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Quantifying Aggregation Dynamics during *Myxococcus xanthus* Development†‡

Haiyang Zhang,† Stuart Angus,‡ Michael Tran, Chunyan Xie,† Oleg A. Igoshin,†* and Roy D. Welch‡*

Department of Bioengineering, Rice University, Houston, Texas 77005,† and Department of Biology, Syracuse University, Syracuse, New York 13244‡

Received 28 April 2011/Accepted 8 July 2011

Under starvation conditions, a swarm of *Myxococcus xanthus* cells will undergo development, a multicellular process culminating in the formation of many aggregates called fruiting bodies, each of which contains up to 100,000 spores. The mechanics of symmetry breaking and the self-organization of cells into fruiting bodies is an active area of research. Here we use microcinematography and automated image processing to quantify several transient features of developmental dynamics. An analysis of experimental data indicates that aggregation reaches its steady state in a highly nonmonotonic fashion. The number of aggregates rapidly peaks at a value 2- to 3-fold higher than the final value and then decreases before reaching a steady state. The time dependence of aggregate size is also nonmonotonic, but to a lesser extent: average aggregate size increases from the onset of aggregation to between 10 and 15 h and then gradually decreases thereafter. During this process, the distribution of aggregates transitions from a nearly random state early in development to a more ordered state later in development. A comparison of experimental results to a mathematical model based on the traffic jam hypothesis indicates that the model fails to reproduce these dynamic features of aggregation, even though it accurately describes its final outcome. The dynamic features of *M. xanthus* aggregation uncovered in this study impose severe constraints on its underlying mechanisms.

A *Myxococcus xanthus* cell is a semirigid rod-shaped delta-proteobacterium that can move in either direction along its long axis on an agar substrate using a complex form of motility called gliding (12). If the agar surface is nutrient rich, *M. xanthus* will grow as a dense and highly motile population called a swarm. Under these conditions, cells move out from the swarm edge in multicellular projections called flares. On a smooth nutrient agar surface, flares move out in all directions, and as they expand and merge with each other, they form a contiguous population that increases the area of the swarm. In this way, a circular swarm expands radially with a relatively even and symmetrical distribution of cells (3).

A swarm placed on nonnutritive starvation agar exhibits a different set of behaviors, collectively referred to as development (9). When development starts, swarm expansion stops, and then the entire swarm appears to contract as movement is redirected inward. Over a period of several hours, thousands of cells accumulate at certain locations within the swarm, and, once there, they arrange themselves into multicellular structures called aggregates. Each aggregate is dome shaped and ~0.1 mm in diameter (2, 10). At the completion of development, the cells in the interior of each aggregate differentiate to become metabolically quiescent and environmentally resistant myxospores. At this point the aggregate is considered to have matured into a fruiting body. The entire process can occur in less than 24 h (24).

Several transient multicellular patterns occur within a swarm during development. Traveling waves (7, 16, 17, 23), swirling vortices (13), and multilevel terraces (2) have all been observed prior to and during the formation of aggregates and fruiting bodies. These patterns occur because development is an emergent process, where global order is produced from the numerous interactions of lower-level components, so that intermediate patterns arise through nonlinear interactions as the system transitions to a new stable, ordered state (1). In developing bacteria such as *M. xanthus*, that stable ordered state has long been recognized to be the fruiting body (14), but a developing swarm typically creates a distribution of fruiting bodies. Under standard experimental conditions, a typical swarm of several million cells will produce hundreds of fruiting bodies over an area of less than 1 cm².

The principles underlying self-organization in swarming cannot be understood solely by available experimental approaches; thus, mathematical modeling has been used extensively to explore these emergent behaviors (4, 7, 8, 18, 19, 20, 21, 25, 26). These models have successfully uncovered sets of intercellular interactions that lead to patterns similar to those observed experimentally in *M. xanthus*. Models with various levels of complexity and with different formalisms have been used to describe development, and several of them are based on the traffic jam principle: cells tend to slow down or stop when entering regions of high cell density (5, 8, 19, 20). This leads to a positive-feedback loop as more cells jam into the region, further increasing cell density so that aggregates are able to grow without any diffusible morphogens or chemoattractants. Thus far, most modeling research has focused on the formation of an aggregate rather than properties associated with multiple aggregates.

† Supplemental material for this article may be found at http://jb.asm.org/.

‡ Published ahead of print on 22 July 2011.

* Corresponding author: Mailing address for Roy D. Welch: Syracuse University, Department of Biology, 107 Campus Drive, LSC RM243, Syracuse, NY 13244. Phone: (315) 443-2159. Fax: (315) 443-2159. E-mail: roweldch@syr.edu. Mailing address for Oleg A. Igoshin: Rice University, Department of Bioengineering, MS142 BRC767, Houston, TX 77005. Phone: (713) 348-5502. Fax: (713) 348-5877. E-mail: igoshin@rice.edu.

† Received 28 April 2011/Accepted 8 July 2011.

‡ Published ahead of print on 22 July 2011.

Received 28 April 2011/Accepted 8 July 2011.

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In this report, we demonstrate that *M. xanthus* developmental self-organization also occurs at the scale of the entire population of fruiting bodies rather than just a single fruiting body. We show that the arrangement of fruiting bodies within the swarm is not random and becomes more ordered with time. Furthermore, the process through which this ordered placement occurs is not reproduced in the traffic jam model for the formation of fruiting bodies.

**MATERIALS AND METHODS**

Liquid culture conditions and microscope culture apparatus. Liquid cultures of wild-type *M. xanthus* strain DK1622 were grown in nutrient-rich CTTYE medium (1.0% Casitone [Difco], 0.5% yeast extract [Difco], 1.0 mM Tris-HCl [pH 8.0], 1.0 mM KH₂PO₄, and 8.0 mM MgSO₄). Development assays were performed using nonnutritive Tris phosphate medium (TPM) agar containing 10.0 mM Tris-HCl (pH 8.0), 1.0 mM KH₂PO₄, 8.0 mM MgSO₄, and 1.5% agar. Cells were harvested between Klett 80 and 120, when they were in mid-log phase. Cultures for the development assay were prepared as previously described (2, 22) with the following modifications: larger silicone gaskets with oval assemblies were used to make the microcinematography culture apparatus; the gasket used to contain the agar was thicker (2 mm) than the gasket used to provide the airspace (1 mm); the initial swarm used for the development assay was created by spotting 5 μl of cells that were grown in shaking CTTYE medium, washed twice in TPM, and resuspended to a final concentration of ~5.0 × 10⁶ cells/μl. Microcinematography was performed at 32°C on a stage warmer (2).

Time-lapse microcinematography and image processing. Time-lapse microcinematography was performed on developing swarms of *M. xanthus* DK1622 cells as previously described (2, 22), with the following modifications: images were acquired using bright-field microscopy with ×20 magnification; images were acquired every minute for 24 h; images were saved as sequential files and processed as a group. Aggregates and fruiting bodies appear as dark spots within the developing swarm.

Sequential time-lapse microcinematography images were processed in several steps to enable the automated identification and measurement of aggregates using the image-processing capabilities of the Matlab software package (MathWorks). The overall image-processing routine is similar to that recently developed by us (27). First, each image was divided into 100 quadrants (10 × 10), so that any inconsistency in light intensity across the image could be isolated and its effect on aggregate identification minimized. The contrast of each quadrant was then adjusted separately before the image was reassembled. A grayscale threshold filter was then applied to each quadrant, and a threshold value corresponding to approximately 60% of the mean intensity of the subimage was selected. In the resulting binary image, pixels with a value of 1 indicate regions of high cell density and therefore represent potential aggregates. To remove noise, a size threshold of 300 pixels (an area of 0.004 mm², or about 1/4 of an average fruiting body at the final stage [see Fig. 3], or an equivalent diameter of approximately 72 μm) was applied, and smaller aggregates were discarded. The remaining pixel clusters with values of 1 were automatically assigned consecutive numbers, so that the total number of clusters corresponded to the total number of aggregates in each image. In some image stacks, small dark regions were present from the first frame, long before the onset of development; these were likely due either to small clumps of cells that were not adequately dispersed in liquid culture or to particles (i.e., dust, hair, etc.) that were trapped during the assembly of the microcinematography apparatus. To prevent this source of experimental error from interfering with the automated analysis of aggregates, these regions were eliminated during processing. Some of these dark regions resolve and thus were eliminated only for the early images.

The position of each aggregate was defined by the center of mass of its binary image (i.e., the values for each set of coordinates x and y are equal to the means of the values of the x’s and y’s for all the pixels within the cluster, respectively). For each image, 100 randomized images were generated in silico as a means of measuring order in the experimental distribution of aggregates, and a comparison of aggregate distributions between random and experimental images was used to determine if the observed distribution was ordered. To produce these randomized images from the experimental one, the position of each aggregate was randomly shifted and the aggregate was rotated by a random angle. The new position and orientation of each aggregate were not allowed to overlap with the other shifted and rotated aggregates; if an overlap occurred, then a new shift and rotation was generated, and this process was repeated until there was no overlap.

CRDF. A radial distribution function is commonly used in statistical mechanics to characterize isotropic distributions in two and three dimensions (15). This function is typically defined as a normalized density of particles in an infinitesimal ring from radius r to r + dr; however, this definition is very sensitive to noise if the number of particles is small, as is the case with aggregates in a developing swarm. To characterize a two-dimensional distribution of aggregates on a swarm, we used a cumulative radial distribution function (CRDF), f(r), as the measurement (11). Starting at the center of each aggregate, we identified the circle of radius r and computed the number of aggregates in the circle, N(r). The aggregate density in this circle, f(r), is computed as [N(r)/πr²]. Periodic boundary conditions are assumed; i.e., the distribution outside the image is assumed to be the same as the distribution in the image. To normalize, the radial density is divided by the average fruiting body density to compute the CRDF, g(r), which is equal to f(r)/N.

For each image, CRDF is computed by averaging the radial distribution functions centered at each aggregate. As defined, g(r) depends on the size of the aggregates in the image. To partially compensate for this dependence, we have chosen to present the distribution as a function of dimensionless radius r/R₀, where R₀ is the effective radius of aggregates in the image and is equal to √(4K/N). A represents the area of aggregates, defined as the average number of pixels per aggregate multiplied by the area of one pixel. This scaling nearly completely eliminates the dependence of g(r)/R₀ on the total area of the visualized control. For each set of images corresponding to a particular parameter setting, the mean and standard deviation of g(r)/r/R₀ for 100 different images are computed.

**Agent-based in silico model of M. xanthus development.** The agent-based model implemented in this work follows the work by Slussarenko et al. (19). The detailed model formalisms are described in the text in the supplemental material. The main components and assumptions are as follows. Each agent represents a *M. xanthus* cell and is defined as a rod on a two-dimensional surface characterized by its length, center coordinates, orientation, and speed. Switching the polarity of an agent’s two endpoints is used to simulate cell reversal. (ii) For each time step, an agent moves and adjusts its orientation to align with neighboring cells. (iii) When local density is higher than a threshold, an agent reduces its speed. This condition is referred to as the traffic jam. (iv) Agents adjust their reversal frequency according to the local cell density. A higher local density leads to longer reversal periods. This corresponds experimentally to a reduction in reversal frequency in streams of cells. (v) Agents signal at their leading poles. These signals affect the reversal period on the basis of a phase-resetting function (see Fig. S1 in the supplemental material) (7, 18).

**Parameter selection and optimization.** We are able to collect some parameters, such as cell dimension, speed, and reversal period, from experimental observations, but many parameters must be estimated. To do so, we employed an optimization algorithm that selects the parameter combination with the best fitness as follows. Parameter values corresponding to those described previously (19) were selected as starting values. Each subsequent generation had 60 mutated parameter sets, and thus, 60 new simulations with these sets were initiated. The results of the 60 simulations were fed into a fitness function that computed agreement between simulation results and experimental observations. Ten parameter combinations with the best fitness were used to generate 60 new sets using the same mutation method. After approximately 10 generations, little improvement in the fitness function was observed, and the parameter combination with the best fitness was used in our agent-based model. This optimization was geared toward finding a local optimum, and further improvement could theoretically be possible in different regions of the parameter space. It must be noted that, in all simulations performed, aggregate fitness was evaluated on each simulation run and the best fitness function was always improved after each mutation step.
RESULTS

Analysis of microcinematography data reveals various stages in the aggregation process. To record the process of *M. xanthus* development, images of a swarm spotted on starvation agar were acquired at 1-min intervals over a period of 24 h, resulting in a stack of 1,441 sequential images. Acquisition was stopped at 24 h because, after this time period and under these conditions, a population of aggregates had largely stabilized. A subset of images from one image stack at different times during development is displayed in Fig. 1 (top row), together with the corresponding processed binary images used for automated analysis (bottom row). The dark spots in both sets of images represent regions of sufficient cell density to be considered aggregates.

The automated quantification of aggregate size, shape, and position is performed on all processed images from each image stack. A plot of aggregate number as a function of time reveals that all aggregates do not follow identical developmental processes (Fig. 2A). Aggregates first appear within a few hours following the onset of starvation, and their number rapidly increases until approximately 7 h into development. At this point there is sometimes a brief plateau, followed by a decrease...
in aggregate number that is almost as rapid as the preceding increase. The rate of decrease becomes lower at between 10 and 20 h, at which point the number of aggregates nearly stabilizes at approximately 25 to 30% of its peak. This loss of aggregates is evident in both the processed and unprocessed images from Fig. 1; there are fewer aggregates in each image past the 6-h time point. These data demonstrate that all of the aggregates within a swarm do not undergo the same developmental process. As observed previously (2, 27), aggregates have different fates: some grow and mature into fruiting bodies, while others grow for a period of time and then regress and disperse. Still others move and occasionally (in less than 10% of the cases) merge, so that two or more aggregates become one. Aggregate merging, regression, and dispersal are clearly visible in the time-lapse microcinematography image series (see Movies S1 and S2 in the supplemental material).

A plot of average aggregate size as a function of time shows that the average aggregate continues to increase unabated past 10 h after starvation onset (Fig. 2B). These data are significant because, at 10 h, the number of aggregates is rapidly decreasing as the average aggregate size continues to increase. The peak in average aggregate size occurs at between 10 and 15 h and is followed by a very gradual decline; since these images are two-dimensional, it is feasible that some of this decline is caused by the upward growth of each aggregate as it matures into a fruiting body. One of the reasons average aggregate size continues to increase is the merging of aggregates. When merging occurs, it reduces the number of aggregates by one, and the merged aggregate is larger than either of its two constituents. This increases the average aggregate size. We note that aggregation dynamics are highly reproducible under the conditions used in this study; as shown in Fig. 2, the timing and peak values vary relatively little between the seven replicates used in this analysis.

Spatial distribution of aggregates shows ordering with developmental time. Results reported thus far indicate a significant rearrangement of aggregates between an early stage, when the majority of aggregates initially form (~7 h after the onset of starvation), and a late stage (~24 h), when the population of aggregates is stable. Could these rearrangements alter the spatial distribution of aggregates on a two-dimensional surface? Specifically, are aggregate distributions at early and late stages random or ordered, and does the distribution of aggregates become more or less ordered as development progresses? To quantify any changes in aggregate order, CRDFs (see Materials and Methods) were calculated for aggregates in experimental images at both early and late stages, and these were compared with the CRDFs for randomized distributions of the same aggregates (see Materials and Methods for details).

Figure 3A and B displays the CRDFs at the early stage, when the number of aggregates was highest (i.e., near the peak of the curve from Fig. 2A and B). These results indicate that the distributions obtained both from an individual image stack (Fig. 3A) and from an average of seven image stacks (Fig. 3B) are very close to those for the randomized controls. To statistically test if the cumulative distributions were different, we computed the differences in the area below the two curves for all the pairs of samples from the 7 experimental and 100
randomized distributions and performed a standard *t* test to see if the mean of this distribution is significantly different from zero. The results indicate that CRDFs computed from images at the early stage of aggregation are not significantly different from randomized distributions (*P* > 0.05). The same analysis applied to images from the late stage (i.e., when aggregates had stabilized) produces very different results; CRDFs computed from images at the late stage (i.e., the final image of the image stack) (Fig. 3C and D) are sufficiently below their corresponding randomized distributions that there is a statistically significant difference (*P* < 1e–8).

**Traffic jam aggregation model reproduces some but not all features of aggregation.** Developmental aggregation of *M. xanthus* has been the subject of significant attention by the modeling community, and multiple mathematical models of aggregation have been published (4, 8, 19, 20, 21). Nevertheless, few of these models explicitly study dynamic aspects of aggregation, such as the merging or dispersal of aggregates or changes in the distribution of aggregates on an agar surface. We decided to test whether a mathematical model of aggregation can reproduce the time dependence of aggregate number and size as well as the nonrandomness of aggregate distribution. To this end, we constructed a two-dimensional model of aggregation based on the traffic jam concept (5, 6); cells slow down or cease motility at high cell density. The nature of our inquiry required us to use a model of aggregation on a length scale at which multiple fruiting bodies will be formed (1 by 1 or 2 by 2 mm). Because of these constraints, we were not able to adopt any of the more sophisticated three-dimensional models of aggregation (4, 20), as it would not be computationally feasible.

Images of the traffic jam model of aggregation (simulated with periodic boundary conditions in a 1-mm² domain) are shown in Fig. 4A, top row. We started with the parameter set used by Sliusarenko et al. (19) and then adjusted some of the parameters (see the text in the supplemental material for details) to ensure that the number, density, and size of aggregates are the same or similar to what were observed in experiments (snapshots of the experimental images cropped to 1 mm² are shown on the bottom row of Fig. 4A for comparison). Although the endpoints of both the model and the experiment appear to be similar, their dynamics are dissimilar. A comparison of changes in aggregate number and average aggregate size as a function of time effectively illustrates this dissimilarity (compare Fig. 4B and C with Fig. 2A and C). In the simulation,
aggregate number increases monotonically with time, the increase is less rapid than in the experiment, and there is no plateau or subsequent decrease. In the simulation, average aggregate size also increases almost linearly with time, it does not plateau at between 10 and 15 h, and it does not show a gradual decrease, all of which are observed in experiments. There is also little or no aggregate movement or merging in the simulation, and very few aggregates regress or disperse. There is no measurement related to the dynamics of development, for which the simulation parallels experimental results.

We also examined the early and late aggregate distributions in the traffic jam model to see if they were similar to those in the experiments. By calculating the model CRDFs in the same manner performed for the experimental data, we determined that the distribution of aggregates in the model was statistically different from random at both early and late stages (Fig. 4D). Therefore, model CRDF data also disagree with the experimental data, which show a random distribution at the early stage and an ordered distribution at the late stage. Although development in both the model and experiment finishes with an ordered set of aggregates, the dynamics are very different.

**DISCUSSION**

Multiple mathematical models with various modeling formalisms, parameters, and levels of complexity that result in aggregation have been created (4, 8, 19, 20, 21). However, for a simulation to accurately represent the collective behavior of cells within a developing swarm, it must capture the observed aggregation dynamics on a variety of scales, from a single cell to a single aggregate to a multiplicity of aggregates. The goal of this work was to quantify features that describe aggregation over the largest scale, which is the entire swarm, and determine if these features agree with features generated by current models.

A comparison of the traffic jam model to the experimental data shows that the model can successfully reproduce end-state aggregation but not the dynamics that lead to that end state. Through parameter optimization we were able to generate a simulation based on the traffic jam model that reproduced the correct aggregate size, as well as the correct number of aggregates per unit area. The simulation also reproduced an ordered aggregate distribution that was similar to experimental observations at the end of development. On the other hand, the simulation was unable to reproduce many important features of aggregation dynamics. Simulated aggregates formed much less synchronously, exhibited a steady increase in size until the end of the simulation, and did not exhibit significant aggregate dispersal. As a result, the distribution of in silico aggregates never exhibited an increase in ordering with time. All attempts to resolve this disagreement through parameter optimization were unsuccessful. Because of these discrepancies between simulation and experiment, we must conclude that the traffic jam model does not sufficiently explain the dynamics of aggregation or elucidate the mechanistic processes underlying *M. xanthus* development.

At the same time, these experimental data and analyses also impose significant limitations on any new model that is proposed to explain *M. xanthus* development. Specifically, there are several experimentally observed and quantified swarm behaviors that now must be represented in a development simulation in order for it to be considered plausible. Aggregation must be rapid and synchronous. Early aggregates must appear within the first few hours, and aggregate number must increase rapidly for no more than 2 h and thereafter must rapidly decrease by more than half over the next 3 h. All aggregates must not share the same fate during the period of decreasing aggregate number; some must grow, others must regress, and still others must move and/or merge with other aggregates. During this period, average aggregate size must steadily increase until it reaches a plateau at between 10 and 15 h, at which point these late aggregates must appear to be stable and exhibit an ordered distribution. This distribution must be more ordered than the distribution of early aggregates, which must be closer to random. In other words, ordering must be imposed on the population during the period between early and late aggregation.

We can speculate as to why the agent-based model is incompatible with the observed dynamics. The model initiates and propagates aggregation through a process that can be described as random capturing; agents are either moving with a regular speed or practically stopped, with the gliding velocity decreased 5-fold. This decrease in velocity occurs when agents encounter high cell density. If the density decreases or if agents move to an area of lower cell density, they resume gliding. These rules result in positive feedback: as more agents slow down, cell density in that region increases and even more cells are captured there. This positive-feedback random capturing is inconsistent with two of the experimental observations described in this report: the rapid rate of increase in average aggregate size and the nonmonotonic behavior of aggregates. Given the average velocity of an *M. xanthus* cell (24), there is no way to simulate the rapid synchronized increase in aggregate size without somehow directing the agents’ movements to fixed points. Also, once the agents are in aggregates, the positive-feedback random capturing produces further growth of the aggregate only until all the cells are captured. There is no way for the current simulation to produce aggregate dispersal and movement. It may be possible to modify the traffic jam model so that a simulation reproduces these two observed behaviors, but it would likely require the incorporation of agent interactions and movement parameters for which there is little or no experimental evidence.

Having stated the limitations of the current traffic jam model, it should be noted that some of the observed swarm behaviors might be sensitive to differences in experimental methods. As the description of swarm dynamics becomes more quantified and detailed, it becomes increasingly likely that at least some behaviors may occur only under a limited set of experimental conditions. Variation in parameters, such as substrate agar concentration, degree of starvation stress, or initial population size and density, will likely result in somewhat different swarm behaviors. Here we have quantified swarm behavior that occurs under a specific set of relatively standard experimental conditions, but we do not yet know which of these behaviors will prove to be robust. Nevertheless, any mathematical model that aims to explain development in *M. xanthus* swarms must be able to reproduce these behaviors using some set of optimized parameters.
ACKNOWLEDGMENTS

This research was made possible through National Science Foundation CAREER Awards (MCB-0845919 to O.A.I. and MCB-0746066 to R.D.W.) and a Syracuse University Undergraduate Research Fellowship to S.A. The simulations were performed on shared research computing clusters at Rice University funded under NSF grants OCI-0959097, CNS-0821727, and EIA-0216467 and a partnership between Rice University, Sun Microsystems, and Sigma Solutions, Inc.

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