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RasA Is Required for *Myxococcus xanthus* Development and Social Motility

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An insertion in the *rasA* gene entirely blocked developmental aggregation and sporulation in *Myxococcus xanthus* while also reducing swarm expansion on a 0.3% agar surface. Data presented here demonstrate that *rasA* is required for extracellular fibril formation and social gliding motility.

Myxococcus xanthus is an unflagellated gram-negative soil bacterium that associates in mobile, expanding swarms during vegetative growth. The mode of motility employed by the individual cells as they move across solid surfaces is termed "gliding," which is characterized by a smooth, nonflagellar motion in the direction of the cell's long axis (12). Motility is integral to the ability of *M. xanthus* to forage for food, which includes predation of other soil-resident microorganisms (24). When nutrients become depleted, cells within the *M. xanthus* colony collaborate to construct a multicellular fruiting body within which a fraction of the constituent cells differentiate into resistant myxospores (for a review, see reference 10). As with nutrient foraging, motility is important for *M. xanthus* to carry out this developmental process, because the complex movements required for fruiting body morphogenesis depend on functional motility apparatuses (for a review, see reference 29).

M. xanthus possesses separate multigene systems for gliding by two distinct mechanisms called adventurous (A) motility and social (S) motility (13, 14, 29). *M. xanthus* does not appear to have any additional means of spatial translocation, because mutants defective for both the A and S systems are entirely unable to swarm (14). This observation has in fact been used as the basis for categorizing motility genes into either system. Hence, if a gene is required for S-motility, then a mutation of that gene in an A^- strain abolishes swarming in the double mutant and vice versa.

Adventurous motility is required for single cells to move in isolation from the colony, although cells within a colonial swarm may also translocate by means of the A system (13). Adventurous motility is thought to be similar to gliding in cyanobacteria, where forward propulsion by the cell is coupled to backward extrusion of a polyelectrolyte gel through specialized nozzles situated at the cell poles (15). Bolstering this hypothesis is the recent discovery by Wolgemuth et al. (36) that *M. xanthus* possesses similar, albeit smaller, nozzle-like structures that are localized at the poles; these are the regions from which ribbons of slime are ejected. Mathematical models predict that the hydration of the cationic polyelectrolyte slime provides sufficient force to propel the *M. xanthus* cell forward at observed velocities (36).

In contrast to A-motility, the S-motility system is a contact-dependent mode of movement that requires cells to be in close proximity to one another, i.e., within a distance of about 2 μm (30). This is about the length of the thin, unipolar organelles called type IV pili (TFP), which are absolutely essential for movement by the S system (16, 37). TFP-mediated gliding appears to involve anchoring the pili onto a solid substratum (which may be a neighboring cell) located ahead, followed by pilus retraction, which has the effect of pulling the cell forward (23). In *M. xanthus*, the *pil* cluster of genes encodes structural and regulatory proteins that are required for TFP assembly and disassembly (37–40).

In addition to the TFP, other cell surface components that appear to mediate the intercellular contacts required for S-motility have been identified. These include the cell envelope-localized, mixed protein-polysaccharide structures called fibrils that are important for cell-to-cell cohesion and interactions within a swarm (5). Recent evidence indicates that fibrils may serve as TFP attachment points (21), which would explain the S^- phenotype of fibril-deficient strains, such as the *dsp* mutants (2, 9, 28). Another cell surface component important for S-motility is the outer membrane O antigen; however, its role in motility has not been clarified (7).

The S-motility components appear to be regulated by signal transduction pathways that are homologous to the chemosensory system of enteric bacteria. One, the Frz system, appears to govern cell reversal frequency at least partly by regulating the switching of TFP bundles from one pole to the other (32). A second, the Dif system, has been shown to be required for fibril biogenesis (41). Recent evidence indicates that some *dsp* mutations are linked to the *dif* locus (19).

Here, we describe the phenotypes of a strain with a mutation in *rasA*, a gene *r*equired for both developmental *a*ggregation and *s*ocial motility. The *rasA* gene, identified as MXAN4150 by The Institute for Genomic Research (TIGR), is predicted to be 1,131 bp in length and to encode a 40.8-kDa protein. We identified the *rasA* gene as part of the characterization of the neighboring *brgE* gene, which is required for fruiting body morphogenesis and sporulation (25). Both *rasA* and *brgE* are predicted to be transcribed in the opposite direction from that

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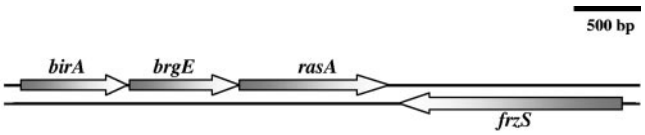

FIG. 1. Physical organization of the *M. xanthus* chromosome in the *rasA* region. Open reading frames and predicted directions of transcription are represented by arrows.

of the downstream *frzS* gene (34) (Fig. 1). The *rasA* gene does not possess any conserved motifs (e.g., signal sequence or transmembrane regions) to aid in its functional prediction, nor does it bear sequence homology to any known genes on public databases as assessed by PSI-BLAST, Pfam, and SMART analyses (1, 4, 20).

A *rasA* insertion mutation abolishes fruiting and sporulation. A *rasA* insertion mutant was constructed by insertion mutagenesis of plasmid pDV678 (Table 1) into the *rasA* open reading frame using previously described methods (26). Southern blot analyses were used to confirm the insertion in *rasA* within colony-purified cells, which were grown on selective media to prevent loss of the inserted plasmid. The developmental defects of the resultant strain, VP1066, were assessed on TPM (Tris-phosphate-magnesium) starvation media by previously described methods (18, 33). As shown in Fig. 2, the *rasA* mutant is unable to form the multicellular assemblages elaborated by wild-type cells over a developmental time course. In addition, it produced fewer than 10 viable spores from an initial input of 5×10^8 cells, compared to wild-type cells, which produce 1×10^7 to 2×10^7 spores from the same number of input cells.

***rasA* is required for S-motility.** *M. xanthus* cells swarm fastest when spotted on a rich medium containing a low percentage of agar (27). The S-motility system operates most efficiently when the substrate is soft and wet, while the A-motility system appears to require a firmer surface over which to power gliding. Thus, the motility phenotype of S^- mutants, which can move only by the A gliding apparatus, is most apparent when cells are placed on a 0.3% agar surface, while A^- mutants display their phenotype most prominently on 1.5% agar media.

TABLE 1. *M. xanthus* strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
DK1622	Wild type	16
DK1217	*aglB1*	14
DK1250	*aglB1 tgl-1*	14
DK3468	*dsp-1680*	D. Morandi, Stanford University
DK10410	Δ*pilA*	40
VP1066	*rasA*::pDV678	This study
VP1145	*rasA*::pDV678 Δ*pilA*	This study
VP1146	*rasA*::pDV678 *aglB1*	This study
Plasmids		
pBGS18	*nptII* (Km^r)	31
pDV678	RasA-F/R[a] amplicon in pBGS18	This study

[a] PCR primers amplifying a 661-bp region of *rasA* between base pairs 277 and 938; RasA-F sequence, CTGAAGCTTGAAGAGGTACAGCCGCAGGT (the underlined sequence is an engineered site for restriction by HindIII); RasA-R sequence, TTCGAATTCCACTCGCGGTTGCTGTAGATG (the underlined sequence is an engineered site for restriction by EcoRI).

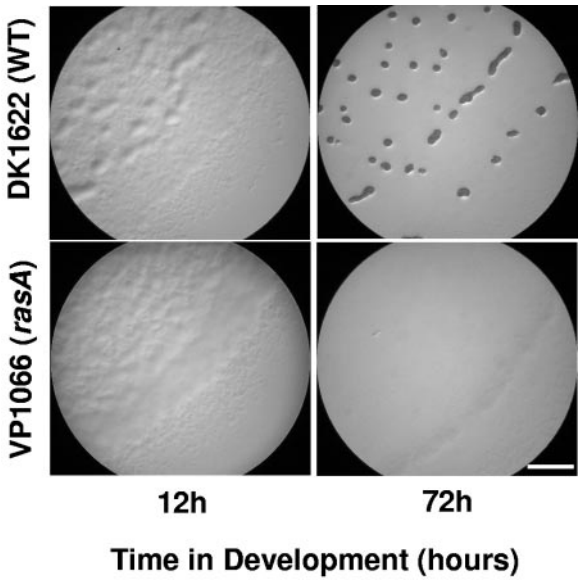

FIG. 2. Light microscopy images showing developmental progression of DK1622 (wild type) and VP1066 (*rasA*) cells on TPM (starvation) agar. Mid-exponential-phase cells were concentrated 10-fold to a density of 5×10^9 CFU/ml and spotted onto TPM agar to induce development. Bar = 1 mm.

Because many developmental defects are caused by impairments in the social motility system (14, 22), the *rasA* mutant was assessed for motility phenotypes on both 1.5% and 0.3% agar containing a rich medium using standard methods (27). No differences between the mutant and its wild-type parent were observed on 1.5% agar (data not shown), which is similar to the phenotype of several *dif* mutants (6). In contrast, on 0.3% agar, the swarm edge of the mutant contained dark, disorganized spikes, in contrast to the translucent "frill" of the wild-type swarm edge (Fig. 3). Moreover, with respect to swarm expansion rates, which are calculated from the difference in swarm radii at 0 and 94 h, the *rasA* mutant expanded at a rate only one-third that of the wild type, which is comparable to the rates for other S^- strains, including DK3468 (*dsp*) and DK10410 (Δ*pilA*) (Table 2). This phenotype is not due to growth defects, because there are no differences between the growth rates of VP1066 and the parent wild-type strain DK1622: both show a doubling time of 5.5 ± 0.085 h.

In order to categorize *rasA* more firmly as an A- or S-motility gene, double mutants containing the *rasA* insertional disruption as well as a mutation in either *aglB* (an A gene) or *pilA* (an S gene) were generated and assessed for phenotypes on the two types of agar surfaces. When spotted onto hard and soft agar media, strain VP1145 (*rasA* Δ*pilA*) displayed no noticeable phenotype on 1.5% agar (data not shown) but formed stubby, rounded extensions at the swarm edge on 0.3% agar that are similar to those of the Δ*pilA* strain (data not shown). In contrast, strain VP1146 (*rasA aglB*) did not swarm on either surface, indicating that both motility systems had been impaired in this strain (Fig. 3). The smooth, nonexpanding swarm edge of strain VP1146 resembled that of the $A^- S^-$ control strain DK1250 (Fig. 3).

TABLE 2. Swarm expansion on 0.3% agar[a]

Strain	Genotype	Swarm expansion (μm/min)
DK1622	Wild type	1.84 ± 0.40
VP1066	*rasA*	0.49 ± 0.17
VP1145	*rasA* Δ*pilA*	0.50 ± 0.17
VP1146[b]	*rasA aglB1*	0.07 ± 0.10
DK3468	*dsp*	0.48 ± 0.10
DK10410	Δ*pilA*	0.37 ± 0.12

[a] Mid-exponential-phase cells were spotted on a rich medium (Casitone-yeast extract) containing low-percentage (0.3%) agar. The radius of each spot was measured at regular intervals for 94 hours. Results shown are for threefold replicate experiments. See reference 27 for A^- S^+ swarm expansion data.
[b] The *aglB1* point mutation causes an A-motility defect.

Cell-to-cell cohesion is dependent on RasA. Close cell-to-cell contact is important for S-motility, and the mediators of such an interaction appear to include cell surface components like TFP and fibrils. Accordingly, mutants lacking these appendages glide in small, loosely cohesive groups or as individuals rather than in the large swarms observed for A^+ S^+ and A^- S^+ cells (28). Cell cohesion can be quantified using the agglutination assay, which measures the turbidity of a dispersed cell culture (28). Over time, the cells form large multicellular clumps that drop out of suspension and reduce the culture's turbidity. The fibril-defective strain DK3468 (*dsp-1680*) was previously shown to fail to agglutinate (28). Strain DK3468 and wild-type strain DK1622 (which is cohesion proficient) were used as controls against which agglutination of strain VP1066 (*rasA*) was compared. As shown in Table 3, the agglutination property of strain VP1066 is very similar to that of agglutination-defective strain DK3468.

A *rasA* insertion mutation causes defects in extracellular fibrils. As mentioned above, cell surface components are important for S-motility, including components that contribute to TFP, fibril, and O antigen production, assembly, and transport (2, 7, 37). To determine which component(s) was deficient in a *rasA* mutant, the mutant's ability to make PilA, FibA, and O antigen was assessed. A Western immunoblot analysis using monoclonal antibody 2105 (11) revealed that strain VP1066 is defective for making the fibril-associated protein FibA (Table 3), which is an autoproteolytic metalloprotease that exists primarily in the 66-kDa form (17). This was similar to the defect of the *dsp-1680* mutant strain DK3468, which is known to lack fibril components (8, 28). On the other hand, no defects were observed for the generation of prepilin (PilA) from whole-cell extracts or for extracellular pili from pilus preparations (Table 3), nor was the *rasA* mutant deficient for O antigen (M. Esmaeiliyan and H. Kaplan, personal communication).

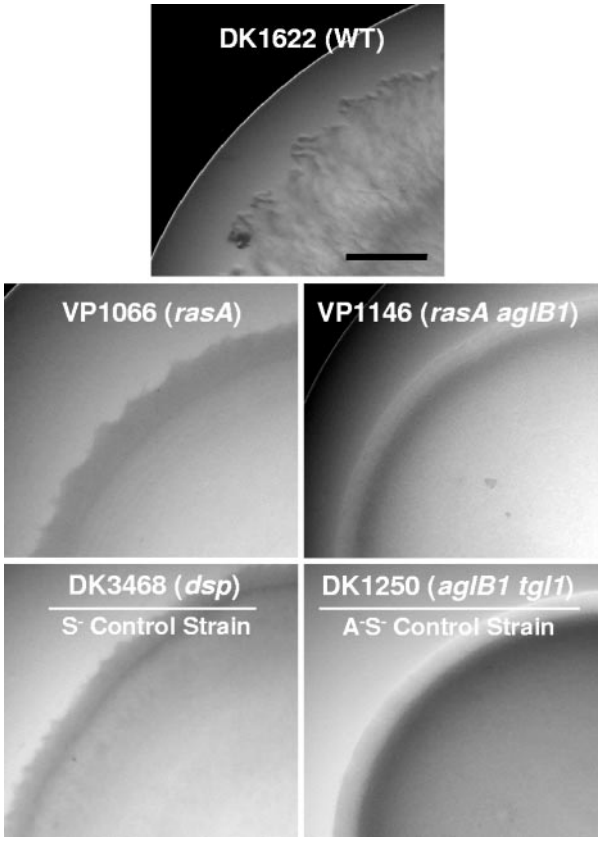

FIG. 3. Swarm edge morphology of motility mutants on rich (Casitone-yeast extract) medium containing 0.3% agar. The strains and their genotypes are displayed within each light micrograph. See reference 27 for equivalent A^- S^+ pictures. Bar = 5 mm.

The fibril defect of VP1066 cells was further corroborated by assays that measure the binding of the fibril-targeted dyes calcofluor white M2R (9), Congo red, and trypan blue (3, 6). As shown in Table 3, strain VP1066 is deficient for binding all three dyes, which is similar to the fibril-defective *dsp-1680* mutant strain DK3468.

TABLE 3. Surface properties of the *rasA* mutant

Strain	Genotype	Agglutination[a]	Surface components[b]			Dye binding%[c]		
			FibA	PilA	O antigen	CW	Congo red	Trypan blue
DK1622	Wild type	0.099 ± 0.062	+	+	+	+	63.3 ± 3.5	66.6 ± 0.8
VP1066	*rasA*	0.897 ± 0.002	−	+	+	−	31.1 ± 2.9	2.9 ± 0.7
DK3468[d]	*dsp*	0.887 ± 0.002	−	+	ND	−	27.9 ± 1.6	6.2 ± 2.3

[a] Turbidity of a suspension of cells was monitored at an optical density of 600 nm (OD_{600}) over a 2-hour period at 6-min intervals. Data shown are the relative absorbance readings at the 2-hour endpoint, which were obtained by dividing the sample readings at each time point by the intial absorbance readings.
[b] Production of FibA and PilA was separately assessed by Western blotting using anti-FibA monoclonal antibody 2105 and anti-PilA polyclonal antibodies, respectively. O antigen production was determined by silver staining lipopolysaccharide preparations separated by 13% deoxycholate-polyacrylamide gel electrophoresis (M. Esmaeiliyan, personal communication). ND, not determined.
[c] Calcofluor white (CW) binding was evidenced by the formation of a white halo around an *M. xanthus* swarm exposed to long-wavelength UV light (365 nm). The Congo red and trypan blue dye binding data shown represent the percentage of bound dye at OD_{490} and OD_{585}, respectively.
[d] Strain DK3468 was used as the fibril-defective S^- control strain against which the *rasA* mutant strain VP1066 was compared.

Concluding remarks. Intriguingly, the *rasA* open reading frame is adjacent to the *frz* cluster of genes (Fig. 1), which are hypothesized to participate in S-motility in the capacity of a signal-transducing pathway (for a review, see reference 35). However, the association between RasA and the Frz pathway needs further study, because unlike RasA, the Frz components are not known to be defective for fibrils. Rather, defects in fibril production are more often associated with the Dif system, which forms another putative signal-transducing pathway that is essential for S-motility (41).

Many questions remain concerning how the various components involved in S-motility, including the Frz and Dif systems, are related to each other. Signaling via the Frz and Dif systems, by virtue of their common involvement in S-motility, may be integrated by a regulator that couples cell reversal frequency with the production of fibrils. RasA is a fitting candidate for such a role, given its requirement in fibril biogenesis and its circumstantial relationship, by virtue of proximal location, with the Frz system. Because it lacks any known functional domains, it is not possible to predict whether RasA carries out a regulatory, enzymatic, or structural function as an integral S-motility component. Experiments to determine whether RasA physically interacts with any of the Frz and Dif proteins, whether it regulates their expression, or whether its own expression is regulated by Frz and/or Dif signal transduction activities may help better define its role in S-motility.

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