Pattern formation: fruiting body morphogenesis in Myxococcus xanthus

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Pattern formation: fruiting body morphogenesis in *Myxococcus xanthus*
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When *Myxococcus xanthus* cells are exposed to starvation, they respond with dramatic behavioral changes. The expansive swarming behavior stops and the cells begin to aggregate into multicellular fruiting bodies. The cell-surface-associated C-signal has been identified as the signal that induces aggregation. Recently, several of the components in the C-signal transduction pathway have been identified and behavioral analyses are beginning to reveal how the C-signal modulates cell behavior. Together, these findings provide a framework for understanding how a cell-surface-associated morphogen induces pattern formation.

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Abbreviations
A-motility system adventurous motility system
GFP green fluorescent protein
h hours
S-motility system social motility system

Introduction
A fundamental issue in developmental biology is how pattern formation is accomplished. Pattern formation results in the spatial organization of cells initially in a uniform suspension or a sheet of cells. Different types of pattern formation are observed in prokaryotic systems. In its simplest form, pattern formation occurs during chemotaxis of swimming bacteria [1]. A more elaborate example is the one-dimensionally patterned formation of heterocysts in filaments of cyanobacteria such as *Anabaena* [2]. In its most elaborate form, cells are positioned in highly complex three-dimensional structures, such as fruiting bodies, in myxobacteria and biofilms [3–5].

Biofilms and fruiting bodies have two features in common: they are formed on solid surfaces in response to external cues and self-generated intercellular signals, and in both structures, the cells are embedded in exopolysaccharide. However, despite these similarities, the mechanisms of pattern formation in these systems are different. Fruiting body formation depends on temporally- and spatially-regulated, organized cell movements. Although cell motility is important for biofilm formation, cell divisions probably also play a role in shaping the pillar- and mushroom-like structures in biofilms. In this review, we discuss how the C-signal, a cell-surface-associated morphogen, regulates *Myxococcus xanthus* cell movements to facilitate fruiting body formation.

Organized cell movements in *Myxococcus xanthus*

*M. xanthus* cells move by gliding, a process in which a non-flagellated cell moves in the direction of its long axis on a solid surface [6]. Gliding in *M. xanthus* is regulated by two genetically separable systems known as the adventurous (A) and social (S)-motility systems, which regulate the movement of isolated cells and groups of cells, respectively. Mutations in A- or S-motility genes inactivate the corresponding system, but the cells are still motile by means of the other system [7]. Components in A- and S-motility systems are currently being identified. However, the mechanism underlying gliding motility remains unknown [8•,9•].

At a high cell density, *M. xanthus* cells can exhibit three types of organized movements, depending on the nutritional conditions: swarming, aggregation and rippling. In the presence of ample nutrients, cells swarm and colonize new areas. When cells at a high density experience starvation, swarming behavior is decreased and cells migrate towards aggregation centers. Initially, these centers are small and asymmetric, but as more cells gather over the next 24 hours (h), they become hemispherical mounds each containing up to 100,000 cells. Inside the mounds, the rod-shaped motile cells differentiate into nonmotile spores, resulting in the formation of the mature fruiting bodies (Figure 1b). Hence, aggregation centers are dynamic multicellular structures, whereas mature fruiting bodies are static, stable, multicellular structures. Rippling precedes and overlaps the aggregation stage of fruiting body formation. During rippling, cells organize into ridge-like structures that move rhythmically over the substrate in a pattern resembling traveling waves (Figure 1a). Thus, ripples are dynamic, transient, multicellular structures.

The C-signal – a key regulator of pattern formation

*M. xanthus* cells are often observed to move into the aggregation centers as streams or chains that move in a spiral pattern [10–12]. Moreover, cells often travel long distances to enter a distant aggregation center rather than entering one close by. This type of behavior seems to contradict the idea that aggregation occurs in response to a diffusible signal molecule that is emitted from the aggregation centers. Several *M. xanthus*-produced compounds have been shown to modulate the behavior of *M. xanthus* cells [13–17,18**]. Among these compounds, the cell surface-associated C-signal is the key candidate for the aggregation-inducing signal.
At least five intercellular signals, known as the A-, B-, C-, D-, and E-signals, are required for fruiting body morphogenesis [19–24]. The C-signal is the latest-acting of these signals, and becomes important for progression of the developmental program after 6 h of starvation [21]. The csgA gene is required for the synthesis of the C-signal [25] and csgA mutants are deficient in rippling, aggregation, sporulation, and developmental gene expression after 6 h [21, 25, 26]. The C-signal was purified from developing wild-type cells on the basis of its ability to restore development of csgA-mutant cells and shown to be a 17 kDa protein, the C-factor protein [27]. C-factor is encoded by csgA and is cell-surface-associated [27, 28]. Overexpression of the csgA gene during development induces premature aggregation, sporulation and C-signal-dependent gene expression, and the rippling stage is bypassed (T Kruse, S Lobedanz, N Berthelsen, L Søgaard-Andersen, unpublished data). Therefore, the C-signal not only is required for four developmental responses, but also appears to directly induce these responses.

### The C-signal-dependent developmental program

**A quantitative model**

How does a single signal induce different morphogenetic responses? The C-signal-dependent responses have different quantitative requirements for C-factor: low, intermediate and high levels of C-signaling induce rippling, aggregation and sporulation, respectively [29, 30]. C-signaling levels are temporally and spatially regulated by two mechanisms, which both result in signal amplification (Figure 2, upper panel). Firstly, C-signal transmission occurs by a contact-dependent mechanism [27, 31]. Therefore, aggregation per se is predicted to result in an increase in the level of C-signaling. Secondly, C-signal transmission is required for full expression of csgA and, consequently, C-factor accumulation [30]. The activity thresholds together with the regulated C-factor accumulation and the coupling between cell position and C-signaling levels have two consequences. Firstly, they allow the C-signal to act as a timer that induces rippling, aggregation and sporulation in the correct sequence. Secondly, they convey positional information to the cells, ensuring the spatial coupling between aggregation and sporulation (see also Update).

**A qualitative model**

The C-signal uses a branched signal transduction pathway to induce morphogenesis and gene expression (Figure 2, lower panel). Little is known about the C-signal transmission event except that it occurs by a contact-dependent mechanism and requires that both cells are motile [27, 31–33]. The requirement for motility is bypassed by forcing cells to align in a pattern that favors end-to-end contacts, suggesting that C-signal transmission takes place at the cell poles and that active cell movements are required to establish these critical contacts [31]. Although the polar localization of C-factor has yet to be shown directly, the polar localization of the *M. xanthus* MbhA protein [34] and type IV pili [35], and the polar activity of the Tgl protein [36] suggest that functional asymmetry is a general feature of the rod-shaped *M. xanthus* cell.

The DNA-binding response regulator FruA has a key position in the pathway [37]. Genetic evidence suggests that C-signaling activates FruA post-translationally — conceivably by activating a histidine protein kinase that phosphorylates FruA — to interact with its downstream targets [37]. Downstream from FruA, the response pathway contains two branches [38]. One branch leads to rippling and aggregation; the proteins in the cytoplasmic Frz signal transduction system are components in this branch [38]. These proteins are involved in the control of directed cell movements and share homology with proteins involved in chemotactic responses in other bacteria [9•]. The C-signal excites the Frz system in a FruA dependent manner and induces methylation of the FrzCD protein [39], a methyl-accepting chemotaxis-protein homolog [40]. The second branch leads to sporulation. In this branch, FruA and C-signal interact to promote transcription of the devRS genes [21, 37]. *devRS*, in turn, are required for the expression of the sporulation gene tagged by the Tn5 lac Ω7536 insertion [41]. The third branch in the C-signal pathway is located upstream from FruA and leads to csgA expression. Recent results suggest that the actI gene product, which shares homology to sigma (σ)-54 activator proteins, is involved in this positive feedback loop [42•].
C-signaling and behavior

A single cell approach

How does the C-signal modulate cell behavior? To address this question we used a motility assay in which cells were starved at a high cell density under starvation conditions that did not allow rippling. Subsequently, cell behavior was followed using time-lapse video microscopy at a low cell density that did not allow cell–cell interactions [18••]. After 6 h, coincidently with the initiation of C-signaling, wild-type cells began to move with increased gliding speeds and in longer gliding intervals, with reduced stop frequencies and with unchanged duration of stop intervals. In contrast, csgA-mutant cells only displayed very limited movements even after 15 h of starvation. However, the motility defects in csgA-mutant cells were corrected by exogenous C-factor protein. This stimulatory effect of the C-signal on cell motility was dependent on the Frz system. Because stimulation occurred in the absence of cell–cell interactions, C-factor directly induces this altered behavior.

A population approach

Fruiting body formation depends on starvation of cells at a high cell density. Therefore, the single-cell motility assay was clearly a reductionist approach to analyze a complex system. To score the full effect of the C-signal on cell behavior, we mixed cells expressing green fluorescent protein (GFP) with nonfluorescent cells at a ratio of 1:400, and then exposed this mixture of cells to starvation (L. Jelsbak, RD Welch, D Kaiser, L Søgaard-Andersen, unpublished data). Using fluorescence time-lapse video microscopy, the behavior of individual GFP-expressing cells was monitored during development. To simplify matters, cells were starved under conditions that did not allow rippling.

In this assay, wild-type cells expressing GFP that were mixed with nonfluorescent wild-type cells displayed an increase in gliding speed and a decrease in reversal frequency after 6 h of starvation, coincident with the initiation of C-signaling. In contrast, csgA-mutant cells expressing GFP mixed with nonfluorescent csgA-mutant cells only experienced a moderate increase in gliding speed during development and the reversal frequency remained unchanged between 0 and 15 h of starvation. Importantly, csgA-mutant cells expressing GFP mixed with nonfluorescent wild-type cells displayed the increased gliding speed and the decreased reversal frequency characteristic of wild-type cells after 6 h. Wild-type cells expressing GFP that were mixed with nonfluorescent csgA-mutant cells displayed a behavior similar to that of csgA-mutant cells mixed with csgA-mutant cells. Therefore, at high cell density, the C-signal induces an increase in gliding speed and a decrease in reversal frequency. The low reversal frequency is only induced at the high cell density, suggesting that other cell–cell interactions are...
required for this effect. A clue to the nature of these cell–cell interactions comes from the observation that the reversal frequency of starving cells correlates inversely with the cell density if the S-motility system is intact [43].

As mentioned before, the C-signal induces methylation of the FrzCD protein [39]. Methylation of FrzCD is thought to be part of an adaptative response to stimulation that regulates the reversal frequency [44,45]. Therefore, the C-signal may only induce a transient decrease in the reversal frequency followed by a return to the higher frequency. However, adaptation may not be observed in the high cell density motility assay because developing cells are continuously exposed to increasing levels of C-signaling.

**C-signal-induced aggregation: think globally, act locally**

At high cell density, the effect of C-signaling on cell behavior is an increase in the distance travelled by a cell per minute. This property is clearly beneficial during aggregation. However, in order for cells to aggregate, they must also move in an appropriate direction. The C-signal transmission mechanism provides a clue to how the C-signal may induce directional cell movements [39].

If C-signal transmission occurs by an end-to-end contact between cells, then the C-signal may induce aggregation as described in the following sentences. In a field of cells, C-signaling is predicted to begin in areas with a high cell density (due to the contact-dependent signal transmission mechanism) and, thus, these areas become aggregation centers. If a cell located in the periphery of a center (cell A in Figure 3a) makes end-to-end contact with C-signal transmission to a cell outside the aggregation center (cell B in Figure 3a), cell B is induced to move with a high speed and a low reversal frequency in the direction of cell A, as long as it maintains contact with cell A. By the same mechanism, cell B may recruit cell C to the chain. Eventually, a moving chain of cells is formed that travels rapidly towards an aggregation center. With time, most cells in a field become organized in chains that are moving towards aggregation centers. Cells that have yet to be recruited to a chain only display limited net movement because they move with low speeds and high reversal frequencies.

Cells in these chains are predicted to be exposed to increasing levels of C-signaling because of the two C-signal amplification loops (Figure 2). The increasing levels of C-signaling may ensure that cells not only experience a transient decrease in their reversal frequency after they have been recruited to a chain, but also continue to move with a low reversal frequency as long as they are in a chain. Once the cells have entered an aggregation center, they continue to move with a low reversal frequency [10] until sporulation is initiated, bringing an end to active gliding movements.

The model for C-signal dependent aggregation suggests that the streams and chains of cells often observed moving towards aggregation centers are a consequence of the mechanism by which cells are recruited to aggregate. The spiraling movements of these streams and chains remain enigmatic. The observation that cells often do not enter the closest aggregation center can be rationalized as described in the following sentences. The direction of movement of a cell in a chain is determined, and conveyed from cell to cell by the direction of movement of the cell at the leading end of the chain. Thus,
once a cell enters a chain, it follows the chain even if it passes an aggregation center. In this model, C-signal transmission is a local event occurring between two cell poles, and the result is a global organization of cells from a disordered field into chains in which the cells move towards aggregation centers (Figure 3b).

Why has M. xanthus adopted this unusual pattern-forming mechanism? M. xanthus is a rather slow-moving organism with an average speed of 5 µm/min for cells in contact with each other [46]. Therefore, the directive properties of a diffusible signal might disperse before the cells can orient in a gradient of the signal [47].

Conclusions
The recent work on M. xanthus fruiting body formation has provided us with models for how the C-signal may coordinate morphogenesis temporally and spatially, and for how the C-signal may act as an organizing signal to induce aggregation. The next challenge will be to understand at the molecular level how different levels of C-signaling are transformed into different responses. Moreover, progress in understanding the functioning of the A- and S-motility systems will be invaluable for a deeper understanding of how C-signaling modulates cell behavior.

Update
The spatial regulation of the C-signaling level was recently illustrated very elegantly using gene fusions between developmentally regulated genes and the gene encoding the GFP [49••]. After 24 h of starvation, fusions between C-signal-independent genes and gfp were expressed independently of cell position and cell density, whereas fusions between C-signal-dependent genes and gfp were expressed in a position-dependent manner. Specifically, the C-signal-dependent fusions were only expressed by cells at a high cell density inside mounds. Thus, cells that have been starved for the same period of time display different expression profiles of C-signal-dependent genes, depending on their cell density.

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