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Gliding Motility in Slide Cultures of *Myxococcus xanthus* in Stable and Steep Chemical Gradients

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A method was devised to construct stable and steep chemical gradients in slide cultures to study the movements of gliding cells. The movement of *Myxococcus xanthus* individual cells and small swarms was studied in these gradients. There was no response to gradients of Casitone and yeast extract that were previously reported to stimulate a positive chemotactic response with *M. xanthus*.

Myxococcus xanthus is a gliding bacterium that feeds communally on other bacteria and *Saccharomyces cerevisiae* and aggregates to form fruiting bodies under conditions of nutrient depletion (3). Whether *M. xanthus* responds chemotactically to nutrient or other chemical signals (e.g., those that may be secreted by developing aggregates) has been a controversial and unresolved question. Several years ago, McVittie and Zahler reported that when aggregates of *M. xanthus* were separated by a dialysis membrane from an overlying population of cells, aggregates formed in the overlying population directly over the previously formed aggregates (9). Fluegel reported similar results with *Myxococcus fulvus* (5). These experiments were interpreted as evidence for the production of positive chemotactic signals by developing aggregates. Ho and McCurdy reported positive chemotaxis of *M. xanthus* towards cyclic GMP and 5-AMP (6), but this could not be confirmed by Dworkin and Eide (4). The latter authors also could not demonstrate chemotaxis towards other compounds, including Casitone. In contrast to the results obtained by Dworkin and Eide, Shi et al. recently reported positive chemotaxis of *M. xanthus* towards a medium containing Casitone and yeast extract (10). An obstacle to studying chemotaxis in *M. xanthus* has been the construction of steep and stable chemical gradients that would allow observation of the slowly moving single cells or swarms entirely within the gradient over long periods. We report here a simple method to construct steep and stable chemical gradients in slide cultures. This has allowed us to study the responses of single cells and microscopic swarms of *M. xanthus* to chemical gradients. Our results indicate that *M. xanthus* does not respond chemotactically to gradients of Casitone or yeast extract.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *M. xanthus* (A^+S^+) DK1622 (obtained from M. Dworkin, University of Minnesota) was grown on a rotary shaker at 32°C in 1% Casitone (Difco) or Tryptone (Difco) with 8 mM $MgSO_4$.

Construction of gradients. Two 300-liter agar (Bacto Agar [Difco]) blocks (17.7 by 7.3 by 2.5 mm) placed 2 mm apart were connected by a 20- to 30-μm-thick bridge of agar on a coverslip (Fig. 1). The agar blocks were made by pipetting 10 ml of the source or sink agar into a Fisher round plastic petri plate (100 by 15 mm). After the agar gelled, the blocks were cut out by pressing the compartments of a four-well LabTek chamber slide system (item no. 177399) into the agar. The two agar blocks were removed onto the edge of each of two coverslips (18 by 18 mm) and positioned 2 mm apart. The agar blocks were allowed to air dry for 1 h. A larger coverslip (22 by 22 mm) was then dipped into molten (60°C) agar, drained on a tissue, wiped clean on one side, and placed agar side down across the two agar blocks resting on the coverslips. After inoculation of the gradient, a glass slide was placed under the agar blocks on the opposite side of the larger coverslip, and the cells were viewed microscopically through the larger coverslip. It made no difference whether the two coverslips on which the agar blocks were initially resting were removed prior to adding the glass slide. Inoculation and sealing of the slide cultures are described below.

Both the source and sink blocks contained 2% agar and HMC (10 mM *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid [HEPES; pH 7.2], 8 mM $MgSO_4$, 1 mM $CaCl_2$). Additionally, the source block contained a putative attractant. When the expansion of swarms was monitored, 100 μM nalidixic acid was present in both the source and sink blocks to prevent swarm expansion due to growth. The thin agar connecting the source and sink blocks contained 1% agar, HMC, and nalidixic acid (when swarms were observed).

Quantitation of the fluorescein gradients. Fluorescein was used as a dye marker to quantitate the gradients and to determine their stability. The source block contained 0.20 g of fluorescein per ml and HMC in 2% Difco agar. The sink block was simply HMC in 2% agar. Fluorescein gradients were equilibrated 20 and 48 h prior to measurement. The intensity of fluorescein in the thin agar layer that contained the gradient was measured as a function of exposure time by using the photometer on an epifluorescent Zeiss Photoscope III (ASA 1600; 1.25× Optivar, 25× Neofluor objective) (2). The agar layer was cut free with a sharp razor blade close to the source and sink blocks (which were then discarded) to determine the readings of the first and final points in the gradient, since light scattering from the source and sink blocks made those measurements inaccurate in the intact apparatus.

To document that the gradient was not disrupted by the introduction of the inoculum, a drop of nonfluorescent fluid similar in volume to the cell inoculum was placed in the fluorescein gradient and allowed to dry. For the purposes of finding the position of the drop and focusing, the suspension contained nonfluorescent latex beads (IDC Spheres; sulfated polystyrene latex, 1.01-μm diameter, 8.2% [wt/vol]; Interfacial Dynamics Corp., Portland, Oreg.) added at a dilution of 10^{-4}. Fluorescein intensity at two positions within the drop was measured at 5 min after the drop dried by use of a 40× objective and the method described above. As a control, measurements at equivalent positions outside of the drop were also made.

Assay of dispersed cells by the developing gradient method. Exponential-phase cells grown in the tryptone medium were diluted with 10 mM HEPES (pH 7.2) to a density of 5×10^7 per ml, and latex beads at a dilution of 10^{-5} were added to the cell suspension; the beads served as a position marker during observations of cell movements. The thin agar layer on the larger coverslip (22 by 22 mm) was dipped into the dilute cell suspension, drained, and lightly air dried (30 s) before the coverslip was placed across the two agar blocks that had been positioned 2 mm apart and had been allowed to air dry for 1 h. The apparatus was placed onto a glass microscope slide with the side containing the thin agar bridge on top. Gradient slides constructed in this manner were sealed around the perimeter with Vaspar (1 part petrolatum, 2 parts paraffin) and incubated at 30°C for 6 to 9 h prior to observation of cell movements. The source block contained Casitone at a concentration of 20 mg/ml.

The behavior of the cells was monitored in three positions within the gradient, and their movements were traced for 6 to 9 h after assembly of the gradient slides. Cells were observed in real time on a Sony Trinitron monitor with a Nikon Optiphot-2 phase microscope and a Sony 3CCD video camera at a magnification of ×3,500 on the video screen; movements were manually traced from the screen. The temperature on the microscope stage was maintained at approximately 30°C. The net movement of each cell in the field at the end of 30 min was resolved into the components of four different directions, arbitrarily designated *X*+, *X*−, *Y*+, and *Y*−. The *X*+ movements were in the direction of increasing concentration of Casitone. Data from 497 cells on 43 different slides were statistically analyzed by Student's *t* test (independent samples with equal vari-

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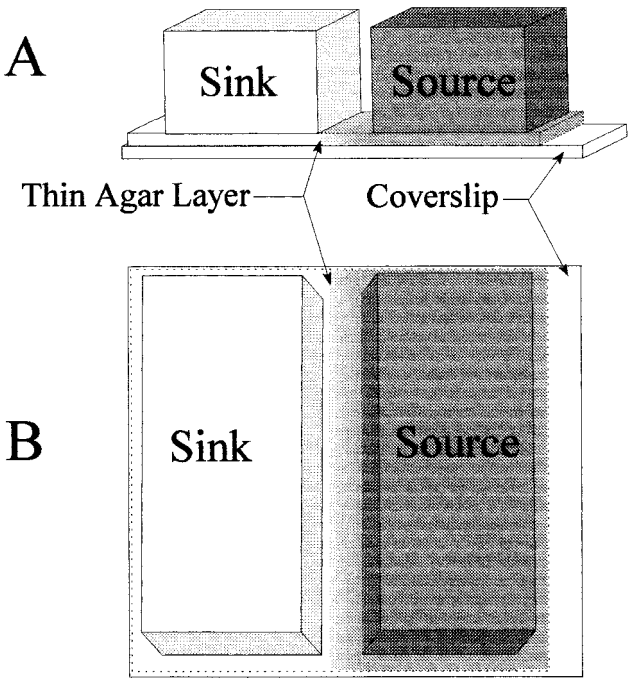

FIG. 1. Diagrammatic representation of the bridge diffusion apparatus. (A) Oblique view. The two coverslips on which the agar blocks were resting while they were being positioned 2 mm apart have been removed. For the established gradient method, the gradient would be incubated as shown in a moist petri dish in the refrigerator for several hours. After inoculation of the thin agar layer between the agar blocks, a glass slide (not shown) is placed on top of the agar blocks. The apparatus is then inverted and the cells are viewed through the coverslip. For the developing gradient method, the thin agar layer is inoculated by dipping the coverslip in a suspension of cells prior to placing the coverslip on the agar blocks. (B) Superior view. The dotted line refers to the perimeter of the thin agar layer. The glass slide which would be on top of the agar blocks is not shown.

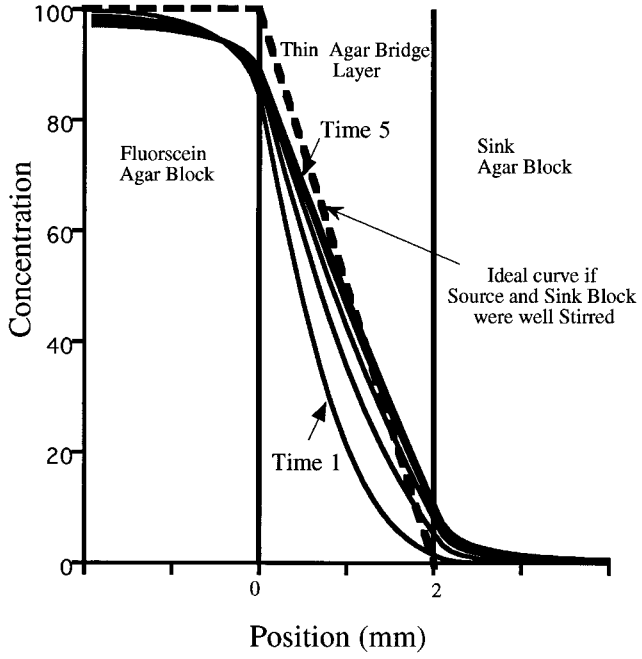

FIG. 2. Computer simulation of gradient of fluorescein. Fick's Law of Diffusion was used to predict the gradient that would form if fluorescein were in the source block of agar. Each line approximates the gradient at various times. On the basis of these calculations, the gradient would be present at 0.41 h, become linear at 4.16 h, and not change for several days. A difference is predicted depending upon whether the source and sink are stirred. If there is no stirring, then the slope of the gradient is decreased by 20%.

ance) to determine if the presence of a gradient influenced the distance and frequency of cell movements in the four directions measured.

Assay of dispersed single cells by the established gradient method. Prior to inoculation, the gradient was allowed to equilibrate refrigerated for 20 h in a closed petri dish lined on the inside with damp paper. The apparatus was placed such that the thin agar layer in the gradient was facing up to avoid condensation. Exponential-phase cells grown in the Casitone medium were harvested by centrifugation (Micro-Centrifuge; Fisher model 235A) for 5 min at 16,720 × *g* at room temperature and resuspended to a calculated 200 Klett units in 10 mM HEPES (pH 7.2) with latex beads at a dilution of 10^{-5}. A drop of cells or a small clump of cells was inoculated with the fine tip of a drawn-out Pasteur pipette approximately in the center of the 2-mm gradient. The cells initially spanned an area of approximately 0.5 mm. After the drop had dried (3 to 5 min), a glass slide was placed over the agar blocks and the gradient was sealed. Movements in the center of the gradient were observed for 30 min by video microscopy and analyzed as described for the developing gradient method.

Assay of swarms: established gradient method. Gradients were equilibrated for 6 h prior to inoculation by the procedure for dispersed single cells. Cells were grown in the Casitone medium, harvested by centrifugation, and resuspended to a calculated density of 3,000 or 300 Klett units with 10 mM HEPES (pH 7.2). Latex beads were included at a dilution of 10^{-3} in the 3,000-Klett-unit suspension and at a dilution of 10^{-4} in the 300-Klett-unit suspensions to mark the original boundary of the cell drop. After the drop had dried, a glass side was placed over the agar blocks. The slides were not sealed with Vaspar, and incubation in the moist petri plate chamber was continued for 18 h at room temperature prior to microscopic observation and photography. Photographs (TriPan 400 black and white film with a green 550-nm filter) were taken with a Zeiss phase Photoscope III. Gradients were constructed with either 10 mg of Casitone per ml or CYE (10 mg of Casitone per ml, 5 mg of yeast extract per ml) in the source block.

Alternatively, small clumps of cells were prepared and inoculated onto established gradients that had equilibrated for 12 h as described for dispersed single cells. Exponential-phase cells grown in the Casitone medium were harvested after the addition of latex beads at a dilution of 10^{-4}. The cells and beads were resuspended in 10 mM HEPES (pH 7.2)–1 mM $CaCl_2$–1 mM $MgSO_4$ to one-half the original volume. After settling for 1 h, the suspension was spun for 10 s at 16,720 × *g* at room temperature to sediment clumped cells and resuspended to a final volume of one-fourth the initial volume with a second addition of latex beads (10^{-3} dilution). A drop of the suspension was placed onto the 2-mm region of the established gradient with the fine tip of a drawn-out Pasteur pipette and allowed to dry in for 3 to 5 min before photography. In this system, it was found that latex beads became associated with the center of the clump of cells and served as an indicator of the initial position of the cell cluster. Other latex beads, presumably the ones added after the cells clumped, marked the perimeter of the drop. Photographs were taken as soon as the drop had dried in (0 min) and at 15 and 30 min after inoculation with a Zeiss phase Photoscope III. The gradients were not sealed, and between photographs, the apparatus was returned to the moist petri dish. The source block contained either 10 mg of Casitone per ml or CYE.

RESULTS

Gradients. The idea for an apparatus with an agar bridge between two much larger blocks was suggested by the knowledge that if a bridge connected two large reservoirs, then a stable linear gradient would develop (7). This is shown by the dashed line in Fig. 2. It would apply if the two reservoirs were well stirred. Because they are not stirred at all, a computer simulation was carried out and used to generate the solid lines in Fig. 2 (8). The simulation shows that stable gradients should be produced quickly and, once established, have 80% of the slope that they would have had if the diffusion within the source and sink blocks was very much faster than it was in the bridge (i.e., if the source and sink were well stirred). The value of 80% was estimated by dividing the concentration difference between source and sink by the bridge length of 2 mm. The gradient would remain stable for an extremely long time because the bridge volume through which diffusion must occur is very small compared with the volume of the source and sink.

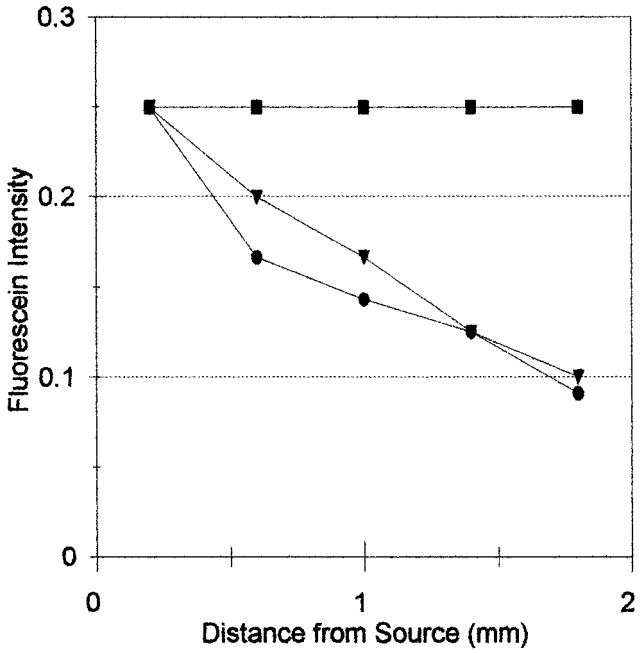

FIG. 3. Fluorescein gradient. Measurements of fluorescein were made at 20 and 48 h. The fluorescein control contained fluorescein in both the source and the sink. The units of measurement are expressed per second. Symbols: ■, fluorescein control; ▼, fluorescein gradient at 48 h; ●, fluorescein gradient at 20 h.

Simulations (data not shown) indicate that it would be weeks before the gradient slope lessened appreciably.

The times given in the computer simulation are for a freely diffusing molecule with the molecular weight of fluorescein. Since Casitone consists of a range of oligopeptides, the diffusion constant for each component will be different. A dipeptide has about the same molecular weight as fluorescein and should diffuse as shown in Fig. 2. Because an oligopeptide of eight amino acids is four times larger, its diffusion constant would be smaller by the square root of four, and the times marked on the curves would apply if they were doubled. Experimental measurements of fluorescein indicated that gradients were steep and remained stable for at least 48 h in agreement with the theoretical predictions (Fig. 3). Placing a drop of liquid on the fluorescein gradient temporarily increased the steepness very slightly compared with measurements in the control area of the gradient. After 5 min, the drop of fluid placed on the fluorescein gradient had altered the gradient steepness very slightly compared with measurements in the control area of the gradient (0.071 versus 0.077 and 0.053 versus 0.059 s^{-1}).

Measurements of motility by dispersed single cells in a developing gradient. As shown in Table 1, dispersed single cells moved an average of 1.7 to 2.8 μm/min and reversed an average of once every 10 to 12 min. The net average distance travelled per cell during the 30-min observation period was 3.4 to 7.7 μm (Table 1). When the movements of 497 cells were measured, the mean cell movement parallel to the Casitone gradient (*X* direction) was not significantly greater than the movement perpendicular to the gradient (*Y* direction) (Table 2). This was true for all three concentration ranges in the gradient where measurements were made. Table 2 shows that there was no statistically significant difference in the mean distance of net cell movement in any of the four directions ($P > 0.05$) except at 1.5 mm from the source where the direction of greatest net cell movement was in the *Y*+ direction ($P < 0.05$). However, the mean net movement was significantly less in the higher Casitone concentrations (closer to the source block).

TABLE 1. Average rate of movement and frequency of reversal of dispersed single cells in a Casitone gradient (developing gradient method)[a]

Position (mm)[b]	Rate of movement (μm/min)			Reversals/30 min		
	Mean	SD	*n*	Mean	SD	*n*
0.5	1.701	0.964	10	2.875	1.586	16
1.0	2.247	1.071	12	2.538	1.128	13
1.5	2.855	1.215	13	3.0	1.468	14

[a] The source contained 20 mg of Casitone per ml.
[b] Distance from edge of source block.

Measurements of motility by dispersed single cells in an established gradient. To further improve the system, the cells were inoculated onto an established gradient to decrease any lingering effects that movement in the absence of a gradient may have had, such as establishment of random slime trails that could have biased cell movements once the gradient was established. As shown in Table 3, the *P* values indicate that the mean cell movement parallel to the gradient (*X* direction) was not significantly different from the movement perpendicular to the gradient (*Y* direction) for both the Casitone and CYE gradients. The mean net movement at the distance of 1 mm from the 10-mg/ml source block (approximately 5 mg of Casitone per ml) was comparable to the mean net movement at 1.5 mm from the 20-mg/ml source block in Table 2. In both of these situations, the cells were moving in approximately 5 mg of Casitone per ml. Table 3 also indicates that the mean value for the CYE gradient in the *X* direction was much larger than its standard deviation. This was because a single cell (of the 32 cells that were observed) moved an extraordinarily large distance in both the *X*+ and *Y*− directions. Therefore, a second test, which tests only the directionality of movement and not the distance in any particular direction, was done. This test is routinely used by behavioral biologists who study the orientational abilities of motile organisms. It was developed by Lord Rayleigh in 1880, and the details of the method are described by Batschelet (1). Basically, the total travel of all observations was set at unity, the directional components *X* and *Y* from the original data were summed, and the mean angle was computed. For the CYE data that generated the numbers in Table 3, the mean vector length was −56°. The root mean square of the *X* and *Y* components combined is called *r*. The *r* value can range from 0 (no orientation) to 1 (precise orientation). For the CYE data, this value is 0.03125, and the *P* value for the hypothesis that movement is random is greater than 0.90, indicating no directionality.

Observations of swarms in the established gradient. Although we failed to detect positive chemotaxis by individual cells, we considered the possibility that swarms might behave chemotactically, i.e., have one edge preferentially moving toward the nutrient. This, in fact, was recently reported by Shi et al. (10). Accordingly, droplets of bacteria were inoculated onto gradients that had equilibrated for 6 h and were photographed after 18 h of incubation. Figure 4 shows that swarms generated from drops of bacteria expanded symmetrically around their circumference when placed in a gradient of Casitone. Similar results were obtained with CYE. The same results were obtained after 58 h of incubation and with gradients that had equilibrated for 16 h prior to inoculation. Similarly, swarms

TABLE 2. Direction of movement of dispersed single cells in a Casitone gradient (developing gradient method)[a]

Position (mm)[b]	*n*	Movement (units)[c] in:				*P* value	Net movement (units)[c]	
		X direction		*Y* direction				
		Mean	SE	Mean	SE		Mean	SE
0.5	115	−0.94	1.36	−0.35	1.11	0.371	11.81	1.104
1.0	205	0.56	1.21	−0.21	1.21	0.328	15.82	1.315
1.5	177	1.26	1.82	5.73	1.78	0.042	26.78	1.613
All positions (0.5 + 1.0 + 1.5)	497	0.47	0.88	1.87	0.86	0.128		

[a] The source contained 20 mg of Casitone per ml. The net movement for each cell in the field at the end of 30 min was resolved into the components of four different directions, i.e., *X*+, *X*−, *Y*+, *Y*−. Movement in the *X*+ direction was towards increasing nutrient concentration. Movement in the *Y* direction was perpendicular to the gradient. Mean net movement was calculated on the basis of both *X* and *Y* values for each position.
[b] Distance from the source block.
[c] One unit = 0.29 μm.

formed from cell clumps placed on gradients of Casitone or CYE that had equilibrated for 12 h expanded symmetrically (Fig. 5 and 6).

DISCUSSION

Shi et al. (10) reported that colonies of *M. xanthus* expanded towards higher concentrations of Casitone and CYE, which they interpreted as positive chemotaxis. We could not demonstrate chemotaxis even though the gradients that we used were steeper than those used by Shi et al. A computer simulation (such as that shown in Fig. 2) shows that the concentration at the sink side of the plastic partition must have been considerably less than the concentration in the source, and consequently, the gradient was much more shallow than they reported (8). Our results support and extend those of Dworkin and Eide, who reported that *M. xanthus* swarms (colonies) did not respond chemotactically (4). The experiments of Shi et al. regarding positive chemotaxis differ from those reported here in two ways. (i) They placed their cells at the edge of the gradients on the nonnutrient side, whereas we placed the cells within the gradients. (ii) They used 0.3% agar, whereas we used 1% agar in the gradient. They reported that 0.3% agar does not support noticeable colony expansion in the absence of nutrient, which may explain their observation of limited expansion into the nonnutrient side. We used 1% agar because it supported swarm expansion even in the absence of added nutrient (11). Thus, we simply asked whether movement was biased towards the higher nutrient concentrations within the gradients. Our conclusion is that it was not, regardless of whether we examined single cells or groups of cells.

TABLE 3. Direction of movement of dispersed single cells in a Casitone or CYE gradient (established gradient method)

Gradient	*n*	Movement (units)[a] in:				*P* value	Net movement (units)[a]	
		X direction		*Y* direction				
		Mean	SE	Mean	SE		Mean	SE
CYE[b]	32	8.37	3.97	4.43	4.52	0.2579	29.9	3.20
Casitone[c]	31	5.59	4.14	1.74	3.65	0.2379	27.4	2.79

[a] One unit = 0.29 m. Net movement for each cell in the field 1 mm from the source block was resolved at the end of 30 min into the components of four different directions, i.e., *X*+, *X*−, *Y*+, and *Y*−. Movement in the *X*+ direction was towards increasing nutrient concentration. Movement in the *Y* direction was perpendicular to the gradient. Mean net movement was calculated on the basis of both *X* and *Y* values for each position. The gradients had equilibrated for 20 h before inoculation.
[b] Source contained 10 mg of Casitone per ml and 5 mg of yeast extract per ml.
[c] Source contained 10 mg of Casitone per ml.

Zusman and his coworkers have shown that *M. xanthus* contains proteins (the Frz proteins) that are similar to the chemotaxis proteins (the Che proteins) of enteric bacteria, both in structure and in ability to be phosphorylated and methylated, and that these proteins influence the frequency of reversals during gliding (reviewed in reference 3). The FrzCD protein, which is the homolog to the enteric MCP protein, is methylated in the presence of nutrients and demethylated in the presence of alcohols (10). Cells mutated in the Frz proteins did not show directed movements towards nutrients or away from

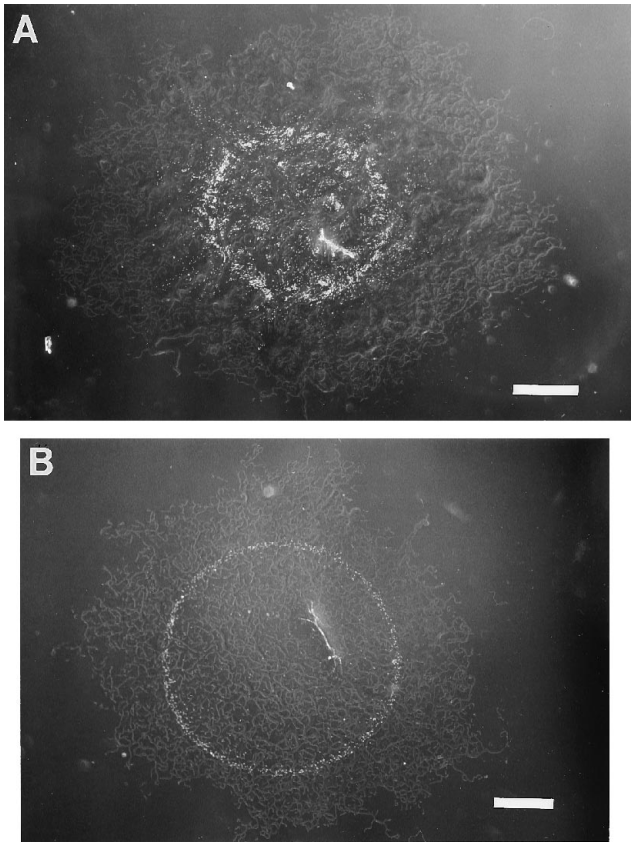

FIG. 4. Expansion of swarms formed from drops of cells in a Casitone established gradient. A drop of cells at 3,000 (A) or 300 (B) Klett units was inoculated onto an established gradient that had equilibrated for 6 h. The photographs were taken 18 h after incubation of the gradients. Bars, 0.1 mm.

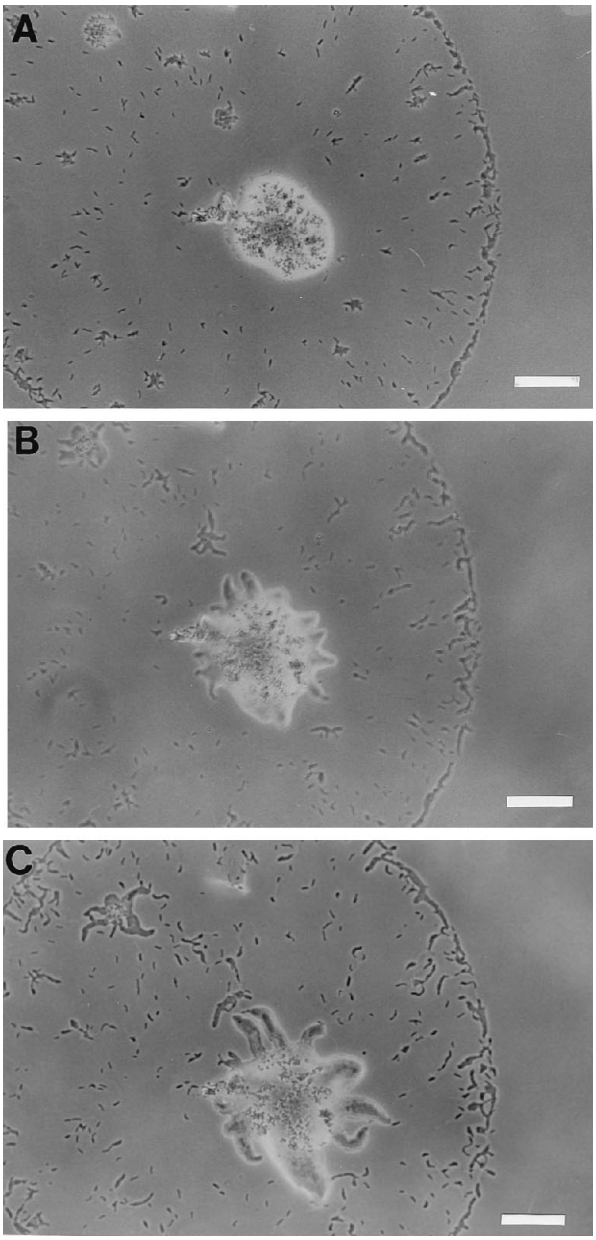

FIG. 5. Expansion of swarms formed from cell clumps in Casitone established gradients. Clumps of cells were formed by allowing the cells to settle in the presence of latex beads and inoculated onto an established gradient that had equilibrated for 12 h. The bead mass in the center of the cell clump marks the original position of the inoculum. Photographs were taken at 0 (A), 15 (B), and 30 (C) min after the inoculum had dried into the agar. The source block contained 10 mg of Casitone per ml. The nutrient concentration increases from left to right. Bars, 0.04 mm.

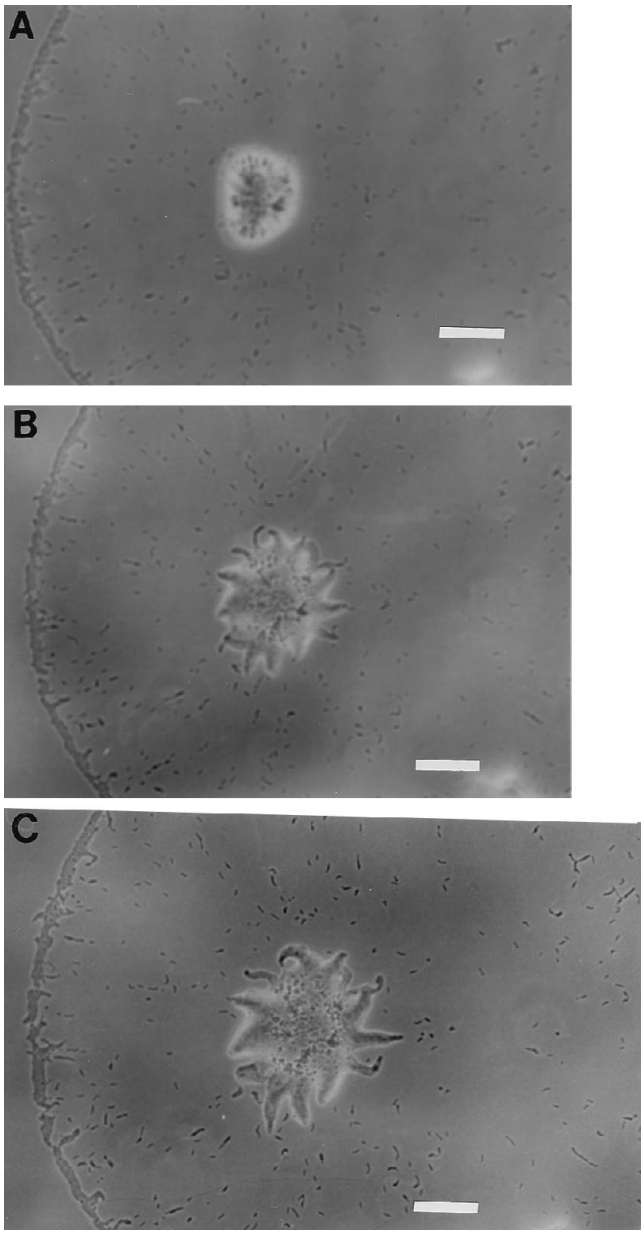

FIG. 6. Expansion of swarms formed from cell clumps in CYE established gradients. For conditions and identification of panels, see the legend to Fig. 5. The source block contained 10 mg of Casitone per ml and 5 mg of yeast extract per ml. The nutrient concentration increases from left to right. Bars, 0.04 mm.

alcohols in their chemotaxis assay (10). They concluded that the Frz proteins function in a signaling system which they interpret to be part of chemotaxis, perhaps analogous to the role of Che proteins in the enteric bacteria. However, we suggest that despite the involvement of the Frz proteins, the evidence does not support the conclusion that *M. xanthus* responds to chemical gradients per se but probably does support its response to the presence or absence of certain chemicals, e.g., nutrients. Additionally, there has not yet been demonstrated an adaptive response to the nutrients, which would be expected if the cells were responding to a gradient of nutrient. In this way, the responses of *M. xanthus* differ from the chemotactic responses of flagellated bacteria. If, indeed, *M. xanthus* does not respond to gradients of diffusible nutrients, this is not necessarily a disadvantage, since its primary sources of nutrients in nature are thought to be intact microorganisms, such as bacteria and *S. cerevisiae* which are lysed when portions of the swarm reach the prey microorganisms.

It has often been suggested that myxobacteria might move towards developing aggregates during fruiting body formation in response to diffusible positive chemoeffectors secreted by the aggregates. No such substances have yet been identified. However, White et al. have suggested an alternative mechanism of aggregate formation in the myxobacterium *Stigmatella*

sp. based upon following the slime trails laid down by the bacteria and a lipoidal pheromone that might keep the cells moving in circles at the site of aggregation, thus trapping the cells there (12, 13), and Koch has written a computer simulation in which aggregation does not rely on a diffusible positive chemoeffector but does depend on random forward and backward motions biased towards higher concentrations of cells (8).

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