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Myxospore Induction in a Nondispersed Growing Mutant of *Myxococcus xanthus*

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Myxococcus xanthus RB5, a rough-colony-forming, nondispersed growing mutant of strain FB_t, forms macroscopic, multicellular masses of radially oriented cells in shake cultures. The cells appear to be held together by slime fibrils. Physical and enzymatic methods to disrupt the spheres were unsuccessful as were attempts to isolate dispersed growing mutants. During incubation of the spheres in starvation medium, the cells within convert to myxospores, indistinguishable from those formed in fruiting bodies. Myxospores were also induced in artificially constructed, dense masses of cells of a nonmotile strain.

The myxobacteria, which demonstrate both fruiting body formation and myxospore development within fruiting bodies, occur naturally on soil particles, bark, and other surfaces (6). When inoculated into shake cultures, most species initially grow in a nondispersed fashion; cells adhere to one another and to the surfaces of the growth vessel. For many myxobacteria, dispersed growth can be achieved by manipulation of medium constituents (7). Also, dispersed growing variants can be selected (10-12).

Myxococcus xanthus is one of the species from which such variants have been isolated. During a search for mutants of FB_t, one of the dispersed growing strains (4), a rough colony was detected among survivors of an ultraviolet mutagenesis experiment. This stable mutant grows in a nondispersed fashion. In this communication the mutant is partially characterized. Its behavior provides some insight into the role of the fruiting body in the life cycle of these bacteria.

MATERIALS AND METHODS

Bacteria. *M. xanthus* RB5 was isolated as a rough-colony mutant of strain FB_t (4). Strain NM was described by Burchard (1). The four strains of *M. fulvus* were isolated by Hans Reichenbach, Walter Fluegel, and myself.

Media. Bacteria were cultured in CT-1 broth (4) on a rotary shaker and on CTE agar (2) at 30 C. Fruiting agar contained the same constituents as CTE, except that the Casitone (Difco) concentration was reduced to 0.02% or omitted entirely.

For myxospore induction, RB5 cell clusters were suspended in 10^{-2}M K_2HPO_4-KH_2PO_4 buffer (pH 7.6) plus 4×10^{-3} M $MgSO_4$ (PO_4-Mg), or in water.

Scanning electron microscopy. RB5 spheres were prepared for scanning electron microscopy by a procedure similar to that of Ridgway and Lewin (13). No membrane filters were required in this case; phosphate buffer (10^{-2} M; pH 7.6) replaced the sea water, and the spheres were shadowed with either aluminum or gold-platinum. Cells were examined with a Cambridge S-600 scanning electron microscope.

RESULTS

M. xanthus RB5 appeared as a rough colony among the smooth colonies typical of the parent strain FB_t after ultraviolet mutagenesis. The colony form is stable during subculture. When inoculated into CT-1 broth and incubated on a rotary shaker, this strain grows in a nondispersed manner. Macroscopic, multicellular spheres (Fig. 1), a heavy ring of cells on the glass at the air-culture medium interface, and a film and/or filaments of cells on the flask bottom develop. A few free cells are observed upon microscopy observation of the clear medium.

Examination of spheres of RB5 cells by scanning electron microscopy reveals that the cells are densely packed in a radial orientation (Fig. 2), as though the spheres are macroscopic rosettes (3). Cells at the sphere surface are less well ordered (Fig. 3); in some fields, they appear to be held together by filaments, presumably consisting of extracellular slime (Fig. 4).

The rate of colony growth, resulting from cell division and gliding motility, was compared with that of the wild type (2). In one experiment, the mean rate of increase of colony diameter was 0.37 and 0.09 mm/h for RB5 and FB_t, respectively. Colonies of the latter are, however, thicker than those of the mutant.

Attempts to disassociate the cells of RB5 spheres by physical and enzymatic methods proved unsuccessful. Ultrasound effectively dis-

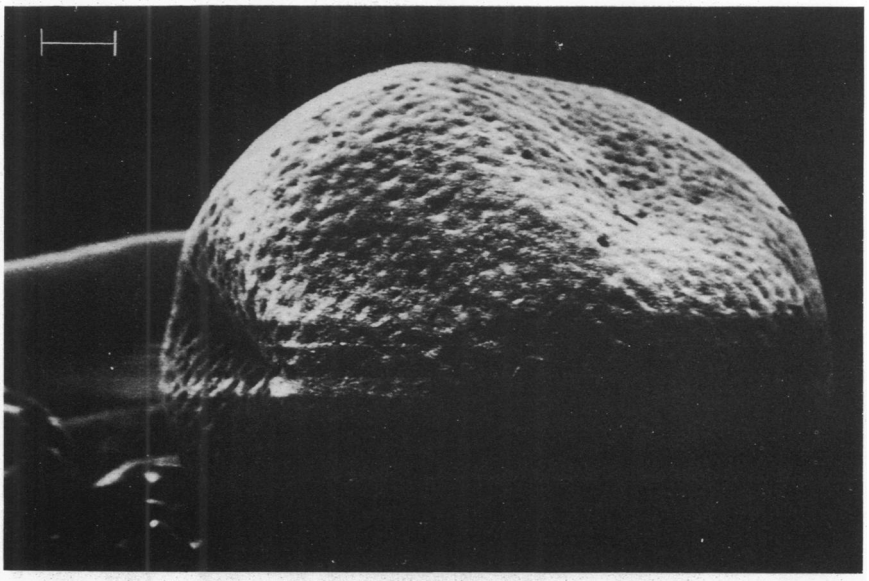

FIG. 1. *Multicellular sphere of M. xanthus RB5 cells as seen by scanning electron microscopy. The indentation of the left side of the sphere is a manipulation artefact. Shadowed with aluminum. Bar represents 100 μm.*

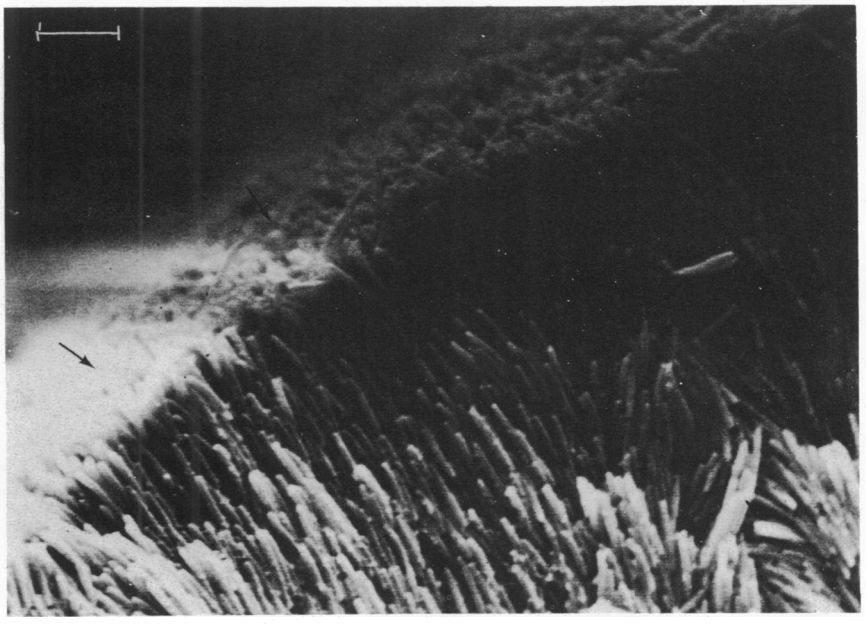

FIG. 2. *Inner structure of an RB5 sphere. Arrows point to the outer surface. Shadowed with gold-platinum. Bar represents 5 μm.*

rupted the spheres and cells within them simultaneously. Presumably, the filamentous interconnections sometimes observed between the cells in the spheres are responsible for holding them together. A variety of hydrolytic enzymes was employed in attempts to degrade these filaments. Pronase, lysozyme, deoxyribonuclease, lipase, cellulase, pectinase, alkaline phosphatase, hyaluronidase, *Cytophaga* L-1 enzyme preparation (BDH), and *Helix pomatia* (snail) gut juice (Indust. Biol. Franca) were ineffective in disassociating the cells.

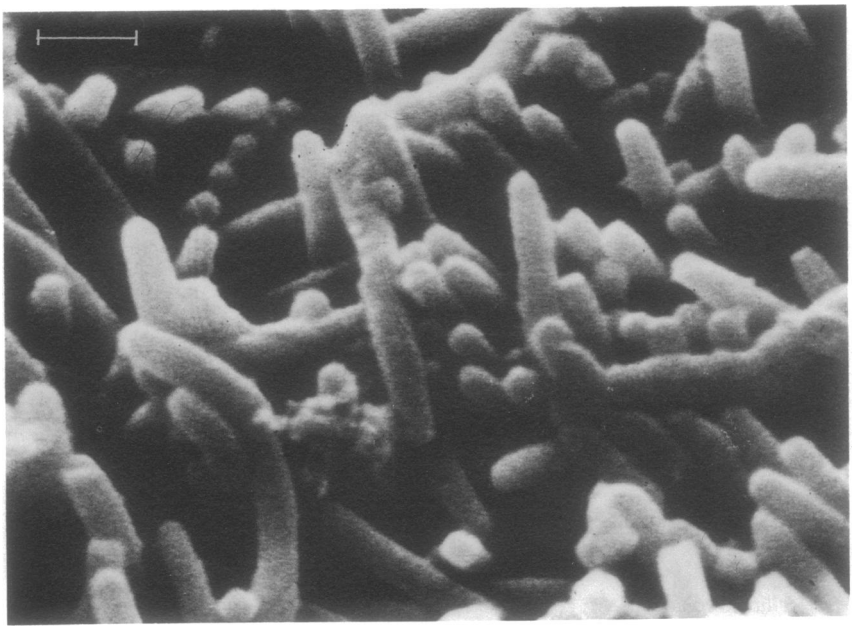

FIG. 3. *Cells at the surface of an RB5 sphere. Shadowed with gold-platinum. Bar represents 1 μm.*

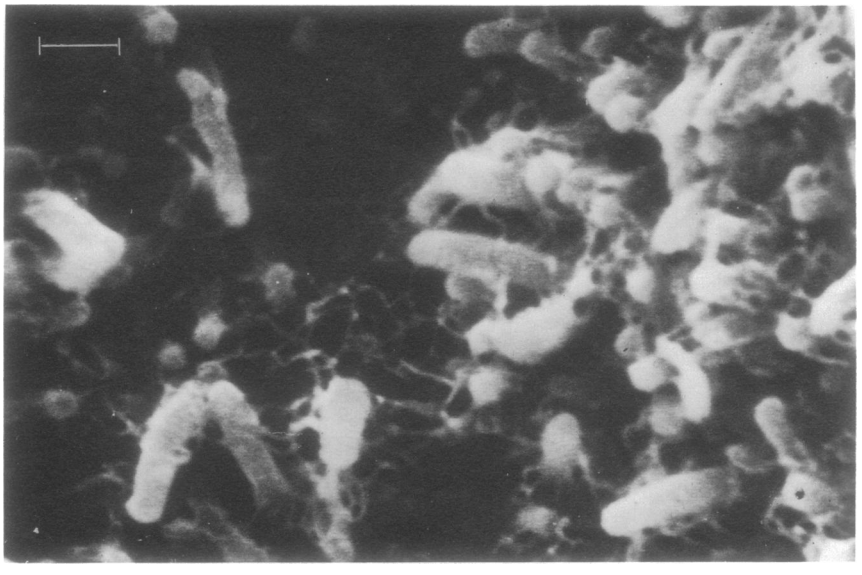

FIG. 4. *Surface of an RB5 sphere. Cells connected by slime filaments are evident. Shadowed with aluminum. Bar represents 1 μm.*

When RB5 spheres are placed on CTE agar, the cells do gradually disaggregate, and a spreading vegetative swarm develops.

To isolate a dispersed growing revertant, enrichment for the few free cells present in RB5 shake cultures was attempted by serial transfer. After 14 daily transfers (approximately 70 generations), the culture appearance was unchanged.

Incubation of strain RB5 on fruiting agar results in the production of very distinct, concentric circles of fruiting bodies (hexenringe). Another nondispersed mutant of *M. xanthus* FB forms fruiting bodies on 1% Casitone agar

(Difco; [14]); RB5 does not. Fruiting bodies are naturally occurring aggregates into which cells glide in response to a presumed chemotactic stimulus (8). In these dense, multicellular structures, cells are induced by an unknown stimulus to form myxospores. To determine whether RB5 spheres can simulate the fruiting body environment, CT-1-grown spheres were transferred to PO_4-Mg or water and were shaken at 30 C. Within 3 to 4 days, myxospores were present in the spheres (Fig. 5); most of the cells eventually differentiated. These myxospores were indistinguishable from those of fruiting bodies by morphological criteria, resistance to ultrasound, and their ability to germinate into viable, vegetative cells. Cell masses making up the rings at the medium-air interface behaved similarly on transfer to PO_4-Mg.

Four nondispersed growing strains of *M. fulvus* tested under the same conditions also demonstrated myxospore formation.

These observations suggest that high cell density itself provides an environment appropriate for cell differentiation under starvation conditions. This hypothesis was also tested by spotting *M. xanthus* NM (1) cells, which had been pelleted at $10^4 \times g$, on fruiting agar. The cells of this nonmotile strain cannot glide away from the inoculation site. Within 1 week myxospores were abundant in these artificially constructed cell masses. When pelleted cells were spotted on a glass surface, myxospore formation did not occur, nor did differentiation occur when dense suspensions of NM cells (5×10^{10} to 10×10^{10} per ml) were shaken in PO_4-Mg ± 0.02% Casitone.

DISCUSSION

The molecular basis of the change in growth behavior characteristic of *M. xanthus* RB5, a rough-colony-forming, nondispersed growing mutant of FB_t, may lie in a qualitative or quantitative change in the undefined, vegetative slime produced by these myxobacteria. The intercellular filaments observed between cells in the macroscopic spheres (Fig. 4) may be composed of this slime.

It is possible that the dispersed growing strains of myxobacteria (5, 10–12) are mutants of the true, nondispersed growing wild types isolated from nature; RB5 may be a revertant. However, the question of dispersed versus nondispersed growth is complicated by intermediate situations. For example, shake cultures of strain FB_t, the parent of RB5, contain mixtures of rosettes and free cells (3).

Under natural conditions, myxospores form within fruiting bodies. They can also be induced by the addition of glycerol and other agents to log-phase cells in culture medium (9,15). This study demonstrates that cell differentiation occurs in both RB5 spheres and NM cell masses in response to starvation and in the absence of exogenous inducers. Myxospore formation in

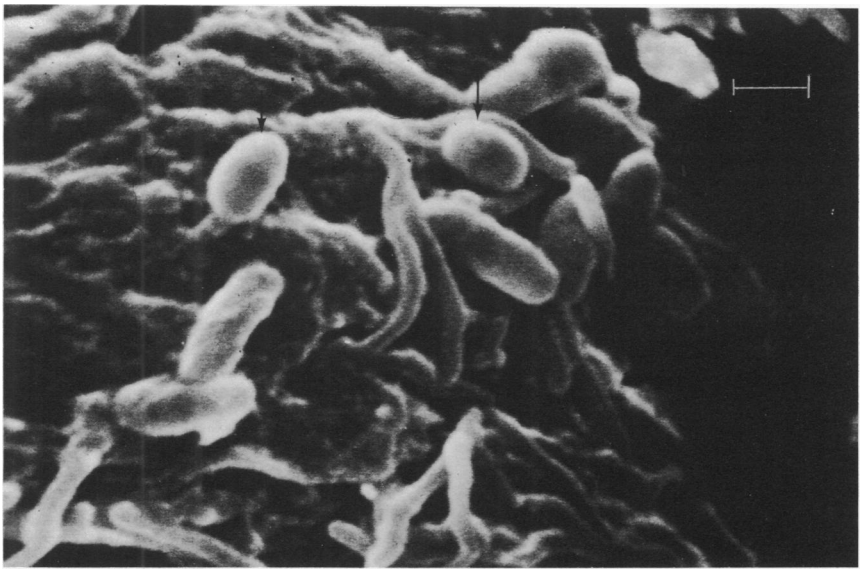

FIG. 5. *Surface of an RB5 sphere after 5 days of incubation in PO_4-Mg. Myxospores (arrows) are intermingled with sinuous, vegetative cells, many of which appear to be embedded in slime. Shadowed with gold-platinum. Bar represents 1 μm.*

these cases is independent of cell aggregation and fruiting body formation. The experiments suggest that differentiation in fruiting bodies and in dense cell masses may be triggered by continuous cell-cell contact under conditions of high cell density and low nutrient levels. The role of cellular interactions in the life cycle of the myxobacteria has been discussed by Dworkin (8).

Dense and easily dissociable cell masses of *M. xanthus* NM might be fruitfully employed in further characterizing the natural mechanism of induction of myxospore formation.

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