Development in *Myxococcus xanthus* Involves Differentiation into Two Cell Types, Peripheral Rods and Spores

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Myxococcus xanthus, a gram-negative bacterium, has a complex life cycle. In response to starvation, most cells in a population participate in the formation of multicellular aggregates (i.e., fruiting bodies) in which cells differentiate into spores. However, some cells do not enter aggregates. In this and the two accompanying reports, the biology and physiology of these nonaggregated cells is examined. A technique to separate aggregated cells from nonaggregated cells was developed; then differentiating cells at stages throughout the course of development were isolated. In this report we (i) describe peripheral rods, those cells which remain outside aggregates after aggregation has ceased in the rest of the population; (ii) document the occurrence of peripheral rods in several wild-type strains; and (iii) characterize the expression of developmentally regulated genes in both aggregated and nonaggregated cells. These studies have shown that myxobacterial hemagglutinin, protein S (Tps), protein S1 (Ops), and several phosphatase activities are expressed in cell-type-specific patterns. These data demonstrate that peripheral rods constitute a cell type distinct from either vegetatively growing cells or spores. The description of a second, late developmental cell type (in addition to spores) opens an entirely new line of investigation in *M. xanthus*, i.e., the regulation of the differentiation of vegetatively growing cells into two cell types that differ significantly in biology, shape, and localization within the population.

Development and differentiation are often perceived and defined in the context of multicellular, eucaryotic organisms. However, *Myxococcus xanthus* is a procaryotic organism that demonstrates both development and differentiation. This rod-shaped, gram-negative bacterium can be found in a variety of soil habitats. It feeds by cooperatively preying on other microorganisms in its environment and through absorption of dissolved nutrients. When the local supply of nutrients becomes depleted, *M. xanthus* forms multicellular aggregates that undergo morphogenesis from flat mounds into raised mounds within 1 or 2 days after the initiation of starvation. Within these fruiting bodies, cells differentiate into spherical, optically refractile spores. The entire cell undergoes this transformation from rod to spore; there are no spore-mother cells. Spore differentiation and maturation continue for at least 7 days after the initiation of starvation (27, 31, 42, 43, 49).

Existing models present development of this organism as a series of events that lead to the differentiation of a single mature cell type, the spore, which is located in a structure known as a fruiting body (12, 42, 43, 49). However, we present evidence in this and two accompanying reports (34, 35), that differentiation of a second cell type occurs during development in *M. xanthus*. We have named this second cell type peripheral rods. These are the cells that remain outside fruiting bodies, dispersed amidst the multicellular fruiting bodies; peripheral rods neither enter aggregates nor sporulate.

Differentiation has been described as "the process in which cells or other parts of an organism become different from one another and also different from their previous condition" (2). If peripheral rods are truly a different cell type arising by differentiation from vegetatively growing cells in response to the induction of development, then they

would be expected (i) to occur in all strains of *M. xanthus*, (ii) to have unique patterns of expression of proteins, (iii) to be structurally dissimilar from the cell types most similar in patterns of protein expression, (iv) to exhibit unique responses to environmental stimuli, and (v) to serve a unique function in the life cycle of the organism. In this report we address points i, ii, and iii. We demonstrate that the proportion of peripheral rods in a developing population is reproducible under constant conditions and that peripheral rods occur in all wild-type strains of *M. xanthus* tested. We also show that the expression of nine developmentally regulated proteins in peripheral rods is different from their expression in vegetatively grown cells, prespores, and spores. Although the developmentally regulated proteins and enzymatic activities examined in this study show peripheral rods to be more similar to spores than to vegetatively grown cells, peripheral rods and spores are obviously different in structure. Point ii is further addressed through the examination by two-dimensional gel electrophoresis of cell-type-specific expression of a large number of proteins (34). Point iv is addressed in a third report, in which the biology of peripheral rods is examined with particular attention to their competence to participate in the multicellular interactions necessary for fruiting body formation and their response to changes in their environment (35). The possible role of peripheral rods in the life cycle of *M. xanthus* (point v) is also examined in reference 35.

In addition to the description of peripheral rods as a significant cell type in *M. xanthus*, it has been found that temperature plays a key role in the regulation of the expression of developmental proteins. The data indicate that development is slower at 28°C and that the cell-type-specific regulation of protein expression is tighter at this temperature. Both 28 and 34°C are well within the growth range of *M. xanthus* (19).

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TABLE 1. *M. xanthus* strains

Strain	Relevant genotype	Notes	Source or reference
DK1622	Wild type	Obtained from D. Kaiser	20
DZF1	<i>sglA</i> ^{leaky}	Mutation in S motility system	9, 15
DZF3353	<i>ops</i>	Tn5 insertion Ω 200 in <i>ops</i>	7
DZF3358	<i>tps</i>	Tn5 insertion Ω 156 in <i>tps</i>	7
DZF3367	<i>ops tps</i>	Tn5 insertions Ω 200 and Ω 156	7
DSW2 ^a	Wild type	Isolated by David Weiss	This paper
DSW4	Wild type	Isolated by David Weiss	This paper
DSW5	Wild type	Isolated by David Weiss	This paper

^a DSW strains were kindly provided by David Weiss, Department of Molecular and Cell Biology, University of California, Berkeley. The strains were isolated from rabbit dung from Colorado as part of his course work in the Microbiology course at Woods Hole Massachusetts in the summer of 1986.

MATERIALS AND METHODS

Bacterial strains and media. Strains of *M. xanthus* are described in Table 1. *M. xanthus* was grown in Casitone-yeast extract medium (CYE) (5) or on CYE containing 1.5% agar. The following buffers were used in working with cells, as described for each type of analysis: TM buffer, 10 mM Tris (pH 7.6) containing 8 mM MgSO₄; TPM buffer, TM buffer containing 1 mM KH₂PO₄; and TPMF buffer, TPM buffer containing 10% Ficoll (molecular weight, 400,000). Clone fruiting medium (CF) (14) containing 1.5% agar was the standard medium used for development on a solid surface. TPM-buffered 1.5% agar was also used in one experiment.

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. ¹²⁵I-protein A was purchased from Amersham, Arlington Heights, Ill. Sheep erythrocytes were purchased from Microtech, Inc., Concord, Calif. The Minibeadbeater and 0.5-mm zirconium beads were purchased from BioSpec Products, Bartlesville, Okla. A Branson model 450 sonicator was used to disrupt cells by sonic oscillation. Nitrocellulose (0.45- μ m pore size) was purchased from Sartorius Filters, Inc., Hayward, Calif.

Fruiting. Cells were grown in CYE liquid medium and then harvested by centrifugation at 12,000 $\times$ g at 4°C for 10 min. Cell pellets were suspended at 2 $\times$ 10⁹ cells per ml in TM buffer. Then 10⁹ 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.

Scanning electron microscopy. Samples were prepared as described previously (32). Coated samples were viewed with an ISI DS-130 scanning electron microscope equipped for Polaroid photography.

Determination of the proportion of each cell type in a population. *M. xanthus* was grown in CYE liquid medium and plated for development as described above. Cells were harvested from plates in TPMF buffer and placed in a hand-operated Wheaton homogenizer (48). Before homogenization, the volume of the harvest was determined (this was used to calculate the total number of cells harvested). (i) Aggregates were allowed to settle to the bottom of the homogenizer (2 to 5 min), and the number of nonaggregated rods was determined by counting (in a Petroff-Hausser counting chamber) an aliquot of the cells remaining in suspension. (ii) The harvested cells were then homogenized to dissociate aggregates. It should be noted that only prespores that have reached a transitional stage in their differentiation in which they are fragile are actually disrupted by

homogenization; all other cells remain intact. The rods present after homogenization were counted. The number of rods released from aggregates was calculated from the difference between the concentration of rods present before homogenization and the concentration of rods present after homogenization. At certain stages of development some unbroken prespores were also found in the homogenized cell suspension. These were included in the total spore count. (iii) The number of spores was determined. The homogenate was centrifuged at 12,000 $\times$ g for 20 min at 4°C. The cell pellet was suspended in 1.0 ml of TM buffer and sonicated on ice for 4 min with a microprobe at a 50% pulse setting to release spores. Sonication-resistant spores were then counted. Both homogenization and sonication break many prespores. At intermediate stages of development, many cells are at this stage of differentiation; thus they are underrepresented in total cell counts (31).

Purification of cell types and sample preparation. Vegetatively growing cells were harvested as described above, suspended in a small volume of ice-cold TM buffer, and then disrupted by sonic oscillation on ice for 2 min at a 50% pulse setting. The sonicated extract was then centrifuged at 14,000 $\times$ g for 10 min at room temperature. The supernatant was drawn off and stored at -70°C. The pellet was washed once with 1.0 ml of TM buffer and centrifuged. The wash was discarded. The pellet was stored at -70°C.

Developmental cells were scraped from CF agar with a razor blade (48) in the presence of 0.5 to 1.0 ml of ice-cold TPMF buffer. A Pasteur pipette was used to gently transfer the harvest to a conical centrifuge tube. Plates were washed with a second aliquot of TPMF, which was added to the first harvest. Care was taken at all times to avoid disrupting aggregates.

The harvested cells were centrifuged at 50 $\times$ g for 5 min at room temperature. The supernatant, containing all nonaggregated cells, was drawn off and placed in a separate tube. The pellet, consisting of aggregates, was suspended in a larger volume of TPMF buffer. The 50 $\times$ g supernatant and pellet were centrifuged separately at 12,000 $\times$ g for 20 min at 4°C. The supernatants were removed and discarded. The pellets were suspended in small volumes of TM buffer. Then both pellets were disrupted by sonic oscillation and centrifuged as described above for vegetatively growing cells. The supernatants were drawn off and stored at -70°C. The pellets were washed once with 1.0 ml of TM buffer and centrifuged. The washes were discarded. The pellet from the 50 $\times$ g supernatant was stored at -70°C.

The washed aggregate pellet was suspended in 1.0 ml of ice-cold TM buffer, transferred to a 2.0-ml screw-cap microfuge tube containing approximately 1.0 g of 0.5-mm zirconium beads, and treated in the Minibeadbeater five

times for 30 s. Microscopy revealed that greater than 95% of the spores were broken open by this treatment. The extract was centrifuged at $8,500 \times g$ for 5 min at room temperature to pellet the zirconium beads; wall fragments formed a layer above the beads. The extract, including the wall material, was drawn off the beads and then centrifuged at $14,000 \times g$ for 10 min at room temperature. The supernatant was recovered and stored at -70°C . The pellet was washed once in 1.0 ml of ice-cold TM buffer and centrifuged. The pellet, consisting of sonication-sensitive aggregated cell walls and sonication-resistant spore walls, was stored at -70°C . The wash was discarded.

This entire protocol results in the isolation of a $14,000 \times g$ soluble fraction and a $14,000 \times g$ pellet fraction from extracts obtained by sonic oscillation of vegetatively growing cells and developing nonsporulated cells and a $14,000 \times g$ soluble fraction and a $14,000 \times g$ pellet fraction from spores disrupted with the Minibeadbeater.

Protein was extracted from pellet fractions by incubating the pellets for 10 min at 100°C in a small volume of TM buffer containing 1% sodium dodecyl sulfate (SDS).

NaOH extraction of spore wall proteins. After sonic oscillation of an aggregate sample (see above), the extract was subjected to centrifugation on a stepped sucrose gradient (16). Spores were removed from the gradient and washed in TM buffer. The rest of the gradient was discarded. After centrifugation, as described above, the spore pellet was suspended in a small volume of TM buffer. NaOH was added to a final concentration of 0.1 N. The spore suspension was incubated at room temperature for 30 min; then spores were removed by centrifugation. The NaOH extract supernatant was stored at -70°C and submitted to polyacrylamide gel electrophoresis (PAGE) without neutralization. Extraction of whole spores with 0.1 N NaOH quantitatively removed outer wall proteins without extracting cytoplasmic proteins (28).

PAGE. A Laemmli gel system was used for conventional one-dimensional SDS-PAGE (22). All gels were 12.5% acrylamide.

Western blots. Western immunoblots of Laemmli gels were performed by standard techniques (1). Sartorius nitrocellulose with a pore size of $0.45 \mu\text{m}$ was used as the blotting matrix. The nitrocellulose was blocked with 5% nonfat powdered milk in 50 mM Tris (pH 7.5)–150 mM NaCl (TBS). Polyclonal antibody to myxobacterial hemagglutinin (MBHA) was prepared by Mike Cumsky (6). Polyclonal antibody to protein S (which recognizes both Tps and Ops) and polyclonal antibody to protein C were prepared by Brent Esmon in our laboratory. Blots were rinsed with TBS containing 0.5% Tween 80. ^{125}I -protein A was used to visualize antigen bands. Blots were washed in 0.2 M glycine-HCl (pH 2.0)–0.05 M KCl–0.02 M magnesium acetate to remove antibody and protein A for probing with a different antibody (23).

Determination of protein concentration. The Bradford assay was used for protein quantitation (4).

Hemagglutination assays. Hemagglutination assays were performed as described previously (6).

Phosphatase assays. Five phosphatase activities were assayed at 37°C with 10 mM *p*-nitrophenylphosphate (Sigma 104) (47). Tris was used at 10 mM (pH 8.5); morpholinepropanesulfonic acid was used at 10 mM (pH 7.2); sodium acetate was used at 1 M (pH 5.2). Assays at pH 8.5 and 7.2 were done in parallel, with and without the addition of MgCl_2 to a final concentration of 10 mM.

Calculation of percentage of aggregated cells that sporulate.

In a typical experiment in which cells were fractionated and counted as described previously (31), 1.52×10^9 cells entered development on a single CF agar plate. At least 80% of these cells went on to survive and complete development (31). Therefore, it could be expected that at least 1.22×10^9 cells were harvested from the plate after 7 days at 34°C . In the harvested population, 1.1×10^8 rods were counted; of these rods, 9×10^7 were nonaggregated. Therefore, 2×10^7 rods were associated with aggregates. Since prespores that have reached a fragile stage in their differentiation cannot be counted accurately (31), the actual number of prespores and spores in an aggregate can only be estimated. This must be done by calculation. No spores or prespores are found outside aggregates (Fig. 1). Therefore the number of spores and prespores in aggregates was 1.22×10^9 total developing cells minus 1.1×10^8 rods, or 1.11×10^9 spores and prespores. The total number of cells in aggregates (rods, prespores, and spores) was therefore 1.13×10^9 , of which the prespores and spores constitute 98% [$(1.11 \times 10^9)/(1.13 \times 10^9) \times 100$].

RESULTS

Description of peripheral rods. A population of cells that had completed the morphogenesis of fruiting bodies was examined by scanning electron microscopy. Figure 1 shows a typical fruiting body surrounded by a number of cells that had not entered aggregates. These nonaggregated cells are rod shaped, in contrast to the spherical spores within aggregates. Previous studies on sliced fruiting bodies have shown that all interior cells differentiate into spores (31, 32). Thus two cell types, which can be distinguished on the basis of structure and localization, exist in the developing population: spores, which are localized within fruiting bodies, and peripheral, rod-shaped cells, which are located outside fruiting bodies.

In establishing a terminology for distinguishing among the cell types seen during development, we have chosen to reserve the term "peripheral rods" for cells that still remain outside aggregates when all aggregation has ceased. The broader term "nonaggregated rods" will be used to describe any rods that are not associated with aggregates, regardless of the stage of development. The distinction in terminology is important in discussing the aggregation phase of development. Until aggregation is complete, the nonaggregated rod fraction is a mixture of cells that will eventually enter aggregates and cells that will not enter aggregates. This latter group of cells will become peripheral rods. Aggregated cells that have not yet differentiated into mature spores are referred to as prespores. At least 98% of the cells that enter aggregates embark upon the process of sporulation (the calculation of this number is given in Materials and Methods). It is therefore appropriate to use the term "prespore" to describe aggregated cells that are not yet mature spores. The term "spores" is reserved for sonication-resistant, spherical cells.

Isolation of peripheral rods. To further study peripheral rods, it was necessary to develop a technique for their isolation. A protocol for the purification of nonaggregated rods and aggregated cells (prespores and spores) is presented in Materials and Methods. It is based upon two characteristics of developing cells. (i) When developing cells are scraped from CF agar in the presence of buffer, aggregated cells remain tightly associated and settle to the bottom of the cell suspension in 2 to 10 min, whereas peripheral rods remain dispersed throughout the buffer. (ii) Mature spores

FIG. 1. Fruiting body of *M. xanthus* (96 h, 34°C) with peripheral rods visible in the background. DZF1 was grown in CYE liquid medium, inoculated for development on nuclepore filters on CF agar, and prepared for scanning electron microscopy as described in Materials and Methods. Arrows point to regions of peripheral rods (PR). Bar, 10 μ m.

are resistant to disruption by sonic oscillation; all other cell types are disrupted. TPMF buffer was chosen as the preferred buffer for harvesting developmental cells because it has been shown to protect prespores from osmolysis (31).

Cell fractions from several steps in the process of cell type purification are shown in Fig. 2. Cells were harvested from CF agar after the completion of fruiting body morphogenesis (Fig. 2A). Immediately after harvesting, both aggregated and nonaggregated cells were present in the suspension (Fig. 2B). After low-speed centrifugation, peripheral rods were localized in the supernatant (Fig. 2C); the pellet was enriched for aggregates (Fig. 2D). Spores were released from aggregates by sonic oscillation; nonspores were disrupted (Fig. 2E). Thus, peripheral rods and aggregated cells were effectively separated. Interestingly, nonaggregated cells could be separated from aggregates when harvested from CF agar as early as 2 to 3 h after plating. Under the developmental conditions we used, raised mounds did not become apparent until 16 to 18 h.

Proportion of peripheral rods in a developing population. The proportion of peripheral rods in developing populations of *M. xanthus* was investigated on CF agar and on TPM-buffered agar (Table 2). The preparation of cells for determining their numbers was slightly different from their preparation for obtaining extracts (see Materials and Methods). The data presented are based upon actual cell counts and are not corrected for loss of prespores at a transitional stage of differentiation when prespores are fragile. The proportion of nonaggregated rods declined during development, in correlation with an increase in the number of aggregated rods. The aggregated rod portion of the population began to decline after aggregation was completed, because these cells were differentiating into spores. As expected, the proportion of the population existing as spores also rose with time until greater than 80% of the developing population had committed to the process of sporulation. The proportion of periph-

eral rods in the population remained constant after the completion of aggregation. The data presented in Table 2 are in very good agreement with the description of the process of development of *M. xanthus* obtained with scanning electron microscopy (32). It should be noted that prespores are not efficiently counted by any of the methods currently in use. Since prespores fall into the category of aggregated cells, the actual number of aggregated cells is higher than that counted. Therefore, the real ratio of peripheral rods to aggregated cells is lower than the ratio calculated from counted cells (Table 2).

Proportion of peripheral rods in different strains of *M. xanthus*. The proportion of peripheral rods in two commonly used laboratory strains (DZF1 and DK1622) and three newly isolated strains of *M. xanthus* were examined. Strains were grown in CYE liquid medium and plated for development at 34°C on CF agar as described in Materials and Methods. After 4 to 6 days of development, cells were harvested in TPMF buffer and counted in a Petroff-Hausser chamber to determine the proportion of peripheral rods in the population. A minimum of 250 cells of each cell type were counted for each strain. Strains DSW4 and DSW5 had very few peripheral rods (4 and 6% of the population, respectively). (Strain DSW5 sporulated in flat aggregates rather than raised fruiting bodies like those seen in Fig. 1A, but it developed at the same rate as other strains of *M. xanthus*.) Approximately 20% of the cells were peripheral rods in strains DZF1 and DSW2 (another newly isolated strain); 30% of DK1622 developing cells were peripheral rods.

Expression of MBHA in developing cells. MBHA has been described as a developmentally regulated protein. Hemagglutination assays of soluble proteins of developing cells demonstrated that MBHA began to appear during aggregation, was present in highest levels just before sporulation, and then decreased to an undetectable amount (6). Ninety to 95% of MBHA is localized in the periplasm and can be

FIG. 2. Isolation of cell types from developing cultures of *M. xanthus*. DZF1 was grown and inoculated for development on CF agar as described in Materials and Methods. After 3 to 4 days at 34°C, cells were harvested in TPMF and separated into cell types as described in Materials and Methods. Cell suspensions were examined by placing 5 μ l of the suspension on a microscope slide. (A) Three days of development, fruiting bodies on CF agar. Bar, 100 μ m. (B) Suspension immediately after harvesting 3-day CF agar plates. Bar, 100 μ m. (C) Four days of development, peripheral rods in supernatant from 50 $\times$ *g* centrifugation. Bar, 10 μ m. (D) Three days of development, aggregates in pellet from 50 $\times$ *g* centrifugation. Bar, 100 μ m. (E) Three days of development, spores after sonic oscillation of aggregates. Bar, 100 μ m.

removed from cells only by disruption of the outer membrane. The remaining 5 to 10% of total MBHA is cytoplasmic (29). The use of TPMF as the harvesting medium minimizes the osmolysis of the outer membrane and consequent release of proteins; thus, prespores should have retained MBHA throughout the isolation of cell types (31).

In our studies, soluble proteins of nonaggregated cells, prespores, and spores of DZF1, obtained as described in Materials and Methods, were analyzed for the presence of MBHA by using hemagglutination assays (Fig. 3). It should be noted that *M. xanthus* developed more slowly at 28°C than at 34°C. At 28°C, aggregates had the morphology of fruiting bodies before many spores had accumulated. In contrast, at 34°C spores began accumulating in the popula-

tion 14 h before aggregates had attained the morphology commonly recognized as fruiting bodies (tightly adherent, well-defined, optically darkened mounds). The data for cells developing at 28°C demonstrate that MBHA is present only

TABLE 2. Proportions of cell types in a population of *M. xanthus* undergoing development^a

Medium	Elapsed time (h)	Aggregate morphology ^b	% of total counted cells ^c		
			Nonaggregated rods	Aggregated Rods	Spores
CF	17	Flat mounds	97	3	0.06
	48	Fruiting bodies	25	14	61
	72	Fruiting bodies	11	15	74
	96	Fruiting bodies	12	19	71
	168	Fruiting bodies	10	2	88
TPM	6	Flat	94	6	0
	12	Mounds	87	7	7
	24	Late mounds, early fruiting bodies	14	45	41
	49	Fruiting bodies	18	12	70
	100	Fruiting bodies	18	5	77
	121	Fruiting bodies	15	3	82

^a DZF1 was grown in CYE liquid medium and plated for development at 34°C on TPM-buffered or CF agar as described in Materials and Methods. Cells were harvested from plates in TPMF buffer.

^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. 1).

^c The proportions of the different cell types in the population were determined as described in Materials and Methods and are expressed as percentages of the total population. Prespores observed in the homogenized sample were included in the total spore count. The process of homogenization or sonic oscillation breaks many prespores. At intermediate stages of development, many cells are at this stage of differentiation; thus they are not represented in cell counts (31).

in nonaggregated cells and that there are two separate cycles of accumulation of MBHA. The first cycle of accumulation occurs as previously reported during the morphogenesis of mounds into translucent mounds (6). The protein has disappeared by the middle of the translucent mound stage. The second cycle of expression corresponds to the time of the appearance of many sonication-resistant spores in the fruiting body. Note, however, that the accumulation of MBHA takes place in cells that have not entered the fruiting body and are not differentiating into spores. The data obtained from cells developing at 34°C differed from those obtained from cells developing at 28°C. At 34°C, the specific activity of MBHA was at least twofold higher in nonaggregated rods than in aggregated rods, although the all-or-none, cell-type-specific pattern of expression was not maintained (Fig. 3). Analysis of Western blots probed with antibody to MBHA indicated that the agglutinating activity present in 34°C samples after 40 h of development was not due to MBHA (see below).

The presence of MBHA was further analyzed by Western blot analysis (Fig. 4 and 5). Western blot analysis can detect lower levels of MBHA than hemagglutinations and is not affected by the presence of inhibitors of agglutination. A Western blot of a Laemmli gel of protein from cells developing at 28°C, probed with antibody to MBHA, is shown in Fig. 4. Since equal amounts of protein were loaded in each lane, the relative intensities of the bands indicate the relative amounts of MBHA present in each sample. There is clearly more MBHA in nonaggregated rod samples than in prespore samples. There is also more MBHA in spores than in prespores (see 57-h samples). Similar Western blots were analyzed for all samples, and the data are expressed in levels from 0 to 4 in increments of 0.5, with 0.5 corresponding to the lowest amount detectable on the longest exposure as

described in the legend to Fig. 5. Western blot analysis of samples from cells developing at 28°C showed that MBHA was present only in soluble proteins, not in pelleted cellular material. It confirmed that much more MBHA accumulated in nonaggregated cells than in prespores. Due to the increased sensitivity of Western blot analysis and its insensitivity to agglutination inhibitors, a third cycle of MBHA accumulation at 80 h of development was detected in addition to the two cycles detected at 30 and 60 h in peripheral rods. MBHA was also detected in soluble proteins from spores; this is the first demonstration of MBHA in spores.

Although hemagglutination analysis proved to be a reliable measure of the presence of MBHA in samples taken from cells developing at 28°C, Western blot analysis indicated that MBHA was not the only hemagglutinin present at 34°C after 40 h of development. In fact, the amount of MBHA present after 40 h of development at 34°C was essentially below the level of detection of Western blot analysis.

In summary, the results of analyses of MBHA in nonaggregated cells, prespores, and spores indicate that MBHA is specific to nonaggregated cells and spores at 28°C and to nonaggregated cells at 34°C. At 34°C there is a single cycle of MBHA accumulation which spans the period of late mound morphogenesis into translucent mounds and ceases abruptly as mature fruiting bodies appear. In contrast, the accumulation of MBHA is cyclic during development at 28°C and occurs in both nonaggregated cells and in spores. The results of the analysis of MBHA in different cell types led us to examine other developmentally regulated markers.

Distribution of protein S in cell types throughout development. It has been shown that protein S can be isolated as a major protein of the outer surface of spores and in soluble proteins from nonspores but not from vegetatively growing cells (16). We obtained extracts from different cell types as described in Materials and Methods and analyzed them by probing Western blots with polyclonal antibody raised to protein S purified from the outer surface of whole spores. The intensities of cross-reacting bands on the autoradiograms of the probed Western blots have been expressed in levels of accumulation as described above for MBHA (Fig. 6). The presence of protein S was detected in both the freely soluble proteins and extracts of pelletable cellular material from all developmental cell types: nonaggregated rods, prespores, and spores. Protein S appeared first in soluble proteins from nonaggregated rods and shortly thereafter in soluble proteins from prespores and extracts of pelletable cellular material from nonaggregated rods. Protein S did not appear in extracts of pelletable cellular material of prespores until later in development. Protein S was in both soluble proteins and pelletable cellular material from spores. These data are consistent with a previous report on the transport of protein S (30).

As was seen for MBHA, in extracts from cells developing at 28°C, the accumulation of protein S was cyclic. In contrast to MBHA, the cycles of protein S accumulation were reflected in both soluble proteins and extracts of pelletable material with the exception of soluble proteins from spores, in which the level of protein S was relatively constant. These cycles did not have the same timing as those for MBHA. At 34°C, cyclic accumulations of protein S were also detected in nonaggregated rod and prespore extracts but not in spore extracts. The initiation of the first cycle of protein S accumulation was earlier at 34°C than at 28°C, and the interval before the onset of the second cycle was shortened. This is in agreement with the observation that aggregation, mound

FIG. 3. Analysis of MBHA in developing cells of *M. xanthus* by hemagglutination. DZF1 was grown and inoculated for development on CF plates; at different points in time, 8 to 10 100-mm plates were harvested and samples were prepared as described in Materials and Methods. Extracts from *M. xanthus* developing at 28°C were taken every 2 h from 2 to 12 h, every 3 h from 12 to 72 h, every 12 h from 72 to 120 h, at 144 h, and at 168 h. Extracts from cells developing at 34°C were taken every 2 h from 2 to 48 h and every 24 h from 48 to 168 h. Hemagglutinations were performed as described in Materials and Methods. Units of hemagglutination are expressed as 1/(last dilution giving a positive result per milligram of protein). The morphological stage of the culture at the time of harvest is depicted in a bar graph below each set of data. Spores accumulate gradually in the population; this is represented by increasing densities of the bars representing spores.

formation, and sporulation proceed more rapidly at 34°C than at 28°C.

Cellular localization of Ops and Tps. The two genes for protein S, *tps* and *ops*, are both expressed but are regulated differently during development. *tps* is turned on early in development, whereas *ops* is not expressed until spore formation (7, 8). It has been shown that *tps* is the gene responsible for the accumulation of large amounts of protein S (Tps) on the outside of spores. Protein S1 (Ops), on the other hand, was detected only in spores that had been broken open (44).

We wished to determine the pattern of expression of Ops and Tps in developmental cell types. Although the antibody used in the Western blot analysis of protein S expression (Fig. 6) was raised to Tps, it also cross-reacts with Ops (Fig. 7). Thus the data shown in Fig. 6 could not be used alone to determine whether there were cell-type-specific patterns of expression for both forms of protein S. However, the existence of null mutations in *ops* and in *tps* makes it possible to examine the expression of each gene indepen-

dently of the other gene. In a *tps* strain, only Ops is expressed; therefore, Western blot analysis of proteins from such a strain could be used to study the distribution of Ops in developing cells. Conversely, in an *ops* strain, only Tps is expressed. Western blot analysis of proteins from this strain would reveal the pattern of distribution of Tps in developing cells. Developmental cell types and extracts were purified from populations of cells in late development as described in Materials and Methods and analyzed by probing Western blots with polyclonal antibody to protein S.

Protein S was present in soluble proteins of peripheral rods in wild-type and *ops* strains but not in a *tps* strain (Fig. 7, lanes A, B, and C). This was also true for extracts of peripheral rod pelletable cellular material (36). These data indicated that Tps was present in both the soluble proteins and pelletable material of nonaggregated rods but that Ops was not present in these cells.

Extracts from the outer surface of spores of the wild type and an *ops* strain contained Tps; however, a *tps* mutant had no Ops on the outer surface of spores (Fig. 7, lanes G, H,

FIG. 4. Western blot of 28°C samples probed with antibody to MBHA. DZF1 was grown and inoculated for development on CF plates; after 51, 54, 57, or 60 h of development 8 100-mm plates were harvested and samples were prepared as described in Materials and Methods. PAGE and Western blots were performed as described in Materials and Methods. Samples of 5 μ g were subjected to SDS-PAGE and electroblotted to nitrocellulose. The blot was probed with polyclonal antibody to MBHA as described in Materials and Methods.

and I). This indicated that the protein S found on the outside of spores was the product of *tps*, in agreement with previous studies (44). In addition, only Tps was found in soluble proteins of spores (Fig. 7, lanes D, E, and F). Ops was found only in spore wall extracts (Fig. 7, lanes J, K, and L). It is likely that the Ops detected in spore wall extracts was deposited on the inner surface of the walls, since it could not be detected in proteins extracted from the outer surface of spores.

Distribution of protein C in cell types throughout development. SDS or NaOH can extract two major proteins from whole spores. One is Tps as described above. The other is protein C (Fig. 7); its presence is developmentally regulated, and polyclonal antibody has been raised to it (28). These characteristics make it a useful developmental marker. The same Western blots used to analyze the presence of protein S during development were probed with antibody to protein C (Fig. 8). Protein C was expressed in all developmental cell types. At 28°C, protein C appeared simultaneously in nonaggregated rods and in prespores, in both soluble proteins and pelletable material, during aggregation. The amount of protein C remained constant in spores and prespores after fruiting body formation. Protein C was more abundant in pelletable material than in soluble proteins and was completely absent in soluble proteins from spores. (Protein C present in Fig. 7, lanes D, E, and F, was from peripheral rods in the mixed population of cells.) The accumulation of protein C in soluble proteins from prespores was cyclic. There were two peaks of expression; the first had a midpoint at 30 h, and the second had a midpoint at 70 h.

The pattern of expression of protein C was somewhat different at 34°C. Although protein C was expressed in the same cells, it was expressed earlier in development, in agreement with the earlier onset of aggregation at 34°C. Less total protein C accumulated at 34°C than at 28°C. Whereas at 28°C the amount of protein C rose to a high level and

remained there in pelletable material, the amount of protein C in extracts from nonaggregated rods and spores developing at 34°C showed a peak of accumulation during mound morphogenesis. The amount of protein C declined to a lower steady state after the formation of mature fruiting bodies. In addition, although protein C was more abundant in pelletable material than in soluble proteins from cells developing at 28°C, the amount of protein C in soluble proteins and pelletable material was approximately equal in cells developing at 34°C. The pattern of accumulation of protein C differs from MBHA and the other spore wall proteins, Tps and Ops.

Expression of phosphatases in developing cells. Alkaline phosphatase was first described as a developmental marker in glycerol-induced spores of *M. xanthus* (10). A recent study described phosphatase activities with pH optima for activity at pH 5.2, 7.2, and 8.5. At pH 7.2 and 8.5, there are both magnesium-dependent and magnesium-independent phosphatase activities. These five phosphatase activities are all developmentally regulated (47). We have extended this study to the analysis of phosphatases in cell types throughout development. Enzymatic activity was assayed in soluble proteins, as described in Materials and Methods.

The results of assays at pH 8.5 (alkaline phosphatase) are presented in Fig. 9. At both 28 and 34°C, magnesium-dependent activity increased during aggregation in both nonaggregated rods and prespores; however, the specific activity was 1.5- to 2-fold higher in nonaggregated rods than in prespores. This magnesium-dependent activity was not detected in spores. Magnesium-dependent alkaline phosphatase had two peaks of activity at 28°C in nonaggregated rods: a peak at 33 h, 10-fold higher than that of the vegetatively growing cell specific activity; and a second peak at 72 h, 15-fold higher than that of the vegetatively growing cell specific activity. About the time mature fruiting bodies appeared, magnesium-dependent activity disappeared in both nonaggregated rods and prespores; only magnesium-independent activity remained (the specific activity of the enzyme remained the same in the presence or absence of magnesium).

In contrast to the magnesium-dependent alkaline phosphatase activity, the magnesium-independent activity appeared late in development and was highest in spores. In prespores it was possible to observe both magnesium-dependent and magnesium-independent alkaline phosphatase activities in the same cell type: samples taken at 40 to 60 h of development at 28°C had 4,000 U of activity when assayed in the presence of magnesium; 2,000 U of this activity could still be detected in the absence of magnesium.

A magnesium-dependent neutral phosphatase activity (with a pH optimum at pH 7.2) has also been described (47). We were unable to detect this activity in our extracts from cells developing at 34°C; however, it was present in cells developing at 28°C. It occurred only in nonaggregated rods, and the profile of the pattern of expression was identical to that seen for pH 8.5 magnesium-dependent phosphatase, including the loss of magnesium-dependent activity at 72 h (36). pH 7.2 magnesium-independent phosphatase activity had the same pattern of accumulation, with similar levels of activity, as pH 8.5 magnesium-independent activity in all three cell types at both temperatures (36).

The acid phosphatase (pH optimum at 5.2 [47]) showed no magnesium-dependent component, so we assayed only magnesium-independent activity. The patterns of expression for this activity were identical to those seen for alkaline magne-

FIG. 5. Analysis of MBHA in developing cells of *M. xanthus* by Western blots. DZF1 was grown and inoculated for development on CF plates; at different points in time, 8 to 10 100-mm plates were harvested and samples were prepared as described in Materials and Methods. Extracts from *M. xanthus* developing at 28°C were taken every 2 h from 2 to 12 h, every 3 h from 12 to 72 h, every 12 h from 72 to 120 h, at 144 h, and at 168 h. Extracts from cells developing at 34°C were taken every 2 h from 2 to 48 h and every 24 h from 48 to 168 h. PAGE and Western blots were performed as described in Materials and Methods. Samples of 5 μ g were subjected to SDS-PAGE and electroblotted to nitrocellulose. The blots were probed with polyclonal antibody to MBHA with detection by 125 I-protein A as described in Materials and Methods. Exposures of two to three different durations were made of each blot. These were analyzed, and values were assigned to the intensities of cross-reacting bands as follows: 0, lack of a detectable cross-reacting band of protein on the longest exposure; 0.5, 1, 2, and 3, increasing intensities of cross-reacting bands. The morphological stage of the culture at the time of harvest is depicted in a bar graph below each set of data. Spores accumulate gradually in the population; this is represented by increasing densities of the bars representing spores.

sium-independent phosphatase in all cell types at both temperatures. The amount of activity of the acid phosphatase was fourfold higher than that of the neutral or alkaline phosphatases in both nonaggregated rods and prespores. In spores, the amount of magnesium-independent activity was the same at all pHs (36).

The patterns of expression of all phosphatase activities in prespores were similar to those in spores. However, the levels of activity of neutral and alkaline phosphatases were

10-fold higher in spores than in prespores. The level of activity of acid phosphatase was the same in both prespores and spores.

DISCUSSION

In response to starvation, *M. xanthus* embarks upon a developmental program that includes the formation of fruiting bodies and sporulation of cells within these structures

FIG. 6. Analysis of protein S by Western blots. DZF1 was grown and inoculated for development on CF agar plates; at different points in time, 8 to 10 100-mm plates were harvested and samples were prepared as described in Materials and Methods. PAGE and Western blots were performed as described in Materials and Methods. Samples of 5 μ g were subjected to SDS-PAGE and electroblotted to nitrocellulose. The blots were probed with polyclonal antibody to protein S with detection by 125 I-protein A as described in Materials and Methods. Exposures of two to three different durations were made of each blot. These were analyzed, and values were assigned to the intensities of cross-reacting bands as follows: 0, lack of a detectable cross-reacting band of protein on the longest exposure; 0.5, 1, 2, and 3, increasing intensities of cross-reacting bands. The tabulations were then graphed for presentation. The morphological stage of the culture at the time of harvest is depicted in a bar graph below each set of data. Spores accumulate gradually in the population; this is represented by increasing densities of the bars representing spores.

(10–12, 32, 42, 43, 49). However, some cells in a starved population do not form spores. It has previously been shown that most nonsporulating cells are also nonaggregating cells. Approximately 80% of cells in the population that have not sporulated after 7 days are peripheral rods; the remaining 20% are on the surfaces of fruiting bodies (see Materials and Methods) (31, 32). This report presents the first direct analysis of these peripheral rods.

Patterns of expression of developmental markers in *M. xanthus*. Biochemical markers of development in different

organisms have many characteristics in common, including the following: (i) a marker need not be exclusive to a single cell type but is expressed at different levels in different cell types, (ii) changes in expression are induced by the same conditions that induce development, (iii) a marker is associated with a cell that has a distinct structure and/or localization (11, 24, 37). An examination of the original descriptions and the data in this report show that activity and protein levels of all nine markers examined increase significantly over vegetative levels (at least fivefold) during development

FIG. 7. Localization of Ops and Tps in developing cell types. *M. xanthus* strains were grown and plated for development and samples were collected as described in Materials and Methods. Lanes: A, D, G, and J, DZF1; B, E, H, and K, DZF3353 (*ops*); C, F, I, and L, DZF3358 (*tps*); M, DZF3367 (*ops tps*). Samples of 5 μ g of protein were loaded in each lane. Lanes A, B, and C are soluble proteins from 7-day peripheral rods from CF agar at 28°C. Lanes D, E, and F are soluble proteins from Minibeadbeater extracts of 7-day cells, including rods and spores, from CF agar at 28°C. Lanes G, H, and I are NaOH extracts of purified, whole 7-day spores from CF agar at 28°C. Lanes J, K, L, and M are SDS- β -mercaptoethanol-solubilized proteins from the purified, broken walls of 4-day spores from CF agar at 34°C. Samples were electrophoresed on 12.5% polyacrylamide gels and electroblotted to nitrocellulose. The blots were probed with polyclonal antibody raised to Tps and with polyclonal antibody to protein C as described in Materials and Methods. Exposure times were not identical among panels. Thus, no conclusions should be drawn about relative amounts of proteins in the four different fractions.

(6, 16, 28, 47). In addition, they are induced under conditions that also trigger development.

The timing of accumulation of MBHA in nonaggregated cells developing at 34°C was exactly as described previously for mixtures of developing cell types (6). However, MBHA was unexpectedly found to be specific to nonaggregated rods. In the initial characterization of the pattern of expression of MBHA in developing cells, spores were not broken open, and consequently MBHA present in the freely soluble proteins of spores was not detected (6). We note that, although our Western blot analysis detected MBHA in spores, our hemagglutination assays were negative for soluble spore proteins. It seems likely that some component of the extract interfered with the hemagglutination assay. It is also clear that a second hemagglutinating activity, not due to MBHA, is expressed during development at 34°C. It is undetectable (although not necessarily absent) in soluble proteins from spores. Although MBHA is less abundant in prespores than in nonaggregated rods, the second hemagglutinating activity accumulates to high levels in prespores late in development. There was no evidence for this second hemagglutinating activity at 28°C, since quantitation by hemagglutination and Western blot analyses agreed within the limits of sensitivity of the assays.

Ops was shown to be specific to the inside of spore walls; this is in agreement with previous work, which showed that Ops was spore specific and expressed late in development (8, 44). Although Tps was detected in soluble proteins from spores, protein C could not be demonstrated in these samples. This predicts that protein C is not translated in spores or is highly unstable in the cytoplasm of spores. Given the

structure of the spore wall, it is unlikely that the absence of protein C in soluble spore proteins is due to transport to the outer wall in mature spores (16).

Five phosphatase activities were also shown to be cell type specific (47). Magnesium-dependent activities were limited to early development, whereas magnesium-independent activities were higher in spores than in nonaggregated rods or prespores.

Temperature strongly influences the patterns of protein expression. Not only is development more rapid at 34°C than at 28°C, but the pattern of expression of proteins during development is also altered. For example, pH 7.2 magnesium-dependent phosphatase activity was present only at 28°C, whereas a hemagglutinating activity not associated with MBHA was detected only at 34°C. We note that both 28 and 34°C are well within the optimal growth range of *M. xanthus* (19). Our data imply that the effect of temperature on gene expression is an important parameter to consider in studying this organism. This is consistent with previous work showing that the expression of protein S is affected by temperature (8). In addition, it has been shown that the *tag* genes are necessary for aggregation at 34°C but not at 28°C (33, 35).

Cyclic expression of developmentally regulated proteins. The expression of some developmentally regulated proteins is cyclic in prespores and/or peripheral rods. Peripheral rods also go through cycles of motility (time-lapse video microscopy [36]). For the most part cells are quiescent, but at 5- to 8-h intervals they become motile for 5 to 20 min. During a period of motility, cells show no net movement because they oscillate between short forward and reverse movements of less than one cell length. This mode of movement is reminiscent of the FrzD phenotype (3). It is not known whether the cycles of quiescence and motility are related to the cycles of rapid and slow rippling noted by Reichenbach et al. (40). It is interesting that peripheral rods demonstrate motility similar to the FrzD phenotype; video microscopy and the movies of Reichenbach show that motility during early aggregation in several myxobacterial species is similar to the FrzA, B, C, E, and F phenotype, in which masses of cells move in circular or spiral patterns, rarely reversing the direction of movement (3, 36, 39, 40). The function and regulation of these cycles are unknown but may be linked to cycles of competence to aggregate in peripheral rods (35).

The process of sporulation. The data show that all developmentally regulated proteins expressed by both prespores and spores demonstrate an initial drop in their amount and/or activity in the earliest stage of sonication resistance in spores. The activity then accumulates as spores mature. For example, Tps was present in the soluble proteins of prespores but was absent in the soluble proteins of early spores (Fig. 6). This was also true for alkaline phosphatase; at 28°C approximately 1,500 U of magnesium-independent alkaline phosphatase were present in prespores, but early spores had no measurable alkaline phosphatase activity (Fig. 9). Analogously, MBHA was present in nonaggregated rods, absent in prespores taken at the same time point, and present in later spores.

It has been shown that MBHA and alkaline phosphatase are localized in the periplasm (29, 45), and Tps must pass through the periplasm on its way to the outer coat of the cell (30). In addition, prespores are very susceptible to osmolysis (31). We therefore postulate that the outer membrane of prespores becomes highly permeable at an early step in differentiation, allowing the release of periplasmic contents into the surrounding milieu. Preliminary work demonstrates

FIG. 8. Analysis of protein C by Western blots. DZF1 was grown and inoculated for development on CF plates; at different points in time, 8 to 10 100-mm plates were harvested and samples were prepared as described in Materials and Methods. Samples of 5 μ g were subjected to SDS-PAGE and electroblotted to nitrocellulose. The blots were probed with antibody to protein C with detection by 125 I-protein A as described in Materials and Methods. Exposures of two to three different durations were made of each blot. These were analyzed, and values were assigned to the intensities of cross-reacting bands as follows: 0, lack of a detectable cross-reacting band of protein on the longest exposure; 0.5, 1, 2, and 3, increasing intensities of cross-reacting bands. Extracts from *M. xanthus* developing at 28°C were taken every 2 h from 2 to 12 h, every 3 h from 12 to 72 h, every 12 h from 72 to 120 h, at 144 h, and at 168 h. Extracts from cells developing at 34°C were taken every 2 h from 2 to 48 h and every 24 h from 48 to 168 h. The morphological stage of the culture at the time of harvest is depicted in a bar graph below each set of data. Spores accumulate gradually in the population; this is represented by increasing densities of the bars representing spores.

that, indeed, periplasmic components accumulate in the medium during development in submerged culture and that these substances show the same pattern of loss and accumulation in spores as is seen for protein S and alkaline phosphatase (36). This model also predicts that the flow of information should be in two directions, increasing the permeability of the outer membrane to incoming molecules as well. This process could play an important role in cell-cell signaling during development.

Peripheral rods are not vegetative cells. It could be hypothesized that peripheral rods have not entered development, since they are not localized in fruiting bodies (the multicellular structures that arise as a result of development) and since they are structurally indistinguishable from vegetatively growing cells when they are examined by light or scanning electron microscopy. However, this hypothesis is not supported by the data presented here. Examination of nine of the proteins and enzymatic activities recognized as

A. Development at 28°C

B. Development at 34°C

FIG. 9. Analysis of alkaline phosphatase in developing cells of *M. xanthus*. DZF1 was grown and inoculated for development on CF plates; at different points in time, 8 to 10 100-mm plates were harvested and samples were prepared as described in Materials and Methods. Extracts from *M. xanthus* developing at 28°C were taken every 2 h from 2 to 12 h, every 3 h from 12 to 72 h, every 12 h from 72 to 120 h, at 144 h, and at 168 h. Extracts from cells developing at 34°C were taken every 2 h from 2 to 48 h and every 24 h from 48 to 168 h. Alkaline phosphatase was assayed as described in Materials and Methods. (A) Analysis of cells developing at 28°C; (B) analysis of cells developing

markers of development revealed that peripheral rods express eight of these markers in patterns associated with development, not vegetative growth (this study). Two-dimensional PAGE of proteins showed global differences in the expression of proteins in vegetatively growing cells and peripheral rods (34). Furthermore, vegetatively grown cells and peripheral rods responded differently to CF agar, conditioned CF agar, glycerol, and suspension in buffer (35).

Peripheral rods are a differentiated developmental cell type of *M. xanthus*. The terms development and differentiation are sometimes used interchangeably. However, in common usage, development is the broader term, applied to organisms as a whole, and includes the processes of morphogenesis, differentiation, and cell-cell interactions. Because procaryotes are unicellular organisms, the distinctions between development and differentiation can become blurred and perhaps moot. Definitions of differentiation are often written to specifically apply to multicellular organisms. For example, *The Chambers Dictionary of Biology* defines differentiation as "those qualitative changes in morphology and physiology occurring in a cell, tissue, or organ as it develops from a meristematic, primordial, or unspecialized state" (46). However, procaryotes and those eucaryotes which do not produce zygotes (e.g., *Dictyostelium discoideum* [24, 38]) have no meristematic or primordial cells from which to begin development. There are no undifferentiated cell types with an unspecialized state. Although vegetatively growing cells are the cells that become induced to develop, these cells are far from unspecialized. Their function is to grow and increase the number of cells in the population. The genes expressed in the cells are those necessary to encourage and permit growth and cellular division. Thus, development in an organism such as *M. xanthus* begins from a differentiated cell with a function (increase in the total mass and number of cells) that is significantly different from the function of cells in the developmental pathway (maintaining the number of cells under adverse environmental conditions).

A textbook on procaryotic development has defined development as "a series of stable or metastable changes in the form or function of a cell, where those changes are part of the normal life cycle of the cell...a strategy such that the organism, rather than consisting of one essential cell type that deals less than optimally with a wide variety of environmental circumstances, consists of multiple cell types each limited in versatility but highly adapted to a particular circumstance" (11). Differentiation is the process of changes in biochemistry, physiology, structure, or function of cells or tissues in the course of development (2, 13, 25, 46).

The following findings support the conclusion that peripheral rods should be considered to be a distinct, differentiated, developmental cell type.

(i) Peripheral rods are part of the normal life cycle of *M. xanthus*. We have demonstrated that peripheral rods occur in each of five wild-type strains of *M. xanthus* tested, including three strains newly isolated from nature. Although the proportion of peripheral rods in a developing population of a given strain was reproducible, it was also found that the proportion of peripheral rods in a population of developing cells varied among the strains tested (4 to 30%). We have no

indications of how the variations in the proportion of peripheral rods among different strains on the same medium are controlled; however, we do know that it is unlikely to involve the social (S) motility system of *M. xanthus* (15). We observed that both DSW4 and DSW5 had a functional S motility system and were therefore *sglA*⁺, as is DK1622. DSW4 and DSW5 fall at either extreme of the range of the proportion of peripheral rods in developing populations. (DSW2 colonies resembled DZF1 [*sglA*^{leaky}] colonies when examined for a motility phenotype.)

(ii) Patterns of expression of developmentally regulated proteins are cell type specific. The expression of previously identified developmentally regulated proteins in nonaggregated cells, prespores, and spores was examined. The distribution of these proteins in soluble proteins and pelletable material of the cells was also studied. If peripheral rods were truly a cell type different from the vegetatively growing cells that they physically resemble, the patterns of expression of developmentally regulated proteins should differ in the two cell types. Indeed, this was clearly the case for all developmentally regulated markers examined. The patterns of expression of the proteins differ strongly among all the cell types examined. A summary of these data is given in Table 3. There are five markers that are absent in vegetatively growing cells but present in peripheral rods. Of the three markers present in both cell types, their expression is increased in peripheral rods during the course of development.

The patterns of expression of a large number of proteins have been examined by two-dimensional PAGE; the patterns of expression were strikingly different among the different cell types of *M. xanthus* (17, 34). In terms of protein expression, peripheral rods are more like spores than vegetatively growing cells, but their morphologies are very different (Fig. 1 and 2).

(iii) The physiological condition of peripheral rods differs from that of vegetatively grown cells. We have studied the behavior of cells in situ and the responses of cells to external conditions. Vegetatively growing cells seem to be principally engaged in activities that lead to growth and cell division; however, peripheral rods do not undergo cell division (31, 35). It has also been shown that peripheral rods do not respond like vegetatively grown cells to CF agar, conditioned CF agar, glycerol, or suspension in buffer (35). Peripheral rods and vegetatively grown cells are in different physiological conditions as measured by these parameters.

Peripheral rods and spores have obvious structural differences despite the similarities in the expression of biochemical developmental markers. On the other hand, peripheral rods look like vegetatively grown cells when examined by light or scanning electron microscopy. However, Tps and protein C are both present in the pelletable material of peripheral rods. Since Tps and protein C are known to be major components of the spore wall, this implies that the wall structure of peripheral rods differs from that of vegetatively grown cells. Peripheral rods and vegetatively grown cells should be examined by transmission electron microscopy.

(iv) The function of peripheral rods differs from that of

at 34°C. The differences in levels of specific activity are significant within a panel. However, differences in the levels of specific activity between panels A and B cannot be analyzed because different batches of substrate were used for the two sets of assays. The morphological stage of the culture at the time of harvest is depicted in a bar graph below each set of data. Spores accumulate gradually in the population; this is represented by increasing densities of the bars representing spores.

TABLE 3. Summary of the expression of developmentally regulated proteins in different cell types of *M. xanthus*^a

Developmentally regulated protein	Vegetatively growing cells		Nonaggregated rods		Prespores (soluble)	Aggregated cells	
	Soluble	Pelletable	Soluble	Pelletable		Spores	
						Soluble	Pelletable
MBHA (6)	No	No	Yes	Rare	Rare	Yes*	No
Tps (18, 44)	No	No	Yes	Yes	Yes	Yes	Yes
Ops (18, 44)	No	No	No	No	ND	No	Yes
Protein C (28)	No	No	Yes	Yes	Yes	No	Yes
Acid phosphatase (47)	Yes	ND	Yes	ND	Yes	Yes	ND
Neutral phosphatase (Mg independent [47])	No	ND	Yes	ND	Yes	Yes	ND
Neutral phosphatase (Mg dependent [47])	Yes	ND	Yes*	ND	Yes	No	ND
Alkaline phosphatase (Mg independent [47])	No	ND	Yes	ND	Yes	Yes	ND
Alkaline phosphatase (Mg dependent [47])	Yes	ND	Yes	ND	Yes	No	ND

^a The data in this table summarize the data in Fig. 3 through 9 and data presented in the text. Note that this table does not reflect the relative amounts of any of the proteins expressed in the cell types. All of the developmentally regulated proteins are expressed at higher levels in developmental cell types than in vegetatively growing cells. ND, no data; No, the protein was not detected in this cell type; Rare, the protein was detected at low levels in a few samples; Yes, the protein was detected in this cell type; *, the protein was detected only in 28°C samples.

vegetatively grown cells. The function of vegetatively grown cells can arguably be said to be to increase both total mass and cell number. Since peripheral rods have not been observed to undergo cell division in situ, they must have a different function. We believe that this function is the same as that of spores; namely, to survive adverse conditions with a minimal decrease in the number of cells in the population. Peripheral rods may be a survival mechanism for resisting short-term adversity. The work of Matin and others has shown that *Salmonella typhimurium* and *Escherichia coli* respond to starvation by changing the physiology of the cells and that this change in physiology results in increased resistance to stresses in addition to starvation (for a review, see reference 26). The function of peripheral rods is further explored in the third paper of this series, in which it is shown that peripheral rods return to vegetative growth in response to levels of nutrients that are not sufficient to induce germination of spores (35).

Development in *M. xanthus*. The results presented in this paper show that vegetatively growing cells differentiate into two cell types during development—spores and peripheral rods. There are therefore three distinct cell types in *M. xanthus*: vegetatively growing cells, spores, and peripheral rods. There are also two transitional cell types that occur in the process of differentiation. There are nonaggregated rods, which are a mixed population of cells including those that will enter aggregates to differentiate into spores and those that will become peripheral rods. Cells within aggregates pass through prespore stages on the way to becoming mature spores.

The discovery of peripheral rods will have a profound impact on our understanding and perspective on the development of *M. xanthus*. For example, in searching for the role of MBHA in development, previous workers were unaware that the protein was absent in cells that had aggregated but had not formed sonication-resistant spores (41). Similarly, Kaiser and co-workers have described a number of Tn5lac insertions in developmentally regulated genes (21). Perhaps some of these will prove to be cell type specific. In an accompanying report, we address some of the questions concerning the biology of peripheral rods (35). What role might they play in the life cycle of *M. xanthus*? What regulates the proportion of peripheral rods in a population? Why do peripheral rods not enter aggregates? Why do peripheral rods not sporulate?

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REFERENCES

1. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. Balinsky, B. I. 1981. An introduction to embryology, 5th ed., p. 574. Saunders College Publishing, Philadelphia.
3. Blackhart, B. D., and D. R. Zusman. 1985. "Frizzy" genes of *Myxococcus xanthus* are involved in the control of rate of reversal of gliding motility. *Proc. Natl. Acad. Sci. USA* **82**: 8767–8770.
4. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
5. Campos, J., and D. R. Zusman. 1975. Regulation of development in *Myxococcus xanthus*: effect of 3':5'-cyclic AMP, ADP, and nutrition. *Proc. Natl. Acad. Sci. USA* **72**:518–522.
6. Cumsky, M., and D. R. Zusman. 1979. Myxobacterial hemagglutinin: a development-specific lectin of *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA* **76**:5505–5509.
7. Downard, J. S., D. Kupfer, and D. R. Zusman. 1984. Gene expression during development of *Myxococcus xanthus*: analysis of the genes for protein S. *J. Mol. Biol.* **175**:469–492.
8. Downard, J. S., and D. R. Zusman. 1985. Differential expression of the protein S genes during *Myxococcus xanthus* development. *J. Bacteriol.* **161**:1146–1155.
9. Dworkin, M. 1962. Nutritional requirements for vegetative growth of *Myxococcus xanthus*. *J. Bacteriol.* **84**:250–257.
10. Dworkin, M. 1973. Cell-cell interaction in the Myxobacteria. *Symp. Soc. Gen. Microbiol.* **23**:134–142.
11. Dworkin, M. 1985. Developmental biology of the bacteria. The Benjamin/Cummings Publishing Co., Reading, Mass.
12. Dworkin, M., and D. Kaiser. 1985. Cell interactions in myxobacterial growth and development. *Science* **230**:18–24.

13. Friday, A., and D. S. Ingram (ed.). 1985. The Cambridge encyclopedia of life sciences, p. 40. Cambridge University Press, Cambridge.
14. Hagen, D. C., A. P. Bretscher, and D. Kaiser. 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. *Dev. Biol.* **64**:284–296.
15. Hodgkin, J., and D. Kaiser. 1979. Genetics of gliding motility in *Myxococcus xanthus* (Myxobacterales): two gene systems control movement. *Mol. Gen. Genet.* **171**:177–191.
16. Inouye, M., S. Inouye, and D. R. Zusman. 1979. Biosynthesis and self-assembly of protein S, a development-specific protein of *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA* **76**:209–213.
17. Inouye, M., S. Inouye, and D. R. Zusman. 1979. Gene expression during development of *Myxococcus xanthus*: pattern of protein synthesis. *Dev. Biol.* **68**:579–591.
18. Inouye, S., T. Francheschini, and M. Inouye. 1983. Structural similarities between the development-specific protein from a Gram-negative bacterium, *Myxococcus xanthus*, and calmodulin. *Proc. Natl. Acad. Sci. USA* **80**:6829–6833.
19. Jansen, G. R., J. W. Wireman, and M. Dworkin. 1977. Effect of temperature on the growth of *Myxococcus xanthus*. *J. Bacteriol.* **130**:561–562.
20. Kaiser, D. 1979. Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA* **76**:5952–5956.
21. Kroos, L., A. Kuspa, and D. Kaiser. 1986. A global analysis of developmentally regulated genes in *Myxococcus xanthus*. *Dev. Biol.* **117**:252–266.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
23. Legocki, R. P., and D. P. S. Verma. 1981. Multiple immunoreplica techniques: screening for specific proteins with a series of different antibodies using one polyacrylamide gel. *Anal. Biochem.* **111**:385–392.
24. Loomis, W. F. 1975. *Dictyostelium discoideum*: a developmental system. Academic Press, Inc., New York.
25. Martin, E. (ed.). 1985. The dictionary of biology derived from the concise science dictionary, p. 70. Warner Books, Inc., New York.
26. Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz. 1989. Genetic basis of starvation survival in nondifferentiating bacteria. *Annu. Rev. Microbiol.* **43**:293–316.
27. McBride, M. J., and D. R. Zusman. 1989. Trehalose accumulation in vegetative cells and spores of *Myxococcus xanthus*. *J. Bacteriol.* **171**:6383–6386.
28. McCleary, W., B. Esmon, and D. Zusman. 1991. Protein C is a major spore surface protein from *Myxococcus xanthus*. *J. Bacteriol.* **173**:2141–2145.
29. Nelson, D. R., M. G. Cumsy, and D. R. Zusman. 1981. Localization of myxobacterial hemagglutinin in the periplasmic space and on the cell surface of *Myxococcus xanthus* during developmental aggregation. *J. Biol. Chem.* **256**:12589–12595.
30. Nelson, D. R., and D. R. Zusman. 1983. Transport and localization of protein S, a spore coat protein, during fruiting body formation by *Myxococcus xanthus*. *J. Bacteriol.* **154**:547–553.
31. O'Connor, K. A., and D. R. Zusman. 1988. A re-examination of the role of autolysis in the development of *Myxococcus xanthus*. *J. Bacteriol.* **170**:4103–4112.
32. O'Connor, K. A., and D. R. Zusman. 1989. Patterns of cellular interactions during fruiting body formation in *Myxococcus xanthus*. *J. Bacteriol.* **171**:6013–6024.
33. O'Connor, K. A., and D. R. Zusman. 1990. Genetic analysis of tag mutants of *Myxococcus xanthus* provides evidence for two developmental aggregation systems. *J. Bacteriol.* **172**:3879–3887.
34. O'Connor, K. A., and D. R. Zusman. 1991. Analysis of *Myxococcus xanthus* cell types by two-dimensional polyacrylamide gel electrophoresis. *J. Bacteriol.* **173**:3334–3341.
35. O'Connor, K. A., and D. R. Zusman. 1991. Behavior of peripheral rods and their role in the life cycle of *Myxococcus xanthus*. *J. Bacteriol.* **173**:3342–3355.
36. O'Connor, K. A., and D. R. Zusman. Unpublished data.
37. Parish, J. H. (ed.). 1979. Developmental biology of procaryotes. University of California Press, Berkeley.
38. Raper, K. B. 1940. Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. *J. E. Mitchell Sci. Soc.* **56**:241–282.
39. Reichenbach, H., H. H. Huenert, and H. Kuckza. 1965. *Myxococcus* spp. (Myxobacterales)—Schwammentwicklung und bildung von protocysten. Film E778. Institut für den Wissenschaftlichen Film, Göttingen, Germany.
40. Reichenbach, H., H. H. Huenert, and H. Kuckza. 1965. *Chondromyces apiculatus* (Myxobacterales)—Schwammentwicklung und Morphogenese. Film E779. Institut für den Wissenschaftlichen Film, Göttingen, Germany.
41. Romeo, J., and D. R. Zusman. 1987. Cloning of the gene for myxobacterial hemagglutinin and the isolation and analysis of structural gene mutations. *J. Bacteriol.* **169**:3801–3808.
42. Rosenberg, E. 1984. Myxobacteria development and cell interactions. Springer-Verlag, New York.
43. Shimkets, L. J. 1990. Social and developmental biology of the myxobacteria. *Microbiol. Rev.* **54**:473–501.
44. Teintze, M., R. Thomas, T. Furuichi, M. Inouye, and S. Inouye. 1985. Two homologous genes coding for spore-specific proteins are expressed at different times during development of *Myxococcus xanthus*. *J. Bacteriol.* **163**:121–125.
45. Voelz, H., and R. O. Ortigoza. 1968. Cytochemistry of phosphatases in *Myxococcus xanthus*. *J. Bacteriol.* **96**:1357–1365.
46. Walker, P. M. B. (ed.). 1989. The Chambers dictionary of biology, p. 84. W and R Chambers Ltd., Cambridge.
47. Weinberg, R. A., and D. R. Zusman. 1990. Alkaline, acid, and neutral phosphatase activities are induced during development in *Myxococcus xanthus*. *J. Bacteriol.* **172**:2294–2302.
48. Wireman, J., and M. Dworkin. 1975. Morphogenesis and developmental interaction in myxobacteria. *Science* **189**:516–523.
49. Zusman, D. R. 1984. Cell-cell interactions and development in *Myxococcus xanthus*. *Q. Rev. Biol.* **59**:119–138.