Cell-to-cell stimulation of movement in nonmotile mutants of *Myxococcus*

(intercellular communication/swarming/gliding/development)

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ABSTRACT A large number of nonmotile mutants of the gliding bacterium *Myxococcus xanthus* have been isolated and partly characterized. About $\frac{1}{10}$ of these mutants are conditional mutants of a novel kind: mutant cells become transiently motile after contact with nonmutant cells or with cells of a different mutant type. These "stimulatable" mutants fall into five phenotypic classes (types B, C, D, E, and F). Most mutants are nonstimulatable (type A) and never become motile, but type A cells (and wild-type cells) can stimulate cells of any of the other five types. Stimulatable mutants of different types are capable of stimulating each other. For example, in a mixture of B and C cells, both become motile. Linkage analysis using a generalized transducing phage has shown that each of types B, C, D, E, and F corresponds to a single distinct genetic locus. Type A mutants, by contrast, belong to at least 17 different loci. Stimulation depends on close apposition of interacting cells, because stimulation does not occur when contact between cells is prevented. It is possible that the stimulatable mutants are defective in components of the gliding mechanism that can be exchanged between cells. Alternatively, they may be defective in a system of cell communication controlling the coordinated cell movements observed in *Myxococcus*.

The myxobacteria are a group of Gram-negative bacteria that exhibit multicellular behavior and can undergo primitive differentiation (1). They provide a useful system for studying cell-cell interaction and development in a simple organism because they resemble more familiar Gram-negative bacteria in their structure and they can be grown and manipulated with ease.

The life cycle of *Myxococcus xanthus*, the best characterized of the myxobacteria, superficially resembles that of the cellular slime mold *Dictyostelium discoideum*, a eukaryote. The cells can grow vegetatively like other bacteria but, when nutrients become limiting, myxobacteria aggregate to form fruiting bodies. Within the fruiting bodies, the cells sporulate, changing from the long vegetative rods to short ovoid spores with refractory properties. Under appropriate conditions, the spores germinate to give vegetative cells once more.

Organized movement is obviously necessary for fruiting, in which thousands of cells enter the same fruiting body, but the mechanism and control of this movement are not understood. Myxobacteria move by gliding, a method of surface translocation found in several unrelated prokaryotic groups and not explained in any of them (2). It seems quite distinct from the flagellar swimming exhibited by most motile bacteria. Gliding occurs only on an interface (usually solid-liquid) and involves the slow, smooth movement of rod-shaped cells in the direction

of their long axis. Time-lapse movies of moving myxobacteria (3) show that cells gliding on agar can stop and reverse direction. The cells also appear to bend and change direction, but this bending may be a passive response to irregularities in the substrate, because vegetative cells in suspension culture invariably are unbent straight rods.

The movement of vegetative cells is also coordinated. Growing cells move together as a swarm, which allows pooling of extracellular digestive enzymes and may thus have adaptive value (1, 4). Time-lapse movies show that cells will move out for a short distance from the edge of an expanding swarm, but they never leave it completely, so that the whole swarm behaves as a coherent unit. Chemotactic signals have been invoked to explain the coordination of myxobacterial movement in fruiting and in swarming, by analogy with the chemotactic signals demonstrated in the slime molds. However, myxobacterial cells are so small ($0.5 \times 7 \mu\text{m}$) and move so slowly ($<5 \mu\text{m}/\text{min}$) that the analogy may not apply. An alternative or additional coordinating mechanism could be provided by direct cell-cell contacts, which are believed to be important in the formation of fruiting bodies (5).

Further evidence of the importance of cell-cell contacts in *Myxococcus* is provided by this work, which began as an attempt at a genetic analysis of motility. Some motility mutants of *M. xanthus* have already been reported (6, 7). In the hope of gaining insight into the mechanism and control of gliding, we isolated a large number of new nonmotile mutants. Among these mutants were several that could be stimulated to move transiently by contact with wild-type cells or cells of a different mutant type. About $\frac{1}{10}$ of the mutants isolated were stimulatable, and these fell into five phenotypic classes. We think it possible that these mutants have the capacity to move but have lost part of a communication system controlling movement and therefore do not move by themselves.

MATERIALS AND METHODS

Strains and Media. The motile parent strain used in this work was DK101, a derivative of *M. xanthus* FB (DK100), which is a standard fruiting strain (8).

Liquid cultures were grown in 1% casitone/8 mM MgSO_4 /10 mM Tris-HCl, pH 7.6/1 mM potassium phosphate, pH 7.6 (CTT broth). Solid media were CTT agar (CTT broth plus 1.5% agar), $\frac{1}{2}$ CTT agar (CTT agar with only 0.5% casitone), or TM agar (CTT agar without casitone or phosphate). When indicated, streptomycin was used at 1 or 2 mg/ml and rifampicin at 5 or 10 $\mu\text{g}/\text{ml}$.

Motility Assay. Cells to be tested were suspended in CTT broth, at a density of about 4×10^8 cells per ml, and a 2- μl drop of this suspension was placed on an agar surface by using an inoculating loop. The drop would quickly dry down to form a small spot of cells, and the edge of this spot was examined 6 or

Abbreviations: CTT broth, 1% casitone/8 mM MgSO_4 /10 mM Tris-HCl, pH 7.6/1 mM potassium phosphate, pH 7.6; TM buffer, 8 mM MgSO_4 /10 mM Tris-HCl, pH 7.6; MeSO_2OEt , ethyl methanesulfonate; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

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more hours later to see how far cells had migrated outward. Sometimes carbon particles were included in the suspension to mark the initial margin of the spot and hence to allow rough measurement of the distance migrated. Cells were examined microscopically and photographed with phase optics and a long working distance condenser.

Mutagenesis. Single motile colonies of DK101 were inoculated into CTT broth, grown to a density of 5×10^8 cells per ml, washed by sedimentation with 8 mM MgSO_4 /10 mM Tris-HCl, pH 7.6 (TM buffer), sedimented, and resuspended in TM buffer at their initial density. For ethyl methanesulfonate (MeSO_2OEt) mutagenesis, the MeSO_2OEt was added to 1% and the culture was shaken at 33° for 60 min. For mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG), freshly dissolved NG was added to a concentration of 100 $\mu\text{g}/\text{ml}$ and the culture was shaken for 20 min. After either type of treatment, cells were sedimented and washed with TM buffer. UV mutagenesis was carried out by exposing 1 ml of the washed cell suspension in a 10-cm glass petri dish to irradiation from a mercury lamp for 2 min at a dose rate of 21 ergs/ mm^2 per sec. Survival was about 30% after MeSO_2OEt treatment, about 10% after NG, and usually less than 1% after UV irradiation. For ICR-191 mutagenesis, DK101 cells growing in CTT broth were diluted to $10^7/\text{ml}$ with CTT broth + 0.2% yeast extract, and ICR-191 was added to 2 $\mu\text{g}/\text{ml}$. The culture was then shaken in the dark for 20–60 hr (ICR-191 was a kind gift from R. Peck). After all types of mutagenesis, cells were diluted 1:10 into CTT broth and grown to a density of 2 to 8×10^8 cells per ml, to allow expression of recessive mutations, before plating on CTT agar for single colonies.

Selection for Motility. To select motile cells from a non-motile background, a spot of cells was grown on nutrient agar. In time, the motile cells migrated out from the smooth margin formed by the nonmotile cells and formed a conspicuous "flare." Reconstruction showed that 1 motile cell can be detected in a background of about 10^6 nonmotile cells. Detection efficiency, however, was decreased in backgrounds of certain nonmotile mutants.

To transduce motility from one bacterium to another, the generalized transducing bacteriophage Mx8 (isolated by Sandra Martin) was grown on the donor strain. Recipient bacteria and phage, irradiated with UV to 10% survival, were mixed and incubated for phage adsorption; then, 55 spots of about 10^6 cells each were distributed on a $\frac{1}{2}$ CTT plate. Spots were examined microscopically for flares (motile transductants) after 5 days' incubation at 33° . Under the conditions used, the number of spots with flares is roughly proportional to the number of motile transductants.

RESULTS

Isolation of Nonmotile Mutants. A colony of motile (wild type) myxobacteria has a characteristic appearance on CTT agar: it is flat and surrounded by a thin sheet of cells migrating outward that forms an irregularly edged "halo" around the colony. Myxobacterial cells move preferentially along lines of stress in agar (9), so that whole colonies are often elliptical in shape as a result of elongation along one axis. By contrast, a colony formed by a nonmotile mutant lacks the halo and is circular in outline, and consequently it is readily distinguished from a wild-type colony by visual examination (Fig. 1).

More than 200,000 colonies of mutagenized *M. xanthus* DK101, a motile strain, grown at 33° on CTT agar were screened in a search for nonmotile mutants, and about 350 mutants were isolated. Most of these were induced by UV irradiation, and the remainder by exposure to MeSO_2OEt , NG,

FIG. 1. Motile and nonmotile colonies of *M. xanthus* grown on CTT agar at 33° . ($\times 30$.)

or ICR-191. Spontaneous nonmotile mutants occur at a frequency of 10^{-4} or less. Not all of the 350 mutants were independently isolated, so usually only one mutant was retained from each mutagenesis. A collection of 87 mutants derived from 61 independent mutagenized cultures is described here.

Mutants were characterized further for motility under changed culture conditions: presence or absence of nutrients (CTT agar or TM agar) and at low and high temperatures (25° or 33°). Most mutants showed no movement of single cells under any condition tested. However, there usually was some movement of groups of cells, manifested as an irregular spot edge, on low nutrient agar or at low temperature. But, in all cases, the irregularity of the edge was less than that of the semimotile mutant described by Burchard (6).

Effect of Mixing Different Mutants; the Stimulation Phenomenon. During attempts to conjugate or fuse different nonmotile mutants with each other, it was found that mixtures of certain pairs of mutants were capable of movement although, in each case, neither member of the pair was motile by itself. The ability to move was transient; no stably motile strain could be obtained from the mixtures. This observation and the results described below suggest that the effect occurs at the phenotypic level rather than by any genetic exchange.

Tests on a large number of pairwise combinations of different mutants divided them into six phenotypic classes, termed types A, B, C, D, E, and F. Each mutant was assigned to a type by a mixing test: for example, all type A mutants failed to produce movement when mixed with each other but did produce movement when mixed with mutants of a different type, etc. The matrix of pairwise combinations of standard mutants of the six types is shown in Fig. 2.

The distribution of mutants into different classes was far from equal: type A mutants accounted for 90% of all nonmotiles isolated. The collection of 87 mutants (in which type A was underrepresented) consisted of 56 type A, 19 type B, 5 type C, 3 type D, 3 type E, and 1 type F. Some of the classes had additional general characteristics—for example, all type D and type E isolates were quite motile at low temperature or on low nutrient agar ($\frac{1}{2}$ CTT or TM). Even under standard conditions, type E had some motility, because the spot edge (Fig. 2) was more irregular than with the other mutants. Nine type A mutants belonging to the *mg1* locus described below were completely nonmotile under all conditions.

The simple mixing test does not reveal whether one or both mutants become capable of movement in a mixture. Accordingly, antibiotic-resistant derivatives of standard mutants were mixed. Moving cells from mixtures were picked up from the

FIG. 2. Matrix of combinations of motility mutants, types A-F, with wild type included for comparison. Exponential phase cultures of six standard mutants (A, DK306; B, DK321; C, DK323; D, DK345; E, DK360; F, DK370) and their wild-type parent (DK101) were diluted to a density of 3×10^8 cells per ml with CTT broth. The matrix was constructed by mixing equal volumes of pairs of mutants. The mixed and unmixed cultures were spotted on CTT agar (2- μ l drops). The edges of the spots were photographed after 18 hr of incubation at 33°. ($\times 34$.)

agar surface with fine capillaries, plated out for single colonies, and then tested for antibiotic resistance and motility type. The results of one series of experiments (Table 1) showed that type A cells do not become motile, that type B and type C cells can stimulate each other, and that there is no detectible genetic exchange. Similar experiments with other combinations showed that mutants of types B, C, D, and E became motile in all pairwise mixtures of different types, but that mutants of type A never became motile. The single type F mutant has not yet been tested in this way.

Table 1. Identification of moving cells in interacting mixtures

Mixture	Colonies derived from single moving cells					
	Motile	A	B rif ^R	B str ^R	C rif ^R	C str ^R
A str ^R + B rif ^R	0	0	64	0	0	0
A str ^R + C rif ^R	0	0	0	0	64	0
B str ^R + C rif ^R	0	0	0	4	60	0

Different cell types were grown to a density of 4×10^8 cells per ml in CTT broth and mixed in equal numbers. Small droplets of the mixtures were spotted on a CTT plate and the plate was incubated for 20 hr at 28°. Groups of moving cells were picked up from the surface of the plate with a fine glass capillary and dispersed in CTT broth before replating on CTT plates. Colonies were grown from these cells and a number were tested for antibiotic resistance and for motility type by mixing with a standard mutant of each type as in Fig. 2. str^R = streptomycin resistant; rif^R = rifampicin resistant. Mutants used were type A (DK306), type B (DK307), and type C (DK323).

Thus, mutants of types B, C, D, E, and F behave as if they are defective in some component of the gliding machinery or in some signal necessary for the initiation of movement, and this component or signal can be donated by mutants of a different type. Mutants of type A, on the other hand, behave as if they are defective in response to either the component of the gliding machinery or the signal because they can act as donors to the other types but never as motility recipients.

Linkage Analysis. How many genes underlie the six different motility types revealed by the mixing test? Table 2 presents results of transductional crosses among nine mutants. The recipient strains had low rates of reversion (column labeled "none") and all were efficiently transduced by phage grown on the wild-type (motile) donor, strain 101. Because mutations in the same gene are closely linked, transduction between two mutants in the same gene should yield fewer motile transductants than transduction from wild type to either mutant.

By this criterion, all the type B mutants occupy one locus—i.e., a single cluster of closely linked mutant sites that could be one gene or a group of adjacent genes. The type B mutant strains 307 and 321 shown in Table 2 were closely linked to each other, and, in other crosses (not shown), all of a total of 17 independent type B mutants were found to be closely linked. Similarly, the two type C mutants in Table 2, strains 323 and 311, were closely linked to each other and to three additional independent type C mutants (not shown), but they were not closely linked to any of the type B mutants. Thus, there is a single type C locus and it is distinct from the type B locus. Type

FIG. 3. Proximity test for stimulation. Log phase cultures of DK321 (type B) and DK322 (type C) were diluted to a density of 4×10^8 cells per ml and spotted on CTT agar. Spots of different types were placed very close to each other (Left) or overlapping each other (Right). Photographs were taken after 18 hr of incubation at 33°. (Bar = 50 μ m.)

D mutants were poor motility transduction recipients but could serve as donors. Both type D mutants showed the same pattern of loose linkage to type B and C mutants, suggesting that the type D mutants are linked to each other and define one locus. Because types B and C are not obviously linked but type D is linked to both, the order is probably B–D–C.

The correspondence of B, C, and D motility types to single loci contrasts with the distribution of type A mutants, which occupy many loci. Type A mutant strain 320 (Table 2) is unlinked from A mutants 306 and 309. Crosses between other pairs of A mutants (not shown) showed that type A mutants are distributed over at least 17 loci. One of these loci contains 306, 309, and the seven other mutants that were nonmotile under all conditions tested.

In sum, transduction reveals single loci each for type B, C, and D and at least 17 loci for type A. This explains why type A mutants occur more frequently than the other types.

Conditions for Stimulation. Stimulation of motility required close apposition of the two interacting cell types. If patches of

two different types (say B and C) were placed close together on a thin sheet of agar, no stimulation of either cell type occurred, even though the spots may have been separated by less than 50 μ m, or about eight cell lengths as they are in Fig. 3. If the spots intersected, however, stimulation occurred. This result suggests that cell contact is necessary for stimulation; but it does not exclude diffusible factors with a short range. Neither culture supernatants nor cell-free extracts, so far tested, have stimulated movement. However, cells killed by a brief formaldehyde treatment can stimulate type C, D, and E recipients but not types B and F.

The following experiment separated stimulation from the movement response. Rifampicin-resistant recipients of type B, C, D, or E and rifampicin-sensitive donor cells were mixed under various conditions to allow stimulation, exposed to rifampicin, and then spotted on rifampicin-containing plates. Rifampicin rapidly kills sensitive cells, so only the resistant recipient cells are left; these were examined 6–20 hr later to see if they had been stimulated to move out from the edge of the

Table 2. Frequency of motile recombinants in transductional crosses between nonmotile mutants

Recipient		Transduction donor										
Motility type	Strain	None	Wild 101	A 306	A 309	A 320	B 307	B 321	C 323	C 311	D 328	D 345
A	DK306	0	36	0	0	52	27	42	37	50	27	24
A	DK309	0	24	0	0	25	15	23	18	22	—	—
A	DK320	0	48	28	36	0	20	43	27	39	—	—
B	DK307	0	25	19	35	28	0	0	19	18	—	—
B	DK321	0	37	18	33	36	3	0	29	11	9	11
C	DK323	1	44	27	30	38	19	28	0	2	4	14
C	DK311	1	40	24	32	35	25	28	2	2	—	—
Donor genotype:			<i>mg1-1</i>	<i>mg1-3</i>	<i>ag1B-1</i>	<i>cg1B-1</i>	<i>cg1B-2</i>	<i>cg1C-1</i>	<i>cg1C-2</i>	<i>cg1D-2</i>	<i>cg1D-1</i>	

All crosses were 5×10^7 bacteria + 3×10^8 phage (MX8). Entries in the table are the numbers of spots that had one or more motile flares after 5 days. Because 55 spots were laid down, the maximum score is 55 (see *Materials and Methods*). The last line in the table shows the motility locus designation and allele numbers for each of the strains.

Table 3. Conditions for types B, C, and D stimulation

Length of interaction, hr	Recipient					
	Type B		Type C		Type D	
	Liq.	Agar	Liq.	Agar	Liq.	Agar
0.5	—	—	±	±	±	±
1	—	—	±	±	+	±
2	—	±	+	++	±	+
3	—	+	±	++	±	+
4	—	+	+	++	±	+

Equal numbers of donor (wild type: DK101) and recipient (rifampicin-resistant type B, C, or D) cells from exponential liquid cultures were mixed, sedimented, and suspended at a density of 2×10^{10} cells per ml in CTT broth. Aliquots (0.1 ml) were shaken in liquid (liq.) suspension or allowed to dry in small drops on CTT agar plates (agar) for $\frac{1}{2}$ –4 hr at 33°, after which they were diluted (liq.) or washed off (agar) into 0.5 ml of CTT broth containing rifampicin at 10 µg/ml. After 1 hr of incubation, 2-µl samples were spotted on CTT plates containing rifampicin at 5 µg/ml. The plates were examined after 12 hr of incubation at 33°. — = no movement; ± = slight movement; + = definite movement; ++ = pronounced movement.

spot. Results of such an experiment (Table 3) show that stimulation occurs more effectively on the surface of agar than in liquid suspension, and that it takes 2–3 hr to develop maximally. This experiment, as well as experiments with formaldehyde-killed donors, shows that wild-type cells (strain 101) are competent to stimulate types B, C, D, and E. Other experiments of the kind illustrated in Table 3 confirm that type A cells cannot be stimulated to move.

DISCUSSION

The products of at least 22 genetic loci are required for gliding motility in *M. xanthus*. Mutations at five loci corresponding individually to types B, C, D, E, and F lead to a conditional defect in gliding. Such mutants do not move by themselves, but they can be stimulated to move by contact with wild-type cells or cells of a different mutant type. Each of these five types is defective in a different stimulation, so pairs of mutant types are capable of mutual stimulation. In each case the stimulation is temporary and seems to depend on close apposition of interacting cells.

There are two kinds of explanation for these phenomena. One is that the mutants are defective in the mechanism of gliding movement. In this case the phenomenon of stimulation would imply that several different (unstable) components of the gliding mechanism can be exchanged between cells.

The alternative explanation that we propose is that swarming myxobacteria stimulate each other to move by exchanging signals between cells in contact. Dependence on these signals could be responsible for the coherence of a swarm. Mutants defective in the emission of a signal might be expected to behave as do the types B, C, D, E, and F mutants: they would fail to move when in contact with cells of the same type but would move transiently after contact with wild-type cells because they would receive the appropriate signal from them. Mutants de-

fective in signal reception or response would, like type A, never move. If all five types of stimulation are for physiologically significant signals, with different meanings, then the communication system must be rather complex. Cells that migrate out alone from the edge of the swarm lose contact with their neighbors, and it may be the decay of the relevant signals that causes these cells to reverse their direction and return to the swarm, rather than a chemotaxis to a diffusible substance produced by the swarm.

Further work has revealed the existence of a second system that controls motility in *Myxococcus* (J. Hodgkin and D. Kaiser, unpublished data). The second system, called system S, operates only so long as cells remain in contact and therefore leads to movement of groups of cells. The system described in this paper (called system A) leads to the movement of individual cells, because temporary contact is sufficient to stimulate movement (compare Table 3). The two systems generate movement independently and, were it not for the fact that the parent strain (101) used in this work carries a mutation in the S system, it would not have been possible to isolate most of the mutants described here. The finding of system S does not seriously modify the results or hypotheses presented here, except to offer additional examples of motility stimulation and to show that many genes in *Myxococcus* control motility.

In conclusion, we have shown that gliding movement in *M. xanthus* can be subjected to genetic dissection and that there are classes of mutants that are possibly defective in cell communication. If this can be proved, then the interactions we have described should provide a way to identify the signals used by myxobacteria (and perhaps other cells as well) to guide their cooperative behavior.

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