JOURNAL OF BACTERIOLOGY, Sept. 1988, p. 4103–4112
0021-9193/88/094103-10$02.00/0

Vol. 170, No. 9

Reexamination of the Role of Autolysis in the Development of *Myxococcus xanthus*

KATHLEEN A. O'CONNOR AND DAVID R. ZUSMAN*

Department of Microbiology and Immunology, University of California, Berkeley, California 94720

Received 23 October 1987/Accepted 16 June 1988

It has been widely reported that 80 to 90% of the cell population undergoes autolysis during sporulation in *Myxococcus xanthus*. A re-evaluation of the techniques used to measure autolysis in *M. xanthus* showed that the methods previously used to draw this conclusion are subject to artifacts, which result in a substantial underestimation of the number of cells present during development. We found that at least 80% of the cells that enter development survive throughout fruiting body formation. The cell loss that did occur appeared to be gradual over a period of at least 7 days. Our results suggest that autolysis is not an obligate stage in the development of *M. xanthus*. The data also showed that sporulating cells pass through a prespore stage in which they become osmotically and physically fragile and therefore difficult to harvest intact. The fragility was correlated with the change from a rod to a spherical shape. As the prespores differentiated into refractile spores, they lost fragility and became amenable to harvesting by standard protocols.

Myxococcus xanthus is a gram-negative, soil-dwelling bacterium which feeds on other organisms and decaying organic matter. Depletion of available nutrients induces cells to aggregate into raised mounds of 10^5 cells. During the next 1 to 2 days, the rod-shaped aggregated cells undergo differentiation into spherical myxospores.

Wireman and Dworkin (18, 19) reported that 80 to 90% of developing cells underwent autolysis during development, specifically during sporulation within the aggregated mounds of cells. As a result of their initial report, "massive cell lysis" has become installed in the literature as an integral step in the process of fruiting body formation (3, 5, 9, 14, 17). As a key focus of discussion of development in *M. xanthus*, autolysis has exerted a strong influence on the experimental design, on the interpretation of data, and on the building of hypotheses. For example, it has been proposed that the lysis of 90% of the population supplies proteins and other substances for the differentiating spore in a role similar to that of the spore mother cell during endosporulation of *Bacillus subtilis* (5). In addition, Teintze and co-workers (8, 16) have suggested that autolysis is responsible for the transport of protein S to the surface of spores, and Zahavi and Ralt (20) have proposed that autolysis functions as a signal and attracts other cells to the sporulating bacterium in a primitive form of cellular collaboration (altruism).

However, we have found little convincing evidence for massive cell lysis when using the methods originally employed to document developmentally regulated autolysis (18, 19): (i) determination of colony-forming units, (ii) microscopic examination of cell numbers, and (iii) detection of [*methyl*-^{3}H]thymidine-tagged DNA throughout the course of development. An improved technique for harvesting and maintaining developing cells has demonstrated that cell survival is much greater than previously reported. This finding has been confirmed by measuring the release of [*methyl*-^{3}H]thymidine by cells developing in submerged culture. Our results show that at least 80% of the cells survive development; the cell loss that does occur is gradual over a period of 7 days. Therefore, we conclude that autolysis cannot be characterized as an obligate stage in development. This conclusion will necessitate a rethinking of some previously formulated hypotheses, most of which can be easily reconciled with the new data.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and growth media. DZF1 was originally designated FB. This strain was used to document developmentally regulated autolysis (18, 19). It is *sglA* (7) and thus grows dispersed in liquid culture. DK1622 was obtained from D. Kaiser. It is *sglA*$^+$, clumps in liquid culture, and can develop in submerged culture. DK2657 is a SpoC mutant (*csg*) of DK1622 which does not sporulate and has been described as failing to undergo autolysis (15). Cells were grown in Casitone-yeast extract (CYE) (2). Clone fruiting (CF) agar was used for development on a solid surface (6). A buffer consisting of 10 mM MOPS (morpholinepropanesulfonic acid), pH 7.2, containing 4 mM $MgSO_4$ and 2 mM $CaCl_2$ (MMC) was used for development in submerged culture (11).

Materials. All medium components were obtained from Difco Laboratories, Detroit, Mich. Salts were purchased from Mallinckrodt, Inc., St. Louis, Mo. Pronase was obtained from Calbiochem-Behring, La Jolla, Calif. All other nonradioactive chemicals were purchased from Sigma Chemical Corp., St. Louis, Mo. [*methyl*-^{3}H]thymidine, 60 Ci/mmol, was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. The Minibeadbeater was purchased from Bio-Spec Products, Bartlesville, Okla. A Biosonik IV sonicator (Bronwill Scientific Inc., Rochester, N.Y.) was used. Plastek treatment M, 50-mm diameter petri plates, were purchased from Tekmat Corp., Ashland, Mass.

Fruiting. The procedure of Wireman and Dworkin (19) was followed except that cells were concentrated in 10 mM Tris, pH 7.6, containing 8 mM $MgSO_4$ (TM buffer). A total of 10^9 cells in 0.5 ml of TM buffer were spread evenly onto each 100-mm CF agar plate which had been dried prior to use. Timing and extent of development were highly reproducible under these conditions. The protocol of Kuner and Kaiser (11) was followed for development of strain DK1622 in submerged culture in MMC.

Scanning electron microscopy. Samples, 5 μl, of DZF1 at 4 × 10^9 cells per ml in TM buffer were applied to Nuclepore

filters (3-μm pore size) resting on the surface of CF agar. Filters were removed at intervals and inverted over a solution of 2.5% glutaraldehyde for 8 h to overnight. After fixation, filters were cut to fit specimen holders; some samples were also sliced with an Exacto-knife to reveal cells within aggregates. Samples were dehydrated in an ethanol series, critical-point dried, and platinum coated. Coated samples were viewed with an ISI DS-130 scanning electron microscope equipped for Polaroid photography.

Harvesting cells. Vegetative cells were harvested from CYE liquid by centrifugation at 12,000 × *g* for 10 min at 4°C. Cells on CF agar were harvested by flooding the plates with 1 to 2 ml of ice-cold, sterile TM buffer. A sterile razor blade was used to scrape the cells from the agar surface. Cells were transferred to a sterile tube. For cells in submerged culture, MMC was poured off; the cell mat was rinsed twice with 2 ml of ice-cold, sterile TM buffer. Then cells were scraped off with a sterile razor blade in 1.5 ml of ice-cold, sterile TM buffer and transferred to a sterile tube.

Glutaraldehyde fixation and aggregate dissociation. Glutaraldehyde was added to harvested cells in TM buffer to a final concentration of 2.5% as described by Wireman and Dworkin (18). Cells were fixed for 8 to 24 h at 4°C. Fruiting bodies were dissociated by sonic oscillation with a microtip. One-half to 1 volume of 15-mm-diameter zirconium beads were added to facilitate dissociation. Several minutes of sonication were required to disperse cells within fruiting bodies. Aliquots for counting in a Petroff-Hausser counting chamber were taken from samples which had been thoroughly vortexed.

Viability and cell number determinations. Harvested cells were thoroughly vortexed to suspend all cells not tightly associated with aggregates. Aggregates were allowed to settle to the bottom of the collection tube. An aliquot of the suspended rods was counted in a Petroff-Hausser counting chamber; another aliquot was serially diluted for plating on CYE agar. The harvest was then subjected to sonic oscillation for four 1-min periods to disperse all spores; all rods and most prespores were disrupted by this treatment. Aliquots of the sonicated harvest were also counted, serially diluted, and plated on CYE agar as described above. Colonies were counted after 7 days at 34°C to allow any slow-growing colonies to appear. This was especially important in counting spore colony-forming units.

Testing stability of cells in liquid media. Several media were tested for suitability for harvesting cells: (i) deionized, distilled water; (ii) TM buffer; (iii) TM buffer containing 1 mM KH_2PO_4 (TPM buffer); and (iv) TM buffer containing 10 g of Ficoll 400 per 100 ml (TMF buffer). Vegetative cells of strain DZF1 were harvested from liquid and suspended at 9.3 × 10^8 cells per ml in 3 ml of each medium. Developing cells were harvested in the specified medium after incubating for 48 h on CF agar at 34°C. The harvested cells and one wash of the CF agar were pooled. An aliquot of the harvested cells was homogenized by hand in a 2-ml Wheaton homogenizer with Teflon pestle (0.125-mm clearance) to release rods from aggregates. Total rods were counted in a Petroff-Hausser chamber. After 15 and 30 min of incubation on ice, 0.5 ml of nonhomogenized aliquots were centrifuged (14,000 × *g* for 5 min) to remove cells. The protein content of the supernatants was determined by the Bradford assay (1). A fresh CF agar plate was washed to obtain background protein content. The protein content of the wash of a CF plate was subtracted from the data presented for 48-h cells.

Improved method for harvesting and counting developing cells. Ice-cold, autoclaved or filtered TPMF buffer (100 ml of TPM buffer containing 10 g of Ficoll 400) was used to harvest cells from CF agar. Harvested cells plus one rinse of the CF agar were placed in a 2-ml Wheaton homogenizer with a Teflon pestle (0.125-mm clearance). The pestle was operated by hand until macroscopic clumps of cells had been dispersed. Cells were counted in a Petroff-Hausser chamber and scored as rods, prespores (ovoid to spherical, nonrefractile cells), or spores (spherical, refractile cells). The homogenate was then centrifuged at 12,000 × *g* for 20 min at 4°C; the cell pellet was suspended in a volume of TM buffer equal to the volume of TPMF buffer removed. The suspension was subjected to sonic oscillation for 1 min to disperse all spores from fruiting bodies. Cells were then counted in a Petroff-Hausser chamber and scored as prespores or spores. No rods and few prespores survive sonic oscillation.

Incorporation of ^3H into cells. The protocol of Wireman and Dworkin (19) was followed to label vegetative cultures of cells with 3 to 5 μCi of [*methyl*-^{3}H]thymidine per ml (50 to 83 nM) in CYE liquid overnight (four to six generations) at 30°C. Cells were harvested and then suspended in 1 to 2 volumes of CYE containing unlabeled thymidine at 250 μg/ml (1.03 mM) and chased for 6 h (1 to 1.5 generations). For submerged culture experiments, labeled cells were inoculated into 50-mm Plastek treatment M (negatively charged) petri plates as described previously (11) in CYE containing 250 μg of thymidine per ml and chased overnight (one to two generations) at 28°C.

Determination of level of [*methyl*-^{3}H]thymidine in cells. Three different methods were used to determine the level of [*methyl*-^{3}H]thymidine in cells: (i) radioactivity in the growth and labeling medium was measured; (ii) total intracellular radioactivity was determined; and (iii) the specific activity of DNA was measured.

(i) [*methyl*-^{3}H]thymidine incorporated by cells was determined from measurements of the ^3H in the extracellular medium. An aliquot of CYE containing [*methyl*-^{3}H]thymidine was counted in a scintillation counter to determine the initial level of [*methyl*-^{3}H]thymidine present in the growth medium. Then the medium was inoculated and cells were allowed to grow as described above. Cells were removed from the growth medium, and an aliquot of the medium was read in a scintillation counter to determine the amount of label in the medium not incorporated by growing cells. The difference between inoculated and remaining label is the amount of label incorporated by the cells. The amount of label released into the CYE supplemented with cold thymidine chase was also determined after cells had been removed by centrifugation. The total amount of [*methyl*-^{3}H]thymidine incorporated in the cells at the start of development is equal to the amount of [*methyl*-^{3}H]thymidine incorporated during growth less the amount released during the chase.

(ii) [*methyl*-^{3}H]thymidine incorporated by cells was measured as total intracellular radioactivity. Three methods were used to release radioactivity from cells. (A) The Wireman and Dworkin protocol (19) was followed for trichloracetic acid (TCA) precipitation of cells; this included rinsing the harvested plates and adding the rinse plus unlabeled carrier cells prior to TCA precipitation. In some experiments, Protosol (Dupont, NEN Research Products, Boston, Mass.) was substituted for NCS (Amersham Corp., Arlington Heights, Ill.) to solubilize the TCA pellet for scintillation counting in 10 ml of PCS (Amersham). (B) Cells were pelleted by centrifugation and then disrupted by sonic oscillation in 0.1 ml of ice-cold, sterile TM buffer containing 0.1 g of acid-washed, 0.5-mm glass beads (The VirTis Co., Inc., Gardiner, N.Y.). Cells were treated on ice with three

45-s bursts with a microtip for a total of 2.25 min of sonic oscillation. The extract, including beads, was transferred to a glass vial for scintillation counting. The tube used for sonication was rinsed with 0.15 ml of water; this rinse was added to the extract in the scintillation vial. (C) The Mini-beadbeater was used to disrupt cells. Samples were treated according to the directions of the manufacturer. Zirconium beads (0.15-mm diameter) were added to the cells in a 2-ml screw-cap Microfuge tube. Spores were treated five times for 30 s each or until the spores were completely broken. Rods were disrupted by one to two 30-s treatments. Cells were inspected for breakage by examining several fields of a 5-μl aliquot at ×400 magnification. If no whole cells were observed, the sample was considered to be fully disrupted. Cells disrupted by method B or C were further treated by enzymatic digestion. Unless otherwise stated, digestions were incubated overnight at 37°C. Samples were first treated with 1,000 Units of DNase I and then with pronase at a final concentration of 1 mg/ml. Lastly, sodium dodecyl sulfate (SDS) was added to a final concentration of 5 mg/ml and samples were boiled for 10 min. Cooled samples, including beads, if present, were transferred to scintillation vials and counted in Ecolume (ICN).

(iii) The specific activity of ^3H in cellular DNA was determined. Cells were harvested as described above. After centrifugation, the cell pellet was suspended in 0.5 ml of 10 mM Tris (pH 7.5)–1 mM EDTA (pH 7.5) (TE buffer) containing 1 mg of pronase, 1 μg of RNase A, and 10 mg of SDS per ml. The cells were suspended and lysed by gentle inversion. The mixture was incubated at 37°C for 3 h and then extracted two or three times with phenol-chloroform-isoamyl alcohol (25:24:1) followed by one extraction with chloroform-isoamyl alcohol (24:1). NaCl was added to the aqueous phase at a final concentration of 0.1 M; 2.5 volumes of cold ethanol were added. The DNA was precipitated in a dry ice-ethanol bath for 20 min. The sample was thawed at room temperature and then DNA was collected by centrifugation at 14,000 × *g* for 10 min at room temperature. The DNA pellet was suspended in 1.5 ml of TE buffer and stored at 4°C overnight. After the DNA was fully suspended, it was quantitated spectrophotometrically by its A_{260} and A_{280}. DNase I (1,000 U) and $MgSO_4$ (final concentration, 0.01 M) were added, and the DNA was digested overnight at 37°C. The digested DNA was analyzed in a scintillation counter. The counts per minute were converted to nanocuries to calculate the specific activity as nanocuries of DNA per microgram. The scintillation cocktail had a counting efficiency of 36 cpm/100 decays per min for [*methyl*-^3H]thymidine.

Recovery of ^3H from submerged culture medium. Submerged culture medium was drawn off the cell layer, and the volume was determined by weight in a precalibrated centrifuge tube. Cells were removed by centrifugation at 12,000 × *g* for 10 min at 4°C. Aliquots, 1.0 ml, of the cell-free supernatant were analyzed in a scintillation counter. The total amount of ^3H recovered in the culture medium was determined by converting counts per minute to nanocuries and multiplying by the total volume of culture medium recovered from the plate.

RESULTS

Microscopic examination of aggregates and fruiting bodies. The lysis of 80 to 90% of the developing cell population should leave visible signs of its occurrence. There should be an accumulation of debris or empty spaces or both within aggregates. The size of aggregates might also decrease. Scanning electron microscopy of cells which had been developing for 24 h (Fig. 1A and B) revealed that, at the onset of sporulation, the aggregates were packed with cells and had no vacant spaces. After 48 h of development, 98% of the cells in aggregates had differentiated into spherical prespores and spores. Figure 1C and D shows that the fruiting bodies were also firmly packed with cells. No particulate debris nor areas of cell depletion were observed within aggregates or in the surrounding, nonaggregated cells.

At the level of magnification afforded by the dissecting microscope, no significant change in the volumes of maturing aggregates was observed during the process of sporulation (Fig. 2). Because these samples were not fixed or prepared in any way for photography, there was no potential for shrinkage artifacts. The volume of a spore is approximately 1.7-fold greater than the volume of a rod. (If the volume of a rod is calculated as the sum of the volume of a sphere having the diameter of the poles of the cell plus the volume of the remaining cylinder, the volume of a rod 5 μm in length and 0.7 μm in diameter [3] is 1.8 μm^3. The average diameter of a spore is 2 μm [Fig. 1D and E]; therefore, the average volume of a spore is 3.1 μm^3.) This difference in cell volume would not be sufficient to maintain a nearly constant volume of aggregates of closely packed cells during aggregation if 80 to 90% of the aggregated, sporulating population were lysing (18, 19).

Development was also studied by time-lapse videotape at ×100 to 200 magnification in a light microscope. Cells were plated on CF agar blocks surrounded by a lake of TPM buffer to prevent dehydration. Development was observed from the time of inoculation until the cells had completed 48 h of development at 35°C. Mature fruiting bodies were present, and most cells had sporulated by the end of the experiment. No lysis was observed at any time. There was no change in the size of aggregates during the period of sporulation nor were cells outside aggregates observed to lyse. These results led us to re-examine the quantitative methods used to document cell lysis in *M. xanthus*.

Cell number determinations during development. The most direct method for determining the number of cells present at different times during development is to follow the change in cell number throughout development. However, aggregated cells are difficult to disperse. One approach used to dissociate aggregates of *M. xanthus* is to fix harvested cells and aggregates in 2.5% glutaraldehyde and then disperse them by sonication (18). The fixed cells were presumed to be resistant to disruption by the sonic oscillation used to dissociate them from aggregates. In our hands, the energy required to dissociate mature fruiting bodies resulted in the disruption of many cells, including spores. DK1622, which had been developing in submerged culture (11) for 5 days at 34°C, was harvested and cells were counted after sonication. Only 27% as many spores were counted in the sonicated glutaraldehyde-fixed samples as in a duplicate, sonicated, unfixed sample. However, no rods or immature spores remained to be counted in the sonicated, unfixed samples. Even though fixed samples contain all cell types, the final number of cells counted after sonication of either fixed or unfixed samples represents only a fraction of the total number of cells present before sonication. The number of cells counted in sonicated, glutaraldehyde-fixed samples after 5 days of development was 9% of the number of cells entering development. This agrees well with previous reports on the extent of developmentally regulated autolysis (18, 19). However, this method

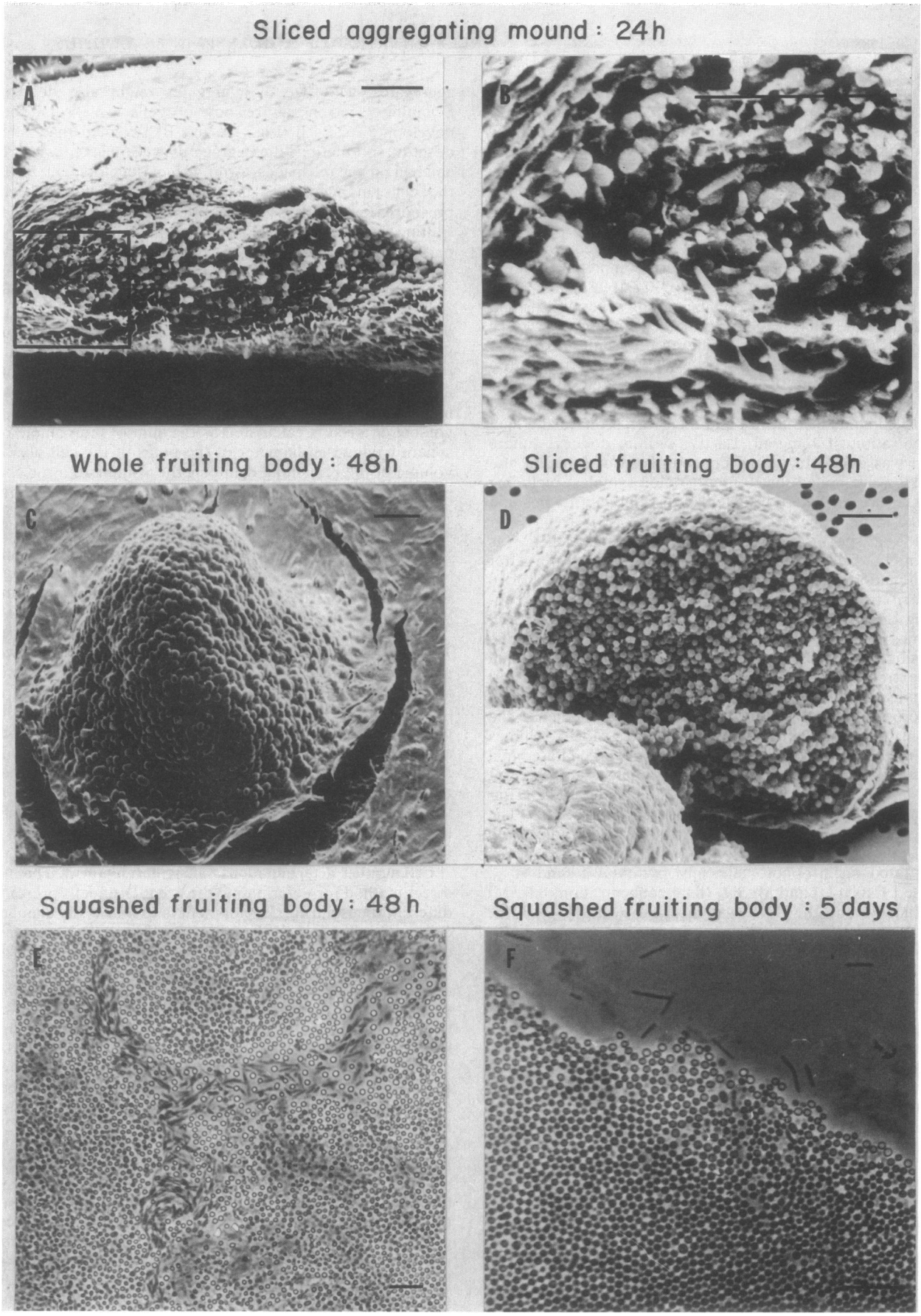

FIG. 1. Microscopy of aggregates of *M. xanthus*. DZF1 was grown and plated for development, as described in Materials and Methods. (A), (B), (C), and (D) were fixed and observed by scanning electron microscopy (SEM). (E) and (F) were not fixed prior to observation by light microscopy. (A) 24-h aggregate sliced longitudinally to reveal cells within the aggregate. (B) Boxed region of panel A. (C) 48-h aggregate (fruiting body). (D) 48-h aggregate sliced to reveal cells within the aggregate. (E) 48-h aggregate, wet mount, squashed under the cover slip to reveal cells within the aggregate; (F) 5-day aggregate, wet mount, squashed under the cover slip to reveal cells within the aggregate. Bar, 10 μm.

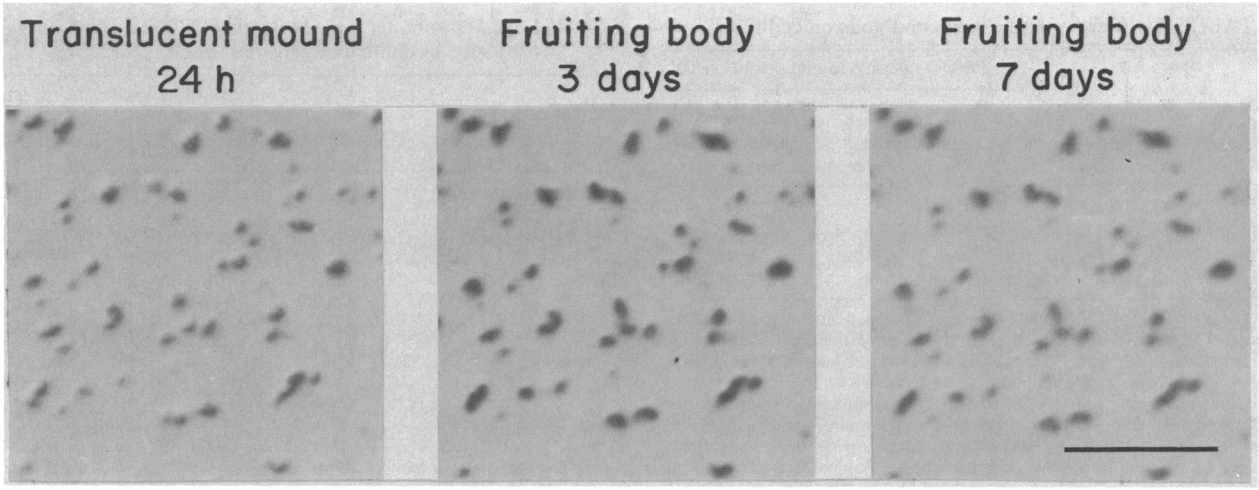

FIG. 2. Constancy of mound size throughout sporulation. DZF1 was grown and plated on CF agar to develop, as described in Materials and Methods. The same region of a plate was photographed over a period of 1 week. Photographs of 24-h translucent mounds (<2% of cells have sporulated) and 3- and 7-day fruiting bodies (75 and 90% spores, respectively) are shown. Bar, 1 mm.

did not accurately determine the number of cells present after sporulation had begun.

We next counted cell numbers without a fixation procedure. Cells were plated on CF agar which contains low levels of nutrients, allowing the cells to undergo approximately one division during the first 16 to 20 h of incubation, after which no further cell division occurs. Cells were harvested in TM buffer at various times during development. Aggregates were allowed to settle to the bottom of the collection tube. Nonaggregated rods remained in suspension and were counted directly. Spores in aggregates were counted following sonication. Aliquots were also plated for colony-forming units (Table 1). The data indicated the following. (i) After cell division has ceased, the number of rods not tightly associated with aggregates declines. This is most likely due to the entrance of rods into aggregates and their subsequent differentiation into spores. (ii) Spores harvested from early fruiting bodies (48 h) form colonies with a low efficiency in comparison to the Petroff-Hausser cell counts. Since no significant clumping of the sonicated spores was observed under microscopy, it seems likely that spores which appear mature by microscopy (i.e., which are sonication resistant, refractile, and spherical) are not all competent to germinate under laboratory conditions. Even after 1 week of development at 34°C, only 43% of the spores were able to form colonies on CYE agar. Thus, colony-forming units alone underestimate the number of cells which survive development. (iii) The Petroff-Hausser cell counts showed that after 48 h of development 20% of the number of cells present at 24 h were recovered from CF agar (50% as rods and 50% as spores). This agrees with previously published determinations of cell survival during development. However, when the experiment was continued for 7 days (3 days longer than other published studies), >36% of the number of cells present at 24 h were counted. This number accounts for spores but does not include rods or sonication-sensitive cells in aggregates. The presence of sonication-sensitive cells in aggregates can be visualized by gently squashing fruiting bodies under a cover slip. In Fig. 1E and F it can be seen that even after 2 and 5 days of development, respectively, fruiting bodies contain many cells which are not mature, refractile spores. Thus, the survival of cells in development is severely underestimated when cells are harvested and counted in TM buffer.

Fragility of developing cells. To study developmentally regulated autolysis, it was necessary to develop a method which permitted more of the rods and prespores (nonrefractile spores) to be represented when developing cells were

TABLE 1. Measurement of developmentally regulated autolysis by counting dispersed cells[a]

Morphology	Time of development (h)	CFU (10^7)		Petroff-Hausser counts (10^7), spores	Total cell no. (10^7)[b]	% of 24-h cell no.[c]
		Rods	Spores			
Vegetative rods	0	32	0	0	32	20
Non-aggregated rods	1	37	0	0	37	23
Early aggregates	12	110	0	0	110	68
Translucent mounds	24	160	0.3	0.9	161	100
Fruiting bodies	48	16	2	16	32	20
Fruiting bodies	160	NA[d]	25	58	>58	>36

[a] DZF1 was induced to develop on CF agar at 34°C as described in Materials and Methods. Cells were harvested from CF agar by scraping and washing with TM buffer. Aggregates were allowed to settle to the bottom of the test tube; nonaggregated rods remained in suspension. An aliquot of the rods was serially diluted and plated on CYE (nutrient) agar plates. The remaining cell suspension, including aggregates, was sonicated to release spores. Spores were counted and plated on CYE agar as described. Colonies on CYE agar were counted after incubation for 1 week at 34°C.

[b] Total cell number is equal to the sum of rod colony-forming units plus the number of spores counted in a Petroff-Hausser chamber.

[c] The maximum number of cells per plate was observed at 24 h.

[d] NA, Not available. The fruiting bodies had begun to deliquesce; therefore, the rod sample contained a number of spores. It was not possible to accurately determine what portion of the cell counts was due to rods.

TABLE 2. Effect of harvesting conditions on cell lysis[a]

Harvesting buffer	Rods recovered per plate (10^7)	Protein released (μg/ml per 10^9 cells)			
		Developmental cells (48 h)[b]		Vegetative cells	
		15 min	30 min	15 min	30 min
H_2O	20	97	135	5	4
TM	21	54	76	2	4
TPM	33	45	41	2	3
TMF	27	35	44	2	3

[a] Vegetative cells of DZF1 were concentrated by centrifugation and suspended at 9.3×10^8 cells per ml in each of the harvesting media. Cells were plated for development on CF agar at 34°C as described in Materials and Methods. After 24 h of development, there were 1.8×10^9 cells per plate. Developing cells were harvested after 48 h of development when sporulation was in progress. One plate was scraped and washed with each of the harvesting media. After 15 and 30 min of incubation on ice, 0.5-ml aliquots of cells were centrifuged to remove cells from harvesting medium. The protein concentration of the supernatants was determined by the Bradford assay (1). Duplicate samples of developing cells were homogenized by hand to release rods from aggregates, and then cells were counted in a Petroff-Hausser chamber. The process of harvesting, dissociating, and counting cells required 15 to 30 min. Buffer ingredients are given in the text.

[b] CF plates contain a low, but detectable amount of protein. Therefore, a CF plate was rinsed with each of the harvesting media and the amount of protein rinsed from a CF plate was subtracted from each value. Thus, each entry reflects only protein released by cells.

counted in a Petroff-Hausser chamber. Wireman and Dworkin (18) reported that a hand-operated tissue homogenizer could dissociate fruiting aggregates. In our hands, dissociation of fruiting bodies with a homogenizer was not complete but was a great improvement upon vortex agitation as a means of releasing intact cells from aggregates. However, we noticed that the concentration of rods in a homogenized sample decreased with time after homogenization (data not shown), suggesting that developing cells were lysing in TM buffer while being stored on ice. The concentration of vegetative cells remained constant under similar conditions. The stability of cells in several different harvesting media was therefore investigated. The data indicated that vegetative cells released very little protein into the medium even when incubated in unbuffered water (Table 2). However, the harvesting medium had a significant effect on the release of soluble protein from 48-h developing cells. The addition of either 1 mM KH_2PO_4 (TPM buffer) or 10% Ficoll 400 (TMF buffer) to TM buffer improved upon the recovery of cells obtained with either water or TM buffer. The improved recovery of cells was correlated with less protein release. We also noted that fewer residual cells remained on plates after harvesting with media containing Ficoll. Some of the protein recovered from cells in this experiment might have been actively secreted. However, this cannot be true for the bulk of the protein released since fewer cells released more protein into water and TM buffer than into TPM or TMF buffer. On the basis of this experiment, cells in subsequent experiments were harvested in TPM buffer containing 10% Ficoll 400 (TPMF buffer).

Direct counting of cells harvested in TPMF buffer to determine extent of developmentally regulated autolysis. An experiment similar to that described in Table 1 was done with TPMF buffer to harvest developing cells (Table 3). The number of rods present rapidly declined from 24 to 48 h but later stabilized at 7% of the number of cells present after cell division had ceased (17 h). The number of spores increased as the number of rods declined. Some 63% of the number of cells present at 17 h were recovered from CF agar at 48 h, and 36% were recovered at 144 h, but 58% were recovered at 168 h of development. These numbers were reproducible in three separate experiments. When cells were harvested in TM buffer (Table 1), 20% of the cells were recovered at 48 h and 36% were recovered at 160 h. Thus, TPMF is an improved buffer for harvesting developing cells.

TABLE 3. Measurement of developmentally regulated autolysis by direct counting of cells harvested in TPMF[a]

Morphology	Time of development (h)	Cell no./plate (10^7)		% of 17-h cell no.[d]
		Rods[b]	Spores[c]	
Early aggregates	17	152	0.1	100
Tight mounds	24	142	7	98
Fruiting bodies	48	37	58	63
	72	16	45	40
	96	15	37	34
	120	13	41	36
		8	45	35
	144	11	46	38
		12	43	36
	168	11	77	58

[a] 10^9 cells of DZF1 were plated to develop on CF agar plates at 34°C. Cells were harvested by scraping in TPMF as described in Materials and Methods.

[b] In 24- and 120-h samples, the number of rods after homogenization was less than that before homogenization. The data report the number of rods in suspension before homogenization. All other entries report the number of rods in suspension after homogenization.

[c] Total number of mature spores plus prespores.

[d] The maximum number of cells per plate was observed at 17 h after cell division had ceased.

After 1 week of development, the number of cells counted was equal to 58% of the maximum cell number (the number of cells counted at 17 h). This is higher than the number of cells counted at 3 to 6 days of development. A trivial explanation of the increase in cell number is that cells were resuming cell division. However, no dividing cells were observed after 17 h of development during the course of this experiment, nor were any cells seen to divide in a videotape of aggregates on CF agar from 7 to 9 days (data not shown). Our observations indicate that two factors contributed to the increased recovery of cells from 7-day aggregates. First, fruiting bodies were beginning to deliquesce by 7 days and dissociated more completely by homogenization; this allowed a higher proportion of sonication-sensitive cells within fruiting bodies to be counted. Second, a higher proportion of the spores were mature and consequently were not disrupted by sonication. (Spore maturation was demonstrated in Table 1 as an increase in the proportion of spores which could form colonies.)

The fragile nature of prespores and the difficulties of disrupting fruiting aggregates must be considered in the interpretation of the data. Microscopy of squashed fruiting bodies showed that many more prespores were present in fruiting bodies than were represented in cell counts (Fig. 1E and F). Prespores constituted 11% of the total counted spore population at 48 h and 20% of the population of spores at 5 days of development. These numbers were less than would be predicted from microscopic observations and therefore represent a large source of error in the measurement of developmentally regulated autolysis by counting the number of cells in the developing population. Although recovery of 58% of the maximum number of cells entering development was significantly greater than the recovery of 10 to 20% described in the literature (18, 19), it was still an underestimation of the total number of cells surviving development.

Recovery of [*methyl*-^{3}H]thymidine from cells as a measure of cell survival during development. As an alternative to the

TABLE 4. Detection of ^{3}H-labeled DNA in vegetative and developing cells[a]

Sample prepn	cpm recovered (%)[b]		
	Vegetative cells	24-h aggregates (<1% spores)	120-h aggregates (90% spores)
TCA precipitation + Protosol	100	100	100
Sonic oscillation	95	95	128
SDS (0.5%)	116	92	149
Sonic oscillation + pronase (0.1%) + SDS (0.5%)	109	104	203
Zirconium bead agitation + pronase (0.1%) + SDS (0.5%)	117	149	266

[a] In a series of experiments, *M. xanthus* DZF1 was grown and labeled during vegetative growth with [*methyl*-^{3}H]thymidine and development was induced on CF agar as described in Materials and Methods. Developing cells were harvested from plates at 24 and 120 h as described in Materials and Methods. Each sample consists of all cells harvested from one plate. Samples were treated as described and then analyzed in a scintillation counter.

[b] For each cell type (vegetative, aggregate, and spore), the ^{3}H recovered as counts per minute by the Wireman and Dworkin procedure (19) was set equal to 100%; all other data are expressed relative to this. The data represent the mean of three to five separate experiments. For all sample preparation procedures, the raw data vary within ±5% of the mean.

estimation of cell numbers during development, an attempt was made to document cell survival in development by measuring the recovery of [*methyl*-^{3}H]thymidine from cells as described by Wireman and Dworkin (19). When samples of DZF1 from different stages of development were analyzed, 10% of the amount of ^{3}H detected in the vegetative inoculum was recovered from 96-h developing cells. The pattern of decrease in recovery of ^{3}H from developing cells was correlated with the pattern of accumulation of spores (data not shown). These results are in strong agreement with the data of Wireman and Dworkin (19). The data led these authors to conclude that 90% of the developing cells must have undergone developmentally regulated autolysis during sporulation. Yet, the results of experiments presented in other sections of this paper strongly argued against this interpretation of the data. We therefore hypothesized that the recovery or quantitation of ^{3}H in samples of developing cells was artificially low.

During routine examination of our samples, it was noted that spores were not solubilized by the tissue solubilization cocktails NCS or Protosol. Several procedures for disrupting cells (especially spores) were therefore tested in an effort to minimize quenching of label due to inadequate solubilization of cells. The results are presented in Table 4. The recovery of ^{3}H from vegetative cells was approximately the same for all five methods of sample preparation. However, disruption of cells with zirconium beads by mechanical agitation followed by pronase and SDS treatments resulted in significantly better recovery of ^{3}H from 120-h aggregates in which spores constitute 90% of the cells harvested. It was also found that digestion with DNase I gave a slight improvement (5 to 10%) in recovery of ^{3}H from 120-h aggregates when used in conjunction with the fifth preparation procedure given in Table 4. Treatment with 0.1 N HCl or 0.1 N NaOH was also tested, but these had no effect on recovery of ^{3}H from sonicated cells.

The experiment described above in which the ^{3}H present in cells undergoing development was analyzed was repeated by using an improved sample preparation procedure (cells were broken with zirconium beads in the Minibeadbeater followed by digestion with pronase and DNase I and boiling in SDS). The experiment showed that, after 3.5 days of development, 34% of 44,000 cpm measured in the vegetative cell inoculum could be recovered in the disrupted samples. This is a threefold-higher level of recovery than was achieved with the original TCA precipitation method (19). However, the samples were still subject to quenching artifacts. Because quenching was still a problem, especially in the spore samples, it would not be possible to distinguish quenching from autolysis in the context of these experiments.

Recovery of [*methyl*-^{3}H]thymidine released by developing cells as a measure of lysing cells. One way to circumvent the problem of intracellular quenching was to measure the release of ^{3}H by developing cells into the extracellular medium. Kuner and Kaiser (11) described a method for inducing *M. xanthus* to develop in submerged culture. This technique has the advantage that both the cells and the extracellular medium can be analyzed. However, this procedure was described only for strain DK1622, a wild-type stain with full motility. Our experiments had been conducted primarily with DZF1 up to this point, and DZF1 is the strain in which developmentally regulated autolysis was originally described. Therefore, strain DK1622 was labeled with [*methyl*-^{3}H]thymidine and inoculated in submerged culture. Cells were harvested and the recovery of ^{3}H was analyzed as described for DZF1. We found that, after 5 days of development, 37% of the 120,000 cpm measured in the vegetative cell inoculum could be recovered in the samples. This recovery was essentially the same as described above for strain DZF1 developing on CF agar. Therefore, it was valid to study developmentally regulated autolysis in submerged cultures of DK1622.

We proceeded to analyze the ^{3}H released into the culture medium during development in submerged culture. Quenching artifacts might be more carefully controlled in a cell-free sample. However, before we could adequately interpret the data from these experiments, it was necessary to demonstrate that developing cells did not reincorporate label released by other cells. The question was addressed in two different experiments.

(i) DK1622 was labeled and inoculated for development. DNA was purified from cells immediately after addition of development buffer (MMC) and from cells after 72 h of development, at which time sporulation was in progress. The specific activity of the DNA at the start of development was 0.24 nCi/μg; after 72 h of development, the specific activity was 0.27 nCi/μg. These data were equivalent within the limits of the accuracy of the assay. If cells were reincorporating the [*methyl*-^{3}H]thymidine released by cells lysing during sporulation, the specific activity of the DNA would have been expected to rise in the absence of unlabeled thymidine in the medium. The presence of unlabeled thymidine would be predicted to prevent the rise in specific activity of the DNA. However, the specific activity of DNA after development for 72 h was the same in the presence (0.26 nCi/μg) or absence of exogenous, unlabeled thymidine. Thus, there is no evidence that thymidine was being incor-

TABLE 5. Measurement of developmentally regulated autolysis by release of [*methyl*-^{3}H]thymidine by developing cells[a]

Strain	Time of development (h)	Morphology	Development in MMC buffer[b]		Development in MMC buffer + thymidine (250 μg/ml)[c]	
			Total ^{3}H recovered (nCi)[d]	% of initial cpm	Total ^{3}H recovered (nCi)	% of initial cpm
DK1622	0	Vegetative rods	0.5 ± 0.3	0.4	0.6	0.3
	24	Early aggregates	5.3 ± 0.5	4	5.3	3
	48	Tight mounds	8.0 ± 0	7	15.8	9
	72	Tight mounds	10.0 ± 1.8	8	20.0	11
	96	Fruiting bodies	11.8 ± 0.5	10	ND	ND
	120	Fruiting bodies	19.0 ± 2.3	16	38	21
	144	Fruiting bodies	16.3 ± 2.3	14	ND	ND
	168	Fruiting bodies	22.8 ± 4.4	19	35	20
DK2657	0	Vegetative rods	0.7	0.9	0.5	0.6
	24	Flat	8	10	8	10
	48	Flat	12	15	10	13
	72	Flat	11	14	11	14
	96	Flat	13	16	12	15
	120	Flat	15	19	13.5	18
	144	Flat	19	24	16	20
	168	Flat	25	31	20	25

[a] *M. xanthus* DK1622 and DK2657 were labeled with [*methyl*-^{3}H]thymidine and inoculated in submerged culture in 50-mm Plastek treatment M petri plates as described in Materials and Methods. DK1622 was inoculated at 120 nCi per plate; DK2657 was inoculated at 80 nCi per plate.

[b] Culture medium was collected at the indicated intervals after the addition of development buffer. Cells were removed by centrifugation; the total amount of ^{3}H present in the total culture supernatant was determined as described in Materials and Methods. Culture supernatant was removed from duplicate plates (one plate of MMC and from one plate of MMCT for DK2657); two 1-ml aliquots were analyzed from each sample. For DK1622, the data are the average of measurements of two aliquots from each of two duplicate MMC samples, i.e., four measurements. In the case of DK2657, the data are the average of two aliquots from the same sample.

[c] In a separate experiment, DK1622 was labeled as described and inoculated at 23 nCi per plate in MMCT. The data for DK2657 were obtained in the same experiment from which the MMC data were obtained. Cells were inoculated at 80 nCi per plate into duplicate plates which contained either MMC or MMCT. In both experiments, samples were collected as described in footnote *b*. The data are the average of two aliquots from the same sample. ND, Not done.

[d] Standard deviation is included when appropriate.

porated from the medium by *M. xanthus* during development.

(ii) Unlabeled DK1622 was inoculated for development in submerged culture. [*methyl*-^{3}H]thymidine (19 nCi) was added to the development medium at the start of the experiment. Culture supernatant was collected and analyzed for ^{3}H in a scintillation counter. Within the first 8 h, 12% of the label was removed from the medium. By the time fruiting bodies had formed at 96 h, 16% of the label had been removed from the medium; this remained constant throughout the next 3 days. ^{3}H could be detected in the cellular fraction, indicating that at least some of the [*methyl*-^{3}H]thymidine was incorporated by cells. (Quenching by cells made exact quantitation of incorporation impossible.) Most of the incorporation of [*methyl*-^{3}H]thymidine occurred during the first 24 h of development when the cells were still undergoing division. The presence of 250 μg of unlabeled thymidine per ml in the medium inhibited the uptake of ^{3}H from the medium for the first 8 h of development (0.3% of the inoculum was removed compared with removal of 12% of the inoculum in the absence of unlabeled thymidine). However, by 48 h of development, 10% of the [*methyl*-^{3}H]thymidine had been removed from the medium. This level remained constant throughout the rest of development. These results suggest that the 10% of the label removed from the medium between 8 and 48 h was not incorporated into DNA since the incorporation occurs in the presence of an excess of unlabeled thymidine. Developmentally regulated autolysis is reported to take place after cell division has ceased and sporulation has begun. It is unlikely that cells would incorporate significant amounts of [*methyl*-^{3}H]thymidine released at the purported time of autolysis since there was little incorporation of exogenous ^{3}H after 24 h of development. The experiments discussed in this and the preceding paragraph indicate that the detection of the release of 80 to 90% of incorporated [*methyl*-^{3}H]thymidine would not be significantly affected by reincorporation of released [*methyl*-^{3}H]thymidine by surviving cells at the time of sporulation and autolysis.

Having demonstrated that cells developing in submerged culture did not incorporate thymidine after cell division had ceased, the release of ^{3}H into the extracellular medium by cells during development was measured. Strain DK1622 was labeled with [*methyl*-^{3}H]thymidine and inoculated in submerged culture; the amount of [*methyl*-^{3}H]thymidine inoculated was calculated from the growth medium. Samples were collected at different times during development (Table 5). The amount of ^{3}H released into the medium was gradual, beginning as early as 24 h. At 96 h, when most cells had differentiated into spores, only 10% of the inoculated ^{3}H had been released into the culture medium. After 7 days of development, 19% of the inoculated ^{3}H was recovered in the culture supernatant. Calculation of the standard deviation of the data showed that 15 to 23% of the ^{3}H incorporated as [*methyl*-^{3}H]thymidine was released over a period of 7 days of development in submerged culture. Similar results were obtained when unlabeled thymidine was added to the developmental medium at a concentration of 250 μg/ml.

The results presented in Table 5 are in striking contrast to the published estimates of the extent of developmentally regulated autolysis in *M. xanthus* and indicate that no greater than 20% of the cells lyse during development. No data exist for estimating the degree to which the released ^{3}H may be due to DNA repair rather than to autolysis. The

[*methyl*-^{3}H]thymidine would be expected to damage DNA, especially after 7 days of exposure. We have noted that cells labeled with [*methyl*-^{3}H]thymidine develop more slowly (approximately 12 h behind) than unlabeled cells. The data therefore suggest that no more than 20% of the cells lysed. The actual amount of autolysis is probably no greater than that expected for any population of starved cells.

It has been reported that SpoC mutants of *M. xanthus* fail to sporulate and do not undergo lysis (15). One would predict that a SpoC strain would release less ^{3}H into the extracellular medium than the wild type (DK1622). DK2657 is SpoC in a DK1622 genetic background. This strain was labeled and inoculated for development in submerged culture. Culture supernatants were analyzed for release of ^{3}H (Table 5). The cells did not aggregate and microscopic inspection detected no mature spores. The pattern of accumulation of ^{3}H in the culture medium was similar to that of the wild-type DK1622; in fact, developing DK2657 released a greater percentage of incorporated ^{3}H than did the wild type. This may be due to the conditions of the experiment; it was observed that cells were being sloughed into the medium. We conclude from this experiment that neither SpoC nor wild-type strains undergo developmentally regulated autolysis as a discrete stage of development, though they do show some gradual lysis over the 7 days of the experiments.

DISCUSSION

The data presented in this paper demonstrate that there is no evidence for a specific, developmentally regulated stage of massive cell lysis in *M. xanthus*. Although all of the available data were consistent with the hypothesis that 80 to 90% of a developing population of *M. xanthus* underwent developmentally regulated autolysis (12, 15, 18, 19), the conclusion was based upon misinterpretations of the data. (i) When the number of cells was determined from colony-forming units (viable counts), clumped cells, fragile cells disrupted by harvesting procedures, and those spores which were unable to form colonies under laboratory conditions were undercounted. (ii) When cells were counted directly by microscopy, cells in clumps and cells too fragile to survive the harvesting technique were undercounted. (iii) When cell number was determined from the recovery of incorporated [*methyl*-^{3}H]thymidine in cellular material, quenching decreased the detection of ^{3}H present in the harvested cells. Different cell types of *M. xanthus* quench ^{3}H to different degrees. In particular, spores quench ^{3}H more severely than other cell types do. We were unable to devise protocols which would eliminate the errors in estimating cell number inherent in the three techniques described above. Although we were able to improve the accuracy of measurement of cell number by cell counts and by incorporated [*methyl*-^{3}H]thymidine, neither technique proved adequately accurate for testing the hypothesis that *M. xanthus* undergoes developmentally regulated massive cell lysis.

In our hands, the best estimate of cell lysis during development was obtained by following the release of ^{3}H into the medium by cells developing in submerged culture. Measuring ^{3}H in cell-free samples made it easier to set up controls and to correct for quenching of the detection of ^{3}H. We were concerned that developing cells might reincorporate label released by other cells. However, it was determined in two control experiments that this was not the case. Furthermore, when unlabeled thymidine (250 μg/ml) was added to the medium, the amount of ^{3}H detected in the culture supernatant was essentially unchanged. Our experiments have shown that the release of ^{3}H into the culture medium was relatively linear throughout development. There was no evidence for a sudden increase in the release of ^{3}H at any stage in development as had been proposed in other reports (18, 19). In addition, the amount of ^{3}H released was much less than predicted: no more than 20% of the incorporated ^{3}H was released during 7 days of development. It should be noted that the release of ^{3}H into the medium is a result of not only cell lysis but probably the metabolism of DNA in the cells as well. Thus, the actual level of cell lysis may be lower than measured in these experiments. The extent of cell lysis during development would seem to be no greater than that expected for any cells which had been under starvation conditions for 7 days.

It is also of interest that the SpoC (*csg*) mutant DK2657 showed a pattern of release of ^{3}H similar to that of the wild-type strain DK1622. SpoC mutants fail to form tight cell-cell adhesions, to sporulate, and to undergo developmentally regulated autolysis (15). The lack of tight cell-cell adhesion and sporulation allows these mutants to be more accurately counted in a Petroff-Hausser chamber, avoiding the artifacts brought about by clumping and breakage in the harvesting procedure. We propose that the ease of counting SpoC mutants throughout development causes it to appear to fail to undergo autolysis. We predict that the measurement of intracellular [*methyl*-^{3}H]thymidine in a SpoC strain would also demonstrate that it fails to lyse. We would interpret both results as indicating that SpoC is blocked in sporulation at a stage prior to the onset of fragility. This is consistent with our microscopic observations of DK2657 and published reports (15).

Two new aspects of development in *M. xanthus* have been elucidated by the studies reported in this paper. (i) Cells pass through a physically and osmotically fragile prespore stage during sporulation. Kottel and White (10) have also documented a fragile stage during differentiation of glycerol-induced spores (4). Prespores were severalfold more sensitive to lysing when incubated in H_2O at 50°C for 15 min than were vegetative cells or mature glycerol spores. The authors interpreted these observations as evidence for lysis. However, the conditions inducing "autolysis" were harsh; cells which remain in the induction medium do not lyse (4). In addition, spores may require more time to mature than was previously assumed. Many refractile, sonication-resistant spores are not capable of forming colonies. This may be because they have not yet become competent to germinate or because they are structurally but not physiologically resistant to sonication. (ii) Approximately 10% of the starved cell population does not sporulate on CF agar. These cells are found on the surfaces of fruiting bodies and in the spaces between them (Fig. 1C and D). Because spores severely quench the detection of the radioactive label, the method of Wireman and Dworkin for assessing cell number by measuring intracellular [*methyl*-^{3}H]thymidine (19) actually reflects the nonsporulated portion of the developing population of cells. An examination of the nonsporulated cells between fruiting bodies indicates that they have significant physiological differences from vegetative rods and from spores. This cell type may be important in the life cycle of *M. xanthus* (O'Connor and Zusman, manuscript in preparation).

We began this series of experiments to resolve to our own satisfaction the discrepancy between what we saw by microscopy and what had been measured by experimentation. We did not set out to disprove the theory of developmentally regulated autolysis in *M. xanthus*. It should be noted that the results obtained by Wireman and Dworkin (18, 19) were

absolutely reproducible in our laboratory. However, our experiences in measuring lysis by techniques standard for *M. xanthus* have led us to conclude that there are valid interpretations of the data which do not invoke the phenomenon of autolysis. We have interpreted the data as evidence for a fragile stage in the process of spore differentiation and for the unfortunate capacity of spores to quench the detection of ^{3}H incorporated as [*methyl*-^{3}H]thymidine. Furthermore, our interpretation is consistent both with the data obtained by measuring extracellular ^{3}H released by developing cells which had incorporated [*methyl*-^{3}H]thymidine during vegetative growth and with the visible evidence obtained by microscopy. Because the concept of developmentally regulated autolysis has played a major role in the interpretation of data since it was first proposed in 1975 (18), our conclusion that there is no developmentally regulated autolysis in *M. xanthus* will have a domino affect on other hypotheses which invoke autolysis as a stage of development. Some existing hypotheses may have to be abandoned. It is difficult to continue to perceive of *M. xanthus* as a model for primitive altruism (20). The role of the autocides (17) will also need to be re-examined. If non-sporulating cells do serve a role in the differentiation of spores as suggested (5), their influence must act through cell-cell contact or active transport of differentiation "factors" or both. The proposal that a spore coat protein, protein S, was deposited on the outside of spores as a result of its release by lysing cells (16) was consistent with the observation that exogenously added protein S would self-assemble on spores (8). It further resolved the question of how protein S was transported to the outside of spores in the absence of a recognizable signal sequence (8). However, Nelson and Zusman (13) have demonstrated that cells export protein S in the absence of sporulation and lysis. Thus, there is no need to invoke lysis in the assembly of protein S on the outside of spores. There are a number of other examples of hypotheses regarding the development of *M. xanthus* which will have to be re-examined. It may be fair to say that nearly every laboratory which has studied *M. xanthus* in the last 13 years has at least one theory which will be affected by our conclusion that massive cell lysis is (was) an artifact.

ACKNOWLEDGMENTS

We thank Dale Kaiser and his laboratory (Stanford University) for helpful suggestions and Mazal Varon, Amy Rosenbluh, and Eugene Rosenberg (Tel Aviv University) for sharing their unpublished findings with us. We also thank Robin Weinberg, Joseph Romeo, Bill McCleary, and Mark McBride for useful comments and discussions.

This research was supported by Public Health Service grant GM 20509 from the National Institutes of Health and grant DMB 85-02000 from the National Science Foundation.

LITERATURE CITED

1. **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.
2. **Campos, J. M., J. Geisselsoder, and D. R. Zusman.** 1978. Isolation of bacteriophage MX4, a generalized transducing phage for *Myxococcus xanthus*. J. Mol. Biol. **119**:167–178.
3. **Dworkin, M.** 1985. Developmental biology of the bacteria. Benjamin Cummings Publishing, Inc., Menlo Park, Calif.
4. **Dworkin, M., and S. M. Gibson.** 1964. A system for studying microbial morphogenesis: rapid formation of microcysts in *Myxococcus xanthus*. Science **146**:243–244.
5. **Dworkin, M., and D. Kaiser.** 1985. Cell interactions in myxobacterial growth and development. Science **230**:18–24.
6. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64**:284–296.
7. **Hodgkin, J., and D. Kaiser.** 1979. Genetics of gliding motility in *Myxococcus xanthus* (Myxobacteriales): two gene systems control movement. Mol. Gen. Genet. **171**:177–191.
8. **Inouye, M., S. Inouye, and D. Zusman.** 1979. Biosynthesis and self-assembly of protein S, a development-specific protein of *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76**:209–213.
9. **Jansen, G. R., and M. Dworkin.** 1985. Cell-cell interactions in developmental lysis of *Myxococcus xanthus*. Dev. Biol. **112**: 194–202.
10. **Kottel, R., and D. White.** 1974. Autolysis activity associated with myxospore formation in *Myxococcus xanthus*. Arch. Microbiol. **95**:91–95.
11. **Kuner, J. M., and D. Kaiser.** 1982. Fruiting body morphogenesis in submerged cultures of *Myxococcus xanthus*. J. Bacteriol. **151**:458–461.
12. **LaRossa, R., J. Kuner, D. Hagen, C. Manoil, and D. Kaiser.** 1983. Developmental cell interactions of *Myxococcus xanthus*: analysis of mutants. J. Bacteriol. **153**:1394–1404.
13. **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.
14. **Shimkets, L. J.** 1987. Control of morphogenesis in Myxobacteria. Crit. Rev. Microbiol. **14**:195–227.
15. **Shimkets, L. J., and D. Kaiser.** 1982. Murein components rescue developmental sporulation of *Myxococcus xanthus*. J. Bacteriol. **152**:462–470.
16. **Teintze, M., T. Furuichi, R. Thomas, M. Inouye, and S. Inouye.** 1985. Differential expression of two homologous genes for spore-specific proteins in *Myxococcus xanthus*, p. 253–260. *In* J. A. Hoch and P. Setlow (ed.), Molecular biology of microbial differentiation. American Society for Microbiology, Washington, D.C.
17. **Varon, M., S. Cohen, and E. Rosenberg.** 1984. Autocides produced by *Myxococcus xanthus*. J. Bacteriol. **167**:356–361.
18. **Wireman, J., and M. Dworkin.** 1975. Morphogenesis and developmental interaction in Myxobacteria. Science **189**:516–523.
19. **Wireman, J., and M. Dworkin.** 1977. Developmentally induced autolysis during fruiting body formation by *Myxococcus xanthus*. J. Bacteriol. **129**:796–802.
20. **Zahavi, A., and D. Ralt.** 1984. Social adaptations in myxobacteria, p. 215–220. *In* E. Rosenberg (ed.), Myxobacteria: development and cell interactions. Springer-Verlag, New York.