Myxococcus xanthus Swarms Are Driven by Growth and Regulated by a Pacemaker

Dale Kaiser1,2* and Hans Warrick1,2

Departments of Biochemistry1 and of Developmental Biology,2 Stanford University School of Medicine, Stanford, California 94305

Received 7 February 2011/Accepted 28 July 2011

The principal social activity of Myxococcus xanthus is to organize a dynamic multicellular structure, known as a swarm. Although its cell density is high, the swarm can grow and expand rapidly. Within the swarm, the individual rod-shaped cells are constantly moving, transiently interacting with one another, and independently reversing their gliding direction. Periodic reversal is, in fact, essential for creating a swarm, and the reversal frequency controls the rate of swarm expansion. Chemotaxis toward nutrient has been thought to drive swarming, but here the nature of swarm growth and the impact of genetic deletions of members of the Frz family of proteins suggest otherwise. We find that three cytoplasmic Frz proteins, FrzCD, FrzF, and FrzE, constitute a cyclic pathway that sets the reversal frequency. Within each cell these three proteins appear to be connected in a negative-feedback loop that produces oscillations whose frequencies are finely tuned by methylation and by phosphorylation. This oscillator, in turn, drives MglAB, a small G-protein switch, to oscillate between its GTP- and GDP-bound states that ultimately determine when the cell moves forward or backward. The periodic reversal of interacting rod-shaped cells promotes their alignment. Swarm organization ensures that each cell can move without blocking the movement of others.

For many years myxobacterial swarming has been viewed as a chemotactic process in which swarm cells are attracted to greener pastures beyond the swarm edge (9, 25, 34, 40). Also, Myxococcus xanthus is said to be attracted to prey bacteria when it forms concentric ripples that advance toward the prey (1), but how ripples imply chemotaxis has not been explained. Investigating chemotaxis in M. xanthus, a Gram-negative deltaproteobacterium, has been motivated by the presence of six frizzy genes, five of which encode proteins with some similarity (28 to 40% identity at the amino acid level) to the core chemotaxis proteins found in Escherichia coli and Salmonella (2). Despite continuing attempts to identify chemotactic attractants, only phosphatidyethanolamine (PE), a normal constituent of M. xanthus membranes, has been found to increase the frequency of reversals of swarved cells, a process called stimulation (18). Stimulation, however, does not depend on the frizzy proteins (23, 39). Since no methyl-accepting chemotaxis (MCP) protein receptor for PE has been identified, extracellular PE might instead be interacting with the cell’s outer membrane and the largely unknown machinery that induces reversal of gliding direction. A serious molecular difficulty with the chemotactic view of M. xanthus behavior is that FrzCD, unlike the methyl-accepting chemotaxis proteins of E. coli, lacks both a transmembrane and an extracellular domain at its N terminus that would respond to attractant or repellent binding to a receptor. FrzCD is, moreover, localized to the cytoplasm (5, 24). The FrzE histidine kinase, like its E. coli homolog CheA, has no sensory domain and is also a cytoplasmic protein. FrzE in addition includes a CheY domain, unlike the corresponding E. coli proteins (25). A recent review of chemotaxis and receptors in E. coli is available for comparison with M. xanthus data (35). The absence of any receptor saddles interested parties with the hope that an “unidentified receptor protein in the cell membrane recognizes external stimuli and conveys this information to FrzCD” (25). In addition to the complete lack of chemotactic receptors, “the time course of methylation or demethylation of FrzCD following stimulation is considerably longer than that observed for the enteric bacteria” (25), which suggests that M. xanthus uses the essential CheB/CheR adaptation apparatus differently and possibly not for adaptation to high levels of attractant. Together, these differences as well as several new observations described below lead us to favor a pacemaker hypothesis of frizzy gene function that can account for M. xanthus swarm expansion. The pacemaker hypothesis states that FrzCD, FrzE, and FrzF constitute a finely tuned, cytoplasmic oscillator that causes cells to reverse their polarity every 8 to 9 min.

Here, we describe evidence that swarming is directed by growth, not by chemotaxis. Second, we offer new genetic evidence that three of the Frz proteins constitute a pacemaker that generates fixed-frequency oscillations. These oscillations cause swarm cells to reverse their gliding direction at regular intervals, thereby enabling the swarm to expand.

MATERIALS AND METHODS

Bacteria. Two wild-type strains of M. xanthus were employed, DK1622 (14) and DZ2 (6), along with several in-frame frz deletion mutants of DZ2 (5). Cultures were routinely grown in CTT medium (1% Casitone, 10 mM Tris-HCl [pH 8.0], 8 mM MgSO4, 10 mM KPO4 [pH 7.6]) at 20°C on 1% agar CTT plates. Cultures were maintained by transfer to a fresh plate at 3-week intervals.

Swarm expansion. In prior studies (15, 38) liquid cultures were grown to exponential phase and concentrated to a calculated density of 2.5 × 109 in CTT medium, and an aliquot of 5 μl of each concentrated culture was spotted on plates that were incubated at 32°C. The average width of the zone of spreading had to be measured using a microscope. Recently, a simpler, more reliable way to initiate and propagate M. xanthus swarms was found: inoculating an agar plate...
with bacteria harvested from the edge of a mature swarm using the tip of a sterilized round toothpick and incubating at 20°C, room temperature. The rate of swarm expansion was quantified by measuring the swarm diameter using a millimeter scale on each of three or four plates daily for 2 to 3 weeks. The average diameter was plotted against time, the best straight line was drawn through the experimental points, and the slope of that line was reported as the rate of swarm expansion. The correlation coefficient, r, of the data points with the line was reported. Despite the procedural difference, results of swarm studies with toothpick inocula have been fully consistent with previous data (15, 38).

Most likely, clusters of aligned cells carried on the toothpick help nucleate the swarm. In the prior experiments a few nucleating cell clusters were most likely present in the high-density liquid suspension that was subsequently diluted to the appropriate density for individual experiments. With the new protocol, bacteria grow within the stab wound and, within 1 to 2 days, glide up the side of the wound and then onto the horizontal surface of the agar, forming a nearly circular disk. Once a disk had formed on the surface, it was found to expand rather uniformly over the surface of the plate for more than 250 h. With the toothpick method, similar rates of swarm expansion were found from experiment to experiment, despite variations in the age of the culture or inoculation density (37). These variations affect the lag before the swarm begins to expand at a constant rate. Though quite reproducible for each genotype, expansion rates with the new protocol do vary with the swarming phenotype; for example, swarms with both adventurous and social motility (A+ S+) expand roughly three times faster than either A− S+ or A+ S− swarms, as had been observed with the 1983 protocol (15).

Low-angle illumination. Swarm plates were examined equipped with edge illumination (Nikon SMZ 1500). Photographs were made using a digital single-frame camera (Sony DSC-575).

Time-lapse photomicroscopy. Swarm plates were examined at various stages of their development. A section of the swarm edge was selected and observed under the microscope (Nikon Eclipse E800) using a 20× phase-contrast objective at 20°C, room temperature. Pictures were taken at 30-s intervals for 60 to 90 min. When possible, the microscope was refocused in the 25-s interval between exposures to compensate for specimen shift due to drying of the agar. Images were collected with a SPOT RT SE Monochrome 6 charge-coupled-device (CCD) camera (Diagnostic Instruments) controlled through SPOT software, version 3.5.6. Serial images were saved as QuickTime movies that could be examined frame by frame using QT Player, version 7.4.5. Particular cells or multicellular clusters were chosen from one of the time-lapse movies. Using several tools of NIH ImageJ (version 1.43g), outlines of areas covered with cells were traced and measured in terms of the numbers of pixels. There were 9,186,47 image pixels per micrometer, established from the photograph of a ruled grid of 1,400 mm². Tracks were measured with the MtrackJ plug-in. The measurements were repeated, averaged, and divided by the number of frames spanned by the movement and the time elapsed per frame (30 s) to determine the speed.

RESULTS

Swarms differ from colonies. Although both swarms and colonies can arise from a single cell and can thus be clonal, the mobility of cells within the two differs markedly. In a colony like the one shown in Fig. 1D, nonmotile cells are displaced only to the extent that they elongate and divide, with the daughters separating from each other. Figure 1A highlights the perfect radial symmetry of a swarm and the gradual drop-off in thickness from a central high plateau toward the edge. Panel B shows the swarm’s leading edge, with the swarm’s flat top dropping down to single cells and an irregular edge. The height-to-diameter ratio of the mature swarm in Fig. 1A is similar to the very young swarm shown in Fig. 1C, whose center has yet to be organized. Their ratios are much less than the corresponding ratio in the colony shown in panel D. Comparison of the swarm diameter (panel A) with the diameter of colonies of a nonmotile (A− S−) mutant of the same age (panel D) implies that most (90%) of the expansion of the swarm diameter arises from cell movement and that only about 10% arises from growth. Thus, the colony is a high-density heap of closely packed cells. In contrast, the continual movement of the swarm cells causes the swarm to spread out. The lower cell density in the swarm as well as the continuous move-
ment of the cells decreases competition between swarm cells for oxygen from the atmosphere and nutrients from the agar beneath. An important consequence is that many cells in the swarm are able to enjoy exponential growth while the very high cell density in a typical heaped up colony implies that cells inside the colony will have slowed or stopped their growth.

The relatively low cell density at the edge of a swarm (Fig. 2) arises from the continuous movement of swarm cells, for which considerable energy is expended. To justify the expenditure, is it possible that the cell movement observed is sufficient to support the exponential growth of some of the swarm cells? To consider that possibility, the growth rate of the swarm was compared with the growth rate of the same strain of M. xanthus (DK1622) measured in aerated (by rotary shaking) liquid 1% CTT medium at 20 to 21°C, room temperature, which are the same conditions used for swarm expansion on solid medium. Exponential growth with a doubling time of 7.9 h was observed in the liquid Casitone medium. It became apparent that the cells in a large, circularly symmetric swarm most favorably positioned to grow would be found within an annulus concentric with the swarm itself. That annulus would include all cells around the edge of the swarm that are at sufficiently low density and that are circulating, both conditions facilitating growth. Since exponential growth of cells within the annulus leads directly to the linear expansion of the swarm, we sought to calculate its width, \( W \), for comparison with the swarm’s density profile. If \( N \) represents the number of growing cells in the annulus, then after 7.9 h of exponential growth, \( 2^N \) cells would be spread across \( 2W \) mm of annulus. That amount of swarm growth should generate 7.9 h \( \times \) 0.13 mm/h, the measured average rate of swarm expansion, or \( W \). Solving gives a \( W \) of 520 \( \mu \)m; the inner edge of the annulus is marked in red on Fig. 1A to relate it to swarm morphology. Noting the 50-\( \mu \)m scale bar in Fig. 2 and positioning a 520-\( \mu \)m-wide annulus over the irregular morphological edge of the swarm shown in Fig. 2 reveal that it would reach farther toward the swarm center than the thick left edge visible in Fig. 2. Such an annulus would include all the low-density edge of the swarm as well as the more central parts that have small multiple (two to five) layers of cells. Compared with a noncirculating colony, circulation

FIG. 2. High-magnification, phase-contrast image showing the distribution of cells at the edge of a DK1622 swarm on 1% agar (14). The swarm is expanding in the radial direction (arrow in image), which is to the right of this small section of the swarm. Scale bar, 50 \( \mu \)m. A 20× phase-contrast objective was used. A multicellular mound with five layers and a large multicellular raft are also identified. This figure is the first frame of movie S1 in reference 37 (reprinted with permission of the publisher).
within the swarm increases the number of cells that are potentially in exponential growth; it also results in an ever-changing spatial distribution of cells.

Figure 3 shows how the diameter of a swarm increases with time after inoculation. Expansion at the characteristic steady state begins rapidly after a new swarm has been seeded with a bit of an old swarm, which has organized its cells in a way that is conducive to swarming. The swarm diameter begins to increase in direct proportion to the time and does so for more than 300 h before any slowing becomes evident. The rate of expansion is linked to their common motility genotype. The graphs of Fig. 4 show the effects on swarm expansion of deleting the Frz proteins tested in principle FrzE in the absence of any one of the three genes are under consideration, the deletion mutants tested have the same set of motility genes (33), the identity of their swarm expansion rates illustrates the robustness and accuracy of the method of measuring the rate of swarm expansion used here. The expansion rate identity suggests that the rate is linked to their common motility genotype. The graphs of Fig. 4 show the effects on swarm expansion of deleting the entire methyl-accepting chemosensory protein (amino acids 6 to 390 of FrzCD [FrzCD_{36-393}]), of deleting 148 amino acids from the N-terminal region of FrzCD (FrzCD_{36-153}), of deleting the histidine protein kinase and response regulator FrzE, of deleting the methyltransferase (FrzF), and of deleting the methyltransferase (FrzG). The rates of swarm expansion are summarized along with the measured reversal periods in Table 1. Together, the swarm expansion rate and the reversal period relate to the pattern of swarm cell movement and the basic mechanics of swarming.

Every one of the mutants tested, including those with longer or shorter reversal periods than the wild type, yields a lower rate of swarm expansion than the wild type. Moreover, all five mutants tested retained some capacity to swarm. Therefore, none of the Frz proteins tested is essential for swarming in the same way as MglA is essential for swarming. Although both the Frz proteins and the Mgl protein change the pattern of cell reversal, they divide the reversal-control pathway into two parts. The Frz part precedes Mgl, which is consistent with Frz knockout mutants retaining the ability to swarm at a low rate because their Mgl is untouched and Mgl is essential for swarming. Significantly, three of the five Frz mutants tested have identical swarming phenotypes in terms of their rates of swarm expansion and their reversal periods: all three have the same long reversal period of 34 min and a swarm expansion rate of 45 μm/h or less. The swarming efficiency (fractional rate of swarm expansion relative to the wild type) of these three mutants is 1/3 or less than that of the wild type (Table 1). In this way, these three frz genes define an Frz pathway that differs strikingly from the E. coli chemotaxis pathway. Since deletions of any one of the three genes are under consideration, the 34-min reversal period can be taken to represent the oscillation of the MglAB switch between its GDP- and GTP-bound states, without entrainment by the Frz proteins to oscillate with the (faster) 8- to 9-min period. In 2004, FrzCD, FrzF, and FrzE were proposed to form an oscillator that was responsible for traveling wave formation early in fruiting body development (12). Subsequently, the Frz oscillator was proposed to be linked to polarity reversals for (growth-dependent) swarming (16). Although both proposals had phosphorylated FrzE (FrzE−P) inhibiting the action of FrzF, in principle FrzE−P could have been stimulating the FrzG methyltransferase to achieve the same effect on the final level of FrzCD methylation as inhibition of the FrzF methyltransferase. Results of our deletion experiments support a mechanism of swarming in which the action of FrzF is maximal when the level of FrzCD methylation is lower than the wild-type level, a mild deficiency that could simply reflect a lower equilibrium level of FrzCD methylation. One straightforward-
ward molecular conclusion to be drawn from the Table 1 data is that FrzE\textsuperscript{P} inhibits the methylation of FrzCD via the FrzF methyltransferase, as diagrammed in Fig. 5. Negative feedback from the downstream to the upstream ends of the pathway from FrzCD methylation to the phosphorylation of FrzE, shown in Fig. 5, would be expected to introduce two time delays, associated with two protein modifications, and to cause the levels of methylated FrzCD (Me-FrzCD) and FrzE\textsuperscript{P} to oscillate out of phase with each other (12, 16). Were FrzCD, FrzF, and FrzE on a linear pathway, the observations would imply that each of the three

---

### TABLE 1. Swarm expansion data

<table>
<thead>
<tr>
<th>Frizzy protein deleted</th>
<th>Function or description</th>
<th>Measured swarm expansion rate (μm/h) ( r )</th>
<th>Reversal period (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Complete Frz oscillator</td>
<td>135 (0.99)</td>
<td>7.2</td>
</tr>
<tr>
<td>FrzCD\textsubscript{ΔN-303}</td>
<td>Deletes entire MCP\textsuperscript{a}</td>
<td>38 (0.99)</td>
<td>34</td>
</tr>
<tr>
<td>FrzE</td>
<td>Histidine kinase</td>
<td>45 (0.99)</td>
<td>34</td>
</tr>
<tr>
<td>FrzF</td>
<td>Methyltransferase</td>
<td>38 (0.99)</td>
<td>34</td>
</tr>
<tr>
<td>FrzG</td>
<td>Methylesterase</td>
<td>87 (0.99)</td>
<td>4.2</td>
</tr>
<tr>
<td>FrzCD\textsubscript{ΔN-153}</td>
<td>Deletes N terminus of MCP</td>
<td>16 (0.94)</td>
<td>1.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MCP, methyl-accepting chemosensory protein.

\textsuperscript{b} \( r \), correlation coefficient to least squares fit lines that are shown in Fig. 4.

---

**FIG. 4.** Expansion of swarms of the A\textsuperscript{+} S\textsuperscript{−} strain DZ2 and five of its deletion mutants. Strains are as follows: DZ4480 (A), DZ4481 (B), DZ4482 (C), DZ4483 (D), DZ4486 (E), and DZ2 (F). For swarm expansion, all strains were inoculated as described in the legend of Fig. 3.
proteins is simultaneously ahead of and behind the others. That paradox is resolved by the feedback loop. Deletion of 148 amino acids from the N terminus of FrzCD (Table 1) decreases the reversal period to 1.4 min and decreases the swarm expansion rate to 16 μm/h, which is more than any of the other mutants tested. This finding confirms the critical role that methylation of FrzCD plays in tuning the Frz oscillator (37).

**DISCUSSION**

The rapid circulation of swarm cells facilitates their growth by enhancing their access to oxygen and nutrients. Reversals of gliding direction are needed for circulation, and Frz proteins control the reversal frequency. *M. xanthus* has five Frz proteins whose amino acid sequences resemble *E. coli* chemotaxis proteins. However, there are eight such clusters of so-called chemotaxis proteins scattered about the *M. xanthus* genome (19). One, the Che3 system, had been shown in 2003 to regulate gene expression, not motility (20). Historically, the Frz system was the first of the eight clusters to be studied, and it seems to have been decided that the system was responsible for a chemotactic process, despite the lack of any N-terminal sensory domain on FrzCD, the MCP. From the results presented here, we conclude that FrzCD and two other Frz proteins, FrzE and FrzF, interact with each other to form a negative-feedback loop, as set out in Fig. 5. They are not part of a chemotactic process. Feedback causes the levels of Me-FrzCD and of FrzE−P to oscillate with a regular period of 8 to 9 min. Although regular, the Me-FrzCD and FrzE−P oscillations are out of phase with each other. Because deletion mutants that lack either FrzCD, FrzF, or FrzE protein, and consequently are not able to oscillate, are nevertheless able to swarm, none of these proteins is essential for swarming. The FrzCD, FrzF, and FrzE proteins do regulate the rate of swarm expansion, however. They set the period of oscillation to a value different from its optimum that is determined by the average speed of gliding. The oscillation period, which is tuned sharply by the extent of methylation of FrzCD, is optimized at 8 to 9 min.

By computing with their swarm model, Wu et al. showed (i) that reversals were essential for swarm expansion and (ii) that mutants with either shorter or longer periods than the 8- to 9-min optimum exhibit lower rates of swarm expansion than the wild type (A+ S+) (37). Remarkably, no single-step mutant has ever been isolated from *M. xanthus* that glides in only one direction, despite the many hundreds of motility mutants isolated and characterized since the first nonmotile mutant was described (4). If swarm expansion were the result of chemotaxis, a unidirectional mutant that adopted new directions by bending might well have been found. In addition, we conclude that methylation of FrzCD tunes the Frz oscillator, giving it a reversal period that maximizes the rate of swarm expansion for cells that move at the *M. xanthus* average rate of 4 μm/min (37). Since the wild type has an 8- to 9-min period, Wu et al. concluded that the period has been tuned by natural selection. Such an evolutionary origin would explain why DZ2 and DK1622 have, within experimental error, the same reversal frequencies and the same rates of swarm expansion, as shown in Fig. 3 and 4. Swarm growth, which provides the pressure for expansion outward, seems to take place in an annulus that includes the entire edge of the swarm and extends a bit into the swarm center, where the cell density is high. Growth is essential for swarming, and swarming stops when the cells are no longer able to grow; instead, they develop fruiting bodies. Several hundreds of hours of exponential growth on petri dishes are made possible by the continuous cell movement found at the swarm edge. Circulation gives cells access to O2 from the atmosphere and other nutrients from the agar substratum. Both nutrients are likely to be limited in the plateau region of the swarm because the cells are competing with those above and below them; circulation mitigates the competition among cells that are heaped on top of each other.

Mutants lacking the MglA protein are the only motility mutants reported thus far that completely lack the ability to swarm (10, 11, 17). When there is no MglB protein, the cells are motile part of the time (38) because the protein stabilizes the MglA protein (8). The mglA gene encodes a small Ras-like protein with a G-loop, and mutations within the G-loop prevent swarming (7, 36), strongly suggesting that MglA switches between GDP-bound and GTP-bound states (3). Recently, Leonardy et al. have provided biochemical evidence that MglB is the cognate GAP protein for MglA G-protein that stimulates its GTPase activity (22). Leonardy et al. also provided evidence that Frz proteins lie upstream of the MglAB switch to govern the frequency of switching (22), consistent with the swarming data presented here. Although the existence of a connection between pulses of FrzE−P and MglAB can be inferred from much of the data, including that presented here, the molecules that make the connection remain to be identified. The connection could be direct, as diagrammed in Fig. 5, for simplicity. Or the pulsing of FrzE−P could be conveyed to MglAB by a dedicated linking protein that is activated when a certain threshold level of FrzE−P is reached. Since FrzE protein has both a kinase/response regulator domain and an additional CheY domain, the FrzE protein itself might, in theory, be able to sense the threshold. Alternatively, the connection might involve either FrzZ that has two CheY domains and is thought to serve as an output component of the Frz system (13) or some other protein. Whatever the link’s identity, Leonardy et al. have shown that the dynamic polar localization of RomR, which is required for A motility, depends on the Mgl switch (21, 22). They also showed that the dynamic polar localization

![FIG. 5. Feedback-induced oscillator that drives the MglAB reversal switch as demonstrated by the phenotypes of gene deletion mutants. Arrows indicate reactions; the action of the FrzF methyltransferase is inhibited (+) by FrzE−P. FrzCD and FrzE constitute a two-component system. A high level of FrzE−P activates the formation of MglA-GTP which causes both the A and the S engines to switch from one cell end to the other.](http://jb.asm.org/)

---

VOL. 193, 2011 REVERSING CELL POLARITY IN SWARMS 5903

Published by the American Society for Microbiology. All rights reserved.

Downloaded from http://jb.asm.org/ on August 29, 2017 by guest
of PiII, which is required for the type IV pilus engines (26), is also downstream of the MglAB switch (22). Together with the Frz oscillator data described above, the data of Leonardy et al. support the circuit diagrammed in Fig. 5. Wu et al. (37) proposed that, with selection for growth, the pacemaker evolved to drive GDP/GTP oscillations to a period of 8 to 9 min that would maximize the swarm expansion rate.

In conclusion, we have linked reversals to the growth of a swarm. Using in-frame deletion mutants, we have shown that loss of FrzCD, FrzF, or FrzE produces quantitatively the same phenotype. With a sensitive swarm expansion assay, the three mutants expand their swarms at the same rates while deletion of FrzG has a much smaller effect. These results are incompatible with the published chemotaxis circuit that includes FrzG, among other Frz proteins. Rather, they support the hypothesis that FrzCD, FrzF, and FrzE constitute a pacemaker, which drives the MglAB reversal switch and organizes the cells appropriately for swarming.

ACKNOWLEDGMENTS

We are grateful to David Zusman and Emilia Mauriello for providing M. xanthus DZ2 and several in-frame frz deletion mutants.

REFERENCES