxyribonucleic acid synthesis does not occur. Irreversible lysis in liquid growth medium or in magnesium-phosphate buffer is initiated on agar plates during nutrient deprivation and such lysis results in the induction of a fraction of the population to myxospores. This induction is dependent upon the concentration of lysis products, thus providing evidence that developmentally induced autolysis is required for myxospore induction.

The myxobacteria are procaryotes that carry out a complex developmental sequence resulting in the construction of a multicellular fruiting body (4, 5). When vegetative cells of the species *Myxococcus xanthus* MD-1 are deprived of specific exogenous nutrients on a solid surface they aggregate into mounds of cells which then construct a raised, slime-covered fruiting body (5). The cells within the fruiting body subsequently convert to the resting-cell type, myxospores. The physiological regulation of the developmental events that occur during fruiting body construction is poorly understood.

Previous observations of the regulation of development of *M. xanthus* have scored only the end product of development, i.e., fruiting body formation, as an assay for the effectors of development (1, 3, 7, 9). We have developed experimental methods that allow a more detailed study of the physiological regulation and timing of specific intermediate developmental events (13). We have observed that massive autolysis occurs during fruiting body formation by three species of myxobacteria, and our evidence suggests that regulated autolysis is an integral part of the myxobacterial development.

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MATERIALS AND METHODS

Bacteria and media. *M. xanthus* MD-1 (formerly strain FB) or MD-4 (a TNS cell type, i.e, tan nonswarmer) were maintained in 2% Casitone growth medium (2) and transferred daily. Strain MD-1 consists of phenotypically distinguishable cell types that are interconvertible (13). To maximize the reproducibility of the cell types present in the growing cultures, new cultures were started weekly from a stock of frozen, glycerol-induced myxospores (6).

Growth and radioactive labeling. Forty-milliliter cultures were grown in 2% Casitone medium to a cell density of 4×10^8 to 6×10^8 cells per ml for unlabeled cultures. Deoxyribonucleic acid (DNA)-labeled cultures were grown in Casitone medium with 2.5 to 5 μCi of [*methyl*-^{3}H]thymidine (Schwarz/Mann) per ml to a density of 2×10^8 to 3×10^8 cells per ml. The cultures were cooled on ice, centrifuged, and suspended in the same volume of fresh medium containing 250 μg of thymidine per ml, and grown for an additional generation (3 h).

Plating for fruiting body formation. Exponential cultures, as above, were harvested by centrifugation, washed with one-fourth volume of cold medium and suspended at 5×10^9 cells per ml in Casitone growth medium. A 0.2-ml sample of this cell suspension was spread uniformly on a 2% agar plate (Difco; 9-cm diameter), dried at 32°C for 1 h, and then incubated at 32°C. To achieve reproducible timing of development, e.g., the time when maximum lysis is occurring, it is essential to control several variables. (i) Different lots of Difco agar result in variability

with regard to the timing of lysis and induction. (ii) The cells must be carefully spread on the agar surface with the end of a smooth-glass rod, since any disruptions of the agar surface result in nonuniform development of a single plate (presumably due to elasticotaxis [13]). (iii) Freshly prepared plates should be used since humidity of the agar surface affects the timing of development. After spreading the cells the plates should be dried in a standard way, i.e., opened for 1 h in a low-humidity incubator and then transferred to a high-humidity incubator. (iv) The density of the cells upon plating affects the timing of developmental events (13); therefore the cell density should be measured accurately before plating, e.g., in a Petroff-Hausser counting chamber. A Wild dissecting microscope at ×50 magnification was used to count the number of fruiting bodies in 10 randomly selected areas on each of three triplicate plates. Each area counted was 0.25 cm^2 and the total surface of the plate was 63 cm^2. Thus, the total number of fruiting bodies in 10 areas (2.5 cm^2) was multiplied by 25 to obtain the average number per plate. Each area counted had a maximum of 60 fruiting bodies.

Measurement of cells per plate. The cells were harvested from plates by scraping with a razor blade and were removed with three successive washes with 3 ml of cold distilled water. The number of vegetative cells or myxospores per plate was determined by cell counts as previously described (13).

Measurement of radioactive label. The amount of trichloroacetic acid-precipitable radioactivity remaining in cells on plates was determined as previously described (13) with modifications. Unlabeled carrier cells (5×10^9 cells) were added to the radioactive cell suspension and precipitated with an equal volume of 20% trichloroacetic acid on ice for at least 1 h. The precipitate was recovered by centrifugation at 12,000 × *g* for 10 min, washed once with 5 ml of cold 5% trichloroacetic acid, and then washed with 5 ml of 75% ethanol. The final precipitate was hydrolyzed in 0.25 ml of distilled water and 1.5 ml of NCS tissue solubilizer (Amersham/Searle) at 50°C for 12 to 24 h. The hydrolyzed samples were transferred to scintillation vials by two rinses of 7 ml of toluene-based counting fluid [2,5-diphenyloxazole–1,4-bis-(5-phenyloxazole)benzene] and their radioactivities were determined.

Lysis in liquid. Cells undergoing development on plates were harvested in distilled water and adjusted to 10 ml in magnesium-phosphate buffer (8 mM magnesium sulfate; 10 mM potassium phosphate, pH 7.6). The cells were either aerated at 32°C in buffer or diluted with concentrated Casitone (Difco) to give a final concentration of 2% Casitone growth medium. The fate of the cells was followed either by cell counts or by their trichloroacetic acid-precipitable radioactivity.

Pulse labeling on plates. To label the fraction of cells on the plates which were synthesizing DNA, a solution of 0.2 ml of [*methyl*-^{14}C]thymidine (50 μg/ml; 10 μCi/ml) was pipetted under the agar 8 h before harvest. Two hours before harvest a solution of 0.2 ml of unlabeled thymidine was added under the agar (5 mg/ml). Incorporation of the [*methyl*-^{14}C]thymidine into DNA began about 1 to 2 h after being added under the agar and continued until 1 to 2 h after addition of unlabeled thymidine. The cells were harvested in the standard way 2 h after adding the unlabeled thymidine and 8 h after adding the [*methyl*-^{14}C]thymidine. The cells that were synthesizing DNA for the 6 h before harvest were, therefore, labeled with [*methyl*-^{14}C]thymidine.

RESULTS

Developmental lysis. The fate of *M. xanthus* MD-1 cells plated by our standard method is shown in Fig. 1. In this experiment cells suspended in Casitone medium were plated on unsupplemented agar plates. The cell number increased during an initial growth period (Fig. 1A) followed by nutrient depletion and a decrease in cell number. Approximately 65% of the maximum number of cells lysed and the survivors were predominantly myxospores in fruiting bodies.

To study the mechanism of lysis or its possible role in development it would be advantageous to have an alternative to direct cell counts for measuring lysis. In the experiment shown in Fig. 1, the cells were prelabeled during vegetative growth in liquid culture with [*methyl*-^{3}H]thymidine (of the trichloroacetic acid-precipitable label in cells, greater than 90% is incorporated into DNA [unpublished data]), and the conversion of this label to soluble form during development can be used as a measure of cell number (Fig. 1B). The calculated radioactivity per cell decreased rapidly during the initial growth phase as expected and then subsequently decreased slightly during the lysis phase. The solubilization of prelabeled DNA during development is thus a reliable criterion of lysis.

Lysis during development of *M. xanthus* is not restricted to this experimental method. Lysis also occurs under all other plating methods that have been used to induce development (Table 1). Growing cells do not lyse in liquid suspension or on a 2% Casitone agar surface, nor do cells lyse when starved in liquid suspension, e.g., distilled water or magnesium-phosphate buffer. In addition, developmental lysis is not unique to this species of *Myxococcus* since lysis also occurs during fruiting body formation by two other species so far studied. Both *M. fulvus* and *M. virescens* lyse during fruiting body formation and the sequence of developmental events appears to be similar to that of *M. xanthus*.

Role of cell types in development. Detailed studies of the mechanism and role of lysis during development requires that the timing of developmental events be reproducible and syn-

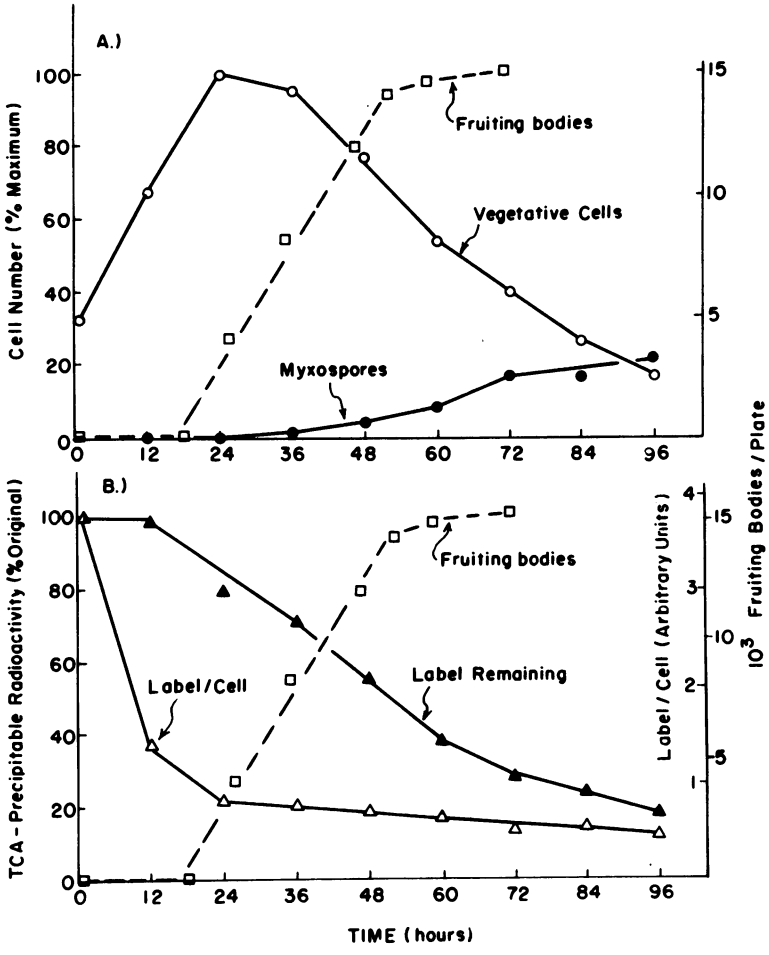

FIG. 1. *Developmental events during fruiting body formation by M. xanthus MD-1 was observed at 12-h intervals. The cells were labeled with [methyl-³H]thymidine (2.5 μCi/ml; 2 Ci/mmol) for four generations during vegetative growth in Casitone medium before plating. (A) Vegetative cell number* (○); *myxospore number* (●); *number of fruiting bodies per plate* (□). *(B) Trichloroacetic acid-precipitable DNA label remaining per plate* (▲). *The 1-h time point (100%) represents 1.07 × 10⁵ cpm per plate. Calculated trichloroacetic acid-precipitable label per cell* (△).

chronous. There are several variables that must be controlled in order to achieve reproducible development (see Materials and Methods) and it is now possible to achieve reproducible timing of development with wild-type populations of *M. xanthus* (MD-1). However, *M. xanthus* has the potential for interconversion among four phenotypically distinguishable colony types that we have designated YS (yellow swarmer), TS (tan swarmer), and TNS (L3). These cell types possess differing developmental potentials. The wild-type population of cells or pure swarmer-cell phenotypes construct a well-defined darkened mound of cells within which the cells are induced to form myxospores. The nonswarmer cell phenotypes do not construct a darkened mound of cells, and myxospore induction occurs first within aggregation centers and slightly later outside of aggregation centers. The nonswarmer phenotypes, YNS and TNS, possess normal motility during development and aggregation: lysis and myxospore induction occur in the same sequence as for the pure swarmer phenotypes or the wild-type population (approximately 10% YS and 90% TNS). Both lysis and induction are rapid and synchronous for the nonswarmer phenotypes and we have used the TNS cell type for the study of the mechanism and role of lysis in development. The obvious experimental advantage in the use of the more synchronous TNS cells (Fig. 2) is that at any time point of devel-

TABLE 1. *Lysis measured by loss of DNA label under several developmental conditions which result in myxospore formation*[a]

Additions	% of label remaining (120 h)[b]	Myxospores (10^6) per plate
None	12.9	3.34
8 mM $MgSO_4$	11.8	3.68
8 mM $MgSO_4$; 5 mM KPO_4, pH 7.6	19.3	4.84
5 mM KPO_4, pH 7.6	17.3	5.86
4 mM $MgSO_4$; 0.1% Casitone; 0.23 mM ADP[c]	13.8	1.64
4 mM $MgSO_4$; 2.5×10^{10} *E. coli* per plate[d]	8.8	8.08
10^9 sonically treated *M. xanthus* per plate[e]	14.3	4.54

[a] *M. xanthus* MD-1 was prelabeled with [*methyl*-^{3}H]thymidine before plating. Aggregation centers which contained mature myxospores were formed in every case, but the morphology of these centers was quite different under the various conditions.

[b] The percent label remaining represents the average of four plates relative to the 1-h plates (100% = 1.54×10^5 cpm per plate).

[c] ADP, Adenosine 5'-diphosphate.

[d] Stationary-phase *Escherichia coli* cells were added to the agar before sterilization (1).

[e] Unlabeled cells were washed and suspended in distilled water at 5×10^9 cells per ml and sonically treated for 2 min until all cells were lysed. A sample of 0.2 ml was spread per plate and dried before spreading of the [*methyl*-^{3}H]thymidine-labeled cells.

opment a greater fraction of the total population is in a similar physiological state.

Irreversible lysis. An additional experimental advantage would be provided by the ability to study certain developmental events in liquid suspension rather than on an agar surface. Therefore, at various times after plating TNS cells, development was interrupted and the cells were suspended in Casitone growth medium. The cells were prelabeled during vegetative growth with [*methyl*-^{3}H]thymidine before plating. Using the solubilization of this DNA label as a criterion of lysis, Fig. 3A shows that lysis does occur in a fraction of the population. For example, 25% of the total population present on the plates at 36 h after plating lysed during a 12-h period in liquid Casitone medium. TNS cells on plates lysed almost quantitatively (Fig. 2) and the only survivors were myxospores. Thus, after 36 h on plates, about 25% of the population that would lyse if allowed to remain on the plate continued to lyse when interrupted and placed in growth medium.

The sequence of events that leads to cell death in the medial epithelial cells of the embryonic rat palatal shelf is characterized by the early cessation of DNA synthesis followed by synthesis of new glycoproteins (8). It is plausible that a cell involved in lysis-specific synthesis would stop replicating DNA at some earlier time. This hypothesis was tested by comparing the fate of cells that were still synthesizing DNA on the plate to the total population (Fig. 3A). Almost all the cells that were synthesizing DNA on the plates during development survived under conditions where 10 to 25% of the total population lysed. This preferential survival of the cells that were synthesizing DNA on the plates was not observed when the cells were interrupted during development and placed in Casitone medium containing chloramphenicol (Fig. 3B). Thus, there are two distinguishable populations of cells. In one population DNA synthesis continues and the cells are reversibly committed to lyse (reversible when protein synthesis is allowed to proceed normally), and in the second population DNA synthesis has stopped and the cells are irreversibly committed to lyse.

Roles of lysis in development. If the above interpretation is correct, then starvation for an energy source after interruption of develop-

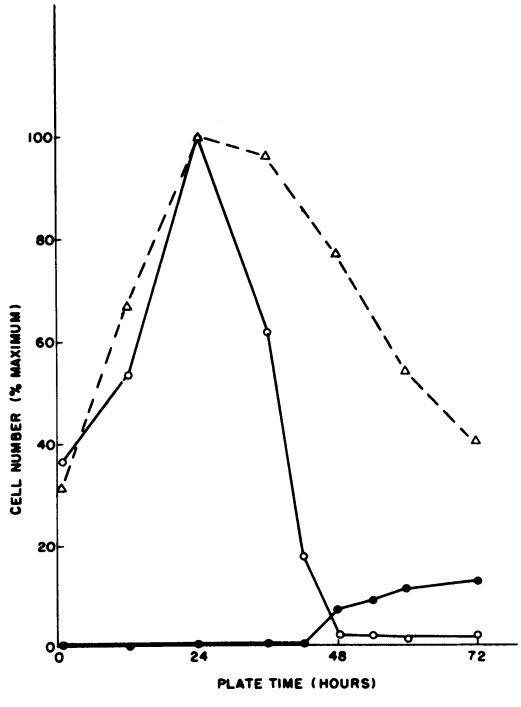

FIG. 2. *Comparative lysis of TNS (MD-4) versus wild-type cells (MD-1). Wild-type vegetative cells* (△); *TNS vegetative cells* (○); *TNS myxospores* (●).

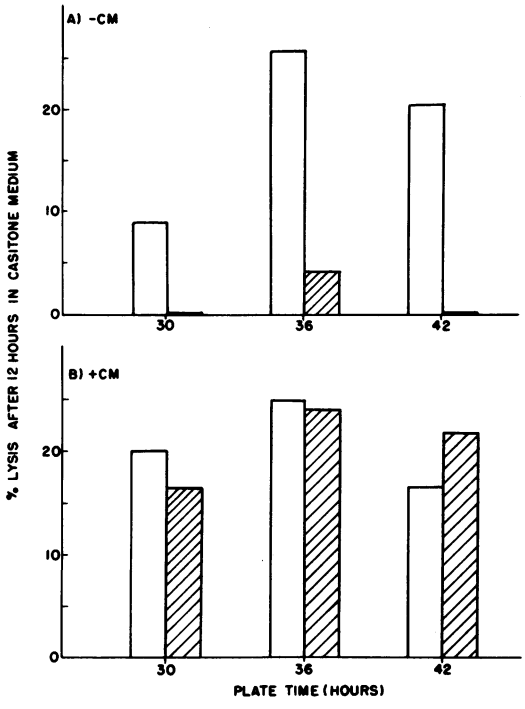

FIG. 3. *Irreversible lysis by MD-4 cells in Casitone medium. The total population was labeled with [methyl-^{3}H]thymidine as in Fig. 1 before plating. The cells were labeled on the plates immediately before harvest. The cells were harvested from triplicate plates for three separate labeling periods (i.e., 24 to 30 h; 30 to 36 h; and 36 to 42 h) and suspended in Casitone medium containing 250 μg of thymidine per ml without chloramphenicol (CM) (A) or with 100 μg of CM/ml (B). This level of CM permits about 5% of the normal level of protein synthesis. The harvested cells were aerated at 32°C for 12 h in Casitone medium and the trichloroacetic acid-precipitable label remaining after 12 h was measured and compared to that present at the time of harvest from the plates. [Methyl-^{3}H]thymidine prelabel (total population) is shown in the open bars as the percent solubilized during the 12 h in Casitone medium and the loss in [methyl-^{14}C]thymidine plate label (population synthesizing DNA on plates) is shown in the hatched bars.*

ment should result in even more complete lysis than that observed in Casitone growth medium. Such an experiment is shown in Fig. 4. TNS cells were interrupted after 36 h on plates and placed in magnesium-phosphate buffer. The fate of the cells almost exactly mimics their fate if allowed to remain on the plate. That is, 90% of the cells lysed and 8% of the cells were induced to form myxospores. This commitment to lysis appeared only after the cells were allowed to undergo development on plates for about 12 to 24 h (unpublished data). Similarly, there is a differentiation into cells that are competent to induce to myxospores that occurs after about 24 to 36 h on plates (unpublished data). The myxospores induced under these conditions are identical to fruiting body myxospores by the criteria of optical refractivity, sonication resistance, and ability to germinate to vegetative rods.

Mechanism of lysis. The mechanism of lysis was studied by measuring lysis at several cell densities. The result of irreversible lysis in magnesium-phosphate buffer at three cell densities is shown in Fig. 5. At 10^7 cells/ml, lysis is approximately the same as at 10^9 cells/ml (Fig. 5A). Lysis is thus not dependent upon cell density nor upon the concentration of lysis products and is likely to be by an autolytic mechanism. In this same experiment, it can be seen that myxospore induction is, however, dependent upon the concentration of lysis products (Fig. 5B), and this suggests that the observed autolysis is in some way required for myxospore induction.

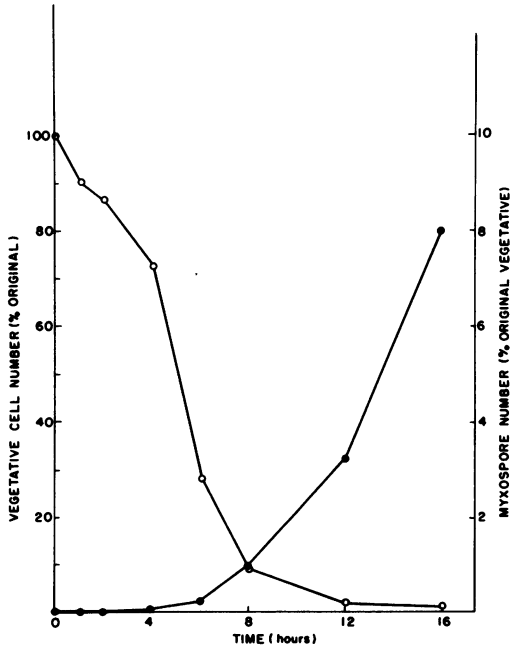

FIG. 4. *TNS cell lysis in magnesium-phosphate buffer. Unlabeled TNS cells were prepared and plated as in Fig. 1. After 36 h on plates at 32°C the cells were harvested and placed in magnesium-phosphate buffer. The resuspended cells were aerated at 32°C, and the vegetative cells number* (○) *and the myxospore number* (●) *were measured, relative to the number present at the time of harvest.*

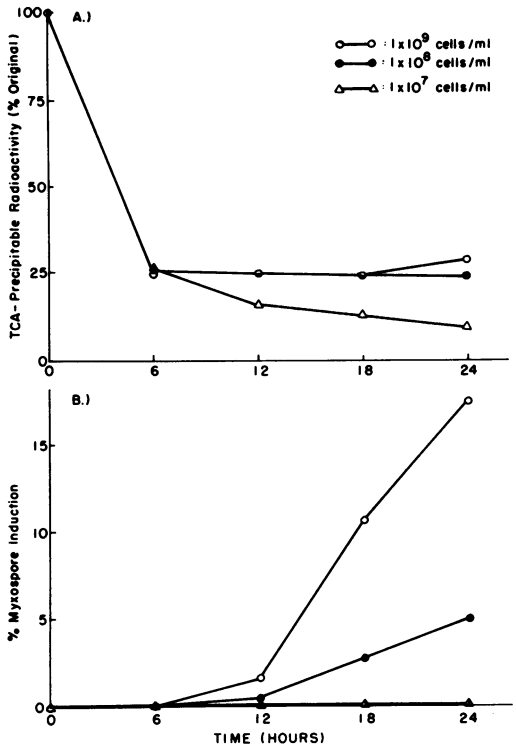

FIG. 5. *Dependence of lysis and induction on cell concentration. TNS cells were prelabeled with [methyl-³H]thymidine harvested and placed in magnesium-phosphate buffer as in Fig. 4. The cells were suspended (10^{10} cells) in either 10 ml, 100 ml, or 1 liter of buffer and aerated at 32°C. At 6-h intervals either 1.5-ml, 15-ml, or 150-ml samples were harvested and diluted to 150 ml. Carrier cells were added (5×10^9 cells), and the cells were concentrated to 1.5 ml by centrifugation. The total survivors was determined by the trichloroacetic acid-precipitable radioactivity remaining (A) and the number of myxospores induced by direct cell counts, relative to the original vegetative cell number harvested (B).*

DISCUSSION

Observations of the temporal sequence of events during the development of *M. xanthus* (e.g., Fig. 1) lead to the conclusion that there is an orderly developmental sequence. The definable stages of development are: Growth → Movement into aggregation centers → Formation of raised, darkened mounds of cells → Autolysis → Myxospore induction.

We have previously suggested that lysis during development of *M. xanthus* is likely to represent a cooperative phenomenon in which a fraction of the population provides, by lysis, some essential requirement for the survival of the remainder of the population (13). The induction of myxospores during development normally occurs under conditions of nutrient deprivation and thus individual cells must either have sufficient endogenous reserves for myxospore induction, e.g., capsule synthesis, or be provided with an exogenous energy source. Induction in magnesium-phosphate buffer of developmentally competent cells to myxospores is dependent upon the concentration of autolysing cells (Fig. 5B). The products of lysis may provide a nutrient or energy source for myxospore induction. Preliminary evidence indicates that the lysis products provide a specific chemical signal for myxospore induction (unpublished data) in addition to an energy source. The initiation of the observed population differentiation into lysis cells and cells that are competent to induce to myxospores is not well understood. Specific nutrient deprivation stimulates development of fruiting bodies (3, 7, 9). Additions to the agar surface, e.g., adenosine 5'-diphosphate or cyclic adenosine 5'-monophosphate, stimulate the number of fruiting bodies formed (1). It is not all clear from these studies which part of development is being affected. It is clear, however, that it is now possible to separate the many complex stages of fruiting body formation into a sequence of events and that a reexamination of the previously described effectors of development (1, 3, 7, 9) can provide further insight into the nature of the molecular regulations involved. The myxobacteria provide an excellent procaryotic experimental system for the study of the mechanism and role of cell death in development. Such studies may provide some insight into the role of cell death in more complex eucaryotic developmental systems and may also provide some insight into the evolution of such important developmental phenomena (10, 11).

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