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Sporulation of *Myxococcus xanthus* in Liquid Shake Flask Cultures

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When suspended in a liquid starvation medium, exponentially growing *Myxococcus xanthus* sporulated within 3 days. These myxospores were similar to spores developed within fruiting bodies, as determined by electron microscopy and the production of spore-specific protein S. This liquid sporulation system may be useful as a means of preparing large quantities of myxospores and extracellular fluid for biochemical studies, including isolation of chemical signals produced during the sporulation process.

Myxococcus xanthus is a gram-negative bacterium which, in response to nutritional downshift, undergoes a complex developmental program (19) culminating in the construction of spore-filled fruiting bodies. These fruiting bodies form from masses of cells that glide along a solid surface to central locations. For many years, *M. xanthus* development has been studied in the laboratory with agar as the substratum; more recently, development has also been carried out on a polystyrene-liquid interface (15). Myxosporulation in the absence of fruiting-body formation has been observed in systems in which glycerol (6), dimethyl sulfoxide (2), or related substances (18) were added to exponentially growing cells in shake flasks. These chemically induced spores, although structurally and biochemically distinct from fruiting-body spores (1, 2, 22), have been an object of genetic and biochemical studies because of the ease with which they are generated and manipulated. However, because of the many dissimilarities between glycerol-induced spores and fruiting-body spores, studies of the glycerol system have their limitations. In the present study, a simple procedure for the production in liquid medium of large numbers of spores which are similar to fruiting-body spores is reported. This system may provide a means of producing large batches of spores and sporulation-conditioned medium for genetic and biochemical studies.

M. xanthus DK1622 (14), a fully motile and developmentally proficient strain, was used throughout the present study unless otherwise indicated. An exponential-phase culture grown in 1CT growth medium (1% Casitone [Difco Laboratories, Detroit, Mich.] containing 0.2% $MgSO_4 \cdot 7H_2O$) was harvested (10,000 × *g* for 5 min), rinsed twice with MCM starvation buffer (10 mM MOPS [morpholinopropanesulfonic acid], 2 mM $CaCl_2$, 4 mM $MgSO_4$ [pH 7.2]), and resuspended in the original volume of MCM buffer. The cell suspension was incubated in a sidearm flask at 30°C on a rotary shaker. Turbidity was determined on a Klett-Summerson photoelectric colorimeter (no. 54 filter). The number of viable spores (MCM spores) was determined by removing samples of the cell suspension, heating them at 57°C for 15 min, sonicating them three times at 100 W for 15 s each time to disperse the spores, and plating appropriate dilutions onto 1CT agar (1CT growth medium with 1.8% agar).

Figure 1 illustrates the decrease in turbidity and the concomitant increase in the number of heat- and sonication-resistant spores during incubation of developmentally proficient (DK1622) and mutant (DK5057) cultures. By 70 h of incubation, approximately 15% of the initial number of DK1622 cells had become mature, resistant spores and had aggregated into large, macroscopic clumps. When *M. xanthus* DK5057, a mutant incapable of fruiting-body formation and sporulation under classic developmental conditions (10), was prepared in the manner described above, fewer than 50 spores per ml were observed, although the vegetative cells remained viable under these conditions for at least 6 days (data not shown).

The rapid decrease in turbidity of the DK1622 culture may be due to lysis of some proportion of the cells, to a change in turbidity due to the formation of macroscopic clumps, or to some interplay of the two processes. The culture containing DK5057 cells gradually decreased in turbidity, although no macroscopic clumping was observed. Cell-cell interactions play an important role in the life-style of *M. xanthus* (7, 13, 20), and it is interesting that, in the absence of a solid surface, the MCM sporulating cells seek an alternative form of multicellular cooperation in order to carry out an altered form of the developmental program. A similar effect was noted by Burchard (3). Vegetative cells of a nondispersed growing mutant formed large clumps in 1CT growth medium; when transferred to starvation buffer, these cells differentiated into spores shaped like fruiting-body spores within these clumps.

In order to compare spores produced in MCM buffer with fruiting-body and glycerol-induced spores, thin sections of spores were prepared for electron microscopic analysis by the procedure of Inouye et al. (11). Both MCM spores and fruiting-body spores displayed thick, multilayered extracellular capsules which were absent in glycerol-induced spore samples (Fig. 2).

The presence of protein S further demonstrated that MCM spores resembled fruiting-body spores and differed from glycerol-induced spores. Protein S is a spore-specific protein whose synthesis is turned on early in development (12) and which is subsequently assembled onto the surface of the spores. Rinsed and sonicated 7-day spore samples at ca. 3×10^7/ml 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 (IgG) prepared in 0.15 M NaCl; 10 mM $CaCl_2$ was added to prevent removal of protein S from the spores by NaCl (11). Samples were centrifuged at 15,000 rpm (Mikroliter; Hettich, Tuttlingen, Federal Republic of Germany) for 2 min, rinsed once, and suspended in 0.15 M NaCl–10 mM $CaCl_2$ at 10 times the original volume. A second incubation was performed at 37°C for 30 min in the presence of 1/10 volume fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. Samples were then removed and

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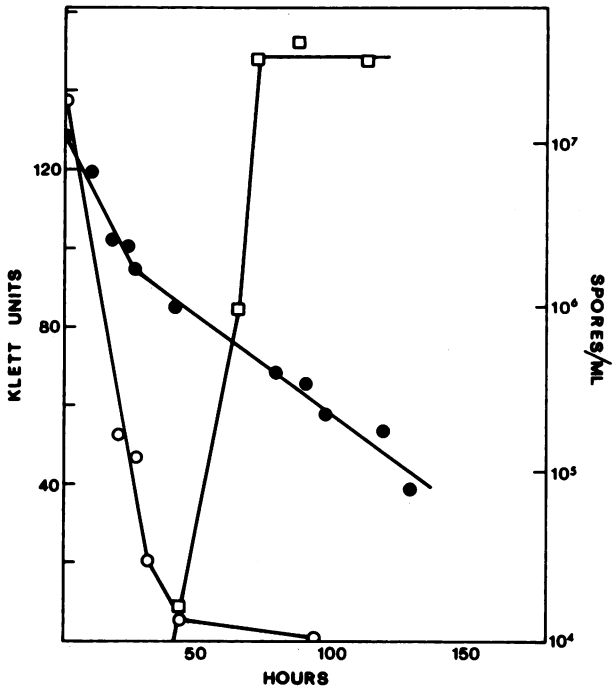

FIG. 1. Behavior of *M. xanthus* cells in MCM liquid culture. The decrease in turbidity of the wild-type strain, DK1622 (○), and the *asg* developmental mutant strain, DK5057 (●), and the appearance of heat- and sonication-resistant spores of DK1622 (□) are shown as a function of time in MCM liquid shake flask culture.

viewed directly by fluorescence optics. In the several fields (ca. 100 cells) of vegetative cells and glycerol-induced spores examined, there was no evidence of fluorescence. MCM spores, however, fluoresced strongly (Fig. 3B). These results indicate that protein S is present on the spore surface of MCM spores, further establishing their resemblance to fruiting-body spores.

Downard and Zusman (5) reported that the *tps* gene, which codes for protein S, was expressed in shake cultures under conditions of nutritional downshift, where aggregation and sporulation were not observed. In the present study, liquid-culture conditions which allowed for aggregation and sporulation and in which protein S assembled on the outside surface of the spores were used. Mutant strains which produce spores lacking protein S have been isolated (4). These spores have been shown to be normally resistant and indistinguishable from protein S-intact spores. This finding has led to the hypothesis that protein S may provide the "glue" which holds spores in place within the fruiting body. In this respect, it is interesting to note that immunofluorescence studies, designed to detect protein S on spores, showed highly fluorescent extracellular material in fruiting-body spore samples (Fig. 3D). This material was seen in conjunction with aggregates of spores which were not dispersed by sonication and might represent the interspore glue.

In comparing spores produced under shake flask conditions with fruiting-body spores, it is important to note that both the kinetics of spore production and the relative yield of spores are similar in both types of systems and are in sharp contrast to those of glycerol-induced sporulation. In the former two systems, differentiation of ca. 10% of the original cell number is a process which takes 2 or more days to

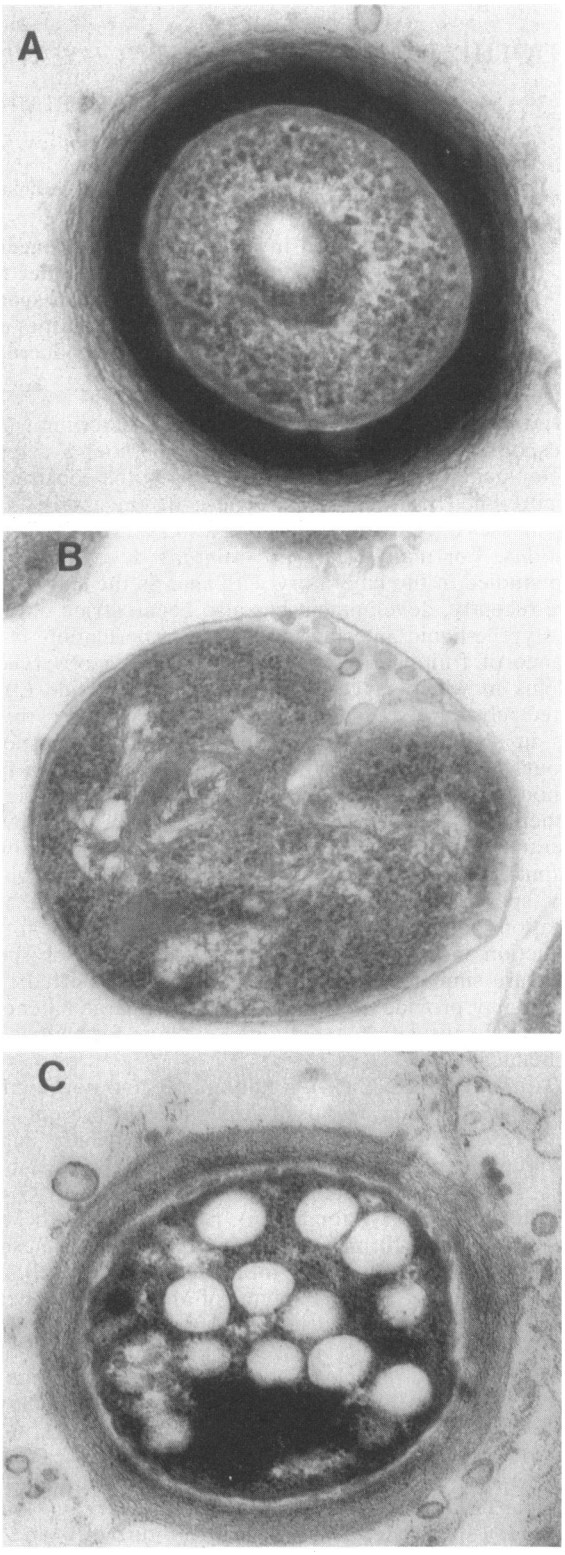

FIG. 2. Thin-section electron micrographs of MCM (A), glycerol-induced (B), and fruiting-body (C) spores. Fruiting-body and MCM spores were prepared for electron microscopy after 1 week; glycerol-induced spores were harvested and fixed after 18 h.

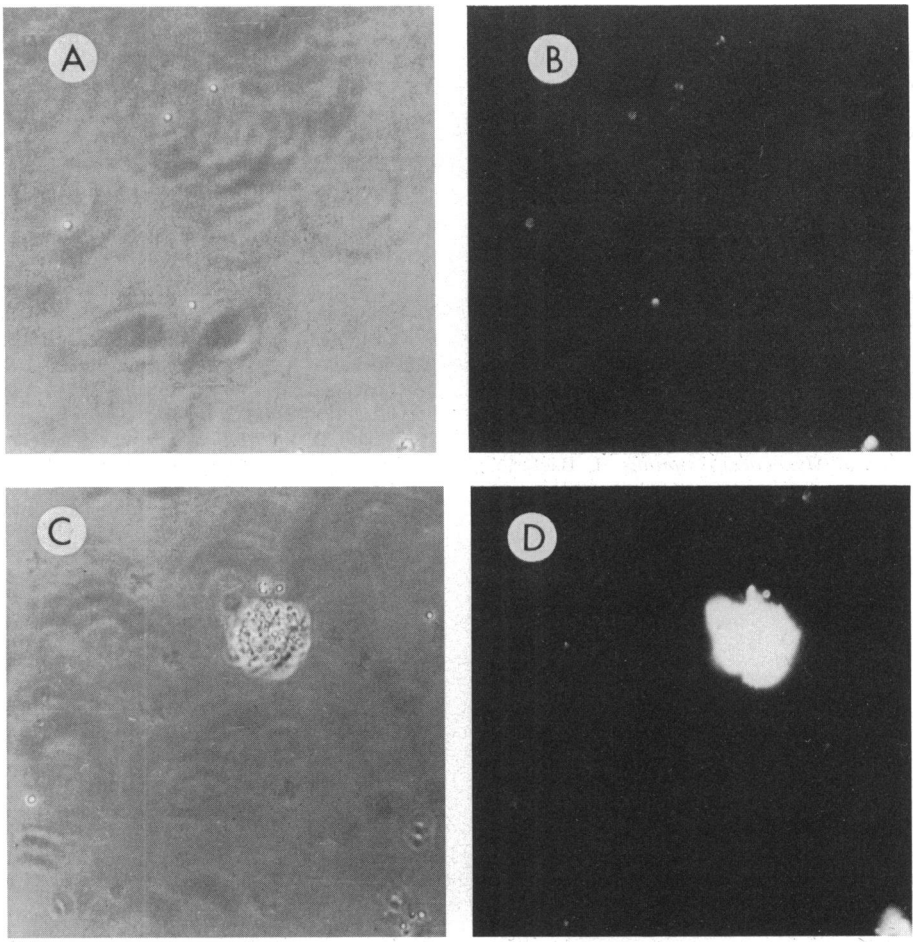

FIG. 3. Photographs of MCM spores after incubation with rabbit anti-protein S IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. (A and B) MCM spores; (C and D) fruiting-body spores. (A and C) Photographed under phase-contrast; (B and D) photographed under fluorescence optics.

complete. In contrast, glycerol induction of spores is rapid and synchronous, with nearly 100% of the cells converting to refractile spores within 6 h (6).

Suspension culture techniques for studying development have proven useful in other systems. Shaking suspensions of aggregation-competent cells of *Dictyostelium discoideum* display oscillations in light scattering and cyclic AMP levels similar to those observed during aggregation on a solid support (8, 9). *Stigmatella aurantiaca*, a myxobacterium which undergoes fruiting-body formation in response to light (17), can be induced to develop by addition of a specific pheromone (21). Although development of *S. aurantiaca* is generally carried out on a solid surface, this pheromone has also been detected in shake flask starvation cultures. In addition, Kuspa et al. (16) have reported the release of A factor, a development-specific signal, from concentrated *M. xanthus* cell suspensions shaking in a MOPS-Ca^{2+} buffer.

The technique described here for inducing myxosporulation in liquid medium allows for the production of large quantities of apparently normal *M. xanthus* spores. In addition, it may be possible to use the large volumes of MCM medium to isolate signal molecules, lytic by-products, and other biochemical markers of development released by aggregating and sporulating cells.

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