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Cell Alignment Required in Differentiation of *Myxococcus xanthus*

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During fruiting body morphogenesis of *Myxococcus xanthus*, cell movement is required for transmission of C-factor, a short range intercellular signaling protein necessary for sporulation and developmental gene expression. Nonmotile cells fail to sporulate and to express C-factor–dependent genes, but both defects were rescued by a simple manipulation of cell position that oriented the cells in aligned, parallel groups. A similar pattern of aligned cells normally results from coordinated recruitment of wild-type cells into multicellular aggregates, which later form mature fruiting bodies. It is proposed that directed cell movement establishes critical contacts between adjacent cells, which are required for efficient intercellular C-factor transmission.

CELL INTERACTIONS ESTABLISH CELL fate during morphogenesis of multicellular organisms (*1–3*). Many cell interactions require correct spatial patterning of cells for proper signaling (*4–6*). A protein called C-factor, which has properties of a morphogenetic paracrine signal, is required for cellular aggregation, spore differentiation, and gene expression induced by starvation of the rod-shaped Gram-negative bacterium, *Myxococcus xanthus* (*7, 8*). C-factor has been purified and identified as the membrane-associated protein product of the *csg*A (ς signal) gene (*7, 8*). Cell motility is required for proper intercellular transmission of C-factor (*9*). Nonmotile cells respond to purified C-factor and produce it at wild-type concentrations, yet they arrest development at a stage similar to *csg*A mutants, which do not produce C-factor. Increased cell density partially restores nonmotile cell sporulation (*10*). These observations suggest that movement might allow cells to establish a spatial pattern that is crucial for subsequent C-factor–dependent sporulation and gene expression. We report here that ordered parallel alignment of nonmotile cells restored both sporulation and developmental gene expression.

On a solid surface, starving *M. xanthus* cells glide to aggregation centers where they build a small, steep-sided mound of about 10^5 cells (*11*). The external simplicity of the mound conceals what scanning electron microscopic studies (*12*) have revealed: cells are patterned inside a mound in organized, coherent arrays. This patterning of cells also can be detected by lower resolution light microscopy (Fig. 1). Coordinated cell movement is apparent early in aggregation, when ridge-like accumulations of gliding cells move with regular periodicity like ripples on a water surface (*13*). Within a circular mound, some cells lyse, while other cells differentiate to ovoid, refractile spores that are resistant to heat and desiccation (*14*). These spores later fill the mature fruiting body. There are similarities in development of myxobacteria and cellular slime molds (*15*).

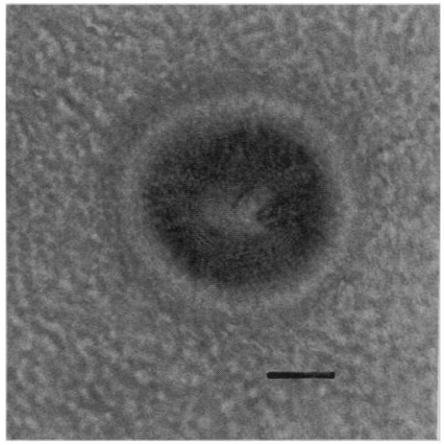

Fig. 1. Internal organization of a nascent *M. xanthus* fruiting body. Wild-type *M. xanthus* strain DK1622 was grown and fruiting body development induced on solid starvation agar (*11, 20*). Aggregated cells were photographed with a Leitz inverted light microscope at 40× magnification. Scale bar, 50 μm.

Control of the development of *M. xanthus* fruiting bodies by cell-cell interactions is implied by four classes of nonautonomous mutants that can sporulate only upon mixture with wild-type cells (*16*). One class of such developmental mutants is the *csg* mutants (*16, 17*), which result from mutation of the *csg*A gene (*18*) and fail to complete aggregation, ripple, lyse, or sporulate (*13*). *csg*A mutants show an altered pattern of developmental gene expression as monitored by transcriptional fusions of *lac*Z to developmentally regulated genes (*19–21*). In *csg*A mutants, expression of *lac*Z fusions is normal for the first 6 hours of development (C-factor–independent expression), but β-galactosidase expression is reduced or abolished after 6 hours (C-factor–dependent) (*21*). All of the developmental defects resulting from mutation of *csg*A are overcome by codevelopment of mutant cells with wild-type cells or by addition of 1 nM purified C-factor (*7, 8*). Biochemical characterization suggests that C-factor is membrane-associated.

Evidence that the transmission of C-factor between cells requires cell movement comes from studies of *M. xanthus* cells that are nonmotile because of mutations in the *mgl*A gene (*22*). Nonmotile cells, like *csg*A mutants, fail to aggregate, ripple, sporulate, or express C-factor–dependent genes (*10*). C-factor (1 nM) purified from wild-type cells restores *mgl*A sporulation and gene expression to wild-type amounts, but admixture of intact wild-type cells does not. Wild-type concentrations of C-factor can be purified from *mgl*A cells, yet intact *mgl*A cells do not rescue *csg*A cells (*9*). These observations support the hypothesis that cells must move for efficient in vivo *csg*A signaling. Proper intercellular C-factor transmission may require a critical spatial orientation achieved only after cells move into the dense aligned cellular organization of a nascent fruiting body.

To test this hypothesis, we simulated the cellular organization found within a fruiting body by artificially aligning nonmotile cells. Nonmotile cells were placed at standard density (5×10^9 per milliliter) on a solid developmental surface that had been scored in one dimension with 5- to 10-μm aluminum oxide abrasive paper to create microscopic grooves. Nomarski optics revealed that cells that settled in these grooves were oriented with their long axes parallel to the

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axis of the groove (Fig. 2A). The cellular organization within the grooves is represented schematically in Fig. 3. In contrast, cells that settled outside grooves formed very small rafts of side-by-side cells but the rafts oriented randomly (out of the focal plane in Fig. 2A). For simplicity this arrangement is called "random." Expression of a C-factor–dependent *lacZ* fusion gene by aligned nonmotile cells was detected by X-gal as discrete stripes of blue-stained cells (Fig. 2B). Cells that were unaligned failed to express the C-factor–dependent *lacZ* fusion gene and appeared as yellow stripes that alternated with the stripes of blue cells. Aligned nonmotile cells sporulated more than unaligned nonmotile cells, achieving 16% of wild-type values (Figs. 2C and 4). Isogenic *csg*A cells similarly aligned failed to sporulate (Fig. 4), suggesting that the position-specific effects observed with nonmotile cells required C-factor.

We also found that nonmotile cells expressed a C-factor–independent *lacZ* fusion gene whether they were aligned or not (Fig. 2, G and H; compare B and H). Thus, cell alignment did not result in a global change in gene expression.

If nonmotile cell density is increased by sedimentation, nonmotile cell sporulation rises to 1% of wild-type values (*10*) (Fig. 4). However, at the highest cell density achieved by sedimentation (5×10^{10} per milliliter), nonmotile cells are still oriented in a relatively random pattern (Fig. 2D).

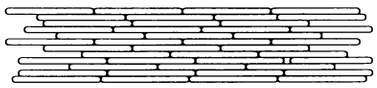

Fig. 3. Schematic representation of nonmotile *M. xanthus* strain DK4170 aligned by a grooved surface. *Myxococcus xanthus* cells are approximately 5 μm long and 0.5 μm wide (*11*). In grooves 5 to 10 μm in width created on a solid developmental surface, nonmotile cells settled with their long axes parallel to the axis of the groove. Cells outside the grooves oriented randomly. β-Galactosidase in aligned cells is depicted by stippling.

Such cells failed to express C-factor–dependent *lacZ* fusion genes (Fig. 2, E and F).

Purified, solubilized C-factor is sufficient to restore nonmotile cell sporulation and C-factor–dependent gene expression (*9*). Rescue of developmental defects in nonmotile cells by simple manipulation of cell position suggests that cell contacts formed by fruiting body morphogenesis are critical for efficient intercellular transmission of C-factor, which is normally bound tightly to the cell surface (*7*). In addition, cell movement may affect developmental cell-cell cohesion, which is critical for fruiting body morphogenesis (*23*). Because our method of cell alignment maximizes both side-to-side and end-to-end contacts (Fig. 3), it is not yet clear whether one or both types of interaction are crucial for C-factor transmission. Intercellular signaling that is restricted by cell position occurs in amphibian mesoderm induction (*1*), antigen presentation in the

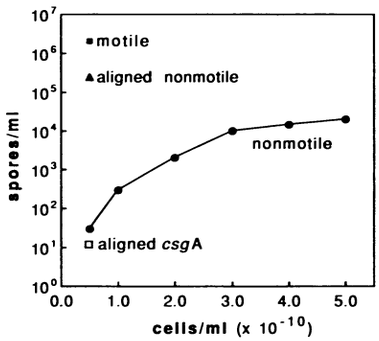

Fig. 4. Effect of cell density and position on *M. xanthus* spore differentiation. Heat-resistant, sonication-resistant, viable spores were quantitated (*20*). The sporulation defect of nonmotile cells is rescued to 1% of wild-type (motile) amounts by a tenfold increase in the density of cells induced to develop (*10*). Motile, wild-type, ■; aligned nonmotile cells in grooves, aligned nonmotile, ▲; unaligned nonmotile cells at increasing concentrations, nonmotile, ●; aligned *csg*A, □.

humoral immune response (*4*), retinal development in *Drosophila melanogaster* (*5*), and *Caenorhabditis elegans* vulva formation (*6*).

Unlike unicellular sporulation (*24*) of bacteria like *Bacillus subtilis*, myxobacteria have developed a program of coordinated multicellular sporulation. Sporulation in cohesive fruiting bodies that contain many thousands of cells ensures that a sufficiently large cell swarm will germinate for efficient feeding and growth (*25*). As measured by C-factor dependent gene expression and differentiation, we have observed that C-factor trans-

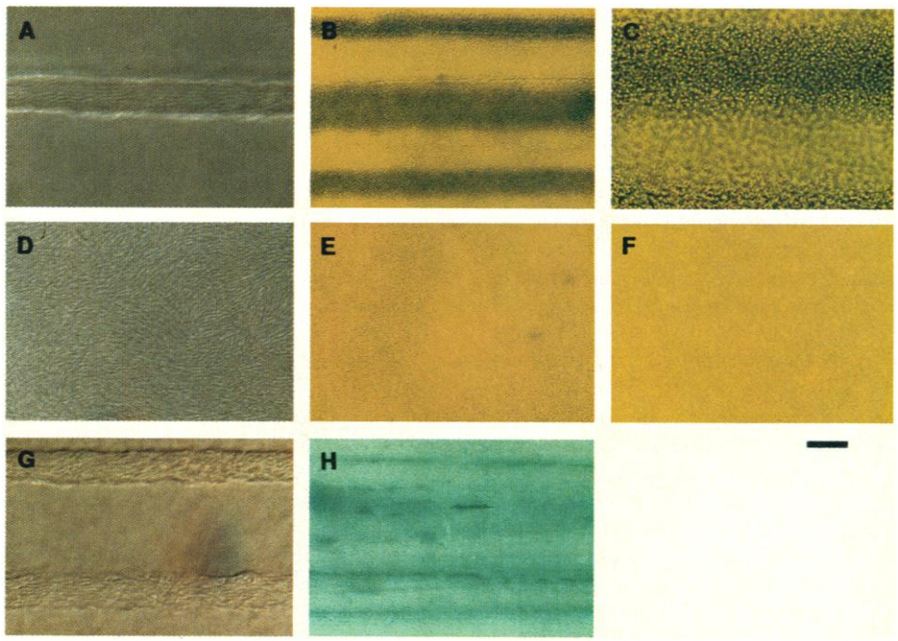

Fig. 2. Effect of cell position on C-factor–dependent gene expression and cell differentiation in nonmotile cells. (**A**) Nonmotile cells (DK4170) at 5×10^9 per milliliter were aligned in a microscopic groove created by scoring a developmental surface in one dimension with 5- to 10-μm Al_2O_3 sandpaper. Cells that settled in these grooves mainly oriented with their long axes parallel to the axis of the groove. Cells that settled outside the grooves (out of the focal plane) oriented randomly (*26*). (**B**) Developmental β-galactosidase expression by aligned nonmotile cells that contain a C-factor–dependent *lacZ* fusion gene was detected by cleavage of the chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Blue stripes of aligned cells alternating with yellow stripes of unaligned cells appeared 3 days after starvation initiated development. (**C**) Detail of a blue stripe of nonmotile cells 5 days after initiation of development, showing that cell differentiation has occurred. Refractile, ovoid spores were mainly observed within blue stripes. (**D**) Nonmotile cells (DK4170) at high density (5×10^{10} per milliliter) on a flat developmental surface formed very small rafts of side-by-side cells, but the rafts oriented randomly. (**E**) Randomly oriented nonmotile cells failed to express C-factor–dependent *lacZ* genes and remained yellow in the presence of X-gal. (**F**) Few refractile spores were observed, confirming earlier observations that densely packed nonmotile cells largely failed to differentiate (*10*). (**G** and **H**) Orientation and developmental β-galactosidase expression of nonmotile cells (DK4176) at 5×10^9 per milliliter that contained a C-factor–independent *lacZ* gene fusion. Note in (H) that no discrete cell stripes of yellow and blue cells were apparent, indicating general expression of *lacZ* both by unaligned and aligned cells. Scale bars: (A), (D), and (G), 8 μm; (B), (E), and (H), 20 μm; (C) and (F), 10 μm.

mission is more efficient when signal-producing and signal-responsive cells have achieved a geometry that favors side-to-side and end-to-end interactions between cells. This suggests that C-factor may function as a developmental timer to trigger sporulation only when multicellular aggregates have achieved the highest possible cell density.

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26. *Myxococcus xanthus* nonmotile strain DK4170 contains a *lacZ* fusion gene at position Ω4401 that is expressed in a C-factor–dependent manner (*10, 21*). DK4176 contains a *lacZ* fusion gene at position Ω4455 that is expressed independently of C-factor (*10, 21*). Cells were grown and concentrated to a density of 5×10^9 or 5×10^{10} cells per milliliter (*10*). Higher cell densities failed to produce uniform suspensions. Clone fruiting agar (*16*) was either unsupplemented or supplemented with X-gal (20 μg/ml), dried for 1 hour at 50°C, and scored in one-dimensional strokes with sterilized Al_2O_3 sandpaper (Sears, #925316). Other developmental surfaces allowed less optimal cell alignment. Cells (10 μl) were spotted on scored and unscored areas and incubated at 32°C. After 3 and 5 days, cells were photographed with an inverted light microscope (Leitz). To photograph aligned cells in grooves, cells were spotted on blocks of 2-mm-thick clone fruiting agar that had been dried and scored on glass microscope slides. These agar blocks were maintained at 32°C in a humid chamber (to prevent agar desiccation) for 24 hours, overlaid with a glass cover slip, and photographed under oil immersion with a differential interference contrast microscope at 100× magnification (Zeiss).

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Interphase and Metaphase Resolution of Different Distances Within the Human Dystrophin Gene

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Fluorescence in situ hybridization makes possible direct visualization of single sequences not only on chromosomes, but within decondensed interphase nuclei, providing a potentially powerful approach for high-resolution (1 Mb and below) gene mapping and the analysis of nuclear organization. Interphase mapping was able to extend the ability to resolve and order sequences up to two orders of magnitude beyond localization on banded or unbanded chromosomes. Sequences within the human dystrophin gene separated by <100 kb to 1 Mb were visually resolved at interphase by means of standard microscopy. In contrast, distances in the 1-Mb range could not be ordered on the metaphase chromosome length. Analysis of sequences 100 kb to 1 Mb apart indicates a strong correlation between interphase distance and linear DNA distance, which could facilitate a variety of gene-mapping efforts. Results estimate chromatin condensation up to 1 Mb and indicate a comparable condensation for different cell types prepared by different techniques.

NONISOTOPIC IN SITU HYBRIDIZATION procedures have been under development because of their greater speed and precision over widely used autoradiographic techniques (*1, 2*). We have previously demonstrated fluorescence in situ hybridization methodology capable of detecting a few kilobases of single sequences in individual metaphase or interphase nuclei (*3, 4*). This high hybridization efficiency and low background resulted from quantitative analysis of in situ hybridization parameters (*3, 5*) applied with the biotin-labeling technique (*2, 6*). In addition to applications for chromosome mapping, previous work (*3*) demonstrated simultaneous resolution in interphase nuclei of two sequences within a single integrated viral genome separated by only 130 kb. This raised the possibility that metaphase and interphase analysis combined could allow physical gene mapping across a broad range of distances, encompassing those approachable by both genetic recombination (*7*) and pulsed-field gel electrophoresis (PFGE) (*8*). The work reported here is based on one-step detection of relatively small genomic sequences (phage clones, 9- to 10-kb inserts) with standard fluorescence microscopy (*9*), without benefit of specialized image processing and computer enhancement techniques.

A sequence may be rapidly localized in terms of its relative position along the length of unbanded chromosomes, as has been recently illustrated for chromosome 11 cosmids (*10*). Cytogenetic banding techniques (including G, Q, or R banding) have been adapted for fluorescence mapping of several human genes (*11, 12*). Figure 1, A to C, provides a direct comparison of the localization of the human *Blast*-1 gene by different approaches. Although both fluorescence techniques provide improved precision and speed over autoradiography, fluorescence detection coupled with banding provided the most accurate and precise placement, independent of chromosome condensation. If measurements were restricted to longer (prometaphase) chromosomes, the range decreased substantially but not, in this case, to the point that it was quite as precise as banding. Moreover, measurements on longer chromosomes placed the gene below the position derived by banding (Fig. 1, B and C). With banding techniques the gene was readily localized to a region encompassing approximately 5 to 7 Mb of DNA. Because banding tends to be time-consuming, we have worked toward rapid simultaneous visualization by two-color fluorescence of bands with hybridization signals (Fig. 1D). Bromodeoxyuridine (BrdU) incorporation

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