Multicellular development in *Myxococcus xanthus* is stimulated by predator-prey interactions

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ABSTRACT

*Myxococcus xanthus* is a predatory bacterium that exhibits complex social behavior. The most pronounced behavior is the aggregation of cells into raised fruiting body structures in which cells differentiate into stress-resistant spores. In the laboratory, monocultures of *M. xanthus* at very high density will reproducibly induce hundreds of randomly localized fruiting bodies when exposed to low nutrient availability and a solid surface. In this report, we analyze how *M. xanthus* fruiting body development proceeds in a co-culture with suitable prey. Our analysis indicates that when prey bacteria are provided as a nutrient source, fruiting body aggregation is more organized, such that fruiting bodies form specifically after a step-down or loss of prey availability, whereas a step-up in prey availability inhibits fruiting body formation. This localization of aggregates occurs independently of the basal nutrient levels tested, indicating that starvation is not required for this process. Analysis of early developmental signaling *relA* and *asgD* mutants indicates that they are capable of forming fruiting body aggregates in the presence of prey, demonstrating that the stringent response and A-signal production are surprisingly not required for initiation of fruiting behavior. However, these strains are still defective in differentiating to spores. We conclude that fruiting body formation does not occur exclusively in response to starvation and propose an alternative model in which multicellular development is driven by the interactions between *M. xanthus* cells and their cognate prey.
INTRODUCTION

Fruiting bodies formed by myxobacteria species, such as *Myxococcus xanthus*, were one of the first examples of social, multicellular behavior in bacteria (16, 27). Fruiting bodies are macroscopic structures that can be as large as a few millimeters in size and can consist of millions of cells. *M. xanthus* cells are predators, growing by lysing prey bacteria and metabolizing the macromolecules released (1, 4, 20). As such, they thrive in soil niches such as herbivorous mammalian dung pellets, and fruiting bodies can be readily observed forming on dung. Fruiting body morphogenesis is thought to require sensing of intracellular nutritional status as well as self-generated extracellular, cell-to-cell communication signals (22). In this study, we examine the hypothesis that direct perception of prey cells could also play an important role in the cell-cell communication process of fruiting body formation.

Vegetative cells of *M. xanthus* are motile on solid surfaces through the use of gliding motility. Gliding motility utilizes both Type IV pili and fixed adhesion sites to propel the cells forward in the direction of the long axis of the cell (14, 26). The direction of cell movement periodically reverses, and modulation of cellular reversals through the chemotaxis-like Frz pathway allows cells to direct their movements (13). Directed cell movement is used during surface colonization, predatory rippling, and fruiting body aggregation. In the laboratory, fruiting bodies can be induced by incubating vegetative cells on a solid surface with low nutrient availability and high cell density. Under these conditions, most cells direct their movement into the formation of hundreds of raised aggregates containing $10^5$-$10^6$ cells each (15). In addition to aggregating, sporulation is induced, with cells inside the fruiting aggregates much more likely to differentiate into spores than cells that occupy the spaces in between.
Several investigations have been performed into the early stages of fruiting body development. Synthesis of (p)ppGpp as an intracellular signal is a common event during the stringent response in bacteria (2). In *M. xanthus*, production of (p)ppGpp during amino acid starvation has been proposed to be sufficient and necessary for induction of fruiting body development (7, 24). The RelA protein is essential for converting GTP to (p)ppGpp during ribosomal stalling that occurs during the stringent response. Ectopic expression of the *Escherichia coli* *relA* gene in *M. xanthus* causes premature aggregation (24). The *M. xanthus* *relA* gene was shown to be required for aggregation, sporulation, and expression of a variety of developmental gene reporters, making it the earliest known requirement for the fruiting program (7, 8). The *relA* gene product is also required for production of another early developmental marker, intercellular A-signal activity (7).

A-signal mutants (*asg*) are considered to be deficient in producing one of the earliest acting signals in fruiting body development (the A–signal). The *asg* mutants have a general defect in protein secretion that includes an inability to secrete at least 2 proteases which are thought to be required for generating the amino-acid and small peptide pool which suffices for complementing developmental reporter gene expression levels in *asg* mutants (17). The A-signal has often been proposed to fulfill a quorum sensing function, providing the cell density information necessary for cells to decide if there is a sufficiently large enough population to be able to construct a fruiting body. However, the A-signal has only a rough correlation to cell density and lacks the specificity of other quorum sensing systems, since the peptide pool produced by starving *M. xanthus* cells can vary significantly from culture to culture (8, 10, 11, 17, 29).
Most experiments studying fruiting body formation examine the process in pure *M. xanthus* cultures incubated with little or no nutrients. However there is evidence that the presence of prey, or prey components, can greatly affect the developmental program (23). Rippling behavior, which has often been interpreted as a discrete stage in fruiting body development, is also a predatory behavior that is observed during growth of *M. xanthus* on prey or large, non-diffusing substrates such as peptidoglycan (1, 19). Starvation-induced rippling has recently been shown to occur in a strain that has a high level of autolysis, but not in a strain with a lower autolytic rate, indicating that the rippling stage of starvation-induced development may be due solely to predation of surviving cells on the lysed cell debris of their sister cells (1). In this report we examine further how the presence of prey alters the developmental program by examining changes in the process of fruiting body formation.
MATERIALS AND METHODS

**Strains and growth conditions.** *M. xanthus* strain DZ2 was analyzed in this study. *E. coli* strain β2155 was supplied as prey since it is a KanR diaminopimelic acid (DAP) auxotroph and prey cell densities can be controlled on any media lacking DAP. For routine culturing, *M. xanthus* was grown in CYE broth and *E. coli* in LB broth (1, 21). Kanamycin was supplied when appropriate at 100 µg/ml and DAP at 100 µg/ml. A modified version of CF agar with reduced nutrient levels termed CFL was utilized for analysis of predation under very low nutrient conditions. CFL contains one tenth the normal levels of pyruvate and citrate, and the recipe is 10 mM MOPS pH 7.6, 1 mM KH$_2$PO$_4$, 8 mM MgSO$_4$, 0.02% (NH$_4$)$_2$SO$_4$, 0.02% citrate, 0.02% pyruvate and 0.1 g/L Casitone. The Casitone level was varied in several experiments to yield 0, 0.1, 1.0, and 10.0 g/L Casitone.

Preliminary experiments on signaling were performed with strains MS1000 (DK101/ΔrelA) and DK4208 (DK1622/asgB-) (Both strains were gifts from M. Singer). Strains JK1701 (DZ2/relA-) and JK1702 (DZ2/asgD-) were constructed by PCR amplifying a ~500 bp internal gene fragment with engineered restriction sites and cloning each fragment into the KanR plasmid pBJ113. The new plasmid constructs were electroporated into *M. xanthus* strain DZ2 and recombinants selected for on CYE plates with 40 mg/ml Kanamycin. Individual colonies were isolated and clones with the correct disruption were confirmed using colony PCR.

**Predation assays.** Cultures of *M. xanthus* and *E. coli* were harvested at mid-log phase and washed 3 times in 10 mM MOPS pH 7.6 buffer. *E. coli* cells were concentrated to a final cell density of 1 x 10$^{11}$ cells/ml and *M. xanthus* cells to a final cell density of 1 x 10$^{9}$ cells/ml. In the initial colony predation assays, 20 µl of *E. coli* cells were pipetted onto the plates and allowed to
dry. *M. xanthus* cells were mixed 2:1 with India ink and 1 µl was added to the center of the *E. coli* prey colony. In the basal nutrient level assays, 10 µl of *E. coli* cells were pipetted onto the plates and allowed to dry. *M. xanthus* cells were mixed with India ink as before and 1 µl was added to the edge of the *E. coli* prey colony. For analysis of step changes in prey, *E. coli* prey cells were pipetted in linear strips consisting of 5 aliquots of 2 µl each that were pipetted close enough that the drops could adhere to each other before drying. Cells were allowed to dry before adding another strip of cells at a different cell density. Last, *M. xanthus* cells mixed with India ink were added to the broad edge of the *E. coli* strip. Plates were incubated at 32˚C for the times indicated in each predation experiment. Assays were performed a minimum of three times, with representative images selected in each case. Fruiting behavior was observed using a Nikon SMZ1000 dissecting microscope and wet mounts were analyzed with a Nikon Eclipse E400 phase-contrast microscope. Images were captured using Q Imaging camera and software. Fruiting body localization was analyzed by creating 100 pixel horizontal slices of images in Adobe Photoshop.

**Sporulation counts.** To determine the level of spores formed in mono-culture compared to co-cultures with prey, cells *M. xanthus* and *E. coli* cells were added to the CFL-based plates listed to yield in aliquots of ~10^7 *M. xanthus* cells only, ~10^8 *M. xanthus* cells only, and ~10^7 *M. xanthus* cells added to a dried colony of ~10^9 *E. coli* cells. After 4 days incubation at 32 ˚C, cells were harvested in MOPS buffer and sonicated at minimum power for 1 second to disperse large aggregates without causing a major change in cell viability. Serial dilutions were added to melted top agar and plated on CYE plates to obtain a total cell count. Dispersed cell suspensions were also incubated at 50˚C for 2 hours to kill vegetative cells. The heat-resistant population was determined through serial dilution and plating of cells on CYE as before. CYE plates were
incubated at 32 °C for 4 days and the colony forming units were determined for the total cell count and the number of heat-resistant spores. Colony forming units were assessed in triplicate on three independent cultures for each condition.
RESULTS

Predation-induced fruiting body formation. *M. xanthus* cell development is routinely analyzed in mono-cultures. It has been proposed that several cell-cell signaling events are required for the development of multi-cellular fruiting bodies (8). Because *M. xanthus* is a microbial predator, we hypothesized that the presence of prey could alter the dynamics of fruiting body construction by providing additional interspecies signals that are lacking in mono-cultures. To examine the impact of prey on fruiting body formation, *E. coli* strain β2155 prey cells were pipetted in 20 µl aliquots containing ~2 x 10⁹ total cells onto a low-nutrient medium designated CFL which contains 0.1 g/L Casitone as the primary carbon and energy source. The β2155 strain is a diaminopimelic acid (DAP) auxotroph, and is useful in predation assays since in any media lacking DAP, the *E. coli* cell density remains relatively constant (1). The prey cell aliquots were pipetted in a variety of patterns and allowed to dry (see Fig. 1). ~1 x 10⁷ cells of wildtype *M. xanthus* strain DZ2 mixed with India ink were pipetted on to the center of the *E. coli* prey colonies and the co-cultures were incubated at 32˚ C. In control samples lacking *E. coli*, *M. xanthus* cells migrate away from the initial inoculum and form fruiting bodies, in a well-spaced but random pattern (see Fig. 1J).

In the presence of *E. coli* prey, the *M. xanthus* swarm expands from the initial inoculum, lysing the *E. coli* cells as they migrate. After 72 hours, many large fruiting bodies are observed forming along the perimeter of the predation zone marked by the original *E. coli* colony, resulting in a fruiting body pattern that mimics the initial *E. coli* inoculum (see Fig. 1C, F, I). The association of fruiting bodies with the predation zone edge occurs regardless of the distance of the edge from the initial *M. xanthus* inoculum, indicating that it is not a timed event. The rate
of swarm expansion is nearly identical in every direction, regardless of whether \textit{E. coli} prey are present or not (data not shown), this indicates that cell movement is not significantly impeded by the presence of prey.

The marked formation of fruiting bodies around the perimeter of prey colonies could be occurring for several reasons. Due to surface tension, cell suspensions do not dry uniformly resulting in slightly more prey cells near the colony edge than the center. This generates an increasing gradient of prey cells directed outward from the center of the inoculum. The presence of more prey cells may lead to localized increases in \textit{M. xanthus} cell density after consuming prey-derived nutrients. If cell density is the major factor in initiation of fruiting body aggregation then there should be a corresponding gradual increase in the number of \textit{M. xanthus} fruiting bodies reflecting the gradual increase of prey cells available from the center to the edge. However, this is not the pattern that emerges. In fact, superimposition of the images captured at time 0 and time 72 h indicates that the fruiting body aggregates form adjacent to, but immediately outside of the predation zone delineated by the original prey colony (see Fig. 1). Additionally, native \textit{E. coli} colonies with a typical convex shape provided as prey will also stimulate fruiting body aggregation along the outside edge of the prey colony perimeter (data not shown).

The presence of prey stimulates fruiting body aggregation at nutrient levels which normally inhibit development. The stimulation of fruiting bodies around the perimeter of prey colonies may also be due to \textit{M. xanthus} cells sensing a sharp decline in nutrient levels as they leave the prey locale. This could cause a localized starvation response which rapidly triggers induction of a developmental program. If this is the case, then raising the basal nutrient level in the medium should result in a more gradual nutrient decline and a spatial dissociation of fruiting
bodies from the perimeter of the predation zone, as cells should be able to travel farther before sensing starvation. If this is not the case, it would indicate that some component of prey cells directs fruiting body formation in a way that is distinct from the sensing of overall nutrient status.

*M. xanthus* cells were added to a series of CFL plates containing varying levels of Casitone as a carbon and energy source. As shown in Figure 2, the Casitone level in the medium has a profound effect on the formation of fruiting bodies under mono-culture conditions. At 0.1 g/L Casitone, fruiting bodies form readily within 72 hours, but both lower (0 g/L) and higher (1.0 g/L) nutrient levels inhibit rapid fruiting body development. In the presence of *E. coli* prey cells, fruiting bodies are observed forming in a ring around the predation zone at 0, 0.1 and 1.0 g/L Casitone. The increased number of fruiting bodies observed at 0 g/L Casitone in the presence of prey may either be due directly to the additional nutrients supplied by prey, providing the energy necessary to develop properly, or indirectly through growth and a consequent increase in the overall cell density of *M. xanthus*. However, neither of these possibilities can be used to rationalize why rapid fruiting body aggregation would occur at higher nutrient levels (1.0 g/L Casitone) without any observed spatial or temporal shift. Induction of fruiting bodies in the presence of prey at nutrient levels which are typically prohibitive of fruiting body formation indicates that prey-induced aggregation is not occurring due to a sharp decrease in nutrient availability. As observed previously, there remains a close association of fruiting body formation adjacent to the edge of the initial *E. coli* colony perimeter.

**Analysis of cellular differentiation in predation-induced aggregation.** The surprising result that the presence of prey is able to induce fruiting bodies at nutrient levels that should be prohibitive, led us to examine the effect of prey on sporulation levels as well. *M. xanthus* cells
were harvested after 4 days incubation on CFL-based media and the total viable colony forming units was determined and compared to the number of colony forming units after exposure to a 2 hour heat stress at 50˚C (see Fig. 3). On CFL plates containing 0 g/L Casitone, spores comprise 19% of the population in the presence of prey, yet < 1% of the population in the absence of prey. The presence of prey in this assay causes an increase in the total number of *M. xanthus* cells harvested, typically about 2 to 3-fold higher. To determine if the increase in sporulation percentage is due solely to the increased cell density achieved by consuming prey-derived nutrients, we repeated the assay with a 10-fold higher inoculum of *M. xanthus* cells. The increased inoculum resulted in more total cells than the samples incubated with prey, yet consisted of only 2% heat-resistant spores. At 0.1 g/L Casitone, the sporulation percentage is again higher in the presence of prey, but in this case increasing the initial *M. xanthus* cell density 10-fold increases the percentage of spore cells in the population to nearly that of the sample incubated with prey. This indicates that a combination of increased cell density and increased nutrient level, either through Casitone or prey cells is sufficient to stimulate higher sporulation levels.

At 1.0 g/L Casitone, conditions in which fruiting body aggregates are observed only in the presence of prey, a measurement of the heat-resistant population indicates that sporulation levels are < 1% under all conditions. This indicates that while the edge of the predation zone serves to stimulate the early stages of fruiting body aggregation, a rapid morphological change to the spore cell type is not concurrently induced. Microscopic examination of wet mounts prepared from the fruiting aggregates formed in the presence of prey after one and two weeks of incubation indicates that the number of spores increases after prolonged incubation, such that they are eventually indistinguishable from fruiting bodies formed under more stringent
conditions (data not shown). These results support the hypothesis that predation-induced aggregation does not require starvation, since sporulation is delayed at 1.0 g/L Casitone and yet aggregation is not.

**Analysis of predation-induced aggregation in developmental signaling mutants.** Two early events in mono-culture starvation-induced development of *M. xanthus* cells are the production of (p)ppGpp during ribosomal stalling, which is produced through the activity of the RelA protein, and generation of the cell density-related A-signal, which requires the *asg* genes for proper expression. If the presence of prey is sufficient to stimulate the early stages of fruiting body formation, then examination of the predation phenotypes of *relA* and *asg* mutants should provide insight into whether or not (p)ppGpp and A-signal production are required for prey-directed fruiting body formation. Preliminary experiments performed with strains MS1000 (∆*relA*) and DK4208 (*asgB*-) indicated that aggregation would still occur at the predation zone edge, but with a severe delay with respect to the DZ2 strain (data not shown). However, both of these mutant strains are known to have motility defects. MS1000 is derived from the DK101 background which harbors a defect in the PilQ Type IV pilus secretin protein (28). DK4208 is a tan-phase locked mutant and displays the reduced motility phenotype common to this phase variant (9, 12).

To eliminate potential motility artifacts, insertion mutants were constructed in the *relA* and *asgD* genes in the DZ2 parental strain. Under mono-culture starvation conditions, both the *relA* and *asgD* mutants are defective in fruiting body aggregation compared to DZ2 (see Fig. 4A-C). In the presence of prey, all three of these strains are able to penetrate the prey colony and lyse the *E. coli* cells with similar timing (see Fig. 4D-O). Fruiting aggregates are observed forming within the predation zone in all strains as well. Both the DZ2 and *asgD*- strains form the
characteristic ring of fruiting bodies around the predation zone edge by 64 hours into the assay. The \textit{relA} mutant has reduced swarm expansion and thus exhibits an aggregation delay with respect to the parent. However, induction of a ring of fruiting aggregates is still observed in the \textit{relA} mutant. This induction can be rapid, since areas that contain macroscopic amounts of prey can be converted to sites of fruiting aggregates in as little as ten hours (see Fig. 4N, O). The final localization of the aggregates is also similar to that observed for DZ2. Microscopic examination of wet mounts prepared by harvesting cells from the fruiting aggregates indicates that neither mutant produces any spherical, phase-bright spore cells (see Fig. 4P-R). This indicates that while the macroscopic developmental process of fruiting aggregation is rescued by the presence of prey, conversion of individual cells to spores is still defective in both the \textit{relA}- and \textit{asgD}- mutant backgrounds. This also demonstrates that the starvation and cell density signals produced by RelA and AsgD are not required for multicellular behavior, only for cellular differentiation.

\textbf{A step-down in prey cell density will induce fruiting body formation.} Because prey-directed fruiting bodies are not dependent on early intracellular or intercellular signals and also not dependent on the overall level of nutrient availability, we hypothesized that the impetus for forming a fruiting body aggregate at the predation zone edge could be due to direct sensing of a loss of prey by the \textit{M. xanthus} swarm. If aggregation occurs due to direct monitoring of prey in the environment, rather than internal metabolic status, then we would predict that a step-down in prey availability could suffice to trigger multicellular aggregation similar to a total loss of prey. To test this hypothesis, \textit{E. coli} cells were added to CFL plates in long, thin adjacent strips containing either of two cell densities, \(\sim 4 \times 10^7\) cells/mm\(^2\) (10 \(\mu\)l of a \(1 \times 10^{11}\) cells/ml suspension) and \(\sim 1 \times 10^7\) cells/mm\(^2\) (10 \(\mu\)l of a \(2.5 \times 10^{10}\) cells/ml suspension). \textit{M. xanthus} cells were pipetted on the edge of these strips such that as the \textit{M. xanthus} swarm expands out from the
initial inoculum, cells that move in the direction of the *E. coli* prey will encounter sharp changes in prey availability (see Fig. 5).

Analysis of control samples in which there is a constant density of *E. coli* cells indicates that fruiting bodies form within the predation zone in vague arcs emanating from the initial inoculum (see Fig. 5A-H). At a constant high cell density of prey, there are more fruiting bodies formed, and the average size of fruiting bodies observed is larger than at the constant low cell density of prey. Under each of these conditions fruiting bodies are distributed evenly, such that when the predation zone is sliced into horizontal sections, no section in either control sample contains more than 15% of the total number of fruiting bodies. When cells are exposed to a sharp step-down in prey availability, however, a corresponding line of fruiting bodies is observed forming along the site of the step-down in prey (see Fig. 5I-L). The horizontal section encompassing the site of the step-down contains 28% of the total fruiting bodies in the sample while the 2 sections immediately following have a combined 0.4% of the total fruiting bodies, indicating that the step-down in prey availability provides a strong aggregation signal such that adjacent areas are devoid of aggregates.

*M. xanthus* cell movement is dependent on the elasticity of the surface, and stressed agar will bias the movement of cells through a process called elasticotaxis (5). Thus, the aggregation of cells at the site of a loss or step-down in prey availability could be due to physical forces such as a change in the overall topology or elasticity of the medium caused by the change in prey. To exclude this possibility, *M. xanthus* cells were also challenged with a step-up in prey availability (see Fig. 5M-P). At the site of a step-up in prey, there is actually a decrease in the probability of forming a fruiting body aggregate, with 5% of the total fruiting bodies found in this section. The neighboring sections contain 16% and 14% of the total fruiting body counts. This indicates that
fruiting bodies formed at the edges of the predation zone or at a step-down in prey availability are not due simply to changes in topology or elasticity. Nutrient depletion should occur everywhere in the sample eventually, and should occur earlier in the sections closest to the initial inoculum of \textit{M. xanthus}, yet the pattern of fruiting body localization does not change significantly after 72 hours. The site of a step-up in prey availability remains nearly devoid of fruiting bodies, even as fruiting bodies are induced in the succeeding sections. The ability of \textit{M. xanthus} cells to react to essentially the same stimulus in two different ways depending on their prior environment indicates that they are responding directly to changes in the availability of prey using a system of cellular memory reminiscent of bacterial chemotaxis.
DISCUSSION

In 1963, Dworkin demonstrated that it is possible to manipulate the life cycle of *M. xanthus* solely by altering the amino acid content of a defined growth medium (4). This was important evidence that the developmental program of *M. xanthus* is a response to environmental conditions rather than an inexorable sequence of a predetermined growth cycle. Further analysis of this laboratory phenomenon has led to a model in which the developmental program of *M. xanthus* is initiated by starvation and then coordinated through a series of self-generated extracellular signals (6, 22). However, there has always been some difficulty in rationalizing why *M. xanthus* would initiate a complex multicellular program requiring significant time and energy after its nutrient supply has been exhausted. One model for starvation-induced development is that *M. xanthus* cells cannibalize each other, taking a step backwards in growth in order to have enough energy to convert a fraction of the population into hardy spores (8). An alternative model, which stems from our analysis of predation-induced development, is that fruiting bodies are induced by a perceived reduction in prey availability. In this model, *M. xanthus* cells initiate multicellular development before nutrients are exhausted and without a requirement for self-sacrifice.

In this study, we examine how fruiting body development occurs in co-cultures with suitable prey. We find that the presence of prey has a dramatic impact on the localization of fruiting bodies. In particular, the boundaries of a prey colony seem to provide a specific signal for fruiting body aggregation. Although this may at first glance seem to be a consequence of starvation, a deeper analysis indicates that this is not the case. By changing the basal nutrient level in the media, we observed that the presence of prey stimulates fruiting body aggregation...
across a wider range of nutrient levels than was observed in mono-culture conditions. We also demonstrate that fruiting body aggregation will occur without immediate commitment to sporulation in the presence of prey and higher nutrient availability. There have been several indications from genetic evidence that the processes of sporulation and aggregation may not be inextricably linked in *M. xanthus*. A DZ2/espB- mutant forms fruiting aggregates but sporulation occurs both outside of the aggregates as well as inside (3). A similar result was observed in the DK1622/rodK- mutant (18). It is now possible to provide a concrete biological rationalization for these genetic observations of how multicellular behavior can be uncoupled from cellular differentiation.

The earliest stages of fruiting body formation are currently thought to be controlled by a combination of sensing starvation and sufficiently high cell density to initiate a developmental program (8). However, the ability of prey to also stimulate multicellular behavior allows for new ways to test the interpretations of mono-culture experiments to determine if they apply to a broader set of environmental circumstances. Two of the early signals for fruiting body aggregation have been proposed to be (p)ppGpp and the self-generated mixture of amino acids and small peptides that comprise the A-signal. The *relA* and *asgD* gene products are required for (p)ppGpp production and A-signal production, respectively, and during mono-culture development cause severe defects in fruiting body formation. However, under predatory conditions, neither of these genetic loci are required for formation of fruiting body aggregates, yet they are still defective in the differentiation of vegetative cells to spores. This indicates that they are not necessary for the early signaling events of fruiting body aggregation, but raises the question of why fruiting aggregates are not observed in mono-cultures of these strains. Close examination of these mutants in mono-culture indicates that small, poorly defined aggregates are
present in both strains. Perhaps the ability to differentiate into spores, which includes the loss of motility at some undetermined checkpoint, is necessary to stabilize the random aggregates formed in mono-culture. Recent work by Sliusarenko et al. supports this idea, where examination of cell movement during the early stages of fruiting body formation showed that reduced cell movement was more important than control of cell reversals for the stability of early aggregates (25). Thus, mutants such as relA and asgD which do not form stable aggregates in mono-culture and are also not able to transition to nonmotile spores may be able to utilize a prey-derived stimulus to halt in order to aggregate under predatory conditions without differentiation.

We propose a model in which cells are able to monitor the availability of prey or prey-derived macromolecules and use this information to initiate multicellular development (see Fig. 6). A previous study demonstrated that the presence of prey or macromolecular growth substrates will induce multicellular rippling behavior in *M. xanthus* (1). In addition to rippling within a predation zone, our results indicate that *M. xanthus* cells respond to a loss of prey by forming fruiting bodies outside the edge of a predation zone. Additionally, this does not appear to be an on/off switch triggered only by a complete loss of prey, as *M. xanthus* cells are also capable of inducing fruiting bodies after a step-down in prey availability and inhibiting fruiting bodies after a step-up in prey availability. The typical laboratory method for inducing fruiting bodies in mono-culture would thus fit into this model as an extreme circumstance in which *M. xanthus* cells suddenly find themselves in the peculiar situation of having a very high density of mid-log phase vegetative cells and absolutely no prey or suitable macromolecular growth substrates. In nature, starvation is not likely to occur so suddenly to such a large population of *M. xanthus* cells, thus it is important to closely examine the relationship that *M. xanthus* cells have with their prey in order to fully understand the multicellular behaviors displayed by this organism.
Determining the specific components of prey which *M. xanthus* detects to make these decisions will be the challenge for understanding how cell-cell communication functions within this predator-prey relationship. An examination of the correlation between rippling behavior in the presence of macromolecular growth substrates and fruiting body aggregation at the edges of prey should also be a focus of study in the very near future.
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REFERENCES


FIGURE LEGENDS

FIG. 1. Prey-directed fruiting body formation. 20 µl of *E. coli* β2155 cells were pipetted and allowed to dry on CFL agar media as prey for 1 µl of *M. xanthus* DZ2 cells mixed with India ink which were pipetted onto the *E. coli* prey. As the *M. xanthus* swarm expands, the *E. coli* cells are lysed and fruiting body formation is induced along the perimeter of the original prey colony. Photographs were captured at 8 x magnification at times (A, D, G) 0 hours, (B, E, H) 48 hours, and (C, F, I) 72 hours. Panel (J) shows a control experiment with no *E. coli* prey at 120 hours.

FIG. 2. Basal nutrient level effect on prey-directed fruiting body formation. *M. xanthus* DZ2 cells were pipetted adjacent to prey colonies (first two columns) and alone (third column) on CFL plates containing (A-C) 0.0 g/L, (D-F) 0.1 g/L, and (G-I) 1.0 g/L Casitone. In the presence of prey, fruiting aggregates are observed at 72 hours under conditions ranging from 0.0 to 1.0 g/L Casitone. In the absence of prey, fruiting aggregates are only observed at 0.1 g/L Casitone.

FIG. 3. Prey effect on sporulation levels. *M. xanthus* DZ2 cells were incubated either in the presence or absence of prey for 4 days on CFL plates with varying levels of Casitone. Cells were harvested and the viable *M. xanthus* cell count was determined through plating serial dilutions before and after a 2 hour 50° C heat stress. Closed bars, $10^7$ *M. xanthus* cells; Open bars, $10^7$ *M. xanthus* cells with $10^9$ *E. coli* cells; Hatched bars, $10^8$ *M. xanthus* cells. The presence of prey increases the sporulation percentage of the population at 0 g/L Casitone, but not at 1.0 g/L Casitone.
FIG. 4. Analysis of early signaling mutants. In a mono-culture starvation assay, (A) DZ2 forms fruiting bodies in 48 hours, whereas (B) \textit{asgD}- and (C) \textit{relA}- strains are defective at aggregation. In a co-culture predation assay, (D-G) DZ2, (H-K) \textit{asgD}-, and (L-O) \textit{relA}- strains are all proficient at inducing fruiting aggregation, in particular at the edges of the predation zone. Pictures were captured at 0, 40, 64, and 74 hours (left to right) during predation. Analysis of wet mounts indicates that phase-bright spores are present in the predation-induced aggregates of (P) DZ2, but not (Q) \textit{asgD}- or (R) \textit{relA}- cells.

FIG. 5. Response to stepped changes in prey cell density. \textit{E. coli} β2155 cell suspensions were pipetted in a series of small drops and allowed to dry to generate relatively straight lines of prey at either $4 \times 10^7$ cells/mm$^2$ and $1 \times 10^7$ cells/mm$^2$. \textit{M. xanthus} cells were mixed with India ink and added to the edge of the dried prey colonies in a 1 µl aliquot. Prey cells were provided at (A-D) a constant low cell density, (E-H) a constant high cell density, (I-L) a step change down from a high cell density to a low cell density, or (M-P) a step change up from a low cell density to a high cell density. The left column portrays a conceptual diagram of the assay followed by images captured at times 0 h and 72 h showing the pattern of fruiting bodies formed. For each assay, the field of view was sliced into 11 equivalent horizontal sections and quantification of the fruiting body localization pattern is provided in the right column. The arrows highlight the location at which a step-up or a step-down in prey availability occurs.

FIG. 6. Model for the initiation of multicellular development. Although a combination of high cell density and starvation will induce fruiting body formation in mono-culture, it is important to consider that \textit{M. xanthus} is a predatory bacterium and that prey availability alters the timing and
localization of fruiting body aggregation as well. The early signals for this process are therefore dependent on interspecies signals, and self-generated signals from the *relA* and *asg* loci are not required until later in development for cellular differentiation.
Casitone (g/L)

0

0.1

1.0
M. xanthus biofilm

High cell density/starvation → random aggregation

Decrease in prey → directed aggregation

rel asg → sporulation