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Behavior of Peripheral Rods and Their Role in the Life Cycle of *Myxococcus xanthus*

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***Myxococcus xanthus* is a gram-negative bacterium with a complex life cycle including a developmental phase in which cells aggregate and sporulate in response to starvation. In previous papers, we have described a heretofore unsuspected layer of complexity in the development of *M. xanthus*: vegetatively growing cells differentiate into two cell types during development. In addition to the differentiation of spores within fruiting bodies, a second cell type, peripheral rods, arises outside fruiting bodies. The pattern of expression of proteins in peripheral rods is different from that of either vegetatively growing cells or spores, and peripheral rods express a number of recognized developmental markers. In this report, we examine four aspects of the biology of peripheral rods: (i) the influence of nutrients on the proportion of peripheral rods in a population of developing cells, (ii) the capacity of peripheral rods to recapitulate development, (iii) the development of peripheral rods on conditioned medium, and (iv) the ability of peripheral rods to resume growth on low amounts of exogenously added nutrients. The results of these studies suggest that peripheral rods play a significant role in the life cycle of *M. xanthus* by allowing the exploitation of low amounts or transient influxes of nutrients without the investment of energy in spore germination. The differentiation of vegetatively growing cells into two cell types that differ significantly in biology, shape, and localization within the population has been incorporated into a model of the life cycle of *M. xanthus*.**

Myxococcus xanthus, a gram-negative, rod-shaped bacterium that can be found in a variety of soil habitats, grows vegetatively by preying on other organisms in its environment and by absorption of dissolved nutrients. When the local supply of nutrients becomes depleted, *M. xanthus* enters the multicellular developmental phase of its life cycle. Cells enter aggregates that undergo morphogenesis from flat mounds into raised fruiting bodies within 1 or 2 days after the initiation of starvation. Aggregated cells undergo whole-cell differentiation into spherical, optically refractile spores (34). Spore differentiation and maturation continue for at least 7 days after the initiation of starvation (18, 22, 38). Ten to 20% of cells induced for development do not enter fruiting bodies. These peripheral rods, which retain their rod shape in situ, can be distinguished from spores by their physical appearance and localization within a developing population. Peripheral rods can be distinguished from vegetatively growing cells by the patterns of expression of a number of polypeptides, including recognized developmentally regulated proteins (25, 26).

A standard textbook on embryology defines differentiation as "the process in which the cells, or other parts of an organism become different from one another and also different from their previous condition . . . the process as a result of which the parts of an organism acquire the ability to perform their special functions" (2). In the course of changes in condition and function, the pattern of expression of proteins in the cell must also change, and that pattern must be significantly different from that of other cell types (2, 8). In the accompanying reports, we show that peripheral rods (i) occur in all strains of *M. xanthus*, (ii) have unique patterns of expression of proteins, and (iii) are structurally dissimilar from the cell types most similar in patterns of protein expression (25, 26). In this report, we extend our study by showing that peripheral rods (iv) exhibit unique responses to environmental stimuli, and (v) serve a unique function in the life cycle of the organism. Particular attention has been paid to the competence of peripheral rods to participate in the multicellular interactions necessary for fruiting body formation and their responses to changes in their environment. We have also examined the influence of nutrient concentration on the proportion of peripheral rods in a population.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* C600 was used in all experiments involving *E. coli* (1); growth was on L broth (20). Strain DZF1 (FB) of *M. xanthus* was used for all experiments (6). *M. xanthus* was grown in Casitone-yeast extract medium (CYE) (3) or on CYE containing 1.5% agar. The following buffers were used: TM buffer, 10 mM Tris (pH 7.6) containing 8 mM $MgSO_4$; TPM buffer, TM buffer containing 1 mM KH_2PO_4; and TPMF buffer, TPM buffer containing 10% Ficoll (molecular weight, 400,000). Clone fruiting medium (CF) (10) containing 1.5% agar was the standard medium used for development on a solid surface. TPM-buffered 1.5% agar, TPM-buffered agar containing 0.05% Casitone (CTPM-buffered agar), and *E. coli* agar (6, 21) were also used to study development. To obtain 34°C conditioned CF agar, cells were allowed to develop on CF agar for 17 h or 2, 3, 4, 5, 6, or 7 days and then scraped from plates in TPMF buffer as described below. The CF agar plates were stored at 4°C in the dark for up to 1 week before use.

Materials. All medium components were obtained from Difco Laboratories, Detroit, Mich. Salts were purchased from Mallinckrodt, Inc., St. Louis, Mo. Other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. SeaPrep agarose and SeaPlaque, a low-melting-point agarose, were purchased from FMC BioProducts, Rockland, Maine.

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Fruiting. Cells were grown in CYE liquid medium and then harvested by centrifugation at 12,000 × *g* at 4°C for 10 min. Cell pellets were suspended at 2×10^9 cells per ml in TM buffer, and 1×10^9 cells were spread evenly onto each 100-mm CF agar plate. Plates were incubated at 28 or 34°C as described below for each experiment. The progress of development was highly reproducible under these conditions.

Purification of cell types. Vegetatively growing cells were harvested as described above. Developing cells were scraped from the agar surface with a razor blade in the presence of 0.5 to 1.0 ml of ice-cold TPMF buffer (22, 39). A Pasteur pipette was used to gently transfer the cells to a conical centrifuge tube. Plates were washed with a second sample of TPMF buffer; this wash was added to the cells. Care was taken at all times to avoid unnecessary or forceful pipetting. Aggregates remained tightly cohered during the harvesting procedure.

The harvested cells were centrifuged at 46 × *g* for 5 min at room temperature. The supernatant, containing peripheral rods, was drawn off and placed in a separate tube. The pellet, consisting of aggregates, was suspended in a larger volume of TPMF buffer and transferred to a fresh centrifuge tube. The supernatant (peripheral rods) and the suspended pellet (aggregated cells) were centrifuged at 12,000 × *g* for 20 min at 4°C. The cell-free 12,000 × *g* supernatants were discarded. The 12,000 × *g* pellets (cells) were each suspended in a small volume of TM buffer. (For some experiments, as described below, peripheral rods were suspended in TPMF buffer.) To obtain purified spores, the aggregate pellet was disrupted by sonic oscillation five times for 30 s with a Branson model 450 sonifier equipped with a microprobe. The extract was then centrifuged at 12,000 × *g* at 4°C for 10 min. The supernatant was discarded; the pellet, containing spores, was washed once with 1.0 ml of TM buffer and centrifuged. This pellet was suspended in a small volume of TM buffer. This sample contains fragments from the walls of sonication-sensitive aggregated cells and whole sonication-resistant spores. For some experiments, spores were further purified on a discontinuous sucrose gradient (12).

Recapitulation of development by peripheral rods. Peripheral rods were isolated as described above. Cells suspended at 4×10^9 cells per ml in TM buffer were distributed on CF agar or conditioned CF agar in 5-μl aliquots. Plates were incubated at 28 or 34°C as described below.

Cohesion assays. Cohesion assays were performed as described by Shimkets (33). Cells were incubated in 10 mM morpholinepropanesulfonic acid (pH 7.2)–4 mM $MgCl_2$–2 mM $CaCl_2$ (MMC buffer) (31) at 28 or 34°C, as specified below. Cohesion was monitored by measuring the A_{625}.

Glycerol induction of sporulation. Vegetatively growing cells at a density of 1×10^8 to 4×10^8 cells per ml were induced by the addition of glycerol to the medium at a final concentration of 0.5 M (7). Samples taken at 1, 18, and 24 h were not separated into cell types for glycerol induction. Cells were harvested from CF agar, dispersed by homogenization, centrifuged to remove TPMF, and then inoculated in CYE containing 0.5 M glycerol at a density of 3×10^8 cells per ml. Peripheral rods were harvested as described above and suspended at a density of 3×10^8 cells per ml in CYE containing 0.5 M glycerol. Cultures were incubated with shaking at 30°C. Samples of cells were examined at ×400 magnification to determine the extent of sporulation: prespores were spherical but nonrefractile, and glycerol-induced spores were both spherical and refractile.

Growth of peripheral rods on *E. coli*. TPM-buffered 1.5% Seaprep agarose in 50-mm petri plates was overlaid with 1 ml of TPM-buffered 1% SeaPlaque agarose (38°C) containing live *E. coli*. After the SeaPlaque had solidified, 10^3 cells of *M. xanthus* in TPM buffer were inoculated in a 2- to 5-μl drop in the center of the plate. Vegetatively growing cells were harvested from CYE broth; peripheral rods and spores were harvested from CF agar at 34°C.

RESULTS

Influence of nutrient levels on the proportion of peripheral rods in developing populations. The influence of the level of nutrients on the proportion of peripheral rods in a developing population of cells was investigated by plating DZF1 on four different media. The proportion of peripheral rods in the population was determined by counting all cells 4 days after plating (fruiting bodies had formed on all media within this time). The proportion of peripheral rods in the populations on CF agar and TPM-buffered agar was similar (16% ± 4.7% over four experiments and 20% ± 2.8% over two experiments, respectively). However, the proportion of peripheral rods on *E. coli* agar was twofold higher (32%). On TPM-buffered agar containing 0.05% Casitone, 57% of the cells were peripheral rods. Thus, the proportion of peripheral rods in the population was higher on media containing higher concentrations of nutrients.

Capacity of peripheral rods to recapitulate development. Cells remaining as peripheral rods after the completion of aggregation have never been observed to spontaneously undergo aggregation when left in situ for several weeks (27). The capacity of peripheral rods and nonaggregated rods from earlier stages of development to undergo aggregation and sporulation was investigated by plating purified rods on fresh CF agar as described in Materials and Methods. If the cells had become irreversibly differentiated or were nonviable, they would not be expected to aggregate or sporulate. On the other hand, if the failure to aggregate and sporulate were due to inhibitors accumulated in the environment during development, then the peripheral rods might be expected to aggregate and sporulate normally when placed on fresh CF agar at appropriate cell densities.

It was decided to study the recapitulation of development at 28 and 34°C because previous work had indicated that both the timing and the physiology of development were affected by temperature (5, 23–25). Vegetatively growing cells and nonaggregated rods were harvested, concentrated to 4×10^9 cells per ml in TM buffer, spotted on fresh CF agar, and allowed to develop at 28 and 34°C for at least 7 days. Sporulation was monitored by scraping cells from each sample 7 days after plating on fresh CF agar and examining the cells with a microscope. Development was monitored at 6- to 12-h intervals. To facilitate the management of the data, we have presented the process of development as involving a progression through three stages. In the first stage, the process of aggregation predominates and is observed as the formation of tight mounds. In the second stage, the morphogenesis of mounds predominates as tight mounds undergo transition to translucent mounds. The differentiation of spores predominates in the third stage as translucent mounds become fruiting bodies. The results of these studies are shown in Tables 1 through 4.

The development of cells harvested from vegetatively growing cultures was monitored to define the normal process under the conditions of this experiment. At 28°C, vegetatively grown cells were slower to establish stable aggregation centers and mounds than they were when incubated at 34°C.

TABLE 1. Development of purified 28°C nonaggregated rods plated on fresh CF agar[a]

Cell type	Time of harvest (h)	Morphology of aggregates at harvest[b]	Time (days) at which the indicated stage of development was reached[c]					
			28°C			34°C		
			Tight mounds	Translucent mounds	Fruiting bodies	Tight mounds	Translucent mounds	Fruiting bodies
Vegetatively grown	0	NA	1	1.5	2.5	0.75	1.25	2
Nonaggregated rods	12	Premounds		1	2		0.5	1
	15	Premounds		1	2	0.75	1.25	Never[d]
	18	Flat mounds		1	2	0.75	1.25	2
	21	Mounds	1	1.5	2.5	0.75	1.25	2
	24	Mounds	3		6		1	3
	27	Mounds	3	Never	Never	1	1.25	3
	30	Early translucent mounds	3	Never	Never	1	1.25	3
	33	Translucent mounds	3	Never	Never	1	1.25	3
	36	Translucent mounds	4	Never	Never		0.75	2
	39	Translucent mounds	3		4		0.75	2
	42	Late translucent mounds	3		4		0.75	2
	45	Late translucent mounds	3		3.75		0.75	2
	48	Late translucent mounds	3		5		0.75	1.75
	51	Early fruiting bodies	3	5	8	1	1.25	2
	54	Fruiting bodies	3	Never	Never	1	1.25	2
	57	Fruiting bodies	3	Never	Never	1	1.25	2
	60	Fruiting bodies	3	Never	Never	1	1.25	2
	63	Fruiting bodies	1	1.5	3		1	4
	66	Fruiting bodies	1	1.5	3		1	4
	69	Fruiting bodies	1	1.5	2.75		1	3.75
	72	Fruiting bodies	1	1.5	4		1	3.5
	84	Fruiting bodies	1	1.5	2.5		1	3.5
	96	Fruiting bodies	1	1.25	3	1	1.25	3
	120	Fruiting bodies	1	1.5	3		2	Never
	144	Fruiting bodies		1	4.5	Never	Never	Never

[a] DZF1 was grown and plated for development on CF agar. At each time point, 8 to 10 100-mm plates were harvested, cell types were isolated, nonaggregated rods were concentrated, and 5-μl samples of cells were aliquotted onto fresh CF agar as described in Materials and Methods. Two aliquots of cells were used for each time point at 28 and 34°C. Development was scored under a dissecting microscope at approximately 6-h intervals.

[b] There are several easily identifiable morphological stages in the development of *M. xanthus*: premound, mound (flat, tight, and translucent, in order), and fruiting bodies (mounds of spores; Fig. 2). NA, not applicable.

[c] Development is presented as a progression through three stages. In the first stage, the process of aggregation predominates and is observed as the formation of tight mounds. In the second stage, the morphogeneis of mounds predominates as tight mounds undergo the transition to translucent mounds. The differentiation of spores predominates in the third stage, during which translucent mounds become fruiting bodies.

[d] Cells did not form the indicated structure.

TABLE 2. Summary of data in Table 1

Stage at time of harvest	Rate of development compared with that of vegetatively grown cells on fresh CF agar[a]					
	28°C			34°C		
	Aggregation into mounds	Morphogenesis into:		Aggregation into mounds	Morphogenesis into:	
		Translucent mounds	Fruiting bodies		Translucent mounds	Fruiting bodies
Early aggregation (12 to 18 h)	Fast		Normal	Varied[a]	Varied	Varied
Midaggregation (21 h)	Normal	Normal	Normal	Normal	Normal	Normal
Early morphogenesis (27 to 36 h)	Slow		Very slow or never	Slow	Fast	Slow
Late morphogenesis (39 to 45 h)	Slow		Fast		Fast	Slow
Very late morphogenesis (48 to 51 h)	Slow		Slow	Normal	Normal	Normal
Early fruiting bodies (54 to 60 h)	Slow	Never	Never	Slow	Fast	Normal
Fruiting bodies (62 to 96 h)	Normal	Normal	Slow		Fast	Slow

[a] The rate of development of vegetatively-grown cells defines "normal." "Fast" and "slow" are defined relative to normal (Fig. 1). "Never" indicates that the cells did not form a given multicellular structure. "Varied" indicates that the rate varied according to the time point.

The process of sporulation also appeared to be slower at 28°C; both the initial appearance of spores in the population and the rate of accumulation of spores were slower (Fig. 1; Tables 1 through 4).

The recapitulation of development by nonaggregated rods plated on fresh CF agar showed complex patterns of behavior. Seven distinct patterns of development were found for nonaggregated rods from 28°C (Tables 1 and 2); six patterns were found for nonaggregated rods from 34°C (Tables 3 and 4). There are three major observations to be gleaned from the data. (i) The 28°C nonaggregated rods went through cycles of competence to make fruiting bodies at 28°C (com-

TABLE 3. Development of purified 34°C nonaggregated rods plated on fresh CF agar[a]

Cell type	Time of harvest (h)	Morphology of aggregates at harvest[b]	Time (days) at which the indicated stage of development was reached[c]					
			28°C			34°C		
			Tight mounds	Translucent mounds	Fruiting bodies	Tight mounds	Translucent mounds	Fruiting bodies
Vegetatively grown	0	NA	1	1.5	2.5	0.75	1.25	2
Nonaggregated rods	12	Premounds	1	1.5	2	0.75	1.25	2
	14	Premounds	2	2.5	4	0.75	1.25	2
	16	Mounds	2	2.5	4	1.25	1.75	3
	18	Mounds	2	2.5	4	0.75	1.25	2
	20	Mounds	2	2.5	4	0.75	1.25	1.75
	22	Mounds	2	2.5	3.75	1.25	1.75	2.25
	24	Mounds	1	1.5	2	0.75	1.25	2
	26	Mounds	1	1.5	2	0.75	1.25	2
	28	Late mounds	1	1.5	2	0.75	1.25	3
	30	Late mounds			2.75	0.75	1.25	2.75
	32	Translucent mounds		1	2.75	0.75	1.25	2.75
	34	Translucent mounds		1	2.75	1.25	1.75	2.75
	36	Translucent mounds		1	2.5	1.5	2	2.5
	38	Fruiting bodies		1	4	1.25	Never[d]	Never
	40	Fruiting bodies		1	4	1.25	Never	Never
	42	Fruiting bodies		1	4.75	1.25	Never	Never
	44	Fruiting bodies		1	4.75	2.5	Never	Never
	46	Fruiting bodies		2	4.5	4	Never	Never
	48	Fruiting bodies		2	4.5	2.5	Never	Never
	72	Fruiting bodies		2	5	Never	Never	Never
	96	Fruiting bodies		3	5	Never	Never	Never
	120	Fruiting bodies	3		5	3	Never	Never
	144	Fruiting bodies		3	5	Never	Never	Never
	168	Fruiting bodies				4	Never	Never

[a] DZF1 was grown and plated for development on CF agar. At each time point, 8 to 10 100-mm plates were harvested, cell types were isolated, nonaggregated rods were concentrated, and 5-μl samples of cells were aliquotted onto fresh CF agar as described in Materials and Methods. Two aliquots of cells were used for each time point at 28 and 34°C. Development was scored under a dissecting microscope at approximately 6-h intervals.

[b] There are several easily identifiable morphological stages in the development of *M. xanthus*: premound, mound (flat, tight, and translucent, in order), and fruiting bodies (mounds of spores; Fig. 2). NA, not applicable.

[c] Development is presented as a progression through three stages. In the first stage, the process of aggregation predominates and is observed as the formation of tight mounds. In the second stage, the morphogenesis of mounds predominates as tight mounds undergo the transition to translucent mounds. The differentiation of spores predominates in the third stage, during which translucent mounds become fruiting bodies.

[d] Cells did not form the indicated structure.

TABLE 4. Summary of data in Table 3

Stage at time of harvest	Rate of development compared with that of vegetatively grown cells on fresh CF agar[a]					
	28°C			34°C		
	Aggregation into mounds	Morphogenesis into:		Aggregation into mounds	Morphogenesis into:	
		Translucent mounds	Fruiting bodies		Translucent mounds	Fruiting bodies
Early aggregation (12 h)	Normal	Normal	Fast	Normal	Normal	Normal
Midaggregation (12 to 22 h)	Slow	Normal	Slow	Normal to slow	Normal	Normal to slow
Early morphogenesis (24 to 28 h)	Normal	Normal	Normal	Normal	Normal	Normal
Late morphogeneis (32 to 36 h)		Fast	Slow	Slow	Normal	Normal
Early fruiting bodies (38 to 44 h)		Fast	Slow	Slow	Never	Never
Fruiting bodies (46 to 168 h)		Slow	Slow	Slow	Never	Never

[a] The rate of development of vegetatively-grown cells defines "normal." "Fast" and "slow" are defined relative to normal. "Never" indicates that the cells did not form a given multicellular structure.

pare 12- to 24-h, 39- to 51-h, and 63- to 144-h samples with 27- to 36-h and 54- to 60-h samples, Table 1). (ii) All of the samples of 28°C nonaggregated rods that could not make fruiting bodies at one temperature could make fruiting bodies at the other temperature (Table 1). (iii) When 34°C nonaggregated rods were taken from cultures that had made fruiting bodies, they could not make fruiting bodies at 34°C but did make fruiting bodies at 28°C (38- to 168-h samples, Table 3). Thus, the stage at which nonaggregated rods were harvested and the temperature at which cells were incubated both before harvesting and after replating were important parameters affecting the development of purified nonaggregated rods on fresh CF agar.

The multicellular processes of aggregation and morphogenesis were altered in most samples. A careful perusal of the data in Tables 1 through 4 shows that any of the three

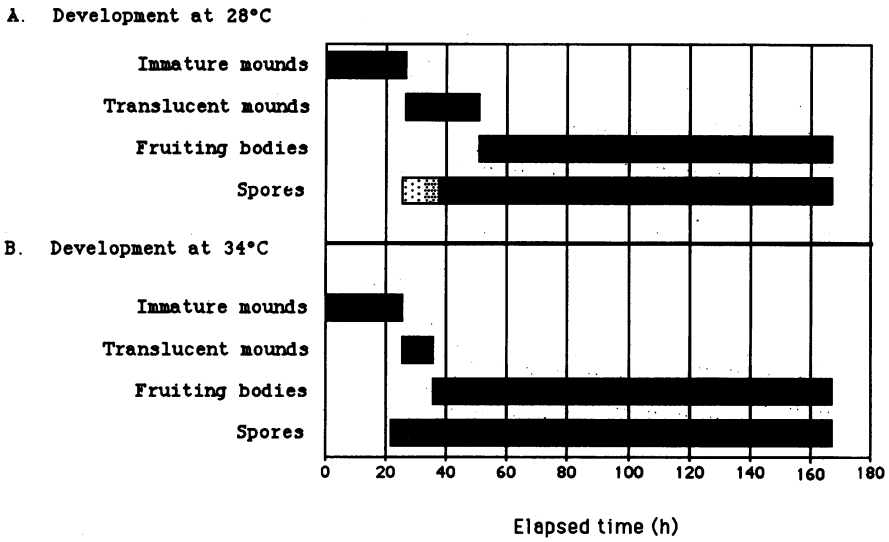

FIG. 1. Influence of temperature on development of vegetatively grown cells. Vegetatively growing DZF1 cells were harvested and suspended at a density of 4×10^9 cells per ml as described in Materials and Methods. Aliquots (5 μl) of cells were applied to fresh CF agar and incubated at 28 or 34°C. Development was monitored at 6- to 12-h intervals. Spores accumulate gradually in the population, as indicated by the increasing density of the bar representing spores.

stages of development could be altered in duration. In some samples, all stages were altered; in others, only one or two stages were affected. Any stage could proceed more quickly or more slowly than normal; the outcome depended upon the stage at which the nonaggregated rods were harvested and the pre- and postharvest incubation temperatures. In fact, nonaggregated rods from very few stages of development were able to develop completely normally. (In contrast, cells that had grown out from the original spot of all samples of nonaggregated rods underwent normal development [27].) In all but two samples (15-h and 120-h 28°C nonaggregated rods), if morphogenesis extended to translucent mounds, then these translucent mounds matured into fruiting bodies. Even though fruiting bodies did not form in all samples, all samples sporulated in apparently normal numbers (27).

The results of this experiment demonstrated that peripheral rods were alive, were not a permanent subpopulation in laboratory strains, and were not irreversibly differentiated. Peripheral rods are capable of undergoing sporulation and, at some stages, are capable of aggregating and forming fruiting bodies.

Development of peripheral rods on conditioned media. The experiments described in the previous section demonstrated that peripheral rods were capable of sporulation on fresh CF agar. Was their failure to do so in situ due to the accumulation of an inhibitor(s) in the medium? We addressed this question by examining the development of peripheral rods on conditioned CF agar. Peripheral rods were harvested from plates at 28 and 34°C after 4 days of development. (Four-day 28°C peripheral rods completed aggregation and morphogenesis at both 28 and 34°C on fresh CF agar, but 4-day 34°C peripheral rods did not aggregate when plated at 34°C on fresh CF agar [Tables 1 through 4].) Aliquots of cells were dispensed on several different media. As standards, vegetatively grown cells were plated on the same media. Development was observed under a dissecting microscope at daily intervals over 7 days. The fruiting morphology at 7 days is reported (Table 5; Fig. 2). Vegetatively grown cells formed fruiting bodies on all media, but the response was temperature dependent on CTPM and *E. coli* agar. Interestingly, 28°C peripheral rods made fruiting bodies under the two conditions that did not promote fruiting body formation in vegetatively grown cells. The 34°C peripheral rods made fruiting bodies only on CF agar at 28°C. They did not complete aggregation on any other media.

The 34°C conditioned CF agar affected the development of all cell types. (i) Vegetatively grown cells formed fruiting bodies of small diameter on 2- to 7-day conditioned CF media at 28°C (Table 5; Fig. 2). (ii) The 28°C peripheral rods did not complete aggregation on 2- to 7-day conditioned CF agar. (iii) The 34°C peripheral rods not only failed to complete aggregation but also failed to complete sporulation at 28°C on 2- to 7-day conditioned CF agar.

Cohesive properties of peripheral cells. Nonaggregated rods taken from certain stages of development failed to aggregate when plated at high cell density on fresh CF agar. Were the cells unable to participate in cell-cell interactions? One measure of the interaction of cells is their cohesiveness. Shimkets has described a method for assaying this cohesiveness in *M. xanthus* (33). In brief, a suspension of cells in MMC buffer is placed in a cuvette, and the A_{625} is monitored over time. The A_{625} is inversely proportional to the degree of cohesiveness of the cells: a decrease in A_{625} is correlated with an increase in the average size of cell aggregates, indicating that the cells are more cohesive.

Peripheral rods were harvested from CF agar after 4 days of development at 28 and 34°C. Vegetatively growing cells were harvested from CYE plates incubated at 34°C for 3 days. The experiment was performed as described in Materials and Methods with incubation at 28 and 34°C (Table 6; Fig. 3). As described previously, vegetatively grown cells formed small clumps of cells (33); the kinetics of cohesion at 28 and 34°C were very similar. Microscopic examination revealed that the cells had formed clumps that were 50 to 100 μm in diameter (Fig. 3). We noted that not all of the aggregates had settled to the bottom of the cuvette. We also discovered that vegetatively grown DZF1 cells harvested

TABLE 5. Fruiting on fresh and conditioned CF agar[a]

Cells and medium	28°C		34°C	
	Morphology	Spores	Morphology	Spores
Vegetatively grown				
CF agar	Fruiting bodies	+	Fruiting bodies	+
17-h conditioned CF agar	Fruiting bodies	+	Fruiting bodies	+
2-, 3-, 4-, 5-, 6-, and 7-day conditioned CF agar	Fruiting bodies*	+	Fruiting bodies	+
TPM-buffered agar	Fruiting bodies	+	Fruiting bodies	+
CTPM-buffered agar	Growth	−	Fruiting bodies	+
E. coli agar	Fruiting bodies	+	Irregular mounds	+
4-day 28°C peripheral rods				
CF agar	Fruiting bodies	+	Fruiting bodies	+
2-, 3-, 4-, 5-, 6-, and 7-day conditioned CF agar	Irregular mounds	+	Irregular mounds	+
TPM-buffered agar	Fruiting bodies	+	Flat	+
CTPM-buffered agar	Fruiting bodies	+	Fruiting bodies	+
E. coli agar	No data		Fruiting bodies	+
4-day 34°C peripheral rods				
CF agar	Fruiting bodies	+	Textured	+
17-h conditioned CF agar	Irregular mounds	+	Flat	+
2-, 3-, 4-, 5-, 6-, and 7-day conditioned CF agar	Irregular mounds	pre	Flat	+
TPM-buffered agar	Mounds	+	Mounds	+
CTPM-buffered agar	Growth	pre	Textured	+
E. coli agar	Mounds	+	Flat	+

[a] Vegetatively grown cells, 4-day 28°C peripheral rods, and 4-day 34°C peripheral rods of DZF1 were harvested and isolated as described in Materials and Methods and suspended at 4×10^9 cells per ml in TPM buffer. Aliquots (5 μl) were dispensed onto plates (see Materials and Methods) and incubated at 28 or 34°C. The morphology was scored after 7 days; cells were scraped from the plate and examined at ×400 magnification to screen for sporulation. Morphological descriptions: flat, no evidence for cell-cell interactions; textured, cell movements and associations cause the surface of the developing colony to appear rough, but no mounds formed; irregular mounds, when mounds are first forming their borders are ill-defined giving them an irregular periphery; mounds, an intermediate stage in aggregation; fruiting bodies, the final stage of development, well-defined mounds of spores. +, refractile spores were present; −, no refractile spores were present; pre, only nonrefractile prespores were present; *, fruiting bodies were smaller than those formed on CF agar.

from CYE plates were more cohesive than vegetatively grown cells harvested from CYE liquid cultures (27).

Peripheral rods from 34°C clumped more rapidly at 28 than at 34°C. A larger portion of this population was recruited into aggregates than was the case with vegetatively grown cells under similar conditions (Table 6). Microscopy revealed that the aggregates of 34°C peripheral rods were similar in size to those of vegetatively grown cells. It was also observed that 34°C peripheral rods had sporulated during the course of the assay (Fig. 3). Prespores were observed within 1 h of the initiation of the assay; sporulation progressed more rapidly at 34°C than at 28°C. This phenomenon may account for the difference in the kinetics of cohesion at the two temperatures. Perhaps spores are not as cohesive as rods. We have never observed sporulation of vegetatively grown cells under the conditions of the cohesion assay. However, sporulation of vegetatively grown cells in MMC buffer was observed when cells were incubated with shaking for 7 days (32).

Peripheral rods grown at 28°C formed an unusual aggregate that could be described as a tube (Fig. 3). Although these aggregates have occasionally been observed in cohesion assays of vegetatively grown cells of strain DK1622, it is not the usual behavior of these cells (35). Four-day 28°C peripheral rods formed a tube every time they were assayed. Tubes formed more rapidly at 34°C than at 28°C. Upon continued incubation, tubes constricted and collapsed upon themselves (Fig. 3). The data in Table 6 reflect tube formation: at 3 h the A_{625} had increased at 28°C due to the formation of the tube and the consequent concentration of cells in the light path of the spectrophotometer; however, the tube had already collapsed at 34°C, giving a lower A_{625}. Examination of the mat of cells in the bottom of the cuvette after 24 h of incubation revealed that cells incubated at 34°C had begun to sporulate. Cells incubated at 28°C had formed aggregates in which cells had begun to sporulate (Fig. 3).

This experiment demonstrated that peripheral rods were at least as cohesive as vegetatively grown cells. Therefore, peripheral rods do not suffer from a complete inability to participate in cell-cell interactions.

Glycerol-induced sporulation of peripheral rods. Vegetatively growing and developing cells of DZF1 were induced with 0.5 M glycerol in CYE growth medium at 32°C (7). Cells were removed from culture at intervals and examined at ×400 magnification to determine the stage of sporulation (Table 7). Vegetatively grown and 18-h developmental cells formed spherical, refractile glycerol-induced spores within 6 h under these conditions (18-h cells were slightly faster than vegetatively grown cells in forming glycerol-induced spores). However, 1-day 34°C cells and 34°C peripheral rods from 2-, 4-, and 6-day CF agar cultures were glycerol inducible, but differentiation occurred at a rate slower than that of vegetatively grown cells. Whereas 1-day 34°C cells were at least 3 h behind vegetatively grown cells, 4-day 34°C peripheral rods lagged behind vegetatively grown cells by no more than 1 h. Four-day 28°C peripheral rods were not glycerol inducible. Note that these cells did form fruiting bodies and sporulated normally when plated on CF agar.

Role of peripheral rods in the life cycle of *M. xanthus*. We hypothesized that peripheral rods might serve a biological role in the life cycle of *M. xanthus* under conditions of low levels or transient influxes of nutrients that would not support the germination and outgrowth of the sporulated portion of the population. To test this hypothesis, we mea-

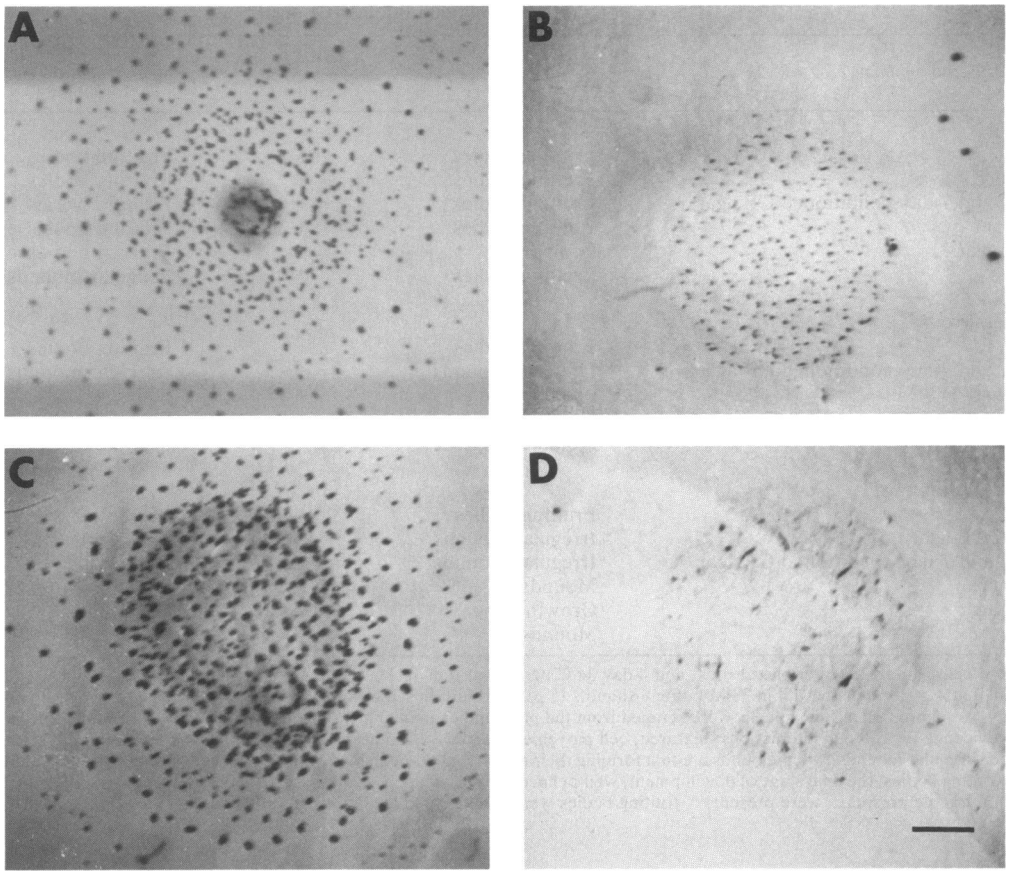

FIG. 2. Development of *M. xanthus* cell types on conditioned CF agar. Vegetatively grown cells (A and B) and 4-day 34°C peripheral rods (C and D) were harvested as described in Materials and Methods. Aliquots (5 μl) of 4×10^9 cells per ml were placed on CF agar (A and C) and on 4-day conditioned CF agar (B and D) and then incubated at 28°C. Plates were photographed after 1 week. All photographs are at the same magnification: bar, 10 mm.

sured the rates of growth of peripheral rods and spores on *E. coli* agar. Peripheral rods and spores were purified from cultures of DZF1 developing on CF agar at 34°C. Vegetatively growing cells were harvested from CYE liquid medium. Cells were inoculated in a drop onto TPM-buffered agarose plates previously overlaid with TPM-buffered low-melting-point agarose containing concentrated, live *E. coli* as described in Materials and Methods. The rate of growth of cells on the plates was measured by recording the diameter of the area of outgrowth of *M. xanthus* from the site of inoculation. The results of these experiments are presented in Table 8.

TABLE 6. Cohesion assay of vegetatively grown cells and peripheral rods isolated from CF agar[a]

Cell type	Time in buffer (h)	% of initial A_{625}	
		28°C	34°C
3-day vegetatively grown cells	3	90	89
	24	66	52
4-day 28°C peripheral rods	3	113	20
	24	20	20
4-day 34°C peripheral rods	4	30	49
	24	14	24

[a] DZF1 was grown in CYE liquid and inoculated for development on CF agar. Peripheral rods were isolated from cultures developing on CF agar incubated at 28 or 34°C for 4 days, and the cohesion assay was performed as described in Materials and Methods. Vegetatively grown cells were obtained from CYE agar incubated at 34°C for 3 days. A_{625} was monitored hourly for 6 to 8 h and again at 24 h. The results of a single experiment are reported. The experiments were repeated a total of three times with similar results.

Peripheral rods grew at the same rate as vegetatively grown cells on both concentrations of *E. coli* tested. However, since this technique could not distinguish the difference in growth rates between 1× and 0.1× inoculums of *M. xanthus* cells, subtle differences in growth rates would not have been detected. By the same token, the data cannot be used to determine whether all inoculated cells resumed growth or whether only a portion of the cells began to grow.

When 4-day spores were plated on 10^9 *E. coli* per plate, spores grew more slowly than peripheral rods. However, 4-day spores plated on 10^{10} *E. coli* per plate grew at the same rate as peripheral rods. This indicates that the spores must have germinated and begun to grow more quickly on the higher concentration of *E. coli*. Since 7-day spores did not germinate on 10^{10} *E. coli* per plate, they were more dormant than spores from cultures developing for 4 days. (We know that 7-day spores are viable because they can form colonies when plated on CYE [22].) This is in agreement with previous observations that spores continue to mature for at least 5 days after the completion of fruiting body morphogenesis (18, 22).

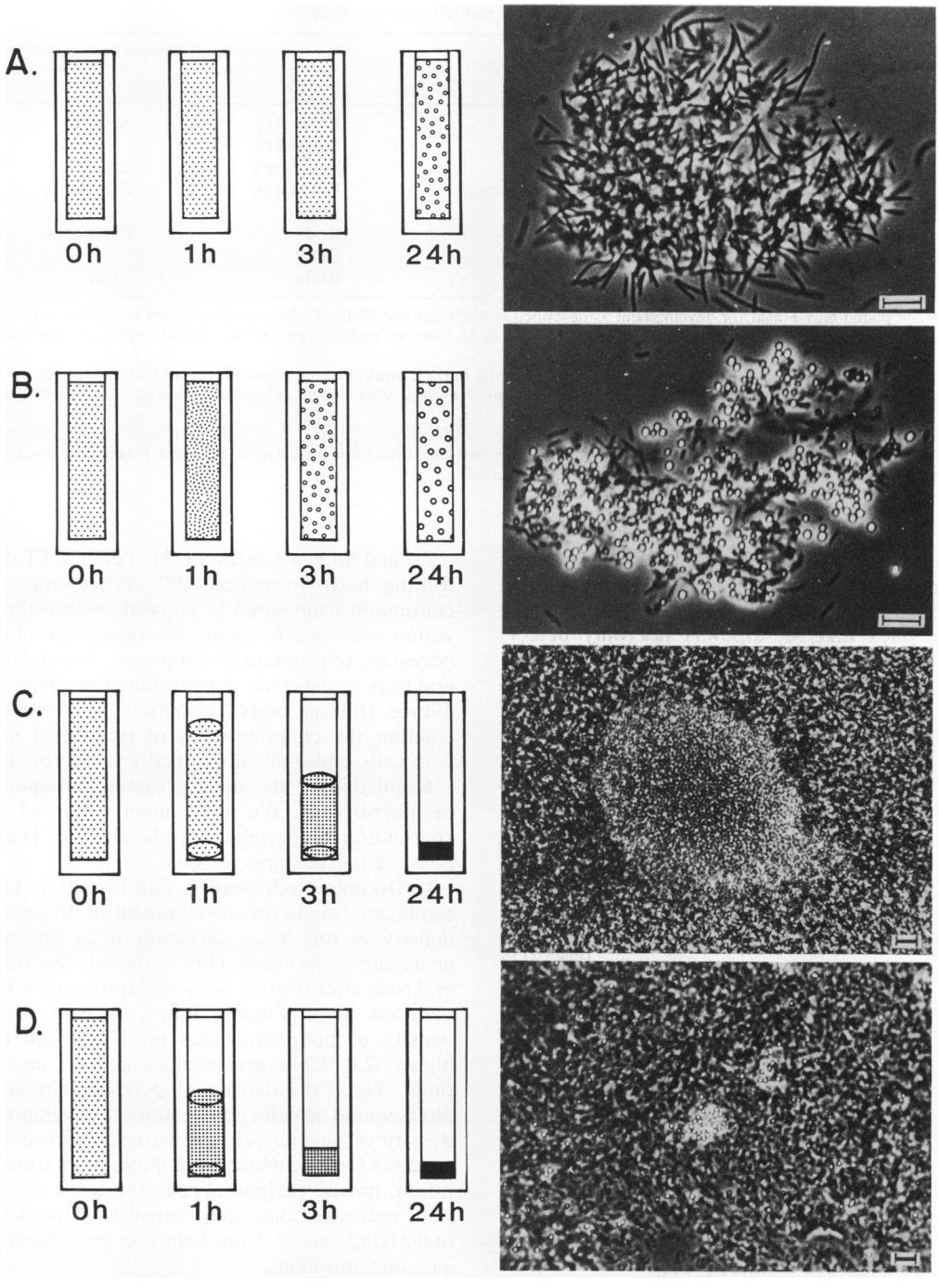

FIG. 3. Cohesion assays. Vegetatively grown cells (A) were harvested from CYE agar after 3 days at 34°C and incubated at 28°C. Peripheral rods were harvested from CF agar after 4 days at 34°C (B) or 28°C (C and D) and incubated at 28°C (B and C) or 34°C (D). Cohesion assays were performed as described in Materials and Methods. Line drawings are based on photographs taken during experiments. After 24 h, 5-μl aliquots of the mat of cells at the bottom of the cuvette were taken for microscopic examination. Bars, 10 μm.

DISCUSSION

Peripheral rods are the cells that remain outside aggregates after the process of aggregation has ceased. In the two accompanying reports, we demonstrate that the patterns of expression of a number of proteins in peripheral rods are distinctly different from the patterns of expression in either vegetatively growing cells or spores (25, 26). These results led us to describe peripheral rods as a separate cell type specific to the developmental phase of the life cycle of *M. xanthus*. In this study, we examined several biological characteristics of peripheral rods.

TABLE 7. Glycerol induction of sporulation[a]

Time	Development temp (°C)	Aggregate morphology[b]	Cellular morphology after glycerol addition[c]		
			3 h	6 h	24 h
0 h[d]	32		Prespores	Spores	Spores
1 h[e]	34	Premounds	Prespores		Spores
18 h[e]	34	Mounds	Prespores	Spores	Spores
24 h[e]	34	Early translucent mounds	Prespores	Prespores	Spores
2 days	34	Fruiting bodies	Rods		Spores
4 days	34	Fruiting bodies	Rods	Prespores	Spores
6 days	34	Fruiting bodies	Rods		Spores
4 days	28	Fruiting bodies	Rods	Rods	Rods

[a] DZF1 was grown and plated on CF agar for development as described in Materials and Methods. Peripheral rods were isolated at the indicated times.
[b] There are several recognizable morphological stages in the development of *M. xanthus*, including premound, mound, translucent mound, and fruiting-body (a mound of spores) stages.
[c] Glycerol was added at a final concentration of 0.5 M to 3×10^8 cells per ml in CYE broth. Cultures were incubated with shaking at 30°C. Aliquots of cells were examined at ×400 magnification to determine the extent of sporulation. Prespores were spherical but nonrefractile. Glyerol-induced spores were both spherical and refractile.
[d] Cells harvested from CYE agar.
[e] Samples taken at 1, 18, and 24 h were not separated into cell types for glycerol induction. Cells were harvested from CF agar, dispersed by homogenization, centrifuged to remove TPMF, then inoculated in CYE containing 0.5 M glycerol.

Nutrients and peripheral rods. The proportion of peripheral rods in a developing population of cells increases with increasing levels of nutrients in the development-inducing medium. This implies that *M. xanthus* not only detects starvation but also monitors the degree of starvation to some extent. Indeed, the developmentally regulated protein myxobacterial hemagglutinin, which is expressed in peripheral rods, is not expressed on CYE agar plates that contain high levels of nutrients (4, 25, 26). However, myxobacterial hemagglutinin is expressed at 0.1 developmental level on 1/2CTT agar, which contains half the amount of Casitone in CYE and no yeast extract (11). Yet 1/2CTT does not induce fruiting body formation (27). There may, in fact, be a continuum from sated to starved, with a threshold of starvation necessary to trigger the expression of all of the genes necessary to promote development. Our definition of peripheral rods applies only to cells that arise under conditions that induce fruiting body formation. It remains to be seen whether the characteristics of peripheral rods are shared with cells under all suboptimal growth conditions.

TABLE 8. Growth of *M. xanthus* with *E. coli* on agarose plates as the source of nutrients[a]

Growth conditions	Cell type	Diameter of growth area (mm)[b]
4 days, 10^9 *E. coli* per plate	Vegetatively grown cells	9
	4-day 34°C peripheral rods	10
	4-day 34°C peripheral rods (diluted 1:9)	8
	4-day 34°C fruiting-body spores	2
1 day, 10^{10} *E. coli* per plate	4-day 34°C peripheral rods	6
	4-day 34°C spores	5
	4-day 34°C spores, gradient purified	6
	7-day 34°C peripheral rods	8
	7-day 34°C spores	0
	7-day 34°C spores, gradient purified	0

[a] DZF1 was grown and plated for development on CF agar at 34°C. Cells were harvested, and cell types were isolated after 4 or 7 days of development. In some experiments, spores were further purified on a sucrose gradient as described in Materials and Methods. Cells were suspended at 10^5 cells per ml in TPM buffer. Then 1,000 in 10 μl of buffer were inoculated in the center of *E. coli* agarose plates, which were then incubated at 34°C. *E. coli* plates were made as described in Materials and Methods. The agarose takes on a cloudy appearance due to the presence of cells.
[b] Plates were periodically removed from the incubator and the diameter of the cleared area (caused the lysis of *E. coli* by *M. xanthus*) was measured under a dissecting microscope at ×12 to ×32 magnification. The data represent the averages of the measurements of two plates. In all cases, the measurement of two plates agreed within 0.5 mm.

Regulation of the nonaggregated, nonsporulated state of peripheral rods. We have never observed aggregation or sporulation of peripheral rods in situ. There are several possible mechanisms for this.

(i) Decreased cell density. Cell density is known to play a significant role in the development of *M. xanthus*. If the cell density is too low, starvation does not induce cells to aggregate or sporulate (39). Although the density of peripheral rods after fruiting body maturation on CF agar is indeed very low (approximately 4.8×10^5 cells per cm^2 [22]), the density of peripheral rods on Nuclepore filters is much higher (23). Cells are in close contact and several layers thick. Thus, the density of peripheral rods on Nuclepore filters would be sufficient to support development of a similar density of vegetatively grown cells. However, no new aggregates form on Nuclepore filters after fruiting body maturation, nor do peripheral rods sporulate (23). This observation indicates that cell density is unlikely to be the underlying cause of the failure of peripheral rods to aggregate and sporulate.

(ii) Subpopulations within laboratory stocks of *M. xanthus*. We observed that peripheral rods harvested at appropriate times during development developed normally on fresh CF agar. The appearance of fruiting bodies and the distribution of peripheral rods in this second round of development were indistinguishable from those of the original population. We conclude that peripheral rods do not constitute a permanent subpopulation of cells incapable of aggregation or sporulation.

We note that most strains of *M. xanthus* undergo phase variation between yellow and tan pigmentation. Although most cells are yellow, as much as 10% of the population can be tan. Among the tan variants are nonswarmers. Tan nonswarmer strains have been isolated; these strains do not

aggregate in response to starvation (40). Although the proportion of peripheral rods in a developing culture is the same as the proportion of tan cells in a growing culture and the phenotype of tan strains is similar to that of peripheral rods in situ, these correlations are only coincidental. Purified peripheral rods could be seen to be predominantly yellow in all strains tested. Furthermore, when peripheral rods were plated on CYE agar, the resulting colonies were not enriched for tan variants (27). Therefore, peripheral rods are not the result of phase variation for pigmentation within the population of cells.

(iii) Loss of intercellular cohesiveness. Another possible explanation for the failure of peripheral rods to aggregate or reaggregate is that they have lost their ability to participate in intercellular interactions. When this was tested by the cohesion assay we observed that all peripheral rods tested were cohesive, more cohesive than cells grown on nutrient agar or in nutrient broth (33). We also observed that samples of peripheral rods that did not form mounds on plates sporulated rapidly in cohesion buffer and that samples of peripheral rods that formed mounds on CF agar formed tubes in the cohesion assay. These structures contracted upon themselves, collapsing into the bottoms of the cuvettes. Fruiting bodies were formed in the collapsed tubes. The formation and contraction of the structures were faster at 34°C than at 28°C. The suspended cells seemed to be going through the cell movements and interactions involved in development on a surface (23). The structure might arise through the formation of a monolayer of cells interacting only through lateral contacts. The subsequent contraction of the tube might result from cellular interactions forming multiple layers of cells that interact at dorsal-ventral surfaces. These phenomena have been documented for development on a surface (23).

(iv) Irreversible differentiation of peripheral rods. The possibility that peripheral rods were irreversibly differentiated into aggregation- and/or sporulation-incompetent cells was investigated by plating nonaggregated rods from many stages of development on fresh CF agar. These experiments are analogous to those performed by Raper on the cellular slime mold *Dictyostelium discoideum* (28). The results were surprisingly complex but did show that peripheral rods were capable of aggregation and sporulation. Therefore, peripheral rods had not reached a state of irreversible differentiation.

Although we do not know why samples of peripheral rods from some stages of development did not complete aggregation, one explanation is that the cells sporulated rapidly when plated on fresh CF agar. Four-day 34°C peripheral rods did sporulate rapidly in MMC buffer and might have done so on fresh CF agar. Sporulation would inhibit aggregation, since spores are certainly unable to participate in the cell movements necessary for aggregation and mound morphogenesis. The timing of sporulation was not monitored in recapitulation experiments and remains to be investigated.

(v) Accumulation of inhibitors of aggregation and/or sporulation. A remaining explanation, consistent with the data, is that inhibitors of aggregation accumulate during development. This hypothesis was tested by observing development on conditioned CF agar. The pattern of aggregation of vegetatively grown cells plated on conditioned CF agar was altered relative to the pattern seen on fresh CF agar. Agar that had been exposed to fruiting bodies caused vegetatively grown cells to form smaller but more numerous fruiting bodies. In contrast, sporulation was apparently unaffected. On the other hand, peripheral rods plated on conditioned CF agar were much more strongly affected than were vegetatively grown cells: Aggregation was reduced or blocked and morphogenesis of fruiting bodies was blocked. Even sporulation was inhibited under some conditions.

Since vegetatively grown cells developed on TPM-buffered agar and 17-h conditioned CF agar, the influence of conditioned media on development is unlikely to be due solely to the depletion of nutrients. Thus, conditioned media must affect the development of vegetatively grown cells and peripheral rods through the accumulation of byproduct(s) in the medium during development. The byproduct(s) is not characteristic of all bacteria, since *E. coli* did not inhibit the development of vegetatively grown cells at 28°C. The data suggest that an inhibitor(s) of aggregation begins to accumulate by 17 h during development of *M. xanthus* and that peripheral rods become more susceptible to the inhibitor as development proceeds. The results with 34°C peripheral rods suggest that an inhibitor(s) of sporulation may also accumulate during late development.

Role of peripheral rods in the life cycle of *M. xanthus*. We have postulated that peripheral rods could take advantage of low levels of nutrients that would not support germination and outgrowth of myxospores. Our studies with *E. coli* as a nutrient source support this hypothesis. Peripheral cells were able to grow on low levels of *E. coli* that were insufficient to support germination of spores. This is consistent with two other observations. (i) After 7 days of development, CF agar plates were overlaid with 2.5 ml of TPM-buffered, low-melting-point agarose containing 5×10^9 *E. coli* per ml. After 6 additional days of incubation, the center of the plate had cleared due to the lysis of *E. coli* cells by *M. xanthus* (*E. coli* did not lyse under the same conditions in the absence of *M. xanthus*). The number of fruiting bodies remained constant in the cleared area, and these fruiting bodies contained spores (27). Thus, under these conditions, spores did not germinate even though *E. coli* cells were obviously lysed and presumably consumed by peripheral rods (27). (ii) After 7 days of development, the nutrient medium CYE was allowed to diffuse into the CF agar. A single microscopic field was videotaped over a period of 8 to 12 h. The first cells to commence cell division were peripheral rods, although spores did eventually begin to germinate and grow. No cell division occurred before the addition of CYE (27).

As we have already noted, there is a direct correlation between the level of nutrients in the medium and the proportion of peripheral rods in the population. It would be advantageous for *M. xanthus* to shift the distribution of cells toward sporulation when nutrient levels were well below those sufficient for growth (e.g., TPM-buffered agar) but to maintain a larger number of peripheral rods when nutrient levels were only slightly below what is necessary to support growth (e.g., CTPM agar). This strategy would ensure that, when a slight increase in nutrient levels would support growth, a number of peripheral rods would be present to take advantage of the situation. On the other hand, when nutrients were so depleted that it would require a long time for them to be replenished (e.g., by repopulation by a prey organism), more *M. xanthus* cells would become spores, thus increasing long-term survival.

Starvation or differentiation? How are we to decide whether peripheral rods are starved vegetative cells or a differentiated cell type? At some point this becomes a question of semantics, because spores also arise as the result of the response of *M. xanthus* to starvation. There is no argument over labeling *M. xanthus* spores a differentiated

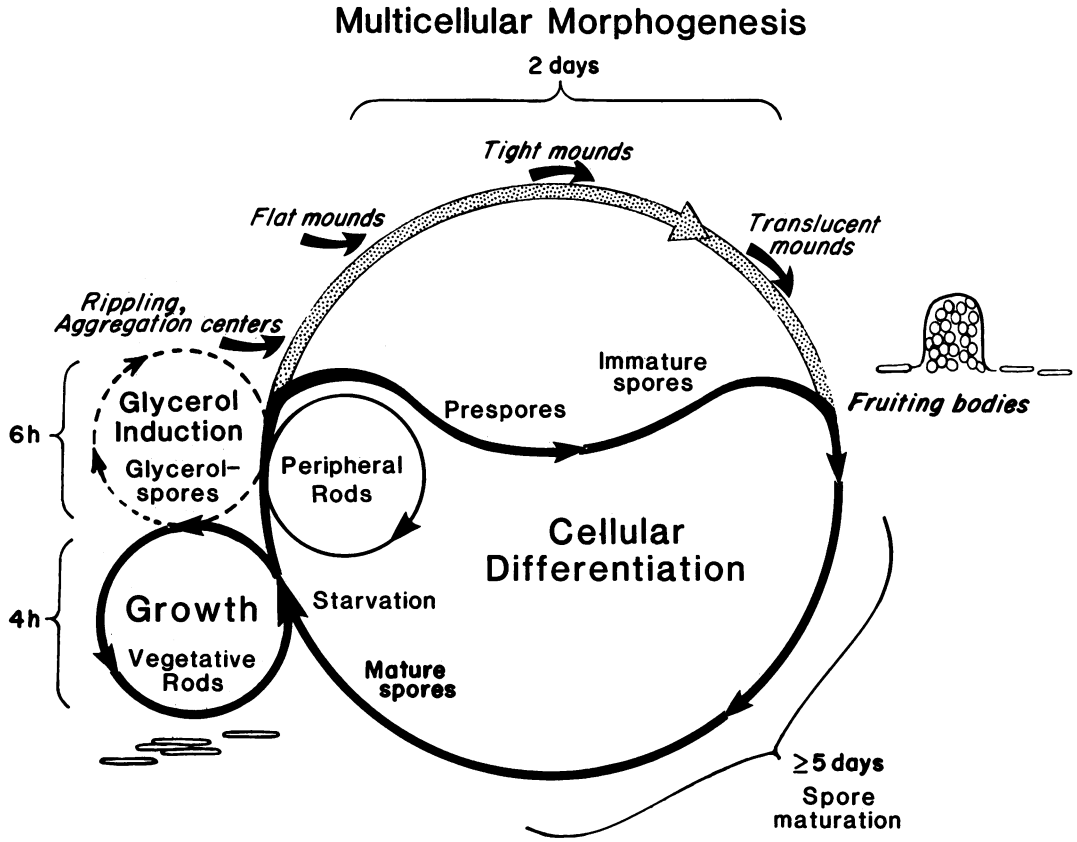

FIG. 4. Life cycle of *M. xanthus*.

cell type. The morphology of spores is so obviously different from that of vegetatively growing cells that few would question that they fit the definition of differentiation in the degree of their biochemical and functional specialization (8). We do not yet know enough about the ultrastructure of peripheral rods and vegetatively growing cells to determine their degree of similarity. We do know that proteins S and C are expressed at high levels in both peripheral rods and spores and that these proteins are associated with insoluble material in both cell types. Neither protein is expressed in vegetatively growing cells (12, 19, 25). This alone suggests that there are ultrastructural differences between peripheral rods and vegetatively growing cells. Peripheral rods express most of the developmentally regulated proteins identified to date, and the global expression of proteins differs as greatly between peripheral rods and vegetatively growing cells as between vegetatively growing cells and spores (25, 26). Could most of the differences between vegetatively growing cells and peripheral rods detected by two-dimensional polyacrylamide gel electrophoresis be due solely to differences in the nutritional states of the cells? The answer is yes, but the same could be said of spores since they also arise in response a change in nutritional state.

Both *An Introduction to Embryology* and *A Dictionary of Life Sciences* refer to change in the function of the cell as a component of differentiation (2, 16). We believe that this component is as important as biochemical and structural specialization (8). Although the biochemistry of *E. coli* growing on maltose is different from that of *E. coli* growing in the absence of maltose, this difference would not seem to constitute differentiation because the function of the organism remains the same: to increase total cell mass and cell numbers. Changes in gene expression that enhance the ability of a cell to perform the same function under different conditions would be better described as accommodation than as differentiation. For example, one of the functions of the liver is to remove toxins; increased production of alcohol dehydrogenase in response to the consumption of alcohol allows the liver to perform this function better. The increased production of alcohol dehydrogenase does not constitute differentiation.

The biochemistry of vegetatively growing cells and peripheral rods differs. The ultrastructure of the two cell types differs (at some level, not yet fully determined). Do the functions of the two cell types differ? We believe that they do. Vegetatively growing cells grow and divide, increasing cell numbers in the population. Peripheral rods are not in the process of cell division. They are in the process of remaining viable days after their last cell division, as are spores. Because peripheral rods arise in response to the same conditions that induce aggregation and sporulation, express proteins peculiar only to peripheral rods and other developing cells, and seem to function more like spores than like vegetatively growing cells, we think that peripheral rods are a differentiated cell type rather than vegetatively growing cells in a different nutritional state.

Life cycle of *M. xanthus*. Figure 4 is a diagram of the life cycle of *M. xanthus* that reflects our increasing understanding of the process of development. Morrison and Zusman have shown by mutational analysis that the processes of aggregation and sporulation are somewhat independent (21). They proposed a tuning fork model for development which

has been incorporated in Fig. 4 as a bifurcation of the circle representing development. However, aggregation and sporulation are not completely independent processes. Hagen et al. have described four classes of mutations that affect both aggregation and sporulation—the Spo mutants (now designated Asg, Bsg, Csg, and Dsg) (10). In addition, we have recently shown that sporulation and aggregation occur concomitantly: the first cells to enter aggregates begin to sporulate, while more cells continue to enter the aggregate (23).

Figure 4 also reflects new information on the rate of maturation of spores. (i) It has recently been shown that the percentage of spores that can form colonies on CYE agar rises for at least 5 days after spores first appear in the culture (22). (ii) It has also been shown that the trehalose content of myxospores continues to rise for at least 4 days after spores first appear in developing cultures (18). (iii) An alkaline phosphatase activity also accumulates with time in maturing spores (25, 38). (iv) We have reported herein that 7-day spores are more dormant than 4-day spores. In addition, 4-day spores germinated within 7 h of exposure to CYE broth in situ, whereas 7-day spores required at least 12 h of incubation to germinate (27). (v) We have found that 100% of 7-day spores were resistant to incubation at 50°C for 1 h, whereas only 57% of 4-day spores were heat resistant (27).

Peripheral rods have been given their own circle within the life cycle because the expression of some functions appears to be cyclical: at 28°C peripheral rods go through cycles of competence to aggregate. Myxobacterial hemagglutinin is also expressed cyclically in peripheral rods (25). Another reason for giving peripheral rods their own circle within development is that their response to starvation is different from that of spores. In an accompanying report, we speculated that peripheral rods might be responding to starvation in a manner analogous to that of *E. coli* or *Salmonella typhimurium* (26). Matin and co-workers have shown that starvation for nutrients alters the pattern of expression of proteins in *E. coli* and that these changes in expression allow the cells to survive starvation for days (9, 17, 29). The response to starvation renders these cells resistant to other stresses, including osmotic challenge (14) and H_2O_2 and heat (15). These studies have led them to propose that, although *E. coli* does not sporulate, it does accommodate to starvation in a manner that may be analogous to sporulation in other organisms. Studies of *S. typhimurium* exposed to four different nutritional deprivations, heat shock, and anaerobiosis demonstrated that the responses to starvation for different nutrients were closely interrelated but that the responses to heat shock and anaerobiosis were not related to the starvation response (37). These workers also propose that the starvation response in *S. typhimurium* is analogous to sporulation in bacilli (36). Further work needs to be done on peripheral rods of *M. xanthus* to determine whether they also are more resistant than vegetatively growing cells to various stresses.

Glycerol-induced spores have been given their own circle separate from development (7). Glycerol-induced spores are very different from myxospores formed in fruiting bodies. For example, glycerol-induced spores do not possess a major fruiting-body spore protein (protein S, Tps), and the outer walls of the two types of spores differ significantly as revealed by transmission electron microscopy (12). *tps* is not expressed under conditions of glycerol induction; however, highly homologous *ops* is expressed (5). Two-dimensional polyacrylamide gel electrophoretic analysis of glycerol-induced spores and developmental cell types has shown that glycerol-induced spores are more similar to 15-h developing cells than to fruiting-body spores (26).

Evidence for two aggregation systems in *M. xanthus* and the role of temperature in their regulation. We have noted that the behavior of peripheral rods plated on fresh CF agar is complex. In some samples, aggregation into tight mounds, morphogenesis of tight mounds into translucent mounds, and morphogenesis of translucent mounds into fruiting bodies containing spores all differed from the behavior of vegetatively grown cells under the same conditions. In other samples only one or two of these stages of development were affected. This indicates that these three stages of development probably express different sets of genes regulated as separate systems. However, there must be some overlap in the regulation of the systems to coordinate the processes.

The development of peripheral rods on fresh CF agar can be interpreted as evidence for two mound formation systems in *M. xanthus*. Peripheral rods grown at 28°C went through cycles of approximately 12 h in their ability to recapitulate development at 28°C. In contrast, these cells retained their ability to form mounds at 34°C through 4 days of development. On the other hand, 34°C peripheral rods lost their capacity to undergo morphogenesis at 34°C at the stage in development when mature fruiting bodies first appeared (38 h), i.e., when morphogenesis was complete. However, they retained their ability to fruit at 28°C through at least 6 days of development. These unexpected results tie into studies of Tag mutants, which are temperature sensitive for the ability to undergo mound morphogenesis but sporulate normally at permissive and restrictive temperatures (21, 24). Strains with mutations in any of eight known *tag* complementation groups develop normally at 28°C but do not form mounds at 34°C. This observation led us to postulate that there were at least two systems for mound formation in *M. xanthus*. One system functioned at 28°C but not at 34°C, and the other system (*tag*) was essential for mound formation at 34°C. The experiments with replated peripheral rods may be a means of studying these two mound formation systems without mutational analysis. During differentiation, 28°C peripheral rods may cyclically shut down the 28°C system such that it cannot be fully reactivated under the conditions of these experiments but the 34°C system may remain functional or at least inducible. Conversely, 34°C peripheral rods may shut off the 34°C mound formation system but the 28°C system may remain functional or inducible. It should be noted that 34°C is well within the optimal growth range of the organism (13); development at 34°C is faster than development at 28°C, and the regulation systems of at least some genes differ at the two temperatures (5, 24, 25).

In addition to temperature, nutrient levels might also affect the two systems for mound formation. Vegetatively grown cells developed on *E. coli* and CTPM agar at 34°C but not at 28°C. This phenomenon has been observed before with 0.1% Casitone–0.1% $MgSO_4$ agar (3, 30). This is a further distinction between the two aggregation systems.

Summary. In this and the two accompanying reports, we have shown that *M. xanthus* differentiates into two cell types, spores and peripheral rods, in response to starvation (25, 26). The two cell types differ markedly in behavior, shape, and physiology as well as localization within a developing culture. We have shown that peripheral rods occur in all laboratory strains tested and that peripheral rods are easily purified to homogeneity for further analysis. Analyses of these cell types have identified cell-type-specific proteins (25, 26). The level of nutrients present at the time of initiation of development contributes to the regulation of the

proportion of peripheral rods in the population. The behavior of peripheral rods is regulated at least in part by the accumulation of one or more inhibitors of aggregation and sporulation in the environment during development.

In addition, we have postulated that peripheral rods play an important role in the survival of *M. xanthus* in its natural habitat. Experiments reported herein support the hypothesis that peripheral rods allow *M. xanthus* to utilize low levels of nutrients. Peripheral rods may be analogous to starved cells of *E. coli* or *S. typhimurium*, which adapt to survive starvation but do not sporulate (17). We do not believe that the differentiation of peripheral rods is related to the altruistic behavior of *M. xanthus* proposed by Zahavi and Ralt (41). Both strategies, the differentiation of peripheral rods and sporulation, are selfish, but the combination may make the organism more adaptable to the vicissitudes imposed by nature. Because of their different capacities to utilize low levels of nutrients and to withstand long-term deprivation, both cell types resulting from development grant an adaptive advantage for survival in *M. xanthus*. Peripheral rods make use of low levels of nutrients but would succumb to dessication or long-term starvation. Spores, on the other hand, survive both dessication and long-term starvation but cannot utilize low levels of nutrients.

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