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Territorial Interactions between Two *Myxococcus* Species

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It is unusual to find fruiting bodies of different myxobacteria occupying the same territory on natural samples. We were thus interested in determining whether myxobacteria establish territorial dominance and, if so, what the mechanism of that interaction is. We had previously observed that vegetative swarms of *Myxococcus xanthus* and *Stigmatella aurantiaca* placed close to each other on an agar surface initially merged but eventually separated. Further studies indicated that these two species also formed separate fruiting bodies when mixed together on developmental agar (unpublished observation). We examined the interactions between two more closely related myxobacteria, *M. xanthus* and *M. virescens*, in greater detail. When mixtures of a kanamycin-resistant strain of *M. xanthus* and a kanamycin-sensitive strain of *M. virescens* were placed together under developmental conditions, the cells sorted themselves out and established separate fruiting body territories. In addition, differential viable counts of a mixture of the two species during development indicated that each strain was producing an extracellular component that inhibited the growth and development of the other. Nevertheless, finally, *M. virescens* invariably outcompeted *M. xanthus* at all input ratios of *M. xanthus*/*M. virescens* tested. This is consistent with the observation that *M. virescens* is by far the more commonly encountered of the two species. The properties of the inhibitory substance from *M. virescens* are consistent with the possibility that it is a bacteriocin. Our working hypothesis is that the bacteriocin plays a role in the establishment of myxobacterial territoriality. If so, this is an example of an ecological function of bacteriocins.

Myxobacteria are unique, gram-negative, gliding prokaryotes found in soil, on bark, and on a variety of plants including lichens and moss (8). Myxobacteria are distinguishable from other prokaryotes by three characteristics. They undergo gliding motility (17); no flagella or any other recognizable means of locomotion have been documented, though explanations for motility have been proposed (1, 2, 4). Additionally, they have complex social interactions mediated by direct contact (5–7). Finally, myxobacteria undergo a complex developmental life cycle culminating in fruiting body formation (3). When development and fruiting body formation are examined on a natural sample, one type of fruiting body invariably dominates a local area. We have been interested in how different populations of these social bacteria interact to establish territorial dominance.

Interactions of cells manifested by sorting of one cell type from another have been documented for mammalian cells (13). In addition, members of the cellular slime molds *Dictyostelium discoideum* and *Dictyostelium purpureum* appear to utilize selective cell adhesion to sort out under conditions in which chemotaxis is unable to function (10, 18, 19).

We first noticed that swarms of *Myxococcus xanthus* and *Stigmatella aurantiaca* initially merged on an agar surface but subsequently separated and established separate fruiting bodies (unpublished results). Additionally, Qualls and White (12) showed that cohesive clumps of *S. aurantiaca* which formed in the presence of other bacteria contained only *S. aurantiaca* and no other bacteria (including other myxobacteria). Taken together, these observations suggested a recognition/interaction process by which the myxobacteria were able to distinguish self from non-self. Such a process could conceivably play a role in the establishment of territories. In order to study interactions during the establishment of territories, *M. xanthus* and *M. virescens*, two closely related myxobacteria, were mixed at various input ratios and allowed to undergo development. The fate of each species was determined by differential viable counting, taking advantage of the transposon-mediated kanamycin resistance of the *M. xanthus* strain. *M. virescens* invariably dominated in these mixtures. Our results are consistent with the finding that *M. virescens* is one of the myxobacteria most frequently isolated from natural samples. The mechanism of dominance is suggested by the finding that an extracellular bacteriocin-like activity produced by *M. virescens* caused spheroplasting and loss of viability in *M. xanthus* (9). Finally, when the mixtures at various input ratios were allowed to complete development and fruiting body formation, localization of fruiting bodies occurred; fruiting bodies of one strain developed in areas excluding those of the other strain. In addition, after we examined approximately 150 fruiting bodies, using kanamycin resistance or sensitivity as a parameter to distinguish the two species, no hybrid fruiting bodies were encountered (data not shown). These results suggest that territoriality does exist in interactions between *M. xanthus* and *M. virescens* and that part of the mechanism may involve bacteriocin-like compounds.

Bacterial strains and growth conditions. *M. xanthus* and *M. virescens* were grown in Casitone-Tris (CTT) broth (14) at 31°C with shaking at 300 rpm. DK4290 is a kanamycin-resistant Tn*5* mutant of *M. xanthus*, while MD715 is a kanamycin-sensitive strain of *M. virescens*. When the viability of DK4290 was determined, 70 μg of kanamycin per ml was incorporated into CTT medium.

Determination of fruiting body territoriality. *M. xanthus* (DK4290 Kan^r) and *M. virescens* (MD715 Kan^s) were grown to 5×10^8 cells per ml, harvested by centrifugation at 12,000 × *g* for 10 min, washed once in Tris-phosphate (TPM) buffer (10 mM Tris [pH 7.6], 1 mM KH_2PO_4-K_2HPO_4, 8 mM $MgSO_4$), and resuspended in TPM buffer to a final density of 10^9 cells per ml. Suspensions of *M. xanthus* and *M. virescens* were mixed

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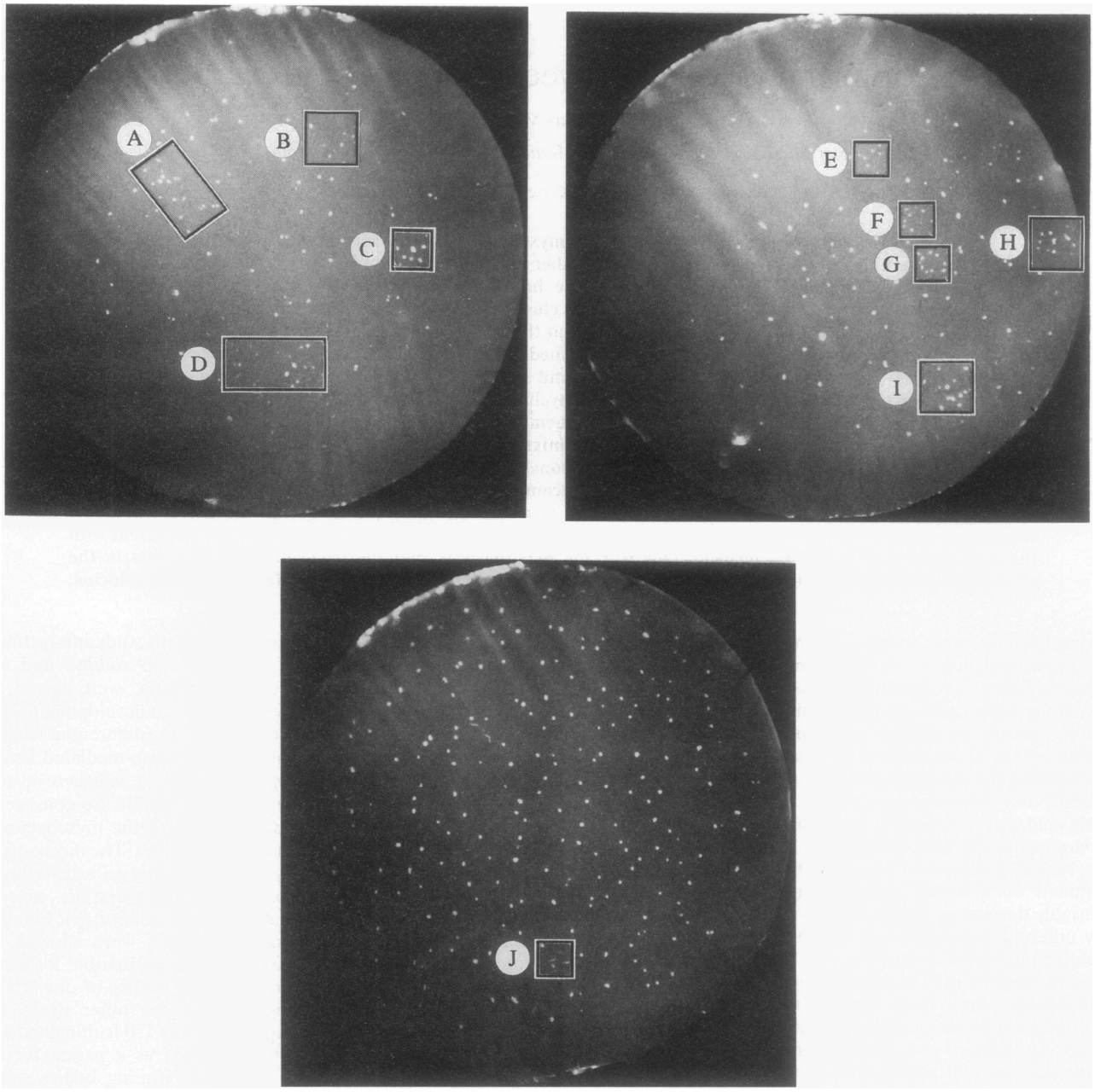

FIG. 1. Territoriality of *M. xanthus* and *M. virescens* fruiting body formation. Each boxed territory, A to J, contains primarily *M. xanthus* fruiting bodies as seen in Table 1. Territories A to D are on the plate at a *M. xanthus*/*M. virescens* ratio of 2:1; E to I, 1:1 ratio; and J, 1:2 ratio. All photos are of single 75-μl spots on developmental agar, and each spot is approximately 10 mm in diameter.

in test tubes at ratios of 1:1, 1:2, and 2:1 (*M. xanthus*/*M. virescens*). Five 75-μl samples of the mixture to be tested were spotted onto agar plates which contained TPM buffer and 1.5% Bacto agar. The spots were dried in a laminar flow hood for 20 min and subsequently incubated at 32°C. Under these conditions, both species undergo a complete developmental life cycle. After incubation for 7 days, pictures were taken to record the location of each fruiting body. In order to identify the individual fruiting bodies as either *M. xanthus* or *M. virescens*, the kanamycin sensitivity of each fruiting body was used as a parameter of its identity. The locations of individual fruiting bodies were noted, and each was then picked and allowed to germinate on CTT agar. After 24 to 48 h, the germinated cells were transferred to CTT agar with 70 μg of kanamycin per ml, and their growth was recorded. The locations of kanamycin-sensitive (*M. virescens*) and kanamycin-resistant (*M. xanthus*) fruiting bodies were then related to the location of that individual fruiting body on the original TPM plate. The patterns of distribution of the two species showed clear evidence of territoriality. This was most obvious in the 2:1 and 1:1 input ratios of *M. xanthus* and *M. virescens* in which clusters of *M. xanthus* fruiting bodies (see boxed areas labeled A to I, Fig. 1) were surrounded exclusively by *M. virescens* fruiting bodies. When the input ratio initially favored *M.*

TABLE 1. Percentages of *M. xanthus* fruiting bodies from boxed areas[a] in Fig. 1

Territory[b]	Input ratio	Fruiting bodies		
		Total no.	No. kanamycin resistant	% Kanamycin resistant
A	2:1	22	20	91
B	2:1	11	10	91
C	2:1	8	8	100
D	2:1	22	21	95
E	1:1	7	7	100
F	1:1	9	8	89
G	1:1	17	14	82
H	1:1	14	13	93
I	1:1	15	15	100
J	1:2	9	7	78

[a] Each boxed area in Fig. 1 contained primarily kanamycin-resistant *M. xanthus* fruiting bodies.
[b] Territory and ratio designations from Fig. 1.

virescens (1:2), fruiting bodies of *M. xanthus* were almost completely excluded; when present, they were still limited in a small cluster (see boxed area labeled J, Fig. 1). Table 1 demonstrates that *M. xanthus* fruiting bodies predominated in all of the clusters labeled A to J in Fig. 1. The percentages of the two species of fruiting bodies seen in a 75-μl spot are presented in Table 2. In general, the number of fruiting bodies of each strain which developed was consistent with the viable count data, especially at the 1:2 input ratio (Table 2). However, since the viable counts were not carried out after 8 h, it is not possible to comment on the relevant events occurring between that time and the formation of the fruiting bodies.

Differential viable counts. *M. xanthus* and *M. virescens* were treated as described above. At 0, 2, 4, 6, and 8 h during development, the cells from the TPM agar plates were harvested by scraping with a sterile spatula and resuspended in 0.5 ml of CTT broth. Cells were counted with a Petroff-Hausser bacterium counter, and appropriate dilutions were made in CTT broth for viable count plating. Standard plating techniques for the quantitative determination of viable cells of *M. xanthus* are generally inadequate; irreproducible and underestimated viable counts are often obtained when cells are plated directly onto agar. This was remedied by embedding the cells in a soft top-agar support. We used CTT medium and the microspot method of Sharpe and Kilsby (16) with 0.4% agar. A 100-μl drop of soft agar containing about 50 cells is deposited directly on the plastic petri dish surface. This is then covered by 100 μl of uninoculated soft agar to lessen drying effects. After incubation, colonies are viewed with a dissecting microscope and counted. Since microcolonies are counted, the incubation time is considerably shortened. The small size of the drops also facilitates multiple replicates. Most important, with soft agar as a mechanical support, cells that might not survive direct plating techniques seem to be protected. To obtain differential counts with mixtures of the two species, viable counts were performed in the presence of 70 μg of kanamycin per ml, which inhibited the growth of *M. virescens* but allowed the growth of *M. xanthus*. The resultant number of colonies was subtracted from the total viable count to obtain numbers of *M. virescens*. Cells in monoculture were harvested at similar time points to determine the recovery efficiency of this technique. All manipulations of the cells were carried out at 4°C. The efficacy of the scraping and soft agar technique was assayed by using monoculture controls, as seen in Table 3. The plating efficiency for *M. xanthus* was the same in the presence or absence of kanamycin (data not shown). The viable count data indicated that each species, when placed together under developmental conditions, inhibited the development and viability of the other (Table 4). This was most clearly evident at the 1:1 input ratios. After 8 h, viable counts of both *M. xanthus* and *M. virescens* in the mixtures were 2.6 and 4%, respectively, of the monoculture controls. The controls showed essentially no loss of viability during the first 8 h of development (data not shown). When twice as many *M. virescens* cells were present (1:2 ratio), no viable *M. xanthus* cells were recovered after about 2 h of interaction. In addition, *M. virescens* numbers were reduced to about 10% of the monoculture control by 2 h. Thereafter, however, *M. virescens* was consistently recoverable at about 30% of the control. Even when there were twice as many *M. xanthus* cells (2:1), *M. virescens* was still recoverable, though in reduced amounts. This was in contrast to the reciprocal ratio (1:2), which resulted in a complete inability to recover viable *M. xanthus* when *M. virescens* was present in greater numbers. These experiments demonstrated reciprocal killing and also the increased competitiveness of *M. virescens*.

Demonstration of bacteriocin-like activity in conditioned medium. *M. xanthus* or *M. virescens* cells were grown to 5×10^8 cells per ml. Cells were removed from the medium by centrifugation at 12,000 × *g* for 10 min. The medium was sterilized by filtration through a 0.2-μm-pore-size filter (Acrodisc; Gelman Sciences, Ann Arbor, Mich.). Collected, sterile medium (conditioned medium) was inoculated with the reciprocal species to a density of approximately 5×10^6 to 1×10^7 cells per ml, and growth of the cells was monitored by using a Klett colorimeter with a green filter. As controls, each species was reinoculated into medium conditioned by its own growth. Activity in the conditioned medium was also tested as described above after heating to 100°C for 30 min to determine the heat lability of the activity. Growth media conditioned by growth of *M. virescens* caused spheroplasting of *M. xanthus* within 2 to 3 h of inoculation. This was also manifested by a decrease in the light-scattering ability of the cells (Fig. 2). *M. xanthus* cells inoculated into medium conditioned by *M. xanthus* (control) showed no spheroplasting and continued to

TABLE 2. Fruiting body territoriality: percentages of *M. xanthus* and *M. virescens* fruiting bodies[a]

Input ratio[b]	% *M. xanthus*	% *M. virescens*
2:1	77	23
1:1	30	70
1:2	0.9	99

[a] These values were obtained from the ability of germinated cells from each fruiting body to grow on agar plates containing kanamycin and are averages of five experiments.
[b] *M. xanthus*/*M. virescens* ratios.

TABLE 3. Efficiency of recovery and plating using monoculture controls during development[a]

Time (h)	% of total cells ± SD	
	M. xanthus	*M. virescens*
0	88.1 ± 2.6	91.5 ± 4.0
2	81.8 ± 4.0	86.7 ± 3.1
4	81.5 ± 0.5	85.3 ± 4.5
6	80.8 ± 2.5	87.6 ± 5.5
8	84.3 ± 2.9	85.1 ± 0.85

[a] Data are percentages of total cells deposited onto agar at time zero.

TABLE 4. Viable counts of *M. xanthus* and *M. virescens*[a]

Time (h)	% of controls ± SD					
	M. xanthus			*M. virescens*		
	2:1[b]	1:1	1:2	2:1	1:1	1:2
0	97 ± 4	100 ± 5.3	87.7 ± 10	95 ± 7.0	99 ± 5.0	90 ± 7.7
2	13 ± 6.8	16 ± 4.2	2.3 ± 1.5	31 ± 8.0	34 ± 12.7	10 ± 5.0
4	12 ± 5.2	5 ± 2.8	0.0 ± 0.0	6 ± 4.5	6 ± 1.4	27 ± 4.7
6	14 ± 1.5	5 ± 2.1	0.0 ± 0.0	2 ± 6.0	3.3 ± 5.5	38 ± 8.0
8	5 ± 7.0	2.6 ± 7.0	0.0 ± 0.0	26 ± 10.2	4 ± 3.0	34 ± 7.0

[a] Values are averages of four experiments and are percentages of monoculture controls.
[b] The ratios are for mixtures of *M. xanthus* and *M. virescens*.

grow. Surprisingly, in the reciprocal experiment, growth medium preconditioned by growth of *M. xanthus* had no effect on the growth of *M. virescens* (data not shown). The inhibitory factor found in medium conditioned by growth of *M. virescens* has at least two properties consistent with bacteriocins found in other myxobacteria (9). It retained activity when heated to 100°C for 30 min (Fig. 2), and it was not active against unrelated bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (data not shown). Part of the basis for the establishment of territoriality may be attributed to the ability of the conditioned medium of *M. virescens* to lyse *M. xanthus* cells within 2 to 3 h (Fig. 2). These properties are associated with bacteriocins, which are known to occur in several myxobacteria and have been designated myxocins (9). Such myxocins may play a role in the establishment of territoriality and, as such, may have an ecological function.

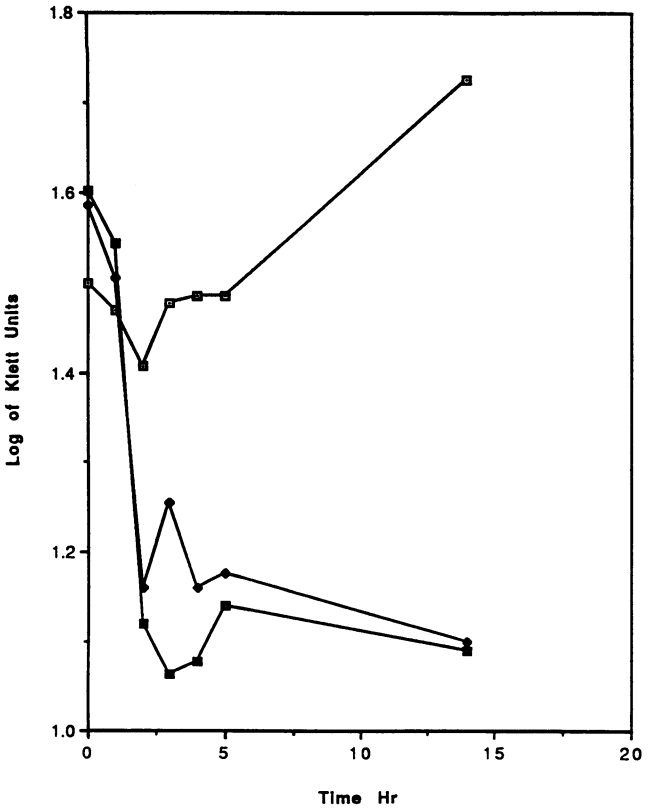

FIG. 2. Growth of *M. xanthus* in CTT broth (control; open squares), in medium preconditioned by growth of *M. virescens* (experimental; closed squares), and in preconditioned medium heated to 100°C for 30 min (heated; closed diamonds).

When myxobacteria have been isolated from natural sources, anecdotal evidence has indicated that any particular area invariably contained one species of myxobacteria to the exclusion of other species. This suggested that myxobacteria may express a rudimentary form of territoriality. Our data now provide evidence for this by showing that in an initially mixed population of *M. xanthus* and *M. virescens*, fruiting bodies of each species developed in defined areas or territories on developmental agar (Fig. 1, Table 1, and Table 2). It is also common experience that *M. virescens* is one of the most frequently isolated myxobacteria. Our data support this by showing that *M. virescens* has a competitive advantage over *M. xanthus*. Part of the mechanism of territory establishment may involve the production of a bacteriocin-like compound.

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