Pattern formation and traveling waves in myxobacteria: Theory and modeling

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Recent experiments have provided new quantitative measurements of the rippling phenomenon in fields of developing myxobacteria cells. These measurements have enabled us to develop a mathematical model for the ripple phenomenon on the basis of the biochemistry of the C-signaling system, whereby individuals signal by direct cell contact. The model quantitatively reproduces all of the experimental observations and illustrates how intracellular dynamics, contact-mediated intercellular communication, and cell motility can coordinate to produce collective behavior. This pattern of waves is qualitatively different from that observed in other social organisms, especially Dictyostelium discoideum, which depend on diffusible morphogens.

Myxobacteria are common components of soil, but their life cycle is far from common. Although they are prokaryotes, their life, in some respects, is similar to that of multicellular organisms (1, 2). Under starvation conditions, a population of myxobacterial cells aggregates by streaming into a number of central foci, eventually forming at the focus a multicellular fruiting body. During this aggregation phase, the cells may pass through a period where the surface is swept by a complex pattern of waves, called the “ripple phase.” These waves are composed of bacteria moving in concert in such a way that colliding waves appear to pass through one another (3). This is quite unlike the seemingly similar phenomenon observed in Dictyostelium discoideum and in chemical waves where colliding wave fronts annihilate one another (4, 5). Here we present a quantitative model for the ripple phase in Myxococcus xanthus that reproduces most of the observed phenomena. A distinguishing feature of this model is that it depends only on intercellular communication by direct cell contact, without any diffusible morphogen signaling.

We shall base our model on the following consequences of experimental observations on Myxobacteria.

(i) Contact Signaling. Myxobacteria signal via the C-signaling system, which operates only when two cells contact one another nearly end to end (3, 6, 7). The ripple patterns can be altered significantly, or even abolished, by manipulation of external C-signal protein concentration or dilution of wild-type cells by mutants that can receive, but not send, C-signal (3). Therefore, we shall base the model on signaling that depends entirely on direct cell contacts, with no diffusible signaling molecule.

(ii) Reversal Cycle. Experiments on individual prerippling bacteria under various conditions show that they glide back and forth, reversing their direction spontaneously about every 5–10 min with a variance much smaller than the mean (see table 1 of ref. 8 and table 2 of ref. 9). Thus the times between reversals are not exponentially distributed, i.e., not Markovian. We interpret this to mean that the internal biochemical circuit controlling reversals contains a delay or cycle time for completion.

(iii) Density Dependence. Measurements show that reversal frequencies depend on the amount of C-signal protein (10–12). Thus cells in a population where C-signaling is transmitted by cell collisions will alter their reversal frequencies in a density-dependent fashion. We will show that this density dependence is nonlinear, indicating a cooperative aspect of C-signaling.

(iv) Refractory Period. The D. discoideum signaling system passes through a “refractory period” after a response to an external signal during which it is insensitive to subsequent signals. This property is essential to its ability to propagate waves. However, there is currently no direct evidence that, after reversal of direction, the C-signaling system passes through a similar refractory period, although there are indications in the reversal histogram for prepping cells measured by Welch and Kaiser that show very few reversals at short times (see figure 4 of ref. 10). We shall demonstrate that a refractory period is necessary for the production of ripple waves and discuss possible experiments required to estimate its length.

We incorporate these properties into a mathematical model as follows.

A Model for the Ripple Phase in Myxobacteria

The motion of a single bacterium in the (x, y) plane can be described by the stochastic equation of motion:

\[
\frac{dx}{dt} = \pm v + r(t),
\]

where \(v = (\pm v_x, \pm v_y)\) are the instantaneous velocities in the x and y directions, \((\pm)\) indicates that individual bacteria glide along their long axes and change their direction by simple reversals rather than turning. \(r(t) = (r_x(t), r_y(t))\) are random terms modeling the variance in individual speeds.

To describe the cyclic internal biochemical state, we define a periodic “phase” variable, \(0 \leq \phi \leq 2\pi\), which locates the state of the C-signal-controlled reversal system in its cycle. We can picture this cycle as shown in Fig. 1, where we have plotted the phase on a circle: \(0 \leq \phi < \pi\) corresponds to right-moving cells, and \(\pi < \phi < 2\pi\) corresponds to left-moving cells. A cell's state can be pictured as a point moving counterclockwise around the circle at a mean speed \(\omega\) (see Fig. 1a). Each cell instantly reverses its direction as it crosses \(\phi = 0, \pi\). For an individual cell, \(d\phi/dt\) advances at a rate given by

\[
\frac{d\phi}{dt} = \omega C_r + r_\phi(t)\]

Here \(\omega C_r\) is the phase velocity, which is affected by C-signaling because of collisions with other bacteria. As we discuss below, the C-signaling intensity, and thus the phase velocity, depends on

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†A model representing the biochemical cycle by a delay, or functional, kinetic equation yields similar results to the model presented here. However, the explicit representation of the C-signal biochemistry by a phase variable is simpler and makes contact with a substantial literature on phase-coupled systems (14).

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density of its neighbors (3, 6).** We describe the motion of a population of bacteria by using the density function \(n(t, x, y, \theta, \phi)\), giving the number of bacteria at time \(t\), position \((x, y)\), moving in the direction \(\theta = \tan^{-1}(v_x/v_y)\), with phase \(\phi\). The density function \(n(t, x, y, \theta, \phi)\) obeys the general conservation law (i.e., the Fokker–Planck equation for Eqs. 1 and 2 of the form

\[
\frac{\partial n}{\partial t} = -\nabla \cdot \mathbf{J},
\]

where \(\mathbf{J}\) comprises the spatial, angular, and phase flux of individuals in \((x, y, \theta, \phi)\) space.

We can simplify considerably this description by taking advantage of the observation that individual cells participating in ripple-phase waves are aligned in the direction of the wave propagation (3, 10). Therefore, we can assume that the cells are aligned parallel to the \(x\) axis and so glide only in \(\pm x\) direction, although they may drift randomly in both \(x\) and \(y\) directions. This eliminates the angular variable, \(\theta\), so that the density equation for \(n(x, y, \phi)\) is:

\[
\frac{\partial n}{\partial t} = -\frac{\partial}{\partial x} \left(-D_x \frac{\partial n}{\partial x} + v_x n\right) - \frac{\partial}{\partial y} \left(-D_y \frac{\partial n}{\partial y} + v_y n\right)
\]

\[
- \frac{\partial}{\partial \phi} \left(-D_\phi \frac{\partial n}{\partial \phi} + \omega \cdot n\right),
\]

where \(\omega\) refers to right- (+) or left- (−) moving cells [i.e., \(\pm 1 = \text{sign} (\pi - \phi)\)]. \(D_x\) is the effective diffusion coefficient caused by the variance in the gliding speed, \(D_y\) is the diffusive flux of cells in the \(y\) direction, and \(D_\phi\) is the effective diffusion coefficient in the biochemical phase space. C-signaling takes place only between cells that meet head-on moving in the opposite direction, so we will need to compute the local spatial density of right- and left-moving cells by integrating the density, \(n(t, x, \phi)\), over \(\phi\):

\[
n_+(t, x) = \int_0^{\pi} n(t, x, \phi)d\phi, \quad n_-(t, x) = \int_{\pi}^{2\pi} n(t, x, \phi)d\phi.
\]

To complete the model, we must specify the dependence of cell reversals on the C-signaling intensity via the local population density; that is, an expression for \(\omega_\pm\) in Eq. 2. For cells in their sensitive phase, the frequency of reversals increases monotonically with the density of opposite moving cells, so that \(\omega_\pm\) must be a function of \(n_\pm\) computed from Eq. 4: \(\omega_\pm(n_\pm)\). This density dependence must ultimately saturate; thus, a convenient mathematical representation is given by the Hill function shown in Fig. 1b:11

\[
\omega_\pm(x, \phi, n, q) = \omega_0 + \omega_\infty \left(\frac{n_\pm}{n_\pm^* + n_\infty}\right)^q F(\phi),
\]

where \(F(\phi) = \{0 \text{ for } \phi \in \Delta \phi_R, 1 \text{ for } \phi \in \Delta \phi_S\}^*\)

**In this model, the C-signaling system behaves in some respects analogous to an "integrate and fire" neuron (13).

*Eq. 5 is a simple and convenient representation for the density dependence of the phase velocity. We will use it, because little is known about how the biochemical phases of the reversal cycle is affected by C-signaling protein. In the supporting information on the PNAS website (www.pnas.org), we discuss modifications of Eq. 5 to address the mutant dilution experiments of Sager and Kaiser (3). However, we show that the results of our simulations are not sensitive to this modification.
Fig. 2. (a) A space–time plot showing a train of right-moving waves (crests are black, troughs white). See Movies 1–3, which are published as supporting information on the PNAS web site. (b) Using Eqs. 1 and 2, one can follow a material point equivalent to a single bacterium as it participates in a wave train moving to the right. The trajectory of a bacterium moves to the right with the wave crest 1→2, then reverses and travels to the left through the trough to the top of the next wave crest, 2→3, then reverses again and travels rightward with the crest 3→4. The dashed portion indicates the refractory period. Because a cell encounters fewer collisions (C-signals) in troughs, the time between reversals is longer when the cell is moving in the troughs against the direction of the wave train. Therefore, there is a slow drift to the left, against the direction of wave propagation. (c) Distributions of right-moving (solid) and left-moving cells (dashed) in a unidirectional wave train moving to the right with velocity v (computed from Eq. 4). Cells alternate between crests with a slow drift to the left: \( v_{\text{drift}} = v (\tau_+ - \tau_-) / (\tau_+ + \tau_-) \), where \( \tau_+ \) is the reversal period in the right- (+) and left- (−) moving wave trains. The wavy line traces the orbit of a single material point (bacterium). Note that the left-going density, \( n \), is nearly constant at its mean value, whereas the amplitude of \( n \) is periodic and large.

Here \( \omega_0 \) is the phase velocity of a single cell in the absence of other cells, and \( \omega_\phi \) is the increase in the phase velocity for a cell in its sensitive period, \( \Delta \phi_S \), when the local cell density is \( n \). Thus the second term in Eq. 5 gives gliding cells a bigger probability of reversing in regions where there are many opposite-moving cells. At small densities, \( \omega \) increases as the \( q^{th} \) power of density; therefore, this exponent characterizes the cooperativity of the signal processing or transduction; \( q = 1 \) corresponds to the case where reversals of a nonrefractory cells increase in proportion to collisions. It turns out that only values of \( q \geq 3 \) can produce rippling, so that the ripple phase is a collective phenomenon. Note that Eq. 5 says that the phase velocity depends only on the local cell density, i.e., we have not introduced any diffusible morphogen analogous to “quorum sensing” in bacteria with diffusible signals (15, 16).

Results
The mean field models described by Eqs. 3 and 5 are solved numerically to produce the characteristic waves observed in the ripple phase. Using the density \( n \) to calculate the phase velocity, we can then solve the stochastic models Eqs. 1 and 2 to illustrate the path of individual cells. With parameters estimated directly from the experiments of Welch and Kaiser (10), we performed parameter scans to determine the range that supported rippling. These are explained in the supporting information on the PNAS web site, www.pnas.org. The analysis presented supports the numerical parameter ranges. Thus rippling is not a robust property of bacterial aggregation and so may provide insight into the properties of the C-signaling system. The numerical simulation procedure based on refs. 17–19 is also described in the supporting information. Movies illustrating the various phenomena can be downloaded from the PNAS web site with the supporting information.

Unidirectional Wave Propagation. The principles underlying the ripple phase can best be illustrated by examining one-dimensional unidirectional wave propagation obtained by setting in Eq. 3 all properties constant in the \( y \) direction and fixed concentration boundary conditions in the \( x \) direction. The waves are best appreciated via the movies; however, a static representation can be illustrated in the space–time plots shown in Fig. 2 (see Movie 1, which is published as supporting information on the PNAS web site). Fig. 2a shows that the solution to Eq. 3 generates a unidirectional wave train. The wave amplitude is such that the density of bacteria in a ripple crest is up to 10 times that in the trough, depending on the ratio of convective and diffusive fluxes in Eq. 3. In the supporting information, we show that the wavelength, \( \lambda \), is approximately

\[ \lambda \sim 2v\tau, \]

where \( v \) is the mean gliding speed and \( \tau \) the mean period between reversals in a trough (where C-signaling intensity is weakest). The reversal time in the trough \( \sim 4.2 \) min, as estimated from the right peak of the histogram in Welch and Kaiser (10). The wave speed is \( -11 \) \( \mu \)m/min, about the same as individual velocity. The resulting wavelength is \( \sim 90 \) \( \mu \)m, in agreement with table 1 in ref. 10.

The necessary condition for wave propagation is given by a parameter inequality:

\[ \frac{q}{4} \frac{\omega_\phi \omega_0}{(\omega_\phi + \omega_0)^2} > \frac{(\Delta x)^2}{2v\tau} \]

(see Eq. 14, which is published as supporting information on the PNAS web site, www.pnas.org). This ensures that the focusing effect of density-dependent reversals that speed the cell to its refractory period can compensate for the drift to maintain the wave train. Here \( (\Delta x)^2 \) is the variance in position caused by the drift in velocity and phase.

Individual Cell Behavior in Unidirectional Waves. Welch and Kaiser also tracked the paths of individuals participating in the ripple phase (see Fig. 4 and accompanying movies in ref. 10). We can
imitate their single-cell tracking experiments by superimposing the trajectories of single bacteria, computed by using the stochastic Eqs. 1 and 2, onto the solution of the density Eq. 3. First, consider individuals from a train of right-moving waves; Movie 1 illustrates the motions dynamically. A static picture is shown in Fig. 2b, where the motion of a single cell is traced on a space–time plot superimposed on a unidirectional wave train. Refractory and sensitive periods are shown dashed and solid, respectively. The waves are generated by cells that oscillate back and forth, corresponding to right angle turns in Fig. 2b. In the crests, more cells are moving in the same direction as the crests (1→2), while in the troughs more cells are moving against the waves (2→3).

When a cell reverses in a crest, it is refractory to signals from oncoming cells and can therefore penetrate the wave and glide against the wave velocity without turning. By the time the cell emerges from its refractory period, it is in the trough, where it encounters few reversal signals, and so it reaches the previous crest in the wave train about the time that its phase is close to its reversal point. Entering the wave, the cell encounters increasing interactions, which advances its phase, so it quickly reverses again, completing the cycle. Thus, wave crests constantly lose cells to reversals but gain new ones from the crest ahead.

Cells entering the wave crest and reversing go into their refractory period, where their phase velocity is at its minimum, which tends to populate the refractory sector, thus synchronizing the cells’ phases. This synchronization maintains the ripple’s stability (a quantitative version of this argument is presented in the supporting information on the PNAS web site). The slow drift of cells in the direction opposite the direction of the wave train (see Fig. 2b) can be understood from Fig. 2c, which plots the density of right- and left-going cells. In a unidirectional wave train, the concentration of left-going cells in the right-going crests is much larger than the concentration of right-going cells in the trough. Therefore, less C-signaling events are received while moving in the trough, so the average time a cell spends there is longer, resulting in a bimodal reversal frequency distribution, as shown in Fig. 4.

**Interpenetrating Waves.** A feature of the ripples that distinguishes them from other developmental waves is that colliding waves do not annihilate but appear to pass through one another (Movie 2a, which is published as supporting information on the PNAS web site). When two wave fronts collide, cells in their sensitive phase increase their reversal frequency because of increased collisions, whereas cells in their refractory phase continue unaffected. Consequently, the outgoing waves consist of a combination of individuals from both incoming waves, as shown in Fig. 3b. This gives the appearance of waves passing through one another, analogous to soliton water waves (ref. 20; see also ref. 21). Movie 2b shows the computed behavior of individual bacteria superimposed on the wave pattern.

Tracing individuals in two counterpropagating wave trains produces a different picture from unidirectional waves (see Movie 2c). Here most of the cells are caught in crests, moving with the right-going crest to the right and with the left-going crest to the left. Cells switch their allegiance from one crest to another when two crests collide, confirming the interpretation of Sager and Kaiser and of Welch and Kaiser that, although wave crests appear to pass through one another, most of the individual cells reverse (3, 10). There is an obvious symmetry between right- and left-going cells, and therefore no drift (net transport of bacteria).
occurs in this situation. Fig. 3c plots the density of right- and left-going cells in a colliding wave train. Most of the time, collisions between oppositely moving cells in the troughs are spatially separated so that C-signaling levels are low. Therefore, even though most of the cells move in crests all the time, their reversal time distribution in Fig. 4 is centered near the “trough” peak of the unidirectional wave. A quantitative analysis demonstrating the stability of the two-dimensional waves is given in the supporting information on the PNAS web site. We will see below when we treat the two-dimensional case that the bimodal reversal frequency distribution measured in figure 7 of Welch and Kaiser (10) can be interpreted as an average of the reversal histograms in Fig. 4.

The Pattern of Cell Reversals. Bi- and unidirectional waves show two distinct types of individual behaviors. Cells in bidirectional waves (“wave alternators”) tend to move within the crest all the time, switching wave trains when they collide. The cells in unidirectional waves tend either to move with the wave in the direction of wave propagation (“crest riders”) or to travel in a trough while going the opposite way (“trough travelers”). Trough travelers (Fig. 2c) are not greatly influenced by C-signaling, because they move in regions with few counterpropagating cells. Similarly, crest alternators (Fig. 3c) are not influenced much by C-signaling, because they spend most of their time “moving with the crowd,” and by the time they encounter a wave collision, they are nearly ready to reverse on their own. Therefore, for both trough travelers and wave alternators, the reversal time is close to τ−τ/ω₀, the time that determines the wavelength (λ = 2π/τ) for both uni- and bidirectional wave trains. The situation is different for crest riders (Fig. 2c), which are constantly advanced in phase as they collide with counterpropagating cells, so their reversal time is much smaller. In the reversal distributions and individual trajectories of Welch and Kaiser, all cell types are represented. Each cell can move for some time with one of the other wave and then commence alternating between two wave crests (10). The bimodal reversal distribution arises as follows. Even though both alternators and crest riders travel in the crests, the alternators arrive at the point of wave collision being ready to reverse, so they are not much affected by C-signaling, i.e., they reverse spontaneously and glide with the oppositely moving crest. However, the “crest riders” are probably not ready to reverse at the collision point, but the C-signaling dose they get during the collision significantly advances them in their cycle, resulting in shorter reversal times for crest riders.

Finally, it is worth noting that the phases of cells participating in a unidirectional wave train are not spatially synchronized: in any volume element, phases of the cells traveling in the same direction are uncorrelated. In contrast, cells participating in two colliding wave trains do have their phases spatially synchronized, so that a cohort traveling together in a crest will also have nearby phases.

Initiation of Waves. When cell density exceeds a critical value, a uniform field becomes unstable: if the cells are aligned, small perturbations initiate an outward-propagating wave. Fig. 7, which is published as supporting information on the PNAS web site, shows how the initial outgoing wave pair triggers “echo” waves that maintain the source until eventually the field is filled with counterpropagating and colliding waves (Movies 3a and b). In a two-dimensional field, this corresponds to a bulls-eye patterns (see figure 4 of ref. 3).

In the experiments of Welch and Kaiser, the waves appeared simultaneously around the entire periphery of the colony. Starting with a field of a small randomly distributed perturbations, several collision times are required to reach a steady-state wave train. Therefore, our numerical solution of the model equations predicts the existence of some transition period that probably took place before the waves became optically visible.
Two-Dimensional Waves. The ripples observed by Welch and Kaiser at the edge of their submerged agar culture consisted of a pair of obliquely oriented oppositely propagating wave trains (see Fig. 5a and figure 2 of ref. 10). Around the periphery of the population, incipient fruiting bodies are forming, spaced roughly one wavelength apart. The radial flux of cells out of colony into the developing fruiting bodies creates a cell density gradient in an annular ring around the colony periphery. If this density gradient is introduced into the two-dimensional model (Eqs. 3–5), it reproduces closely the observed wave pattern. This is shown in Fig. 5b and c as cell density and perspective wave plots. Movies 2a–c show the pattern of counterpropagating waves that appear to pass through one another as well as the tracks of individual cells.

Spacing of Fruiting Bodies. The wave pattern generated by the model also shows how the spacing of fruiting bodies around the periphery evolves. Where the oblique wave trains intersect, the local bacterial population is almost twice that in the individual waves (see Fig. 5b and Movie 2a). As the waves counterpropagate, these intersection regions move radially outwards (downwards in Fig. 5b). Thus the colony edge is “pulsed” with extra bacteria each time an intersection hits the boundary. Because the intersections are spaced about a wavelength apart, the wavelength of the counterpropagating waves will be echoed by the spacing of the peripheral aggregations that form the incipient fruiting bodies. Fig. 5c shows a perspective view of how the wave intersections determine the fruiting body locations.

Discussion

There are many examples of pattern formation in microorganisms and a large literature on mathematical models describing them. A few notable examples, among many, include periodic patterns in colonies of Proteus mirabilis (22, 23), Salmonella typhimurium (24), Escherichia coli (25), Bacillus subtilis (26), and D. discoideum (27–29). The signature feature of these patterns is that they depend on diffusion-mediated chemotaxis and/or growth and death of the cells. This is quite different from the rippling pattern in myxobacteria, which depend on contact-mediated signaling and adective motion rather than diffusion. Although some aspects of swimming behavior of myxobacteria colonies were considered in (30–33), to the best of our knowledge, rippling behavior has not been successfully modeled before.

The mathematical model presented here has four critical ingredients: (i) each bacterium possesses an internal biochemical cycle whose progression controls the time between gliding direction reversals. (ii) Contact-mediated C-signaling alters the probability of reversal by modulating the rate of the biochemical cycle. (iii) Immediately after reversal, the C-signaling system enters a refractory phase, where it does not respond to collisions with countermoving cells. (iv) The response to C-signal depends nonlinearly on local cell density.

The model successfully reproduces the principal features of the ripple phase, both qualitatively and quantitatively. For example, the spacing of the waves obeys the predicted relationship (Eq. 6), the location of the nascent fruiting bodies, and the behavior of individual cells as they move with and against the wave directions. Most importantly, the model demonstrates that no diffusible signals are necessary: the ripple patterns can be generated by density dependent intercellular signals relayed by cell contact alone.

Indeed, the success of the model leads us to view the four assumptions on which the model is based as predictions that can be addressed experimentally: the existence of a refractory period, nonlinear density dependence, and wave tilt being determined by the transverse density gradient. A crucial prediction of the model—confirmed by the experiments of Welch and Kaiser (10)—is the correlation between the behavior of individual cells with the macroscopic properties of the waves.

The importance of rippling behavior for myxobacterial cell development is unknown. Although rippling precedes fruiting body formation, fruiting body formation can proceed without the ripple phase preamble. However, when rippling is present, the cells are able to distribute themselves with a nearly constant time-averaged spatial density that fosters the formation of equidistant fruiting bodies. The model shows how an even distribution arises in the experimental system of Welch and Kaiser (10). The cells also align in ripples, enhancing the formation of streams into nascent fruiting bodies. Changes in the quantitative characteristics of the C-signaling and gliding systems can trigger the transition from rippling to aggregation (11). Thus, rippling patterns are not robust in the same sense that the swelling and aggregation patterns are. Their importance to biologists may lie in their value as a sensitive spatiotemporal assay for cell signaling and motility. That is, rippling is a transient pattern that reveals important information (e.g., refractory period and cooperativity) about intercellular signaling.

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Cell behavior in traveling wave patterns of myxobacteria

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Cells in the early stages of starvation-induced fruiting body development migrate in a highly organized periodic pattern of equi-spaced accumulations that move as traveling waves. Two sets of waves are observed moving in opposite directions with the same wavelength and speed. To learn how the behavior of individual cells contributes to the wave pattern, fluorescent cells were tracked within a rippling population. These cells exhibit at least three types of organized behavior. First, most cell movement occurs along the same axis as the rippling movement. Second, there is a high degree of cell alignment parallel to the direction of rippling, as indicated by the biased movement. Third, by controlling the reversal frequency, cell movement becomes periodic in a rippling field. The periodicity of individual cells matches the period of macroscopic rippling. This last behavior is unique to a rippling population and, on the basis of Myxococcus xanthus genetic data, we conclude that this periodicity is linked to the C signal, a nondiffusible cell contact-mediated signaling molecule. When two cells moving in opposite directions meet end to end, they transmit the C signal to each other and in response reverse their gliding direction. This model of traveling waves represents a new mode of biological pattern formation that depends on cell-contact interactions rather than reaction diffusion.

Traveling waves are often found in multicellular development, where they may create a pattern in a previously undifferentiated sheet of cells. One well studied example is the aggregation of Dictyostelium discoideum amoebae to assemble highly structured fruiting bodies. The Dictyostelium waves can be understood in terms of chemotaxis toward cAMP and the induced release of cAMP (1, 2). Traveling waves are also observed in the developing retina of insects (3) and mammals (4). Using time-lapse cinematography, Reichenbach discovered that many species of myxobacteria propagate multicellular waves in the early stages of fruiting body development (5).

When Myxococcus xanthus is starved, the cells transmit developmental signals to each other as they build multicellular fruiting bodies and finally differentiate into spores (6). As aggregates begin to form, cells between the aggregates often migrate in a highly organized periodic pattern of equispaced ridges that move as traveling waves, called ripples (5, 7). In one closely monitored experiment designed to identify the different behavioral stages of fruiting body development, 100% of starved cultures showed rippling during the first 22 h of fruiting body development (8). Later, cells leave these ripples and stream into nascent aggregates. Myxobacterial genetic studies have revealed a series of cell–cell signals necessary for fruiting body development and rippling (9, 10). Mutants defective in either the early A or B signals cannot form fruiting bodies, yet they can ripple (7). In contrast, the C signal, a cell envelope-associated protein encoded by the csgA gene, is required both for fruiting body formation and for rippling (11). Microscopy revealed that the ripple crests were organized heaps of cells (7). C signal was found to change the behavior of cells by altering their reversal frequency (12). C-signaling was also found to depend on cell arrangement, requiring end–end contact between cells (13). csgA mutants, when combined with wild-type cells in different proportions, altered the ripple wavelength (12). These findings led to a proposal that cells travel a distance equal to the ripple wavelength without reversing. Contact with countermigrating cells in a neighboring ripple would then transmit the C signal, triggering cell reversals (12). However, cell tracking in ripples showed that cells reversed direction in ripple troughs, albeit less frequently than in ripple crests, indicating that more was involved than collision of cells in ripples (12).

Ripples offer an opportunity to investigate the role of signaling in the formation of patterns involving thousands of cells. To identify the cell movement behavior that underlies ripples, we first sought conditions under which the pattern is consistently observed. Under these reproducible conditions, cells were tracked and their trajectories analyzed. In collaboration with others, we compared cell tracks with predictions of a mathematical model that was developed to explain rippling behavior (14). Here we report a set of orderly cell behaviors that are sufficient to produce rippling according to that model.

Materials and Methods

Submerged Agar Culture (SAC). The wild-type strain DK1622 (15) and DK00547, a derivative with green fluorescent protein (GFP) transcriptionally fused to the highly active pilA promoter, constructed by E. Licking (16) were used. In preparation for SAC, cells were grown in nutrient liquid media (1% casitone/10 mM Tris/8 mM MgSO4/1 mM KPO4) to midexponential phase (Klett 100 or ~5.6 × 10⁶ cells/ml). These bacteria were pelleted at 12,000 × g, washed in nutrient-deficient medium (CF medium: 0.015% casitone, 10 mM Tris, 8 mM MgSO4, 1 mM KPO4), resuspended at 2.4 × 10⁹ cells/ml in CF.

A sterile 0.5-mm-thick silicone rubber gasket (Grace Biolabs, Bend, OR) was placed on top of a flame-sterilized glass microscope slide, creating a small well. Molten 1.5% agar in CF medium was poured into the opening in the gasket, covered by a second flame-sterilized microscope slide, clamped to the agar, and then cooled to room temperature. While the agar hardened, the M. xanthus culture was prepared for spotting as described. A second gasket apparatus was then set up, consisting of another sterile gasket, a flame-sterilized glass coverslip, a 4 × 4-cm piece of Parafilm (American National Can, Chicago), and another glass slide. The coverslip was placed on top of the gasket, creating a watertight well. This was placed on top of the slide, coverslip down, with the Parafilm between the coverslip and the slide to prevent the two from sticking to each other. The Parafilm and slide supported the second gasket to keep it from flexing and breaking the coverslip. The well created by the second gasket and the coverslip was filled with CF liquid medium. When the agar in the first apparatus hardened, the clamp and second slide were removed. A 0.5-μl aliquot of resuspended liquid culture was placed on the now-exposed agar disk and allowed to dry for ~5 min. The apparatus containing the agar was then inverted and removed. A 0.5-μl aliquot of resuspended liquid culture was placed on the now-exposed agar disk and allowed to dry for ~5 min. The apparatus containing the agar was then inverted and

Abbreviations: SAC, submerged agar culture; PSD, power spectral distribution; GFP, green fluorescent protein; IMQ, Iglohim, Moglinier, and Oster.

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placed on top of the gasket holding the liquid medium so that the positions of the two gaskets match. The resulting sandwich was clamped for at least 1 h so the two gaskets attached to each other. Finally, the clamp was removed, along with the Parafilm and associated support slide. The final layers of a SAC apparatus consist of the coverslip, gasket containing CF liquid, gasket containing agar, and slide. The M. xanthus population is between the agar and the CF liquid (diagrammed in Fig. 1).

**Microscopy.** During the acquisition of time-lapse images, SAC cultures were maintained at 25°C with a heated stage (Brook, Lake Villa, IL). All video microscopy was performed on a Nikon Eclipse E800 microscope (Nikon) by using long working distance objectives. Digital images were acquired from an analog video source by using a Scion LG-3 video capture card and SCION IMAGE software (Scion, Frederick, MD). Phase-contrast images were generated with a charge-coupled device (CCD) camera (Optronics Engineering, Goleta, CA), whereas fluorescent images were generated with an intensified charge-coupled device video camera (Video Scope International, Sterling, VA). Images were saved at regular intervals (either 20 or 60 sec per frame), and background noise was reduced in the fluorescent images by averaging the video-rate images over a period of 2 sec. The SCION IMAGE macro language was used to create scripts for automated hardware control, image acquisition, and data analysis. Images were saved as sequentially numbered Tagged Image File Format (TIFF) files and assembled into time-lapse movies by using QUICKTIME (Apple, Cupertino, CA).

**Tracking Cells.** Images of cells were acquired from prerippling and rippling populations at least 48 h, respectively, after their placement in SAC. Fluorescent images were acquired at 20-sec intervals for periods of 2 h. Consecutive images were assembled into stacks (NIH IMAGE, Bethesda, MD), and individual cells were tracked manually. Four criteria were used to select cells for tracking. During the entire 2-h period: (i) A cell must be continually visible; (ii) a cell must not undergo cell division; (iii) a cell in a rippling population must remain within the rippling field; and (iv) a cell in a prerippling population must remain in the area where future rippling will occur. Cells were picked from the first frame in the stack and then were observed to see whether all of these criteria were met.

The two-dimensional path of each cell was divided along the x and y axes, with the x axis running tangent to the edge of the cell spot, which is parallel to the direction of ripple movement. Cell reversals, defined as the switching of a cell's leading pole, were determined both systematically and manually. The systematic determination of reversal events exploited the fact that a reversal is followed by an abrupt change in cell direction. Therefore, each cell reversal was initially marked as a point of change in the trajectory from a negative to a positive slope (or the reverse) in either the x or y axis of a cell path. All reversals determined by this method were confirmed visually from the assembled stacks. The time between reversals, or reversal period, was determined from the number of 20-sec frames between reversal events.

**Results**

Earlier experimental designs had limited the observation of ripples to brief periods of time and to uncertain locations (see, for example, ref. 8). To find the cell behaviors that underlie ripples, cells were observed in SAC. A spot containing about 1 × 10⁷ starving M. xanthus cells was placed on a thin layer of agar then covered with liquid medium (Fig. 1). These cells eventually complete fruiting body development with spore formation. During the first few hours in SAC, cells from the center accumulate at higher density in an annulus around the edge of the circular spot (Fig. 2a and b). Such accumulations have previously been observed (17) and arise from the reproducible tendency of M. xanthus cells to migrate from the center of a swarm toward its periphery (18). The cells remain within the edge of the initial spot in SAC and form a peripheral ring of higher cell density than the center 4–8 h later (Fig. 2b). Once the cells have reached a certain density, seen as a darkening of the annulus, rippling initiates. Rippling persists with constant wavelength and period for several days in SAC, offering long periods for photography. As rippling proceeds, cells slowly migrate outward from the ripples into the round fruiting body aggregates that form at the very edge of the spot in Fig. 2d (arrows). As cell density decreases in the ripples they fade, and the fruiting body aggregates enlarge and darken. The ripples are evident in Fig. 3a as a series of lines that extend from the round nascent fruiting bodies toward the center of the culture. The lines are dark at the edge and become progressively lighter toward the center. Thus SAC provides conditions favorable for quantifying the behavior of cells within ripples.
Ripples have several remarkable properties that distinguish them from *D. discoidium* waves. First, ripples move in opposite directions around the perimeter of the spot as two sets of opposing waves (see Movie 1, which is published as supporting information on the PNAS web site, www.pnas.org). Each set of waves consists of parallel crests angled toward the perimeter; the opposing set of crests is angled in the complementary direction (Fig. 3). Second, ripples do not spread outward from a selected point. Rather, the entire annulus behaves as one coherent field around which the ripples propagate continuously in both directions (Fig. 3b–e), and this pattern remains coherent for hours as the ripples move (see Movie 1). *D. discoidium* waves lead to slug formation. In contrast, myxobacterial cells do not accumulate anywhere within the ripple field despite the incessant movement of the ripples (see Movie 1). *D. discoidium* waves lead to slug formation. In contrast, myxobacterial cells do not accumulate anywhere within the ripple field despite the incessant movement of the ripples (see Movie 1). *D. discoidium* waves lead to slug formation. In contrast, myxobacterial cells do not accumulate anywhere within the ripple field despite the incessant movement of the ripples (see Movie 1). *D. discoidium* waves lead to slug formation. In contrast, myxobacterial cells do not accumulate anywhere within the ripple field despite the incessant movement of the ripples (see Movie 1). *D. discoidium* waves lead to slug formation. In contrast, myxobacterial cells do not accumulate anywhere within the ripple field despite the incessant movement of the ripples (see Movie 1).

Finally, when two countermigrating ripple crests collide, they do not annihilate but appear to interpenetrate one another (Figs. 3a and 4a; see Movie 1). Four sequential stages of ripple crossing are shown in Fig. 3b–e. Even though the ridges are dense heaps of cells (7), the shape of each ridge emerges unchanged as it leaves an intersection. The preservation of shape suggests that colliding waves are reflecting cell by cell, not interfering (see Movie 2, which is published as supporting information on the PNAS web site).

To learn how the ripple pattern is created, individual cells were tracked as they moved within the dense rippling population. DK10547 cells that express GFP were diluted 1/500 into a population of unlabeled cells, and the mixture was allowed to develop in SAC. In epifluorescence mode with a 10×20 objective lens, GFP-expressing cells are visible as uniformly fluorescent rods, ∼0.5 μm × 5 μm. The higher-density ripple crests are visible by their autofluorescence as diffuse bands, because *M. xanthus* cells have carotenoids and other fluorescent pigments (Fig. 4a and b). The autofluorescence is too weak to show individual cells. By using GFP fluorescence for identification, two qualitative observations were made on Movie 3 (which is published as supporting information on the PNAS web site). First, the labeled cells in a crest moved in the direction of the crest, and second, the cells tended to reverse when the crest collided with an oppositely moving crest. For quantitation, a total of 20 GFP cells were tracked at 20-sec intervals for 2 h in the time-lapse series (see Movie 3). Plots of the trajectories of six cells are shown in Fig. 5a–f. The x axis is defined as the direction tangent to the edge of the cell spot, the same axis along which the macroscopic ripples move. It is evident from the tracks in Fig. 5 that the cells were displaced many-fold more in the tangential (x) than in the perpendicular (y) direction. Because the long *M. xanthus* cells move by gliding in the direction of their long axis, this indicates that the cells are predominantly oriented in the x direction. It is likely that the cells became aligned in that direction.

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Fig. 3. A field of *M. xanthus* traveling waves. (a) A phase-contrast image showing most of a rippling population in SAC. Wave crests appear as darker bands. As shown dynamically in Movie 1, crests move in opposite directions around the perimeter of the spot as two sets of opposing waves. (b–e) Crossing patterns of opposing waves are shown in magnified images from a time-lapse series of the boxed area of the spot perimeter (Left), with an interpretive diagram of the crests (Right). The intersection points of opposing ripple crests are indicated by arrows. Each pair of adjacent frames represents a time difference of 180 sec. (Bar = 0.5 mm.)

Fig. 4. Tracking of individual *M. xanthus* cells in a rippling population. (a) Phase-contrast view of a rippling population. (b) Inverse-contrast fluorescent image of the same field. Individual cells are tiny dark spots, and the ripple crests appear as diffuse dark bands because of autofluorescence. The difference in fluorescence intensity between GFP-labeled cells and unlabeled cells is evident. Fluorescent cells were tracked in time-lapse images acquired at 20-sec intervals (Movie 3).
direction because of the asymmetry of the cells, when they were initially packed into the annulus by their outward movement (Fig. 2a and b).

The cell trajectories along the x axis plotted over time in Fig. 5 show rounded sawtooth paths, indicating motion alternately to the left and the right. There is little motion in the y direction. Each trajectory in Fig. 5 is different, showing a stochastic element in the movement. Nevertheless, a regularity in the oscillatory movements in the x direction was revealed by Fourier analysis. The power spectral distribution (PSD) for movement along the rippling x axis has a peak rising above background (Fig. 6b) with a period of 7.5 ± 1 min. Therefore, the cells are not moving randomly on a line. The 7.5-min. peak was reproduced in two experiments and was absent when movement along the y axis was measured (Fig. 6c). In a separate set of experiments and several hours before macroscopic rippling had started, an area within the annulus was photographed in time-lapse photography, and individual GFP cells were tracked to see the behavior of prerippling cells. The 7.5-min peak was also absent from these prerippling cells in either the x (Fig. 6a) or y direction (data not shown). For comparison with the data for individual cells, the wavelength, period, and speed of the macroscopic ripples were measured on magnified images of Movie 3, and the data are summarized in Table 1. The 7.5-min period of individual cell movement in the x direction is not significantly different from the period of macroscopic ripple movement of 8.2 ± 0.6 min.

To relate the periodic movement of the population along the x axis to the behavior of individual cells, the time interval between pairs of reversals was measured for each tracked cell. For example, each of the six cells shown in Fig. 5 shows alternating ups and downs in its x trajectory; each up-and-down cycle represents a pair of reversals. The time interval between two reversals for each of those oscillations was measured as described in Materials and Methods, and the interval data are compiled in Fig. 7. For rippling cells (Fig. 7b), the distribution of interval lengths between reversals is asymmetric: it appears to be bounded on the left side of the distribution, with no reversals less than 40 sec. It also appears to be bimodal, with a right mode at 3.8 min, approximately one-half of the first. Because

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<th>Table 1. Movement of ripple crests in SAC</th>
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<td><strong>Parameter</strong></td>
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<td>Speed</td>
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<tr>
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The speed of ripples was measured from the time required for a particular crest to move a known distance. Wavelength was measured as the distance between adjacent ripple crests, using their points of intersection with an oppositely moving crest as the mark. The period was measured as the time required for succeeding ripple crests to pass the same point. The data for each measurement were taken from two independent experiments. n represents the number of measurements.
alignment, movement along the x axis of macroscopic ripple movement. These orderly behaviors aligned and moving back and forth along the reversals of individual cells. Moreover, rippling cells tend to be periodic (within error) as the macroscopic rippling of 8.2 min. periodicity is only in the x spectrum (Fig. 6) is present only in rippling cells, and that during rippling but not before it. The 7.5-min peak in the power peak (one-half cycle) for individual cells in Fig. 7 is present multicellular behavior of rippling cells. The 3.8-min reversal period (imo, a 2-min peak dominates the prerippling distribution. In place of a right peak at 3.8 min, there is a shoulder extending from 3.5 to 6 min with a long tail that extends beyond 13 min. The prerippling distribution had 19 intervals longer than 10 min, but there were no such intervals in the rippling cell distribution. This difference, as well as the 3.8-min peak, distinguishes rippling cells from prerippling cells.

**Discussion**

Several results reported here provide a quantitative basis for the multicellular behavior of rippling cells. The 3.8-min reversal peak (one-half cycle) for individual cells in Fig. 7 is present during rippling but not before it. The 7.5-min peak in the power spectrum (Fig. 6) is present only in rippling cells, and that periodicity is only in the x direction. Both peaks have the same period (within error) as the macroscopic rippling of 8.2 min. Thus macroscopic rippling is temporally correlated with the reversals of individual cells. Moreover, rippling cells tend to be aligned and moving back and forth along the x axis, which is the axis of macroscopic ripple movement. These orderly behaviors—alignment, movement along the x axis, and periodic reversals—provide a cellular mechanism for rippling. What generates the periodic reversals needed for rippling? Other experiments have shown that C-signaling regulates rippling behavior (7, 8, 12). Because both cells have been moving at the same average speed; the period is one-half that of two ripples moving in the same direction (which we observed to be 8.2 min). The 3.8-min peak also includes cells from a crest that travel through a trough and reverse when their cycle has completed. These dynamics also explain why two colliding ridges do not annihilate each other. Instead, they partly reflect from each other and partly exchange cells (14). These properties may also explain the absence of net cell transport in a rippling field of bidirectional

Importantly, null csgA mutants are unable to ripple (7). Moreover, neither fruA (20), frzCD, nor frzE mutants make ripples (12, 20). By contrast, deletion of devT, a C signal-dependent gene, does not block rippling (21), because devT is in the sporulation branch of the C-signaling pathway, as distinct from the branch that controls cell movement. Particular mutations in frzCD change the reversal frequency in growing cells (22), showing that the average time interval between reversals is under genetic control. The genes essential for rippling define the signal transduction pathway from C signal reception to frz gene control of cell reversal (8, 23, 24). Another experiment shows that C is critical to the propagation of ripples: diluting C signal-proficient cells with C signal-deficient cells that can still respond to the signal increased the ripple wavelength in a regular way. This is as expected, because the frequency of collisions that result in a C signal response from both colliding cells would decrease in proportion to the dilution (12).

These patterns of cell movement and C-signaling were recently used by Igochik et al., to construct a complete quantitative model of rippling (14), which we call IMO for Igochik, Mogilner, and Oster. The key features of IMO are: (i) reversal of gliding direction is controlled by a biochemical cycle internal to the cell; (ii) contact-mediated C-signaling induces an increase in cycle phase velocity, thereby increasing reversal probability; (iii) after a reversal, the cell enters a refractory phase of the cycle temporarily insensitive to collision; and (iv) response to C-signaling depends nonlinearly on the local cell density. In light of this model, we suggest that the prominent 7.5-min period of individual cells (Figs. 6 and Fig. 7b) reflects a complete reversal cycle initiated by C-signaling in the high density of a ripple crest. The result of dilution with C signal-deficient cells, described above, is quantitatively predicted by theory (14). Fig. 7 shows a distribution of time intervals between reversals that falls rapidly to zero on the left and has no reversals at less than 40 sec, consistent with a refractory period after a cell has responded to C signal. The peaks observed in the distribution of reversal intervals for rippling and prerippling cells (Fig. 7) are consistent with the model as follows. The faster peak at 2 min is present in both cell types but dominates the prerippling distribution. A 2-min peak in prerippling cells would result from C signal-induced reversals arising from random end-to-end collisions between aligned cells. Its period, which must exceed the cycle refractory period, reflects the average time to accumulate sufficient C signal to trigger reversal. The prerippling population is assumed to have a uniform cell density in the x direction, so that the likelihood of the cell colliding and C-signaling is constant as the cell moves at constant speed in that direction. This condition is reflected in the prerippling distribution of reversal intervals, which lacks a 3.8-min peak and decays from its 2-min peak to a tail that extends beyond 13 min (Fig. 7a).

In the rippling population, according to the assumptions of IMO, a 2-min peak arises from cells that move with a crest and reverse when their cycle has reached the end of its period. A broad peak at 3.8 min is prominent only in the distribution for rippling cells and is broad because it reflects two patterns of movement according to IMO. One pattern is the movement of a cell from one ripple crest that meets a cell in a countermoving crest and reverses after collision, as Sager and Kaiser had proposed (12). Because both cells have been moving at the same average speed; the period is one-half that of two ripples moving in the same direction (which we observed to be 8.2 min). The 3.8-min peak also includes cells from a crest that travel through a trough and reverse when their cycle has completed. These dynamics also explain why two colliding ridges do not annihilate each other. Instead, they partly reflect from each other and partly exchange cells (14). These properties may also explain the absence of net cell transport in a rippling field of bidirectional
waves. The ability of the IMO model to produce traveling ridges of cells that appear to pass through each other and to explain the distribution of reversal times shows that the set of behaviors proposed in the model is sufficient to produce the traveling wave pattern. The traveling waves are thus propagated by cell-contact signaling and by cell movement directed by that signaling: the signal need not diffuse.

Rippling may contribute to the spacing pattern of fruiting bodies. Fig. 3a shows that the nascent fruiting bodies are spaced about a ripple wavelength apart around the edge of a submerged agar culture. The two-dimensional IMO model predicts the locations where fruiting bodies are most likely to form (14). Jelsbak and Søgaard-Andersen show that C-signaling also directs another pattern of cell movement, namely cell streaming into nascent fruiting bodies (L. Jelsbak and L. Søgaard-Andersen, personal communication), demonstrating the power of C-signaling to modify cell movement. Streaming behavior follows rippling, because streaming requires a higher level of C-signaling than rippling, and the intensity of C-signaling increases during this period of development (8, 11).

Comparison of the behavior of individual cells with the macroscopic ripples revealed the cell behaviors that underly rippling. These data and other considerations were used by Igoshin et al. (14) to construct a mathematical model, IMO, of rippling. IMO and quantitative data from this study may account for the unusual properties of these traveling waves. IMO shows that the observed cell behaviors, together, are sufficient to generate traveling waves. The waves depend on cell-contact signaling and organized cell movement, not on a diffusible signal.

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