Spatial restriction of cellular differentiation

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Myxococcus xanthus cells differentiate into myxospores within a fruiting body, an aggregate of $\sim 10^5$ cells. Previous work had discerned an inner and outer domain within the fruiting body differentiated by cell density and cell alignment. To test whether the two domains might play different roles in spore differentiation, developmentally regulated gene fusions were screened for expression restricted to one domain or the other. Transcriptional *lacZ* fusions to 80 developmentally regulated genes were examined and eight fusions were found that restricted expression to the inner domain, while one fusion, $\Omega 7621$, showed initial expression in the outer domain. Initial $\Omega 7621$ expression coincided with patches of spore precursors evident in bright-field microscopy. Later in development, both $\Omega 7621$ expression and the patches expanded inward, eventually filling both the inner and outer domains. Previous work had also shown that high cell density and cell alignment are required for transmission of the C-signal, which is needed to initiate spore differentiation. Evidence is presented for a novel morphogenetic mechanism in which C-signaling in the outer (high density) domain initiates spore differentiation. It is proposed that spore precursors are passively transported from the outer to the inner domain by the movements of undifferentiated rod cells. Reconstruction experiments showed that developing rod cells move with sufficient force to displace spores. Spore precursors thus accumulate in the inner domain where they express spore-specific genes at high levels and account for inner domain specific expression.

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Myxobacteria are primitive multicellular organisms. Upon starvation, *Myxococcus xanthus* cells actively move to assemble a hemispherical fruiting body, within which cells differentiate from elongate rods into spherical and environmentally resistant myxospores (Shimkets 1990). Internally, a nascent fruiting body is organized into two concentric domains: an outer hemispherical shell of ordered cells at high cell density surrounds an inner hemisphere of less ordered cells at one-third the cell density (Sager and Kaiser 1993).

Nonmotile mutants fail to aggregate. They are also defective in both developmental gene expression and sporulation, producing only 10^{-5} as many spores as a motile strain (Kroos et al. 1988). Either genetic or phenotypic rescue of motility rescues aggregation, gene expression, and sporulation (Kroos et al. 1988). Because cell movement is required to build the fruiting body, the sporulation and gene expression defects of nonmotile cells could result from loss of spatial cues normally derived from the fruiting body architecture. Increased cell density achieved by sedimentation or mechanical alignment of cells rescued the developmental defects of nonmotile cells (Kroos et al. 1988; Kim and Kaiser 1990a).

A high cell density and alignment of cells are required

for cell-to-cell transmission of C-signal. This signal that is carried by the 17-kD C-factor promotes aggregation and initiates spore differentiation (Kim and Kaiser 1990a,b,c; Li et al. 1992). Because the conditions for C-signal transmission, cellular order, and cell density are highest in the outer domain of the fruiting body (Sager and Kaiser 1993), spore-related gene expression and sporulation were expected to initiate within the outer domain. In contrast to expectation, late expression of eight C-factor dependent spore-specific genes was found in the inner domain of the nascent fruiting body. To resolve this apparent conflict, the expression patterns of sporulation-related genes and of differentiating spores were examined in time and in space within nascent fruiting bodies. In fact, spore differentiation is initiated in the outer domain, as expected. However, spore precursor cells lose motility as their differentiation proceeds. These nonmotile cells are then passively transported to the inner domain, where they express high levels of C-factor-dependent genes and complete their morphologic differentiation into spherical spores.

Results

Spatial patterns of gene expression within a fruiting body

M. xanthus strains have been isolated that contain *lacZ*

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transcriptional fusions to the promoters of genes that are induced during aggregation and fruiting body formation (Kroos et al. 1986; Kroos and Kaiser 1987; H. Kimsey, unpubl.). Spatial patterns of β -galactosidase expression were examined in a set of eighty different fusion strains. Cells of each strain were induced to form fruiting bodies on nutrient-deficient agar containing the chromogenic β -galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl β -D-galactoside), and the fruiting bodies were viewed by bright-field microscopy at 16, 26, 46, and 72 hr after the initiation of development. Blue color indicative of X-gal hydrolysis was not detected in fruiting bodies at either 16 or 26 hr, but was detected at 46 hr. At that time, two distinct patterns of β -galactosidase expression were evident. In one set of fusion strains, blue color was limited to the inner domain of the fruiting body (Fig. 1A). In another set of fusions, both the inner and outer domains stained blue (Fig. 1B). The remaining fusion strains showed no color in either domain at 46 hr. Presumably, these fusions express too little β -galactosidase to produce a visible color. By 72 hr, all fusions that displayed the inner domain-localized staining pattern at 46 hr became blue throughout both the inner and outer domains of the fruiting body, resembling the pattern of Figure 1B.

Eight different fusions localized their β -galactosidase expression to the inner domain at 46 hr (resembling Fig. 1A): Ω 4401, Ω 4406, Ω 4459, Ω 4499, Ω 4506, Ω 4480, Ω 4451, and Ω 4435. These eight fusions have other properties in common. Their β -galactosidase activity rises to levels more than fivefold higher in spores than in rod-shaped cells by 46 hr (Kroos et al. 1986). Second, their β -galactosidase specific activity ultimately reaches a level between 180 and 1600 Miller units [1 Miller unit represents 1 nmole of ONP (*O*-nitrophenol) produced per minute per milligram of protein (Kroos et al. 1986)]. These fusions initiate expression after C-signaling has begun, and all eight fusions are C-factor dependent (Kroos and Kaiser 1987; Kroos et al. 1988). In contrast, those fusions, Ω 4445, Ω 4469, and Ω 4473, with β -galactosidase expression visible in both domains at 46 hr (resembling Fig. 1B), express similar levels in spores and rod cells. The onset of expression in these fusions precedes C-signaling by 3–6 hr (Kroos et al. 1986).

These experiments revealed that β -galactosidase expression was not visible unless a peak level of 180 or more units of β -galactosidase specific activity was produced at 34 hr and allowed to accumulate through 46 hr. This visibility threshold applied to blue color at 25 $\times$ optical magnification.

Changes in gene expression within a fruiting body

Intercellular C-signaling regulates spore-related gene expression during the development of fruiting bodies (Kroos and Kaiser 1987). C-signal requires cell alignment and high cell density for its transmission (Kroos et al. 1988; Kim and Kaiser 1990a). Because the inner domain of a nascent fruiting body contains unorganized cells at relatively low density compared with the outer domain (Sager and Kaiser 1993), expression of C-signal-dependent genes localized to the inner domain was unexpected. If, however, their expression initiated in the outer domain but remained below the color detection threshold, and if the expressing cells then moved to the inner domain where their β -galactosidase expression rose above the detection threshold, the observed pattern might be explained. The following experiments were performed to examine this proposal.

If gene expression can initiate in the outer domain and the initiated cells move inward, then there might be a time for some *lacZ* fusion when its β -galactosidase activity in the outer domain would be above the optical detection threshold. To search for such a pattern, the set of 80 developmentally regulated *lacZ* fusion strains described above was reexamined at 32 hr, a time just before expression of inner domain only. These specimens were examined at a high light intensity to increase the sensitivity for detecting blue color. Weak outer domain expression could be detected in fusions Ω 4401, Ω 4403, Ω 4411, Ω 4451, and Ω 4469. These fusion strains develop at a rate similar to wild type. Strong expression localized to the outer domain was found in strain Ω 7621 (Fig. 2B).

The insertion of Tn5 *lac* at genomic site Ω 7621 also slows fruiting body development, presumably by decreasing the function or expression of the target gene. On X-gal-containing agar, the first Ω 7621 aggregates are seen

Figure 1. Spatial differentiation of β -galactosidase expression in 46-hr developing fruiting bodies examined in bright-field microscopy (25 $\times$). (A) Inner domain-restricted expression of gene fusion Ω 4459 (strain DK4299). (B) β -Galactosidase expression evident in both the inner and outer domains for gene fusion Ω 4445 (strain DK4296). Size marker, 50 μ m.

at 30 hr, whereas the first wild-type aggregates form at 10 hr. This reduction of roughly threefold in the rate of development may prolong otherwise fleeting intermediate developmental states, such as the initial differentiation of cells in the outer domain, and reduce the synchrony of development from one fruiting body to another. Ultimately, however, both the number of heat-resistant spores (10^4 spores/ 5×10^8 developing rod cells; cf. with Fig. 6, below) and the morphology of 5-day old $\Omega 7621$ fruiting bodies (resembling Fig. 2D) approach wild-type levels. At 48 hr of development, strain $\Omega 7621$ simultaneously displays four different β -galactosidase expression patterns in the inner and outer domains of different fruiting bodies (Fig. 2). Figure 2A depicts one of the fruiting bodies with no visible β -galactosidase expression; Figure 2B shows a fruiting body in which expression is most intense in the inner half of the outer domain; Figure 2C shows a fruiting body in which expression is most intense in the inner domain and the inner half of the outer domain. Expression patterns were classified as inner-domain or outer-domain specific as described in Materials and methods. Figure 2D depicts a fruiting body showing expression throughout both domains of the fruiting body.

The relative numbers of the four patterns changed with time, as shown in Figure 3. At 32 hr of development, most fruiting bodies showed no expression. At 48 hr of development, the most common pattern was expression in the inner half of the outer domain (like Fig. 2B). At 64 hr of development, expression was restricted

primarily to the inner domain (as defined in Materials and methods), though expression less intense than that of the inner domain could also be detected in the inner half of the outer domain (like Fig. 2C). By 80 hr, most fruiting bodies express throughout both domains (like Fig. 2D). The data of Figure 3 imply that expression of the $\Omega 7621$ fusion first reaches the threshold of blue color detectability in the inner part of the outer domain. While $\Omega 7621$ expression continues in the outer domain, it also moves into the center, where it intensifies. Eventually, high-level expression spreads throughout the fruiting body. When spores from X-gal-stained fruiting bodies of strain $\Omega 7621$ were purified in a sucrose gradient as described by Inouye et al. (1979), the spore-containing band had an indigo color. Microscopic examination of spores taken from the gradient showed indigo dye within individual spores. Taken together, the changes in the most common $\Omega 7621$ expression pattern (Fig. 3) and the spore color data suggest that the shift in the pattern of $\Omega 7621$ staining reflects a movement of spore precursors (or spores) from the outer domain to the inner domain, as their differentiation proceeds.

Changes in the spatial distribution of spores

Optically, spores refract more light than rod cells and, hence, spores are visible in bright field (without phase contrast). To investigate the site (or sites) at which optically refractile cells first appear, fruiting bodies were examined by time-lapse videography on agar with bright-

Figure 2. Four spatial expression patterns of the *lacZ* fusion $\Omega 7621$ within fruiting bodies. Fruiting bodies were examined under bright-field optics (25 $\times$) at 48 hr of development. (A) No expression; (B) expression in the inner part of the outer domain; (C) expression in the inner domain; (D) expression throughout the fruiting body.

Figure 3. Distributions of the four spatial patterns of $\Omega 7621$ gene expression as a function of developmental time. More than 100 fruiting bodies were classified at each time. (Open bars) No expression; (horizontally hatched bars) expression only in the outer domain (like Fig. 2B); (diagonally hatched bars) inner domain expression (like Fig. 2C); (solid bars) expression throughout both domains (like Fig. 2D). Gene expression patterns within fruiting bodies were classified as inner or outer domain specific as described in Materials and methods.

field optics. Under these experimental conditions, cell aggregates were first detected at 8 hr; mounds formed at 10 hr and retained an approximately constant shape from 10 to 14 hr. At ~ 14 hr, patches of light refractile material appeared in the outer domains of the mounds (Fig. 4). The inner domains are white at this time (see below). At 16 hr, the patches became more elongate; by 23 hr, they enclosed the entire nascent fruiting body within its outer domain. With time, the darkness invaded the inner domain; and by 46 hr, the fruiting body had become uniformly dark.

The white patch evident in Figure 4 at 14, 16, 23, and 26 hr at the center of each fruiting body is the inner domain, which is not devoid of cells, but rather contains cells at $\sim 30\%$ the density of the outer domain (Sager and Kaiser 1993). To allow for greater resolution of the dark patches in the outer domain, videograph contrast was enhanced. Under enhancement, the inner domain became white as a result of the nonlinear brightness dependence of the enhancement process.

Three lines of evidence correlate the dark patches with clusters of spores and spore-like cells. First, when the preparations were examined at a magnification sufficient to resolve individual spores, dark patches had more spores than light areas, as shown in Figure 5. In the inner domain of a fruiting body developed for 36 hr, where the fruiting body appears light at low magnification (Fig. 5A), few spores are visible at high magnification (Fig. 5B); in the outer domain, where the fruiting body was dark at

low magnification (Fig. 5A), many spores were evident at high magnification (Fig. 5B). The mean diameter of the round, opaque objects in Figure 5B ($1.9 \pm 0.21 \mu\text{m}$) is comparable with that of the purified spores in Figure 5C ($1.6 \pm 0.26 \mu\text{m}$). Second, when nascent fruiting bodies were dried in air (a procedure found to lyse virtually all of the rod-shaped cells but to preserve spores), the dark patches remained. Third, the increase in total patch area per fruiting body parallels and precedes the increase in total spore number, measured as heat- and sonication-resistant colony-forming units (Fig. 6).

On the other hand, the dark patches in the outer domain are not unusually high peaks on the aggregate mounds, because when fruiting bodies containing fluorescently labeled cells were optically sectioned by confocal microscopy, dark patches did not correspond to localized areas of increased height (data not shown). Nor are the dark patches shadows cast by the mound, because 180° rotation of the specimens in the optical light path of the microscope left the patch morphology unchanged.

Spore transport

These experiments suggest that sporulation begins in the outer domain, because that is where optically refractile cells first appear. Spores have been detected at the periphery of nascent fruiting bodies examined by scanning electron microscopy (O'Connor and Zusman 1989). The outer domain is also the locus of intense cell movement, where dense streams of cells that reverse infrequently circle within the nascent fruiting body (Sager and Kaiser 1993). Spiral arrangements of cells were reported during aggregation of *Stigmatella aurantiaca* (Vasquez et al. 1985). Apparently, the differentiation of rod-shaped cells into spherical spores takes place within a torus of moving, circling cells. Gliding motility is lost during the formation of spores, and regained upon spore germination. This process is illustrated graphically in the time-lapse films of Reichenbach (1965).

The invasion of $\Omega 7621$ β -galactosidase expression into

Figure 4. Development of dark patches within wild-type fruiting bodies. Contrast-enhanced bright-field videographs (at $10\times$) showing development on agar at various times. Size marker at lower right, $100 \mu\text{m}$.

Figure 5. Dark patches and spores. (A) A dark patch (at $6.3\times$) within a 36-hr-old, air-dried wild-type fruiting body (size marker, $25\ \mu\text{m}$); (B) the spore distribution within that fruiting body (at $25\times$; size marker, $25\ \mu\text{m}$); (C) purified fruiting body spores photographed at the same magnification as B.

the inner domain (Fig. 2) and the high level expression of C-signal dependent, spore-associated genes within the inner domain late in development (Fig. 1A) suggests the movement of spore precursors or spores within the fruiting body. The circling movements of rod cells provides a mechanism by which spore precursors could be transported toward the inner domain. Cells could begin their differentiation in the densely packed outer domain; then, as these cells rounded up and lost their own motive power, they could be passively transported to the inner domain by the circular movements of the remaining motile rod-shaped cells.

A critical aspect of this hypothesized transport process was reconstructed. To test whether moving rods had sufficient power to displace spores, a mixture of rod cells (strain DK5204 cells harvested at 8 hr of development) and mature, 7-day-old spores (strain DK1622 spores har-

vested from mature fruiting bodies) was prepared. Purified DK1622 spores (see Materials and methods) were deposited onto developmental agar and overlaid with DK5204 rod cells to give a final 3 : 1 rod-to-spore ratio. At the start, the entire agar surface appeared to be covered rather uniformly with spores even after the rods were added (Fig. 7A). However, as the rod cells aggregated, they formed masses that were predominantly white in bright-field microscopy (Fig. 7B). The dark rings in the fruiting bodies shown in Figure 7B are not regions of accumulated preformed DK1622 spores, as the bands are present in nascent fruiting bodies developing in the absence of preformed spores. Nor are the rings regions of differentiating DK5204 spores, because at 22 hr, spore maturation has not yet begun (Fig. 6). The areas between the aggregates in Figure 7B retained the (dark) spore-related optical refractivity. Apparently, the aggregating

Figure 6. Average total patch area per fruiting body (○) and spore number (●) as a function of developmental time.

Figure 7. Reconstruction of the displacement of mature spores by moving cells. A mixture of developing rods (strain DK5204) and mature spores (strain DK1622) in a 3 : 1 rod-to-spore ratio was prepared. (A) Photograph taken just after deposition of the rod and spore mixture; (B) the same field 22 hr later after aggregates have formed. Bright-field microscopy at $6.3\times$. Size marker, 100 μm .

cells that formed mounds had swept away most of the spores that initially lay beneath them. Such sweeping action was also observed when 2-day-old spores (as opposed to 7-day-old spores) were overlaid with developing rods.

In this experiment strain DK1622 is sensitive to kanamycin, whereas strain DK5204 is kanamycin resistant; both strains are capable of forming fruiting bodies and spores (Kroos et al. 1986). Mixtures (3 : 1) of DK5204 rods at the start of development and DK1622 preformed, dispersed spores were developed together for 22 hr (before appreciable sporulation was expected from the DK5204 strain). Microscopic analyses revealed that fruiting bodies contained mostly rods; spores were not visible within the aggregates. Fields of fruiting bodies and the nonaggregated cells between them were then scraped from five plates, and this harvest was treated for spore counts as described in Materials and methods. No colonies formed on any of the kanamycin-containing CTT plates (see Materials and methods). Thus, the spores harvested within and between fruiting bodies at 22 hr were sensitive to kanamycin. Because the preformed spores were sensitive to kanamycin while the admixed rod cells were resistant to kanamycin, the spores that were displaced in this mixture must have been derived from the population of preformed spores and not mixed at 22 hr with newly formed spores. Thus, the reconstruction clearly demonstrates that moving, developing cells can transport spores.

If rod cell movements in the outer domain continually transport spore precursors toward the inner domain, then the inner region of nonmotile cells should expand with time as more and more cells are transported inwardly and the inner domain becomes packed with spores. Time-lapse videomicroscopic examination of the bases of three representative wild-type fruiting bodies did reveal an expanding central region of nonmoving cells. This circular region initiated in the center of the inner domain at 28 hr after the start of development. The radius of the region then increased progressively, encompassing all of the inner domain and the inner part of the outer domain by 50 hr (Fig. 8). A qualitatively similar pattern was observed for >100 fruiting bodies.

Discussion

The finding of strong inner domain β -galactosidase expression from spore-specific, C-signal-dependent *lacZ* fusions is consistent with the hypothesized transport of spore precursors from an outer domain area of induction to an inner domain area of accumulation. The first cells to sporulate are likely to be the earliest to enter the inner domain, both because they have the most time to undergo transport into the center of the fruiting body and because they experience a greater transport-driving force, as most of their neighbors are still undifferentiated, motile cells. For similar reasons, the last cells to sporulate are likely to be the last to enter the inner domain. While spores are accumulating, the inner domain should thus contain predominantly older spores, whereas the outer domain should contain relatively younger ones. β -Galactosidase of spore-specific fusion strains would be expected to accumulate to the highest levels within the oldest spores (Kroos et al. 1986), which have been expressing *lacZ* for the longest time. As the oldest cells are concentrated in the center of the fruiting body, the inner domain of the fruiting body would be expected to show the most intense β -galactosidase staining for these fusions, as observed (Fig. 1A).

Because spores are nonmotile, motility must be lost somewhere along the pathway of spore differentiation. Transport of such nonmotile cells requires an external driving force. The circular orbits of rod cells within the outer domain, maintained by a reduced frequency of cell reversal (Sager and Kaiser 1993), could supply this force. In mixtures of spores and aggregating rod cells, the movements of rod cells displaced the spores that initially occupied the sites at which fruiting bodies later formed (see Fig. 8), demonstrating that rod cells move with sufficient power to transport spores laterally.

In the course of fruiting body formation by the mechanism proposed, the lateral displacement of spores would have to be inward, not outward. There are several reasons why inward-only displacement would occur in normal fruiting bodies. First, the fruiting body is covered by a tight-fitting coat of slime that is evident in scanning electron micrographs (cf. Kuner and Kaiser 1982). The

Figure 8. Expansion of the region of nonmoving cells with developmental time. The bases of three representative wild-type fruiting bodies were examined by time-lapse videomicroscopy at 25 $\times$. The ordinate indicates the average ratio of the radius of the circular region of nonmoving cells to the radius of the fruiting body perimeter, expressed as a percentage. Average fruiting body radius was 60 μ m.

slime coat might constrain spores arising in the outer domain from outward displacement. Second, the density of moving rod-shaped cells is maximum in the outer domain of the fruiting body and decreases threefold toward the center (Sager and Kaiser 1993). Only the inner domain has space within which the maturing spores could accumulate. Finally, the spiraling movements of cells (O'Connor and Zusman 1989; Sager and Kaiser 1993) creates a center-directed shear gradient, resulting in centripetal accelerations throughout the fruiting body.

Mutants defective in *csgA*, the gene that encodes C-factor, are unable to sporulate (Shimkets et al. 1983; Hagen and Shimkets 1990; Kim and Kaiser 1990b). Addition of purified C-factor to *csgA* mutants rescues their sporulation (Kim and Kaiser 1990b,c). These data argue that sporulation requires C-signaling. Cell motility, high cell density, and regular cell orientations have all been shown to be required for efficient cell-to-cell transmission of C-signal (Kroos et al. 1988; Kim and Kaiser 1990a,d). Initially, the outer domain of the fruiting body contains a densely packed and aligned stream of moving cells (O'Connor and Zusman 1989; Sager and Kaiser 1993). The architecture of the outer domain appears to satisfy the conditions necessary for efficient intercellular C-signaling. The inner domain does not. Spore differentiation would therefore be expected to initiate in the outer domain.

As C-signaling induces cells in the outer domain to differentiate into spherical spore precursors and lose their motility, the rounding cells would reduce the efficiency of further intercellular C-signaling between still undifferentiated rod cells. These more spherical cells would both reduce contact between adjacent rod cells

and impede the movements of a stream of circling rod cells. Thus, removal of the more-rounded and nonmotile spore precursors from the stream of C-signaling rod cells in the outer domain would maintain high signaling efficiency to the end of fruiting body development. The transport of spores from the outer to the inner domain may have been selectively advantageous in the evolution of fruiting body construction. Because spheres are more likely to achieve close packing under external pressure than are rods, the mechanism proposed would lead to a high spore density within the mature fruiting body.

In sum, the nascent *M. xanthus* fruiting body consists of two concentric hemispherical domains, an outer domain of densely packed cells surrounding an inner domain of lesser cell density. Gene expression and the differentiation of spores initiate in the outer domain, where densely packed and aligned rod cells move in circular orbits. As undifferentiated cells continue to circle, differentiating spores lose their motility and accumulate in the inner domain of the fruiting body. These findings suggest that once spores differentiate into nonmotile spheres, they are passively transported from the outer to the inner domain by the movements of still undifferentiated cells (Fig. 9). There may be parallels between the circling movements of cells in the outer domain and the spiraling migrations of prestalk cells in the tips of *Dicystostelium discoideum* slugs (Siegert and Weijer 1992).

Materials and methods

Strains, growth, and development

Wild-type strain DK1622 was described previously (Kaiser 1979). The *lacZ* transcriptional fusions Ω 4401 (DK4293), Ω 4403

Figure 9. A model for the transport of spores within the wild-type fruiting body. The circular bidirectional movements of undifferentiated cells (depicted by the solid black arrows) drive the passive transport of spores (depicted by the stippled arrow) laterally, toward the center of the fruiting body. The outer concentric circle represents the perimeter of the fruiting body; the inner concentric circle represents the boundary between the outer and inner domains of the fruiting body.

(DK4368), Ω 4406 (DK4294), Ω 4411 (DK5200), Ω 4435 (DK5204), Ω 4445 (DK4296), Ω 4451 (DK4297), Ω 4459 (DK4299), Ω 4469 (DK4469), Ω 4473 (DK4473), Ω 4480 (DK4480), Ω 4499 (DK4494), and Ω 4506 (DK4506), all in the genetic background of DK1622, were described previously (Kroos and Kaiser 1984; Kroos et al. 1986). Bacteria were grown in CTT broth (Hodgkin and Kaiser 1977) and harvested during early exponential phase of growth. Development on agar was initiated by spotting an aliquot of cells resuspended in TPM buffer (10 mM-Tris-HCl, 1 mM KH_2PO_4 , 8 mM MgSO_4 at pH 7.6) (Kroos et al. 1986) at 5×10^9 cell/ml onto TPM agar-coated slides. Development was carried out in submerged culture by resuspending a 25- μ l aliquot of cells at 5×10^9 cell/ml into a 700- μ l total volume of MC7 buffer (Kuner and Kaiser 1982) in one well of a Nunc (Naperville IL) model 4804 Lab-Tek tissue culture four-well chamber whose base and cap had each been replaced with a 24 $\times$ 60-mm coverslip (no. 1), both of which were sealed with high vacuum silicone lubricant (Dow-Corning, Midland, MI) to the rest of the chamber. Cells were incubated at 33°C in the dark.

Analysis of β -galactosidase activity

Temporal expression of β -galactosidase was measured spectrophotometrically by ONPG (*o*-nitrophenol- β -D-galactoside) hydrolysis as described previously (Kroos et al. 1986). The spatial pattern of β -galactosidase expression was examined using bright-field microscopy of fruiting bodies formed on TPM agar containing 30 μ g/ml of X-gal. High microscopic light levels were required to detect blue color at 25 $\times$ within the fruiting bodies.

A spatial expression pattern was classified as inner domain localized when the blue color indicative of X-gal hydrolysis was constrained to a disk centered at the base of the fruiting body, and the radius of the blue disk was approximately half the ra-

dius of the fruiting body. Expression was classified as outer domain localized when blue color was constrained to a ring-shaped band within the outer half of the fruiting body base. Fewer than 10% of the fruiting bodies showed expression patterns intermediate between those of the inner and outer domain classifications.

Spore purification and measurement

Spores were purified in a single, four-step sucrose gradient of 7 ml as described by Inouye et al. (1979). More than 99% of cells in spore fractions were spherical and optically refractile.

Spore titers (Fig. 6) were obtained from 20- μ l aliquots of growing cells, spotted at 5×10^9 cells/ml onto TPM agar to induce development and incubated at 33°C in the dark (Kroos et al. 1986). At each time indicated in Figure 6, five spots of developing cells were suspended into 400 μ l of TPM buffer and frozen at -80°C . To disrupt fruiting bodies and rod cells, the frozen suspensions were thawed and then sonicated for 45 sec (in three 15-sec bursts) using a Microson Ultrasonic Cell Disrupter (Heat Systems Ultrasonics, Inc., Farmingdale, NY) at 90% power. Sonicates were heated at 48°C for 120 min to kill rod cells, serially diluted in TPM buffer, and plated out on CTT plates with or without 40 μ g/ml of kanamycin. Microcolonies were counted after 3–4 days of incubation at 33°C.

Spores within dark patches were examined by inducing development of 20- μ l spots of 5×10^9 cells/ml on TPM-agar-coated slides in humidified chambers. After 36 hr of development, the agar was dried by removing the culture from its humidified chamber and incubating it in air for 6 hr at 33°C. Dried cultures were examined with bright-field optics. Rods could be seen before the fruiting bodies were dried, but only spores were visible afterward.

Video microscopy

Video microscopy with bright-field optics using a Leitz Labovert inverted microscope has been described previously (Sager and Kaiser 1993). Contrast was enhanced with an Argus-10 Image Processor (Hamamatsu, Japan).

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References

- Hagen T.J. and L.J. Shimkets. 1990. Nucleotide sequence and transcriptional products of the *csg* locus of *Myxococcus xanthus*. *J. Bacteriol.* **172**: 15–23.
- Hodgkin, J. and D. Kaiser. 1977. Cell-to-cell stimulation of movement in nonmotile mutants of *Myxococcus*. *Proc. Natl. Acad. Sci.* **74**: 2938–2942.
- Inouye, M., S. Inouye, and D.R. Zusman. 1979. Biosynthesis and self-assembly of protein S, a development-specific protein of *Myxococcus xanthus*. *Proc. Natl. Acad. Sci.* **76**: 209–213.
- Kaiser, D. 1979. Social gliding is correlated with the presence of

- pili in *Myxococcus xanthus*. *Proc. Natl. Acad. Sci.* **76**: 5952–5956.
- Kim, S.K. and D. Kaiser. 1990a. Cell alignment required in differentiation of *Myxococcus xanthus*. *Science* **249**: 926–928.
- . 1990b. C-factor: A cell-cell signaling protein required for fruiting body morphogenesis of *M. xanthus*. *Cell* **61**: 19–26.
- . 1990c. Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein. *Proc. Natl. Acad. Sci.* **87**: 3635–3639.
- . 1990d. Cell motility is required for the transmission of C-factor, an intercellular signal that coordinates fruiting body morphogenesis of *Myxococcus xanthus*. *Genes & Dev.* **4**: 896–905.
- Kroos, L. and D. Kaiser. 1984. Construction of Tn5*lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. *Proc. Natl. Acad. Sci.* **81**: 5816–5820.
- . 1987. Expression of many developmentally-regulated genes in *Myxococcus xanthus* depends on a sequence of cell interactions. *Genes & Dev.* **1**: 840–854.
- Kroos, L., A. Kuspa, and D. Kaiser. 1986. A global analysis of developmentally regulated genes in *Myxococcus xanthus*. *Dev. Biol.* **117**: 252–266.
- Kroos, L., P. Hartzell, K. Stephens, and D. Kaiser. 1988. A link between cell movement and gene expression argues that motility is required for cell-cell signaling during fruiting body development. *Genes & Dev.* **2**: 1677–1685.
- Kuner, J. and D. Kaiser. 1982. Fruiting body morphogenesis in submerged cultures of *Myxococcus xanthus*. *J. Bacteriol.* **151**: 458–461.
- Li, S., B.U. Lee, and L.J. Shimkets. 1992. *csgA* expression entrains *Myxococcus xanthus* development. *Genes & Dev.* **6**: 401–410.
- O'Connor, K.A. and D.R. Zusman. 1989. Patterns of cellular interactions during fruiting-body formation in *Myxococcus xanthus*. *J. Bacteriol.* **171**: 6013–6024.
- Reichenbach, H. 1965. Rhythmische vorange bei der schwarzmentfaltung von *Myxobakterien*. *Ber. Dtsch. Bot. Ges.* **78**: 102–105.
- Sager, B. and D. Kaiser. 1993. Two cell-density domains in the *Myxococcus xanthus* fruiting body. *Proc. Natl. Acad. Sci.* **90**: 3690–3694.
- Shimkets, L.J. 1990. Social and developmental biology of the *Myxobacteria*. *Microbiol. Rev.* **54**: 473–501.
- Shimkets, L.J., R.E. Gill, and D. Kaiser. 1983. Developmental cell interactions in *Myxococcus xanthus* and the *spoC* locus. *Proc. Natl. Acad. Sci.* **80**: 1406–1410.
- Siegert, F. and C.J. Weijer. 1992. Three-dimensional scroll waves organize *Dictyostelium* slugs. *Proc. Natl. Acad. Sci.* **89**: 6433–6437.
- Vasquez, G.M., F. Qualls, and D. White. 1985. Morphogenesis of *Stigmatella aurantiaca* fruiting bodies. *J. Bacteriol.* **163**: 515–521.

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References

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