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Cell-Density-Dependent Lysis and Sporulation of *Myxococcus xanthus* in Agarose Microbeads

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Vegetative cells of *Myxococcus xanthus* were immobilized in 25-μm-diameter agarose microbeads and incubated in either growth medium or sporulation buffer. In growth medium, the cells multiplied, glided to the periphery, and then filled the beads. In sporulation buffer, up to 90% of the cells lysed and ca. 50% of the surviving cells formed resistant spores. A strong correlation between sporulation and cell lysis was observed; both phenomena were cell density dependent. Sporulation proficiency was a function of the average number of cells within the bead at the time that sporulation conditions were imposed. A minimum of ca. 4 cells per microbead was necessary for efficient lysis and sporulation to proceed. Increasing this number accelerated the lysis and sporulation process. No lysis occurred when an average of 0.4 cell was entrapped per bead. Entrapping an average of 1.7 cells per bead resulted in 46% lysis and 3% sporulation of survivors, whereas entrapping an average of 4.2 cells per bead yielded 82% lysis and 44% sporulation of the surviving cells. Sporulation and lysis also depended upon the cell density in the culture as a whole. The existence of these two independent cell density parameters (cells per bead and cells per milliliter) suggests that at least two separate cell density signals play a role in controlling sporulation in *M. xanthus*.

Multicellular development and differentiation comprise a complex set of processes that have been studied in many systems. Among the simplest organisms capable of the cell communication and interaction necessary to carry out such a sequence of events are the members of the order *Myxobacterales*, of which *Myxococcus xanthus* is the most extensively studied (11, 15).

In this species, development into heat-resistant spores proceeds within macroscopic fruiting bodies. Initiation of these events depends upon the fulfillment of several prerequisites: (i) a solid surface must be available to support the developing fruiting bodies, (ii) a downshift in the availability of certain nutrients must occur, and (iii) the cell culture must have achieved a critical density prior to starvation (14).

Close to 15 years ago, Wireman and Dworkin reported that the development of *M. xanthus* was accompanied by cell lysis (18, 19). This developmental lysis has played an important role as a marker of development (see, e.g., reference 17). However, an easily reproducible methodology for measuring this lysis has been difficult to develop. In an effort to examine developmental lysis by using new methods, at least one group has obtained results which have led to a reevaluation of the very existence of developmental lysis (9). The present study presents evidence for a strong correlation between lysis and sporulation in *M. xanthus* and indicates that both of these phenomena are cell density dependent, as has been shown for fruiting-body development (4, 16).

A recently developed technique (R. Nir, R. Lamed, and E. Sahar, manuscript in preparation) was used for studying various aspects of the myxobacterial life cycle. This technique involves entrapping a predetermined number of *M. xanthus* cells within small agarose beads; thus, cell density dependence studies may be performed without the complications present under the usual culture conditions (e.g., clumping and aggregation).

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MATERIALS AND METHODS

Strains and growth conditions. Unless indicated otherwise, *M. xanthus* ER1500 was the wild-type strain used in this study. It is a derivative of the dispersed-growth strain DZ15 (P. D. Nathan, M.Sc. thesis, Tel Aviv University, Ramat Aviv, Israel, 1980) and contains the transposon Tn*5* inserted in a gene which is nonessential for growth or development. DZ15 and ER1500 are antibiotic TA-overproducing strains; both strains sporulate and form fruiting bodies like the wild type. *M. xanthus* DK5057 is an *asg* mutant strain (2), kindly provided by D. Kaiser. It is a derivative of the semidispersed-growth strain DK1622 (5) and contains Tn*5* in a gene essential for normal development and sporulation. The Tn*5* insertions confer kanamycin resistance on ER1500 and DK5057.

Strains were maintained in 1% CT growth medium (1% Casitone [Difco Laboratories, Detroit, Mich.] containing 0.2% $MgSO_4 \cdot 7H_2O$) and on 1 CT agar plates (1 CT medium plus 1.8% agar). The incubation temperature was 30°C.

Production of agarose microbeads. Agarose microbeads were produced in a system based on the vibrating-nozzle technique, described in detail elsewhere (Nir et al., in preparation). Briefly, *M. xanthus* cells were suspended in melted 0.6% agarose (type VII, low gelling temperature; Sigma Chemical Co., St. Louis, Mo.), made up in 1 CT growth medium or in sporulation buffer. This suspension was then ejected through a vibrating orifice, and the beads thus formed were cooled rapidly. The two variables which were manipulated in the present study were the number of cells per bead at the initial time, t_0, and the number of beads per milliliter of culture. The former value was controlled by varying the absolute number of cells introduced into the melted agarose (at 39°C) before bead formation proceeded: the more cells introduced into the constant volume of agarose, the larger the average number of cells per bead. The latter value was in direct proportion to the length of time that the beads were collected into the growth or sporulation medium; i.e., the longer the beads were collected into the

constant volume of collection fluid, the larger the number of resulting beads per milliliter.

Preparation of *M. xanthus* cells for entrapment in agarose microbeads. Cells were grown to the exponential phase in 1 CT growth medium containing 50 μg of kanamycin per ml, washed with sporulation buffer (MCM buffer: 10 mM morpholinepropanesulfonic acid [MOPS], 2 mM $CaCl_2$, 4 mM $MgSO_4$ [pH 7.2]), and concentrated by centrifugation and suspension to approximately 10^9 cells per ml. The cell titer of the concentrated culture was determined by using a Petroff-Hausser counting chamber, and then the culture was added to the melted agarose in the specific concentration which would ensure the desired average number of cells per bead. *M. xanthus* DK5057 cells were passed through four layers of sterile 3MM chromatography paper (Whatman Ltd., Maidstone, England) before the concentration step, to eliminate the microscopic cell clumps characteristic of strains which grow in a semidispersed manner. The cells were then introduced into the bead preparation apparatus and collected in sporulation buffer, in the presence of 250 μg of kanamycin per ml. For growth experiments, the cells were not washed before being concentrated and were collected in 0.5 CT growth medium containing 250 μg of kanamycin per ml.

Determination of the number of cells entrapped in agarose microbeads. Samples (0.5 ml) were withdrawn from the microbead suspension at the indicated time points and fixed in 2.5% glutaraldehyde at 4°C for at least 18 h. The number of cells per bead was determined directly by phase microscopy. Samples were also counted immediately after removal from the microbead suspension, omitting the fixation step, to verify that fixing the samples did not alter the results. Refractile ovoid or refractile round bodies were counted as spores. The number of beads counted for a single measurement varied from 140 (for samples with a large number of cells per bead) to 800 (for those with a small number of cells per bead).

Determination of spore viability in microbeads. Samples (1 ml) were removed from incubating microbead suspensions and heated for 15 min at 57°C. The heating killed vegetative cells and melted the microbeads, releasing the spores. Since the spores did not form aggregates within the beads, the sonication step, usually necessary to disperse the spores from fruiting bodies or clumps, was not required. Appropriate dilutions were performed, and 10-μl drops were applied to 1 CT agar. Plates were incubated for 3 to 5 days, and the number of CFU was determined.

Protein S assay. Seven-day spores, developed in agarose microbeads, were incubated for 60 min at 37°C in the presence of an equal volume of a 12-mg/ml stock solution of rabbit-anti-protein *S* immunoglobulin G, prepared in 0.15 M NaCl; 10 mM $CaCl_2$ was added to prevent removal of protein S from the spores by NaCl (3). Samples were centrifuged at 12,000 × *g* for 2 min, rinsed once, and suspended in 0.15 M NaCl–10 mM $CaCl_2$ in 10 times the original volume. A second incubation was performed at 37°C for 30 min in the presence of 1/10 volume of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. The samples were then removed and viewed directly under fluorescence optics.

Photography and electron microscopy. Photographs of the microbeads and the cells contained therein were taken with a phase-contrast microscope (no. BH-2; Olympus Corp., Lake Success, N.Y.) under phase and fluorescence optics. Thin sections were prepared for electron microscopy as described by Inouye et al. (3).

RESULTS

Entrapment and growth of *M. xanthus* in agarose microbeads. *M. xanthus* ER1500 was grown to the exponential phase and entrapped in agarose microbeads, as described in Materials and Methods. In the example shown in Fig. 1, an average of ca. 11 vegetative cells per bead were entrapped (Fig. 1A). The cells appeared intact and healthy at the completion of the entrapment process. After incubation in a rotary shaker at 30°C in 0.5 CT growth medium for approximately 24 h, the cells multiplied and migrated within the beads (Fig. 1B). This movement was apparent from the characteristic arrangement of cells along the periphery of a partially filled bead. After 40 h of incubation in growth medium, the cells completely filled the beads (Fig. 1C). Cells were observed escaping from the microbeads only after the beads were filled to capacity.

Sporulation and lysis of *M. xanthus* in agarose microbeads. When developmentally proficient *M. xanthus* cells were entrapped in agarose microbeads and incubated in sporulation medium, refractile spores were observed in the beads within 48 h (Fig. 2). The spores were well dispersed within the beads, and the beads themselves remained dispersed in the liquid buffer. In the past, clumping of cells and spores has made measurements of cell numbers and the extent of cell lysis difficult to determine. With the clumping problem eliminated by inducing sporulation within microbeads, the question of developmental lysis was addressed.

The results of a sporulation-lysis experiment, in which the average number of cells per bead at t_0 was 4.2, are summarized in Fig. 3. As some of the cells sporulated, a shift in the distribution of cell density within the beads was observed. Whereas the highest percentage of beads contained four cells at t_0, most of the beads were devoid of cells by 40 h. The proportion of empty beads increased and then leveled off by 8 days. At intermediate times, many beads were observed to contain disintegrating cells. Cells and cell debris, clearly evident within the beads, were not observed outside the beads in the surrounding medium. The asymmetric distribution of cells per bead at t_0 occurred because, for technical reasons, the beads themselves were not perfectly uniform in size.

Cell density dependence of sporulation and lysis of *M. xanthus* in agarose microbeads. In an effort to determine the effect of cell density on sporulation and lysis in microbeads, the average initial number of cells per bead was varied from 0.37 to 4.18 (Table 1). In experiment 1, with an average of 0.37 cell per bead at t_0, no spores were observed by 8 days. The number of cells per bead increased and plateaued at a level 73% higher than the initial value. This increase was observed at early time points in all sporulation experiments carried out with microbeads. The number of cells per bead did not decrease from this elevated level throughout experiment 1.

In experiment 2, the initial average of 1.66 cells per bead increased 37% by 42 h and then decreased (46% decrease by 192 h, from a maximum of 2.27 at 42 h). Concomitant with this decrease in cell density within the beads was a low level of sporulation (3% of surviving, intact cells).

In experiment 3, beads were prepared with an initial average number of 4.18 cells per bead. By 25 h this number had increased by 12% (data not shown), and then it rapidly and dramatically decreased to 35 and 18% of the maximum by 42 h and 8 days, respectively. This large decrease in the average number of cells per bead was accompanied by a high level of sporulation. By 8 days, 44% of all visually intact

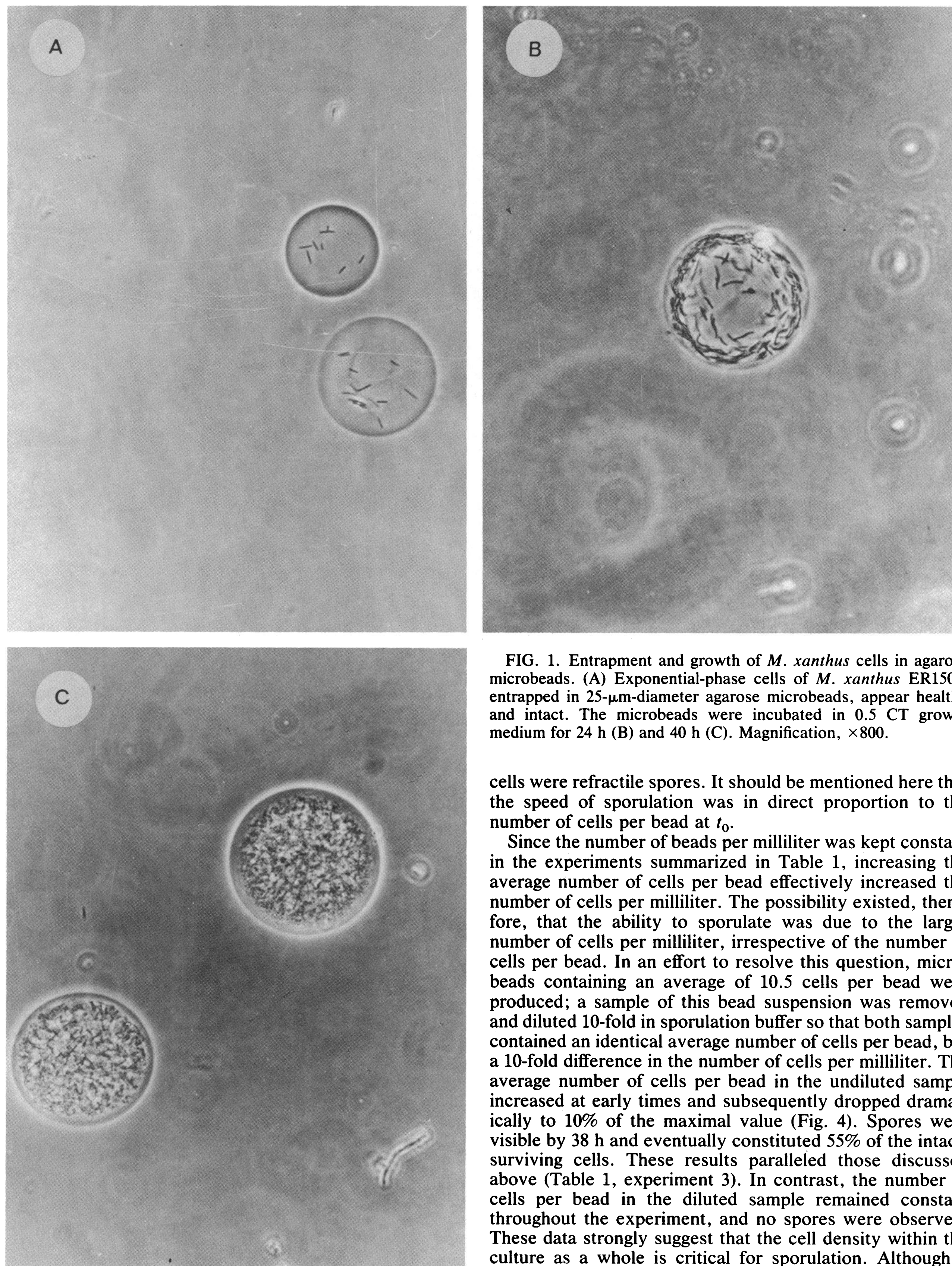

FIG. 1. Entrapment and growth of *M. xanthus* cells in agarose microbeads. (A) Exponential-phase cells of *M. xanthus* ER1500, entrapped in 25-μm-diameter agarose microbeads, appear healthy and intact. The microbeads were incubated in 0.5 CT growth medium for 24 h (B) and 40 h (C). Magnification, ×800.

cells were refractile spores. It should be mentioned here that the speed of sporulation was in direct proportion to the number of cells per bead at t_0.

Since the number of beads per milliliter was kept constant in the experiments summarized in Table 1, increasing the average number of cells per bead effectively increased the number of cells per milliliter. The possibility existed, therefore, that the ability to sporulate was due to the larger number of cells per milliliter, irrespective of the number of cells per bead. In an effort to resolve this question, microbeads containing an average of 10.5 cells per bead were produced; a sample of this bead suspension was removed and diluted 10-fold in sporulation buffer so that both samples contained an identical average number of cells per bead, but a 10-fold difference in the number of cells per milliliter. The average number of cells per bead in the undiluted sample increased at early times and subsequently dropped dramatically to 10% of the maximal value (Fig. 4). Spores were visible by 38 h and eventually constituted 55% of the intact, surviving cells. These results paralleled those discussed above (Table 1, experiment 3). In contrast, the number of cells per bead in the diluted sample remained constant throughout the experiment, and no spores were observed. These data strongly suggest that the cell density within the culture as a whole is critical for sporulation. Although a minimum cell density (4×10^6 cells per ml) within the culture was necessary for sporulation and lysis, it was not sufficient: cells did not lyse or sporulate when prepared at 5×10^7 cells

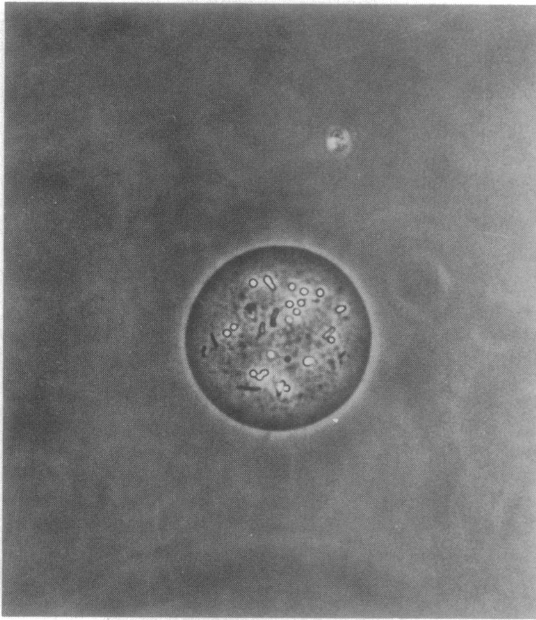

FIG. 2. Sporulation of *M. xanthus* in agarose microbeads. Microbeads containing vegetative cells of ER1500 were transferred to MCM sporulation medium. By 48 h of incubation, refractile spores had formed within the beads. Magnification, ×800.

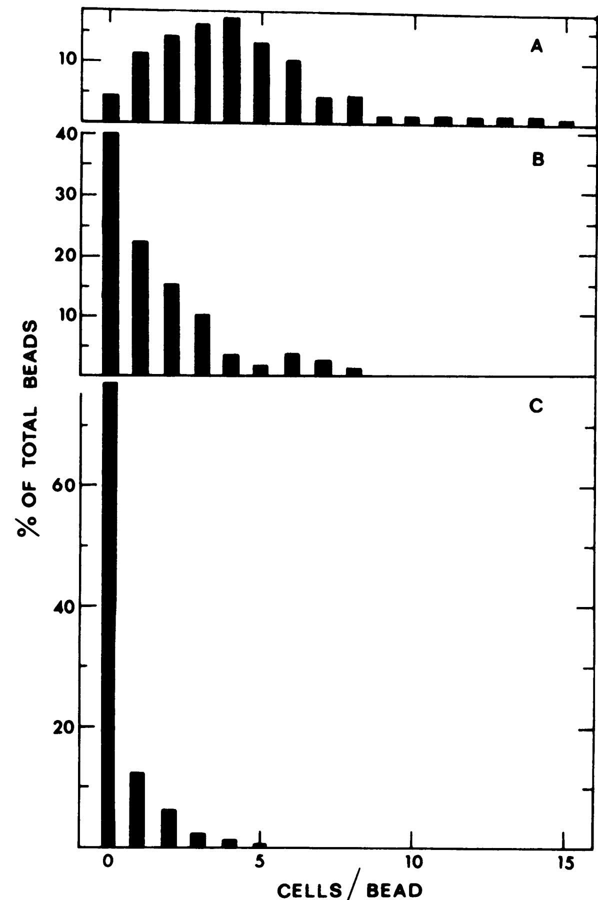

FIG. 3. Lysis during sporulation in microbeads. The number of cells per microbead (strain ER1500) was determined by counting samples in a microscope. The results were arranged according to category, based on the number of cells in each particular bead. Plotted here is the distribution of the cell population within the beads at t_0 (A) and after 40 h (B) and 192 h (C) of incubation in sporulation medium. The total numbers of beads analyzed at t_0 and at 40 and 192 h were 474, 369, and 695, respectively.

per ml but at a small average number of cells per bead (2.1 cells per bead). Apparently, both the overall cell density and the number of cells per bead play a role in determining sporulation proficiency in this system.

Viability of spores formed in agarose microbeads. The viability of spores produced in agarose microbeads was demonstrated in two ways. A suspension of beads containing spores was plated on 1 CT agar after being subjected to heat treatment, as described in Materials and Methods. The number of CFU agreed with the theoretical value based on the average number of refractile spores per bead and the number of beads per milliliter (data not shown). In addition, when beads containing spores were stored for up to 6 weeks at 4°C and then incubated in 0.5 CT growth medium at 30°C, the spores germinated within 24 h and became foci of microcolonies within the beads (Fig. 5).

Existence of an extracellular capsule and protein S on spores produced in agarose microbeads. Figure 6 is an electron micrograph of thin sections of an agarose microbead containing mature, refractile spores. The thick extracellular capsule characteristic of fruiting-body spores can be seen. In addition, membrane fragments, ostensibly from lysed cells, surround the spores.

After the protein S assay was performed, as described in Materials and Methods, spores which developed within agarose microbeads were shown to have significant amounts of protein S on their surface (Fig. 7). Glycerol-induced spores and vegetative cells did not stain under these conditions (data not shown). The data presented indicate that spores produced within agarose microbeads are similar in two critical characteristics to fruiting-body spores, to be distinguished from glycerol-induced spores.

Inability of a developmental mutant to sporulate or lyse in agarose microbeads. *M. xanthus* DK5057, a sporulation-deficient mutant from the *asg* class, failed to sporulate or lyse when entrapped in agarose microbeads and incubated in sporulation medium (Table 2). The number of entrapped cells increased by 30% at early times, similar to the values obtained with the wild type. This value leveled off and did not decrease significantly, even after 12 days of incubation. In addition, no sporulation was observed, either visually or

TABLE 1. Distribution of cells and spores of ER1500 in microbeads under sporulation conditions

Expt	Time (h)	Total no.			Cells/bead	Spores/bead
		Beads	Cells	Spores		
1	0	801	300	—	0.37	—
	42	389	207	—	0.53	—
	68	418	262	—	0.63	—
	192	392	251	—	0.64	—
2	0	618	1,027	—	1.66	—
	42	331	752	—	2.27	—
	68	242	458	—	1.89	—
	192	535	652	21	1.22	0.04
3	0	474	1,982	—	4.18	—
	42	369	604	—	1.64	—
	68	234	307	52	1.31	0.22
	192	695	591	259	0.85	0.37

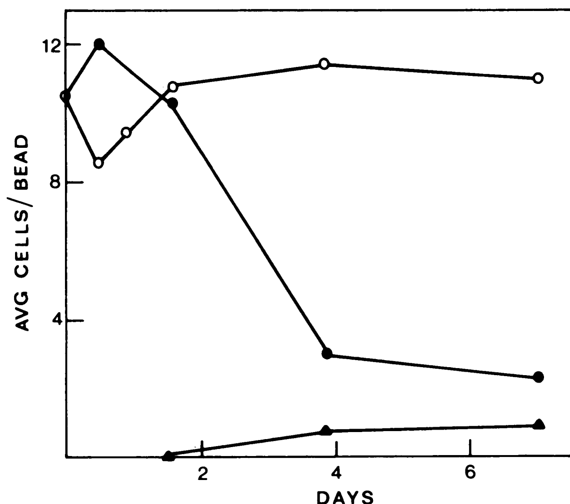

FIG. 4. Average number of cells per bead as a function of time and overall cell density. ER1500 cells were prepared in agarose microbeads, with an average of 10.5 cells per bead at t_0. A sample was diluted as described in the text, and the average number of cells per bead was plotted as a function of the time of incubation in sporulation buffer, for the diluted (○) and undiluted (●) samples. The number of spores in the undiluted sample (△) is indicated. Only a small number of spores (less than 0.05 spore per bead) was observed in the diluted sample.

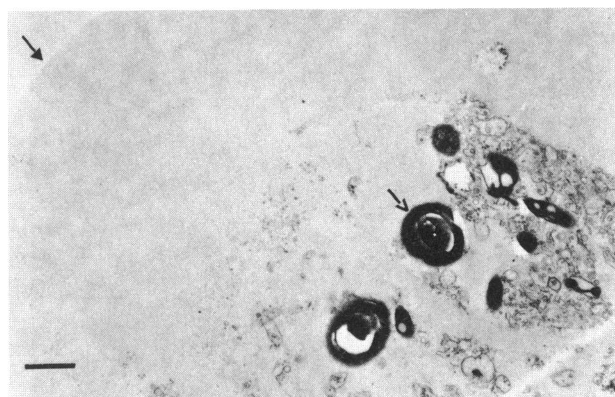

FIG. 6. Electron micrograph of thin sections of 6-day-old spores developed in agarose microbeads. Symbols: ◄—, boundary of the agarose microbead, within which the spores can be seen; ←, spore capsule. Bar, 1 μm.

as determined by heat resistance, after 12 days of incubation in sporulation medium. This failure to sporulate in microbeads was obtained even when the beads were filled to capacity with the DK5057 mutant cells, a condition under which wild-type cells sporulated in less than 48 h. It should be pointed out that DK5057 cells remained viable within the beads and were capable of growing and filling the beads when incubated in 0.5 CT growth medium.

DISCUSSION

Multicellular development in *M. xanthus* is a complex process, culminating in the formation of spore-filled fruiting bodies. One of the conditions necessary to initiate development is a critical cell density. In this paper we have described a new technique which has been exploited to study this cell density dependence phenomenon. Results suggest a dual dependence on cell density based on local, as well as overall, cell concentration. The data also indicate a strong correlation between cell lysis and sporulation.

In every experiment in which sporulation was obtained, it was preceded and accompanied by a decrease in the average number of cells per bead. This decrease can best be explained by invoking the phenomenon of cell lysis. The cells did not appear in the surrounding supernatant fluid, nor were they observed leaving the beads at any time during the sporulation process, precluding an explanation based on cell migration out of the beads. In addition, disintegrating cells were visualized in the beads during cell loss, preceding and concomitant with the appearance of spores in the beads.

In samples in which sporulation was not observed, either because of a low cell density within the beads or within the culture as a whole or because a sporulation-deficient strain was used, there was no evidence of cell lysis. In experiments containing an intermediate concentration of cells (e.g., 1.7 cells per bead and 9×10^6 beads per ml), a low level of sporulation was observed in conjunction with a low level of lysis, further underscoring the relationship between the two phenomena.

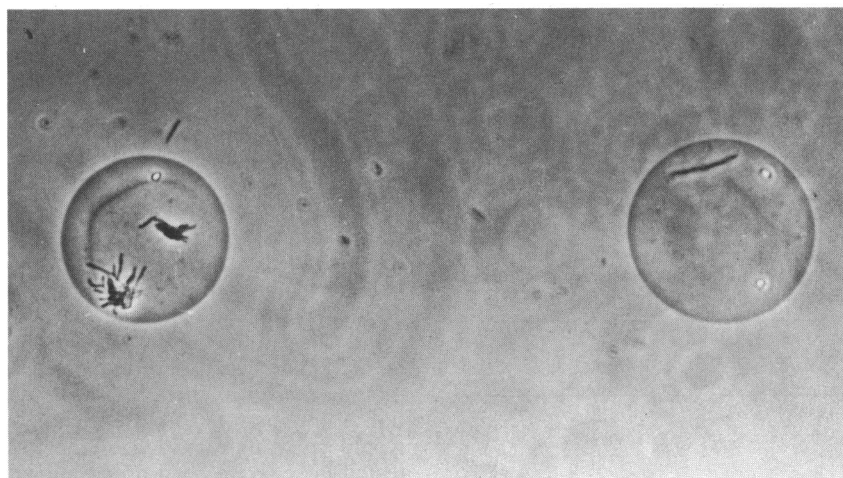

FIG. 5. Germination of agarose microbead spores in situ. Agarose microbeads, containing refractile spores, were incubated in 0.5 CT growth medium. By 20 h, partial germination was observed. Magnification, ×800.

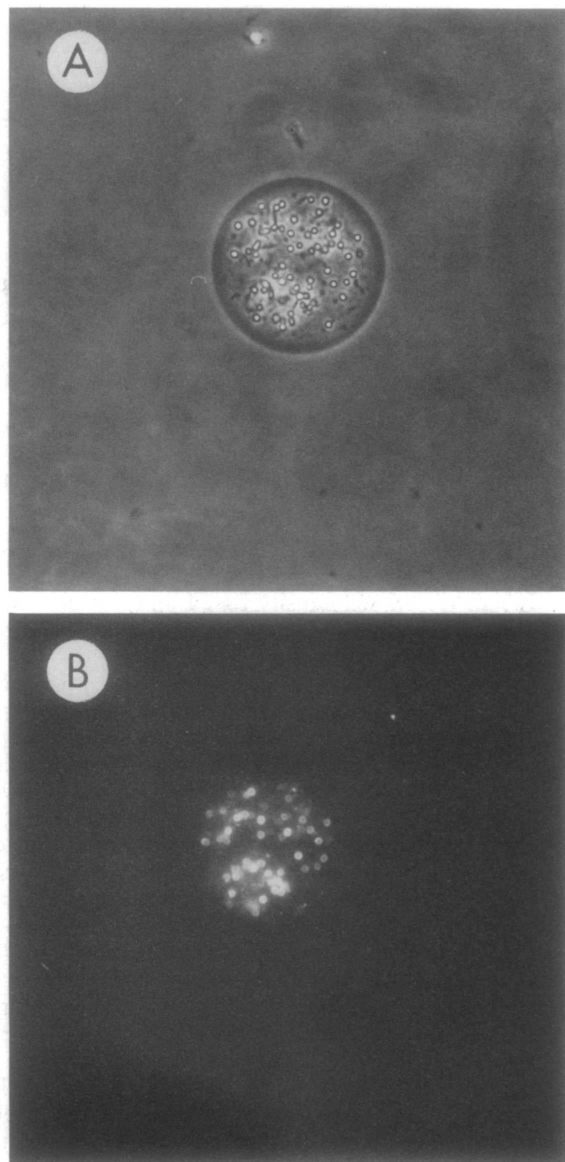

FIG. 7. Protein S assay of spores produced within agarose microbeads. The figure shows photomicrographs taken in phase (A) and fluorescence (B) optics of 7-day-old spores in agarose microbeads, assayed for protein S as described in the text. Magnification, ×800.

A major problem with previous attempts at evaluating developmental cell lysis has been the level of manipulation necessary to prepare dispersed samples for analysis, and at least some of the lysis has been reported to be investigator induced (9). Use of the present technique has obviated the need for such manipulations, because the cells and spores were dispersed throughout the bead and the beads were dispersed evenly throughout the culture. Beads were simply removed from a flask and placed directly onto a glass slide for microscopic analysis. Lysis measured by this technique clearly is not an artifact of manipulating the sample.

TABLE 2. Distribution of cells and spores of DK5057 in microbeads under sporulation conditions

Time (h)	Total no.			Cells/ bead	Spores/ bead
	Beads	Cells	Spores		
0	261	1,393	—	5.3	—
41	237	1,634	—	6.9	—
96	198	1,384	—	7.0	—
168	251	1,608	—	6.4	—
288	240	1,518	—	6.3	—

In light of the results presented in this study, two categories of cell density must be taken into account: local cell density, expressed as the average number of cells per bead, and overall cell density, i.e., the number of cells per milliliter of culture. The results reported here indicate that a local concentration of approximately four cells per bead is necessary to promote efficient sporulation. This value is the equivalent of 5×10^8 cells per ml of agarose, based on an average bead diameter of 25 μm. This local cell density is comparable to that necessary for development of *M. xanthus* to proceed in other systems (7, 19).

In addition to a minimum local cell density, a minimum overall cell density of approximately 4×10^6 cells per ml must exist for sporulation to occur. There appears to be a complex interplay between local and overall cell density, which interact to determine whether sporulation will proceed. One explanation for the observed results is that at least two types of signals control sporulation: a readily diffusible signal and a poorly diffusible or nondiffusible signal. The poorly diffusible or nondiffusible signal may be a large molecule or a molecule adhering to the cell surface. This signal could serve to indicate to the starving cells their concentration in the immediate vicinity (within a bead or within the aggregate on a solid surface). The readily diffusible signal may be a small molecule that can travel easily through the medium, announcing the overall cell concentration in the liquid culture or on a solid surface. Both pieces of information would therefore be important to the starving culture. Development would proceed only if the total number of cells in the larger volume was sufficient to produce a minimum population of spores and if the local concentration was sufficient to support the process. The latter condition assumes that cells, both developing and vegetative, communicate on a local scale and interact to support these cell-density-dependent processes. Social motility in *M. xanthus* is one well-studied process which depends upon such local interactions (5).

Four classes of developmental mutants with cell signaling defects have been identified (2) and are categorized according to their ability to extracellularly complement one another during development. Two mutants capable of complementing each other are, by definition, placed in separate classes. This complementation involves the transfer of extracellular signals between cells of the different mutant strains. Some of these signals have recently been identified (8; S. Kim, L. Kroos, and D. Kaiser, personal communication). A study of complementation in the microbead system might further clarify the mechanisms by which these signals are transmitted. As indicated above, at least two cell density signals can be differentiated in this system, one based on the local cell density within the bead and the other based on the overall cell density in the culture as a whole. The nature of complementation between different mutants might be tested, exploiting these parameters. Two ways to test complementation in the microbead system come to mind. One approach would be to entrap each of the two mutant strains to be tested in separate bead preparations. Mixing beads of each kind under sporulation conditions would test the existence of a freely diffusible signal. A second approach would be to

entrap cells of the two mutant strains within the same bead. The ability of two mutant strains to complement under these conditions when they are incapable of complementation when each strain is in a separate bead would indicate that the necessary signal is not readily diffusible and that cells must be in close proximity to ensure complementation.

"Normal" myxobacterial development has been studied by using systems which provide a solid support for fruiting-body formation, and the spores produced in fruiting bodies have been the object of genetic and metabolic studies. However, numerous investigators have observed sporulation outside the fruiting body (see, e.g., reference 10). In the present study and in previous studies (12, 13), spore formation has been observed in the absence of fruiting bodies. We propose that this sporulation may also be "normal" and may constitute an alternate route taken by starving *M. xanthus* cells when they find themselves outside fruiting bodies. This idea is supported by the way in which development is now understood, as being composed of two parallel pathways, one leading to fruiting-body formation and the other leading to sporulation (15, 20). The data presented here demonstrate that sporulation and/or lysis occurs in microbeads under appropriate conditions in a cell-density-dependent fashion. It is possible that developing cells, stabilized by the conditions found inside a fruiting body, do not lyse to the same extent as do developing cells entrapped in agarose. However, in general, cells are stabilized in polymeric porous networks (1, 6).

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