

Using a Phase-Locked Mutant of *Myxococcus xanthus* To Study the Role of Phase Variation in Development

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The bacterium *Myxococcus xanthus* undergoes a primitive developmental cycle in response to nutrient deprivation. The cells aggregate to form fruiting bodies in which a portion of the cells differentiate into environmentally resistant myxospores. During the growth portion of the *M. xanthus* life cycle, the organism also undergoes a phase variation, in which cells alternate between yellow and tan colony-forming variants. Phase variation occurs in our laboratory strain (M102, a derivative of DK1622) at a frequency high enough that a single colony of either the yellow or the tan phase already contains cells of the alternate phase. In this study we demonstrate that tan cells within a predominantly yellow population of phase variation-proficient cells are preferentially recovered as heat- and sonication-resistant spores. To further investigate the possibility of a differential role of tan and yellow cells during development, a tan-phase-locked mutant was used to compare the developmental phenotypes of a pure tan population with a predominantly yellow, phase variation-proficient population. Pure tan-phase populations did not produce fruiting bodies or mature spores under conditions in which predominantly yellow wild-type populations did so efficiently. Pure populations of tan-phase cells responded to developmental induction by changing from vegetative rod-shaped cells to round forms but were unable to complete the maturation to heat- and sonication-resistant, refractile spores. The developmental defect of a tan-phase-locked mutant was rescued by the addition of phase variation-proficient cells from a predominantly yellow culture. In such mixtures the tan-phase-locked mutant not only completed the process of forming spores but also was again preferentially represented among the viable spores. These findings suggest the intriguing possibility that the tan-phase cells within the vegetative population entering development are the progenitors of spores and implicate a requirement for yellow-phase cells in spore maturation.

Myxococcus xanthus is a heterotrophic, gram-negative soil bacterium, which exhibits a complex life cycle (4, 5). When induced by nutrient limitation at a critical cell density, *M. xanthus* vegetative cells begin to aggregate into mounds. Between the mounds, the layer of vegetative cells forms uniform patterns of rippling, made from discrete waves of cells (15, 19). The mounds gradually enlarge, forming dense, compact fruiting bodies covered by extracellular material. The rod-shaped vegetative cells inside the fruiting bodies then differentiate into round, dormant, environmentally resistant spores. When the fruiting body encounters favorable nutrient conditions, the spores germinate into vegetative cells and repeat the cycle. In the vegetative state the organism is capable of forming two distinct colony types which differ in pigmentation and colonial morphology, a phenomenon described as phase variation (2). The predominant colony type is yellow pigmented, with a rough, swarming, medusoid appearance. The alternate colony type is tan, unpigmented, with a smooth, mucoid appearance. There is great variability in the frequency of phase variation among the common laboratory strains of *M. xanthus* (6a). For our wild-type developmentally and phase variation-proficient strain (M102, a derivative of DK1622), a freshly isolated yellow colony typically contains 1 to 5% tan variants, while a freshly isolated tan colony contains an average of 25% yellow cells (12).

The majority of work on *M. xanthus* development has been done with yellow-phase variants. These yellow, swarming pop-

ulations formed well-defined spore-filled fruiting bodies, whereas tan, mucoid variants produced more rudimentary aggregates (20). Recall, however, that these are not pure yellow and tan populations but are instead mixtures of the two variants. The possibility that phase variation plays a role in development has been suggested by several observations. Colonies resulting from germinated spores produced by wild-type predominantly yellow-phase cells are enriched for tan-phase variants compared with the population which entered development (6a, 11). Janssen and Dworkin showed that for certain sporulation mutants (*spoC*), the efficiency of extracellular complementation was greatly enhanced when the *spoC* population included a few percent tan-phase variants (7). Furthermore, Mueller and Dworkin (13) reported that glucosamine-induced sporulation requires the presence of tan cells and that tan cells were more resistant to glucosamine-induced lysis than yellow cells.

A thorough investigation of the role that phase variation may play in development has been hindered by the lack of phase-locked mutants. Such mutants could provide a pure population of cells in either the tan or yellow phase so that comparisons between their respective developmental behaviors can be made. We recently described a phase-locked mutant, M678, which is very stable in the tan phase (11, 12). This strain can be propagated as essentially pure tan-phase variants and so affords us the unique opportunity to determine the developmental phenotype of a tan population in the absence of yellow variants. Although this strain is quite stable in the tan phase, rare yellow variants do arise (12). Surprisingly, these yellow variants undergo phase variation from yellow to tan at a nearly wild-type frequency. The second-generation tan variants so formed are again stable and phase locked, indicating that the

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TABLE 1. *M. xanthus* strains used in this study

Strain	Description	Relevant phenotype(s)	Source or reference
M102	Single colony isolate of DK1622	Standard wild-type laboratory strain; fully motile, Fru ⁺ Spo ⁺ Var ⁺	Dale Kaiser (5)
M226	M102::Tn5ΩDK2466. Tn5ΩDK2466 is near the <i>bsgA</i> locus but has no effect on development or frequency of phase variation	Km ^r Fru ⁺ Spo ⁺ Var ⁺	This lab
M226-Tc	M102::Tn5-132ΩDK2466. Contains Tn5-132 encoding Tc ^r at the same chromosomal site as M226	Tc ^r (Km ^s) Fru ⁺ Spo ⁺ Var ⁺	This lab
M678	<i>var-683</i> mutation in M102-derived genetic background; tan phase	Var ⁻ , tan phase. Phase variation defect in rate of switching from tan phase to yellow phase; tan variant is stable. Km ^r expressed in yellow phase	12
M678-yv	Yellow phase variant of M678, <i>var-683</i>	Retains tan-to-yellow switching defect, but yellow variant undergoes yellow- to tan-phase variation at near normal frequency. Km ^r expressed in yellow phase	12
M683	M678 which has spontaneously lost the integrated plasmid pREG1541. Km ^s in both phases. <i>var-683</i>	Retains all phase variation properties of M678 Km ^s	12
M697	M683 containing pREG1666 integrated at Mx8 attachment site	Km ^r Var ⁻ , tan phase	11, 12, this work
M1040	M683 containing Tn5 at an uncharacterized location in chromosome	Km ^r , Var ⁻ , tan phase; retains all phase variation properties of M678	8, 9, this work
M632	M102::Tn5Ω620. Tn5Ω620 is at an uncharacterized location but has no effect on development or frequency of phase variation	Km ^r Fru ⁺ Spo ⁺ Var ⁺	This work
M632-Tc	M102::Tn5-132Ω620. Contains Tn5-132 encoding Tc ^r at the same chromosomal site as M632.	Tc ^r (Km ^s) Fru ⁺ Spo ⁺ Var ⁺	This work

rare yellow variants retain the *var-683* mutation and are not simply phase variation-proficient revertants. The consequence of this phenotype is that M678 can be used to grow populations of pure tan-phase cells, but its yellow variants grow as mixtures of yellow and tan cells as does the wild-type parent strain.

There were two primary goals of this study. The first goal was to quantitate any bias in the recovery of tan-phase variants as viable spores produced from predominantly yellow, phase variation-proficient, wild-type cells. Such a finding would help to substantiate a role for phase variation in development. The second objective was to use the tan-phase-locked mutant to produce uniform populations of tan cells and to compare the developmental properties of these pure tan cells with those of predominantly yellow, phase variation-proficient, wild-type cells. Our results are indeed consistent with a bias in the production or germination of spores, such that cells entering development in the tan phase are preferentially found among the viable spores. Furthermore, we found that tan-phase cells alone were extremely inefficient at fruiting body formation and were unable to form heat- and sonication-resistant spores. Instead, the tan-phase cells formed only phase-dark, rounded cells in response to starvation-induced development. This developmental defect could be rescued by the addition of cells from a wild-type culture containing predominantly yellow cells. Surprisingly, the spores produced from these mixtures were derived predominantly from the tan-phase-locked parent.

MATERIALS AND METHODS

Bacteria and media. All strains used in this study (summarized in Table 1)

were derived from *M. xanthus* M102, our single colony isolate of DK1622 (8). M102 is a fully motile, developmentally proficient strain, capable of undergoing phase variation (Var⁺) between the yellow swarming colony type and the tan mucoid colony type. In Var⁺ strains derived from M102, phase variation occurs at a frequency high enough that a single colony of either the tan or yellow phase already contains cells of the alternate phase. Exponentially growing cultures derived from fresh yellow colonies typically contain 1 to 5% tan variants; those derived from tan colonies typically contain 20 to 30% yellow variants (12). Although there are considerable differences in the frequency of phase variation among common laboratory strains of *M. xanthus* (6a), the phase variation and developmental properties of M102 are taken to be wild type in this report.

M678 is a tan-phase-locked mutant containing the mutation designated *var-683* (12). This mutation results in a defect in the rate of switching from the tan-to-yellow phase, resulting in an extremely stable tan phase population. Rare yellow variants of *var-683* strains do arise; M678-yv is such a yellow-phase variant of the mutant M678 used in this study. The rate of yellow-to-tan switching of yellow variants of M678, including M678-yv, is nearly that of the wild-type. Therefore, M678-yv grows as mixtures of yellow and tan cells. The second-generation tan isolates of M678-yv are again phase locked and quite stable, verifying the presence of the *var-683* mutation in this strain, rather than being simply a Var⁺ revertant.

M683 is a derivative of M678 from which the integrated plasmid pREG1541 had spontaneously been lost, such that the strain no longer expressed Km^r in the yellow phase (12). Southern analysis indicated that the plasmid sequences had been excised precisely from the chromosome, leaving the locus intact (11). M683 retains the *var-683* mutation and associated phase-locked phenotype and is otherwise phenotypically indistinguishable from its parental strain. M226-Tc and M632-Tc are Tc^r Km^s strains, which are isogenic with M226 and M632, respectively, but contain Tn5-132 at the same locations as the Tn5 present in M226 and M632, respectively. They were constructed by using P1::Tn5-132 as previously described (1).

The bacteria were grown vegetatively in CTT medium containing 1% Casitone, 10 mM Tris-HCl (pH 7.6), 8 mM MgSO₄, and 1 mM potassium phosphate (pH 7.6) (2) at 32°C with vigorous aeration. For the solid medium, 1.2% Acumedia agar was added. When appropriate, kanamycin was added to a final concentration of 50 µg/ml.

Plating analysis. To determine the percentage of yellow- and tan-phase cells

in a given population, a sample was removed from the culture, diluted, and plated in an overlay of soft agar on solid medium. After the plates were incubated for 4 to 5 days, the number of tan and yellow colonies were counted.

Developmental assays. Development of fruiting bodies on solid medium was induced by spotting 10 μ l of a suspension of washed, logarithmically growing cells on TPM (10 mM Tris-HCl [pH 7.6], 8 mM MgSO₄, 1 mM potassium phosphate [pH 7.6]) agar containing 0.2% sodium citrate and 0.1% sodium pyruvate. Plates were incubated at 30°C and harvested at the times specified in the text.

The submerged culture assay (9) was performed by adding 15 ml of cells at 2.5×10^7 cells per ml in CTT broth into a plastic petri dish and incubating the mixture at 30°C overnight. The medium was then removed from the adherent cells by aspiration and replaced with developmental medium (TPM containing 0.1 mM CaCl₂ and 0.1% sodium pyruvate), and the cells were incubated at 30°C without shaking.

For the liquid shake culture assay, exponentially growing cells were washed in 10 mM MOPS (morpholinepropanesulfonic acid) and resuspended in MCM medium (10 mM MOPS, 2 mM CaCl₂, 4 mM MgSO₄ [pH 7.2]) at a concentration of 5×10^9 cells per ml (16). Cultures were incubated at 32°C for 3 to 6 days, with shaking.

For glycerol induction of spore formation, cells were grown vegetatively in CTT to a density of 5×10^8 cells per ml. Glycerol was added to a final concentration of 0.5 M, and cells were shaken at 32°C for 24 h (3).

For each of the above assays, cells were harvested at the times indicated in the text. The number of viable spores was determined by heating the samples at 57°C for 15 min, by sonicating them 1.5 min to kill vegetative cells and to disperse aggregated spores, and then by plating appropriate dilutions on CTT agar. Plates were incubated for 5 days, and the number of CFU was determined (10).

Cell cohesion assay. Cell cohesion was assayed by the method of Shimkets (17). Cells were grown to a concentration of 5×10^8 cells per ml in CTT medium. The cells were harvested by centrifugation at $10,000 \times g$ for 5 min at 4°C, washed once in 10 mM MOPS, and resuspended in 10 mM MOPS (pH 6.8)–1 mM MgCl₂–1 mM CaCl₂. The cell suspensions were incubated in tubes at 32°C without shaking, and the turbidity was measured at 10-min intervals.

RESULTS

Identification of parentage of viable spores produced by wild-type cells. There have been numerous anecdotal observations made by our lab and others that the viable spores arising from strain DK1622 and other strains of this lineage are enriched for tan-phase variations, compared with the composition of the input population. This observation may reflect a developmentally induced phase switch or a phase bias in either the formation or germination of spores.

To determine whether a phase bias does indeed exist in the formation of germination of viable spores, we performed a mixing experiment which allowed us to separately monitor the developmental fate of preexisting yellow- and tan-phase cells within the mixed, predominantly yellow-phase population. For these experiments we used the genetically marked but otherwise isogenic developmentally and phase variation-proficient strains M226 and M226-Tc. These strains are resistant to kanamycin and tetracycline, respectively. Yellow- and tan-phase cultures of the two strains were mixed so that the yellow-phase parent composed 95% of the mixture; the tan-phase parent composed the remaining 5%. Droplets (10 μ l) containing 5×10^9 cells per ml were applied to TPM–2% agar and incubated at 30°C to induce development. After 5 days the fruiting bodies were harvested and sonicated to disperse the spores and kill any remaining vegetative cells. The spores were then diluted and plated on CTT agar to allow germination, and the resulting colonies were transferred to CTT containing kanamycin or tetracycline to determine the parentage of spores. Colonies growing on both media (<0.2%) were presumed to have arisen from a clump of spores and were not considered.

In the two experiments for which the results are shown in Table 2, and 18% of the total input cells, respectively, were accounted for as spores. Among the germinating spores, approximately 37 and 35%, respectively, were derived from the subpopulation of predominantly tan-phase cells, even though this tan-phase parent accounted for only 5% of the input cells.

TABLE 2. Parentage of spores produced from mixtures of yellow- and tan-phase wild-type cells

Experiment	Yellow-phase parent ^a	Tan-phase parent ^b	% of spores from tan parent ^c
A	M226-Km	M226-Tc	36.8
B	M226-Tc	M226-Km	35.2

^a In each experiment, the yellow-phase parent constituted 95% of the cells in the mixture.

^b In each experiment, the tan-phase parent constituted 5% of the cells in the mixture.

^c Reported values are the averages of two determinations.

This sevenfold enrichment corresponds to nearly quantitative recovery of the tan-phase parent as viable spores.

The enrichment for tan-phase cells among the viable spores cannot be accounted for by differences in the genotypes of the parental strains. Each parent strain has a Tn5 insertion at the same chromosomal location, and this Tn5 has not been observed to affect the growth or developmental proficiency of the strain. Furthermore, the experiments were done in a reciprocal fashion; in experiment A, the tan strain was the tetracycline-resistant strain M226-Tc, while in experiment B the tan strain was the kanamycin-resistant strain M226-Km.

These data are consistent with the qualitative observations that have been made for a number of strains from the DK1622 lineage and suggest a sporulation mechanism in which there is indeed a phase bias in the parentage of the cells recovered as viable spores. This bias appears to be based on the phase of the individual cells entering development, such that individual cells which enter development in the tan-phase are preferentially recovered as viable spores.

Fruiting body defect in tan-phase-locked mutant. The increased representation of tan input cells among the viable spores has led us to speculate whether this bias reflects different roles for yellow- and tan-phase cells in development and, therefore, a requirement for cells of each phase for normal fruiting body and spore formation. If this is true, then one might expect that pure populations of either yellow- or tan-phase cells will exhibit a developmental defect. We have not been able to address this question directly because phase variation occurs at a frequency high enough that a single colony of either the tan- or yellow-phase already contains cells of the alternate phase, making it impossible to grow and test the developmental phenotypes of pure populations.

We recently reported the isolation of a phase-locked mutant (M678) which is very stable in the tan phase (12). Very rare yellow-phase variants do arise, and in its yellow phase, this mutant undergoes phase variation back to the tan state at nearly a wild-type frequency. The second-generation tan variants so formed are again phase locked in the tan phase. A corresponding mutant locked in the yellow phase has not been isolated. Nevertheless, using this tan-phase-locked mutant, we are now able to reproducibly grow cultures of pure populations of tan-phase cells and to determine their developmental phenotype in the absence of yellow variants.

In the following sections of this report, we compare the developmental behavior of the tan-phase-locked mutant with wild-type phase variation-proficient cells and with a yellow variant of the phase-locked mutant strain. The wild-type strain used in these experiments, M102, was grown from fresh yellow colonies. Cultures contained predominantly yellow-phase variants, but because the strain is phase variation proficient, 1 to 5% of the population were tan-phase variants. The latter strain, M678-yv, contained the *var-683* mutation but gave rise

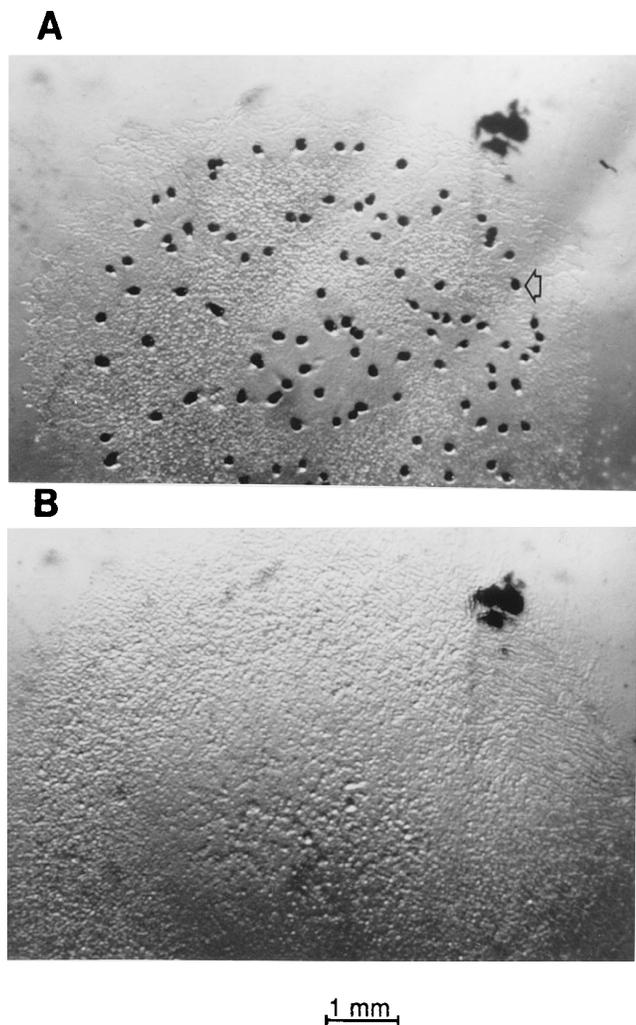


FIG. 1. Light microscopy of formation of fruiting bodies on solid medium. (A) Cells from a yellow-phase M102 culture formed dense fruiting bodies (arrow). (B) Cells from the tan-phase-locked mutant M678 culture failed to form fruiting bodies.

to cultures which were a mixture of yellow and tan variants. This strain was used to assess whether the *var-683* mutation (or an additional cryptic mutation in M678) affects the developmental phenotype of the tan-phase-locked strain in a way other than that which can be accounted for by its inability to undergo phase variation.

The ability of the tan-phase-locked mutant to form fruiting bodies was assessed in TPM starvation agar (Fig. 1) and in submerged culture (Fig. 2). On TPM agar, the yellow-phase wild-type cells swarmed into aggregation foci, forming dense, uniform fruiting bodies (Fig. 1A). These are seen as the black spots in the photograph. The fruiting bodies were filled with refractile spores, visible by phase microscopy. The few surviving vegetative cells formed swirling patterns between the fruiting bodies. Wild-type yellow-phase populations also displayed the distinct rippling patterns characteristic of *M. xanthus* (15, 19). In sharp contrast to the wild-type yellow strain, the tan-phase-locked mutant, M678, failed to form distinct fruiting bodies (Fig. 1B). Microscopic examination showed that M678 formed a mat of cells which lacked a distinct pattern or rippling

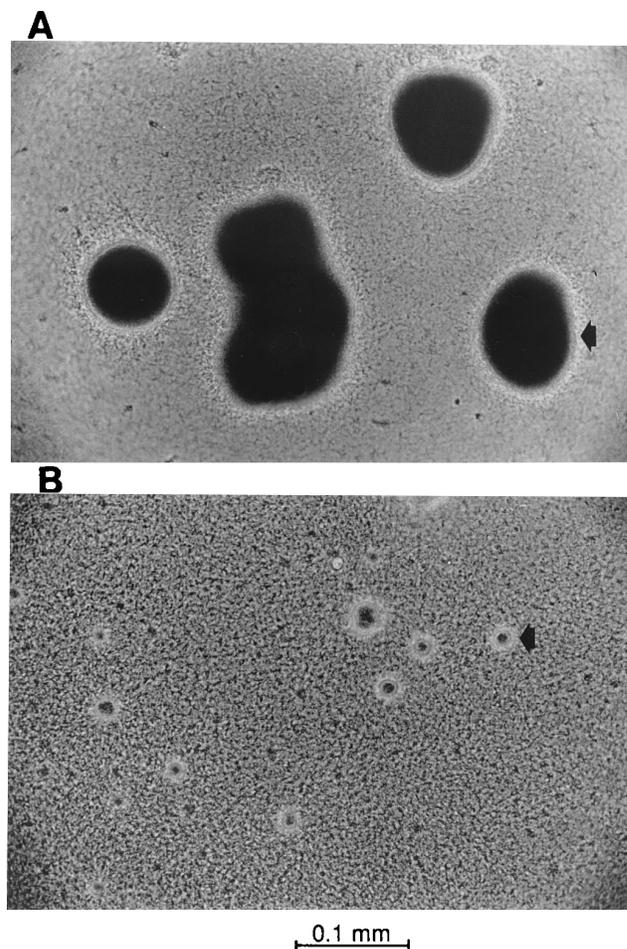


FIG. 2. Submerged culture developmental assay. (A) The yellow-phase, Var^+ M102 culture formed well-defined fruiting bodies (arrow). (B) The tan-phase-locked mutant M678 culture made only very small, rudimentary fruiting bodies (arrow).

behavior. Virtually all the cells became rounded, but no refractile spores were seen.

It is important to point out that, M678-yv, the yellow variant of the stable tan-phase-locked mutant M678, made spore-filled fruiting bodies which were indistinguishable from those of wild-type M102 (data not shown). This comparison is important, since it suggests that the inability of the tan strain to form fruiting bodies is likely due to the composition of the population and not to a consequence of a direct effect of the mutation on development or of a secondary mutation in the strain.

Fruiting body formation was also assessed in submerged culture (9). In this assay, cells adhere to the plastic surface of the petri dish and develop under a layer of starvation buffer. Under these conditions the wild-type cells aggregate into foci forming large, dense fruiting bodies. The very few vegetative cells remaining adhered to the plastic surface surrounding the fruiting bodies (Fig. 2A). The tan-phase-locked mutant formed only rare, very small, loose fruiting bodies. The remainder of the cells became rounded and adhered to the plastic surface (Fig. 2B). As noted above, this defect is apparently not a general property of the mutant strain. M678-yv, the yellow variant of M678, was indistinguishable from wild-type cells in this assay (data not shown).

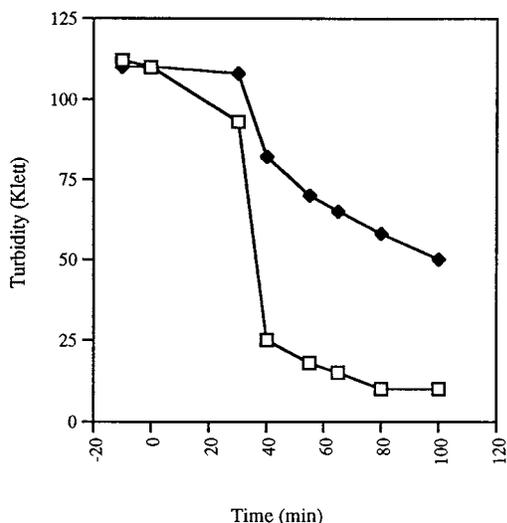


FIG. 3. Cohesion assay of yellow-phase Var⁺ (□) and tan-phase-locked (◆) strains. Turbidity was measured at 10-min intervals. The -10-min time point indicates the concentration of the vegetative culture prior to the initiation of the experiment. The yellow-phase Var⁺ M102 culture consisted of approximately 99% yellow-phase cells; the tan phase-locked mutant M678 contained 0.2% yellow cells. These results represent the average values for two experiments.

Cohesive properties of tan-phase-locked cells. Cell-to-cell cohesion is an energy-requiring property of *M. xanthus* which is associated with, and perhaps necessary for, fruiting body formation (13, 14). We next determined whether the fruiting body formation defect observed for the tan-phase-locked M678 strain was correlated with changes in its cohesive properties. The simple cohesion assay described by Shimkets (17, 18) was used: the vegetatively grown cells were washed, resuspended in a buffer containing Mg²⁺ and Ca²⁺, and allowed to clump in the test tube. A decrease in turbidity of the suspension indicates that the cells are undergoing cohesion. The results of cohesion assays using the tan-phase-locked mutant M678 and predominantly yellow wild-type cells are shown in Fig. 3. The wild-type yellow-phase cells cohered very efficiently and formed large, nondispersible strands of cells. The cohesiveness of the tan-phase culture, however, was greatly reduced. The portion of tan cells that settled out of the suspension was easily resuspended and had a cloudy, granular appearance. This lack of cohesiveness may contribute to the inability of tan-phase cells to form fruiting bodies.

Sporulation defect of tan-phase-locked mutant. The ability of the tan-phase-locked strain M678 to form myxospores upon starvation was assessed in the submerged culture assay. Exponentially growing cultures of M678, M102, and M678-yv were each placed in submerged culture and induced for development. After 3 days of incubation under starvation conditions, the cell mat was harvested, examined by phase microscopy for the presence of refractile spores, and tested for the presence of heat- and sonication-resistant spores. Figure 4A is a photograph of refractile spores formed in submerged culture by wild-type strain M102. These spores were also resistant to heat and sonication (data not shown). The cell mat harvested from submerged culture of M678 did not contain refractile spores, nor were the cells heat and sonication resistant. Instead, they became dark, rounded forms (Fig. 4B). These structures were distinctly different from both vegetative cells (Fig. 4C) and refractile spores (Fig. 4A) but appeared identical to the M678 cells harvested from starvation agar (data not shown). An

important control for the interpretation of the behavior of M678 is to consider the developmental behavior of its yellow variant, M678-yv. Under the conditions of this assay, M678-yv produced refractile, heat- and sonication-resistant spores, which appeared identical to those of the wild-type strain (data not shown). Since M678-yv still contains the *var-683* mutation but grows as a mixture of yellow and tan variants, these results strongly suggest that the developmental defect observed for M678 is due to the composition of the population rather than the *var-683* mutation or a secondary mutation in the strain.

If fruiting bodies are required for spore production, tan-phase cells may not have made spores simply because they were unable to form fruiting bodies. The liquid shake culture assay is a method of assaying for spore production in response to starvation but in the absence of fruiting bodies (16). Therefore, we next tested whether the tan-phase-locked mutant could sporulate under these conditions. Exponentially growing cultures of M678 and a predominantly yellow culture of wild-type M102 were induced in liquid developmental medium and tested for the production of heat- and sonication-resistant spores at daily intervals. Strain M102 yielded 8×10^3 viable spores per ml by day 2 and 1×10^6 spores per ml by day 3. Spores were not detected in the tan-phase-locked culture even after 4 days postinduction. However, instead of rod-shaped vegetative cells, the tan-phase-locked mutant appeared as dark, rounded structures similar to those found on solid starvation medium or in submerged culture. Consistent with the results of the cell cohesion assay, M102 formed macroscopic clumps, which settled out of suspension, accompanied by a decrease in turbidity. In contrast, M678 failed to form clumps and instead retained a cloudy, granular appearance. As was observed in each of the previous assays, M678-yv, the yellow variant of the phase-locked mutant, behaved the same as the wild-type M102 strain (data not shown).

It has previously been reported that spores, although morphologically distinct from myxospores which form within fruiting bodies, can be induced by the addition of glycerol to vegetatively growing cultures (3). Formation of glycerol spores does not require the normal, complete pathway of signaling steps followed in starvation induction. To determine whether the tan-phase-locked mutant was able to complete this form of cellular morphogenesis, strains M678 and M102 were induced with 0.5 M glycerol and assayed after 24 h for production of heat- and sonication-resistant spores. Unlike starvation-induced sporulation, both the tan-phase-locked mutant and wild-type strains formed glycerol spores and did so at nearly equivalent efficiencies. Glycerol spores produced by the two strains appeared identical by phase microscopy.

Restoration of myxospore production by tan phase-locked mutant by extracellular complementation with yellow-phase cells. The results of the experiments reported in the above sections indicate that tan-phase input cells are preferentially recovered as viable spores from mixed populations of yellow- and tan-phase wild-type cells. However, the tan-phase-locked mutant, which grows as pure populations of tan cells, is unable to produce fruiting bodies and viable spores in response to starvation. These developmental defects were not a direct consequence the *var-683* mutation or of an additional cryptic mutation in the strain, since the yellow variant of the phase-locked mutant was developmentally proficient. Rather, it appears that the composition of the population is the critical feature, and it seems possible that formation of viable spores by tan-phase cells requires the presence of yellow cells in the population. Therefore, the next question asked was whether the block in the development of the tan phase-locked strain could be overcome by the presence of wild-type, predominantly yellow cells.

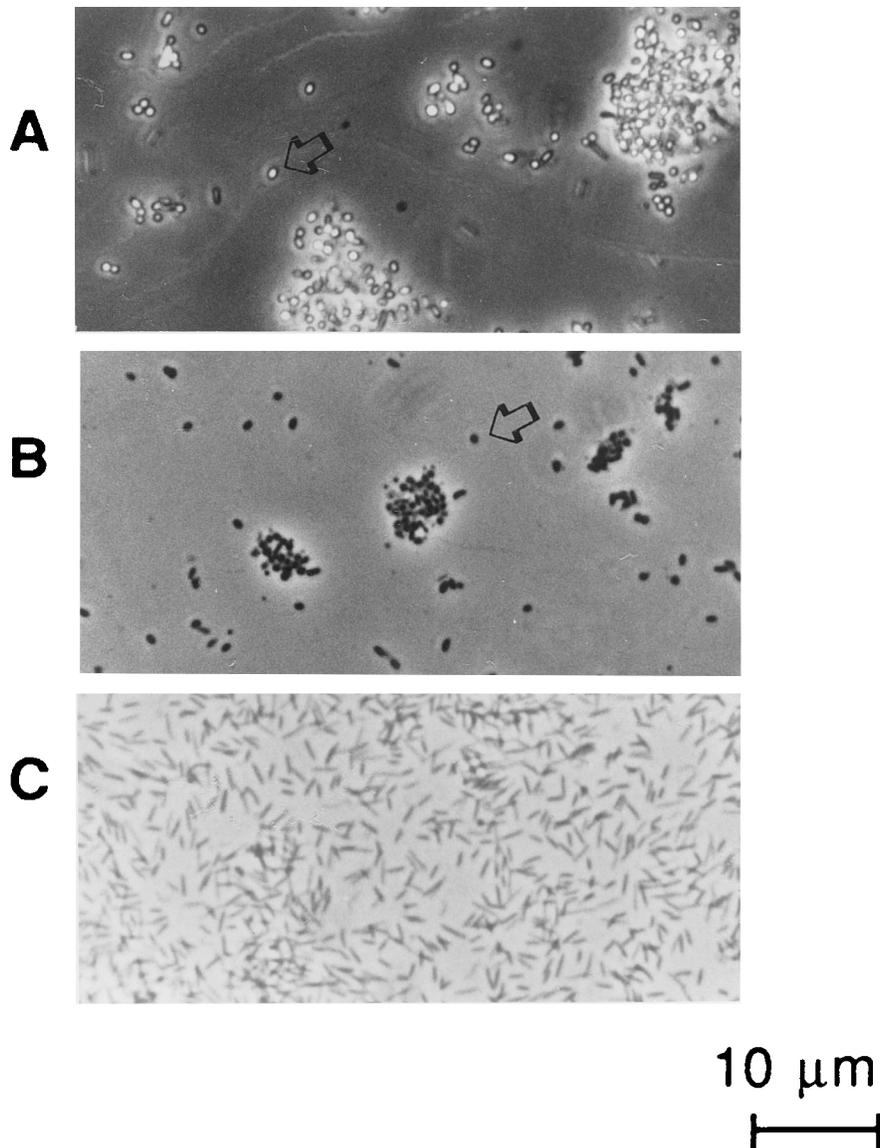


FIG. 4. Morphology of cells during submerged culture development observed by phase microscopy. (A) Yellow-phase Var^+ M102 cells. Refractile spores are indicated by an arrow. (B) Tan-phase-locked M678 cells. Phase-dark, rounded cells are shown by an arrow. (C) Rod-shaped, exponentially growing cells prior to developmental induction.

To address this question, a kanamycin-resistant derivative of the tan-phase-locked mutant (either M1040 or M697) was mixed at various ratios with a tetracycline-resistant derivative of M102 (M632), and the mixture was induced to develop in submerged culture at a constant total cell density. Since the two strains in the mixtures were differentially marked, the parentage of the viable heat- and sonication-resistant spores could be determined on the basis of their respective antibiotic resistance. The compiled results of several such experiments are shown in Table 3. Consistent with the previous results, the tan-phase-locked mutant alone produced no detectable heat- and sonication-resistant spores (Table 3, line 1). However, the ability of the tan-phase parent to produce mature spores was dramatically restored by the incubation of the tan-phase cells with yellow-phase cells at a ratio of 1:1 or greater (Table 3, lines 4 to 7). Furthermore, not only was the ability of the tan-phase-locked mutant to sporulate rescued by the presence

TABLE 3. Rescue of sporulation of tan-phase-locked mutant by wild-type cells

Composition of original mixture (% of total cells)		No. of viable spores/ml at:		% of viable spores from tan <i>var-683</i> mutant
M1040 (<i>var-683</i>)	M632-Tc (<i>var</i> ⁺)	Day 3	Day 4	
100	0	ND ^a	— ^b	
93	7	ND	—	
87	13	ND	—	
50 ^c	50	ND	5×10^4	78
13	87	2×10^5	—	97
7	93	1×10^4	—	75
0	100	2×10^4	1×10^6	NA ^d

^a ND, none detected.

^b —, not determined.

^c M697 used as the Km^r *var-683* parent in this experiment.

^d NA, not applicable.

of wild-type cells, but also the vast majority of the viable spores which were detected were derived from the phase-locked mutant. The optimal ratio of parental cells for the formation of viable spores was 13:2 (wild type to mutant; Table 3, line 5). At this ratio, 97% of the viable spores were derived from the tan-phase-locked parent, even though this strain constituted only 13% of the total input cells. These data indicate that the sporulation defect of the tan-phase-locked mutant can be rescued by the presence of wild-type cells in the developing population and support the idea that there is a requisite interaction between tan- and yellow-phase variants during sporulation.

DISCUSSION

In this study, we report the results of our investigation of the possible role of phase variation in the developmental process of *M. xanthus*. We found that in our standard laboratory strain (M102, a derivative of DK1622), which is developmentally and phase variation-proficient, tan-phase variants within the population entering development were overrepresented among the viable myxospores which were recovered (Table 2). These experiments used mixtures of differentially marked tan and yellow variants to determine the parentage of the resulting myxospores. The preferential recovery of viable spores derived from the tan input parent cannot be explained on the basis of genetic differences between the two strains. The yellow and the tan parents each had the same Tn5 insertion and differed only by the antibiotic resistance encoded by Tn5. Furthermore, since the experiments were done in a reciprocal fashion, with similar results, it is unlikely that the antibiotic resistance element itself skewed the results in any way.

There are alternative explanations for the results shown in Table 2. One possibility is that spores derived from tan cells simply germinate more efficiently than spores derived from yellow cells. Unfortunately, there is currently no way to separate or differentiate between spores that may have been derived from tan or yellow cells; so, we cannot test this possibility. However, the conclusions drawn from this experiment are based on the recovery of viable spores, which was intended to include this particular contingency. An alternative explanation for the results in Table 2 is that spores derived from tan cells mature more quickly than the corresponding spores derived from yellow cells. Since spores were sampled in these experiments at only a single, relatively early time point, the data presented cannot directly address this possibility. The time at which spores were harvested was chosen to minimize other artifacts that we have observed. For example, during later times after starvation, it becomes progressively more difficult to distinguish between the first and subsequent rounds of sporulation in which growth is a confounding issue since tan and yellow variants do not grow at the same rate under low-nutrient conditions. The data presented are nevertheless consistent with a phase bias in the cells which appear as viable spores, and this bias is based on the phase of the cell as it enters development, particularly when these data are considered together with the data for mutant strains discussed below. Differences in spore germination efficiency or spore maturation rates cannot satisfactorily account for all of the data presented.

One particularly intriguing possibility is that the data in Table 2 reflects different roles for yellow- and tan-phase cells in development and perhaps a requirement for cells of each phase for normal fruiting body and spore formation. If this hypothesis is true, then one might expect that pure populations

of either yellow- or tan-phase cells will exhibit a developmental defect.

We found that the tan-phase-locked mutant M678 showed a behavior quite distinct from that of the predominantly yellow-phase populations of our Var⁺ strain. The yellow Var⁺ strain efficiently formed discrete fruiting bodies under starvation conditions on agar and in submerged culture. These fruiting bodies contained refractile spores which were heat and sonication resistant. On the solid surface surrounding the fruiting bodies, the remaining vegetative cells displayed the swarming and distinctive rippling patterns which are characteristic of *M. xanthus* (15). In liquid-shaking developmental culture, yellow cultures formed macroscopic clumps, which may represent the equivalent of fruiting bodies and which also contained refractile spores. In contrast, the tan-phase-locked cells were unable to make either well-developed fruiting bodies or refractile, heat- and sonication-resistant spores when starved on solid medium or in submerged culture. Instead, the cells became phase-dark, round forms that were viable but not optically refractile or heat and sonication resistant. In liquid starvation medium, tan-phase cells did not form mature spores or macroscopic clumps but did become phase-dark, round forms similar to those observed on solid medium.

We believe that this is a valid and significant comparison. We recognize that the *var-683* mutation has not been fully characterized and that use of incompletely characterized mutations may lead to misinterpretations. However, the defect in the tan-phase-locked mutant does not appear to be simply a developmental mutation. In this respect it is important to recall that the tan-phase-locked mutant does give rise to rare yellow-phase variants. These yellow variants retained the *var-683* mutation, in that their second-generation tan variants are again phase locked. However, the yellow *var-683* strains have a frequency of variation back to the tan phase close to that of the wild type and as such grow as mixtures of predominantly yellow cells and a smaller proportion of tan cells. The developmental phenotype of the yellow *var-683* variants was identical to that of yellow wild-type cells. These strains made well-formed fruiting bodies which contained refractile, heat- and sonication-resistant spores. Taken together, these data argue strongly that the developmental defect in the tan-phase-locked mutant is neither a general property of the mutant strain nor a consequence of a secondary mutation but is likely a reflection of the composition of the population. The data support the interpretation that pure populations of tan-phase cells are unable to complete starvation-induced cellular morphogenesis to become heat- and sonication-resistant spores. This is true even in light of the observation that in mixed yellow and tan populations of Var⁺ cells, the tan cells are preferentially represented among the viable spores.

The data presented in this report also indicate that the developmental defect observed with tan-phase-locked cells can be rescued by the presence of predominantly yellow Var⁺ cells in a mixed population. As the proportion of Var⁺ cells added to the phase-locked tan culture was increased, the total number of spores also increased. The maximum production of spores occurred when yellow-phase Var⁺ cells constituted approximately 87% of the initial population. As was observed for Var⁺ cells, again the tan-phase cells were preferentially represented among the viable spores.

It is intriguing to consider the possibility that tan-phase cells are the progenitors, perhaps the only progenitors, of mature, viable spores and that the phase-dark rounded forms made upon starvation by the tan-phase-locked cells are prespores, an intermediate between vegetative cells and fully mature spores. The existence of prespores was suggested by O'Conner and

Zusman (14), who observed similar round cell forms, although a detailed comparison has not been made. The prespores that they described were seen by scanning electron microscopy by slicing through a normal fruiting body during the early stages of morphogenesis. The prespores appeared in less than 24-h fruiting bodies but gradually became heat- and sonication-resistant spores in 3 to 5 days. By this scenario, completion of sporulation by the tan-phase-locked cells absolutely requires the presence of Var⁺ cells and implicates a specific role for the yellow cells within the population. The exact nature of the requirement of Var⁺ cells can only be speculated at this time. It may be at the level of cell-to-cell interactions, resulting in a deficiency of signaling among cells of a pure tan-phase population. Alternatively, the tan-phase cells may be incapable of synthesizing all the components, such as spore coat proteins, necessary for production of viable resistant spores.

Validation of this hypothesis will require the isolation of a mutant which is phase locked in the yellow phase. If our hypothesis is correct, we expect that such a yellow-phase-locked mutant will also be unable to form spores but that the mixture of the two phase-locked strains will sporulate and all of the spores will result from the tan-phase-locked parent. We also expect that expression of certain developmental genes will be phase specific and will be indicative of the existence of two specialized populations of cells.

Organisms such as *M. xanthus* survive as a social community and exhibit cooperative behavior. We seem to have identified the need of one cell type for another. This may provide an explanation for why *M. xanthus* maintains the capability of undergoing phase variation. The phase variation mechanism during vegetative growth would guarantee that, at any given time, cells in both phases were available. The existence of two cell types within a population, each providing a separate function to assist the other is an unusual, but not unique, property among prokaryotes. It is similar to the developmental process in *Bacillus subtilis*, in which the mother cell and the forespore serve separate functions, each of which is required for spore maturation (6). In *M. xanthus*, the tan and yellow variants may be the functional equivalents of *B. subtilis* forespore and mother cell, respectively. In *M. xanthus*, the two cell types may not require the physical attachment found in *B. subtilis* because of their tight packing within the fruiting body. Also, in contrast to *B. subtilis*, the *M. xanthus* cell types coexist during the vegetative cycle, and both are capable of reproduction.

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