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Localization of MglA, an essential gliding motility protein in *Myxococcus xanthus*

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Abstract

MglA, a 22 kDa protein related to monomeric GTPases, is required for the normal operation of the A (Adventurous) and S (Social) motility and for multicellular development of *Myxococcus xanthus*. To determine how MglA controls A- and S-motility, MglA was assayed biochemically and its cellular location was determined. His-tagged MglA hydrolyzed GTP slowly *in vitro* at a rate nearly identical to that of Ras showing that MglA has GTPase activity. Immunofluorescence microscopy of fixed cells from liquid showed that MglA was associated with helical track similar to the MreB spiral that spanned the length of the cell. The distribution pattern of MglA depended on the type of surface from which cells were harvested. In cells gliding on 1.5% (w/v) agar, the helical pattern gave way to punctate clusters of MglA-Yfp at the poles and along the long axis (lateral clusters). The lateral clusters emerged near the leading pole as the cell advanced coincident with a decrease in the intensity of the MglA-Yfp cluster at the leading pole. Newly formed lateral clusters remained fixed with regard to the substratum as the cell moved forward, similar to focal adhesion complexes described for AglZ, a protein partner of MglA. Lateral clusters did not form in cells gliding in methylcellulose, a polymer that stimulates S-motility at low cell density; rather MglA-Yfp was diffuse in the cytoplasm and more concentrated at the poles. The results suggest that conditions that favor S-motility prevent the formation of lateral clusters of MglA, which are associated with A-motility functions.

Keywords

GTPase; gliding; multicellular development; cytoskeleton

INTRODUCTION

The Gram-negative soil bacterium *Myxococcus xanthus* is a model prokaryote for understanding the complexity of intercellular interactions that occur during development. When the supply of nutrients is limited, large numbers of *M. xanthus* cells ($>10^5$) aggregate and assemble into fruiting bodies. Within these elaborate, multicellular structures, a subset of cells differentiates to form resting spores which are resistant to heat, ultraviolet light, and desiccation (Shimkets 1999). Both the aggregation of cells during the morphogenesis of fruiting bodies and the differentiation of heat-resistant spores are dependent on subsets of genes involved in the ability of *M. xanthus* to glide over surfaces.

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M. xanthus is endowed with genetically distinct mechanisms, A (for adventurous) and S (for social) that allow it to glide over surfaces. Phenotypically, *M. xanthus* strains with single A gene mutations form smaller colonies than the wild-type (WT) but motile “flares” of cells are visible at the colony edge because S-motility is retained (Hodgkin and Kaiser 1979a). Strains with single S gene mutations likewise form smaller colonies than the WT but are surrounded by halos of single cells at the colony edge because A-motility is retained (Hodgkin and Kaiser 1979b). Double mutants ($A^- S^-$) form colonies with sharp edges and are nonmotile when viewed by time-lapse microscopy (Spormann and Kaiser 1999). Most $A^- S^+$ strains swarm poorly on 1.5% (w/v) agar but show robust swarming on 0.3% (w/v) agar, which suggests that A-motility is optimal on hard, dry surfaces (1.5% (w/v) agar). The converse is true for $A^+ S^-$ strains placed on 0.3% (w/v) agar which suggests that S-motility is optimal on soft, moist surfaces (Shi and Zusman 1993). It remains unclear whether WT cells use both motility systems simultaneously or alternately and if there is a sensing system that enables cells to distinguish hard and soft surfaces.

Several models have been proposed to understand the two gliding mechanisms. One model proposes that A-motility is powered by secretion of slime from the lagging pole (Yu and Kaiser 2007). RomR, an A-motility protein that localizes to the lagging pole, has been proposed to induce the polarity switching of the A-engine (Leonardy et al. 2007). An alternate model proposes that focal adhesion clusters propel the cells by making transient attachments to the substratum to provide traction (Mignot et al. 2006). The essential A-motility protein AglZ, which interacts with MglA (Yang et al. 2004), participates in the focal adhesion clusters along the long axis of the cells. AglZ also interacts with the methyl-accepting chemotaxis protein FrzCD (Mauriello et al. 2009b), which, like AglZ, forms clusters in gliding cells (Mauriello et al. 2009a).

S-motility in *M. xanthus* requires type IV pili (T4P), LPS, and extracytoplasmic matrix (ECM; fibrils) (Bowden and Kaplan 1998; Li et al. 2003; Wu and Kaiser 1995). Other prokaryotes, such as *Neisseria* and *Pseudomonas*, use T4P for twitching motility (Maier 2005; Mattick 2002) and *Clostridium* uses T4P for gliding (Varga et al. 2006). During twitching motility, pili attach to a surface and are then retracted, thus pulling the cell toward the attachment point. In *M. xanthus*, T4P are elaborated at the leading pole of an actively gliding cell and retraction is thought to be stimulated when pili contact components in the ECM of an adjacent cell (Li et al. 2003). The ATPases that power extension and retraction of pili co-localize in twitching bacteria, which is consistent with their reciprocal activities (Chiang et al. 2005). *M. xanthus* deviates from the twitching paradigm because the two ATPases, PilT and PilB, have been shown to reside at opposite poles of the cell (Bulyha et al. 2009) which calls the simple extension-retraction model into question. Although the role of LPS has not been elucidated in *M. xanthus*, it may play a role in swarming as a wetting agent, similar to that reported for swarming in *Salmonella* (Toguchi et al. 2000).

mglA mutants form colonies with smooth edges that resemble those formed by $A^- S^-$ double mutants. The *mglA* gene is predicted to encode a small (22 kDa) protein similar in sequence to members of the Ras (p21) superfamily of monomeric GTPases. Some, but not all, of the defects caused by an *mglA* deletion mutation can be complemented by the expression of the *Saccharomyces cerevisiae* GTPase, Sar1p, in place of *mglA* (Hartzell 1997). A Sar1 mutant that cannot hydrolyze GTP does not complement the Δmgl mutant. By extrapolation, these results suggested that GTPase activity of MglA is critical for gliding movement.

Genetic and biochemical studies have confirmed the central role that MglA plays in motility. MglA is required for the correct localization of RomR, AglZ and FrzS, key components of A and S motility (Leonardy et al. 2007; Zusman et al. 2007). Its known protein partners include AglZ (Yang et al. 2004), a component of the A motility system, and Mask

(Thomasson et al. 2002), a component of the S motility system. In addition, interactions with proteins that share identity with the Frz chemotaxis proteins have been proposed (Kaiser and Yu 2005; Sliusarenko et al. 2006; Spormann 1999). These data suggest that MglA plays a role in coordinating the simultaneous or alternating operation of the two motility systems. To test the hypothesis that MglA might function as a GTPase to coordinate the A- and S-motility systems, we studied MglA biochemically and used fluorescence microscopy to monitor its cellular location and migration. Here we demonstrate that MglA is a GTPase that has a slow intrinsic rate of GTP hydrolysis, similar to that of Ras. Furthermore, we report that MglA-Yfp is associated with an internal helical structure and it can reorganize to form clusters in an actively moving cells. The distribution and timing of the clusters is similar to those described for AglZ (Mignot et al. 2006), a protein partner for MglA.

MATERIALS AND METHODS

Strains, plasmids, media and reagents

Strains, plasmid DNA and oligonucleotides used for this project have been listed in Table 1. *E. coli* cells were grown at 37°C on LB (Sambrook et al. 1989). *Myxococcus xanthus* strains were grown at 32°C on CTPM (1% (w/v) of Casitone, 10mM Tris, 1mM potassium phosphate, 5 mM MgSO₄, final pH 7.6) broth with vigorous shaking. Solid media for routine use contained 1.5% (w/v) agar. Where applicable, media were supplemented with 40 µg/ml kanamycin sulfate (Km).

Strain construction

Plasmid pAGS120 is a derivative of the expression vector, pET24a, and expresses the entire WT *mglA* coding sequence from a phage T7 early promoter. The MglA protein made from this plasmid is predicted to have an additional 14 N-terminal amino acids and an additional six C-terminal histidine residues, to facilitate the purification of MglA. To ensure that additional residues did not impair function, the recombinant version of the *mglA* gene from plasmid pAGS120 was subcloned into plasmid pPLH325 (Hartzell and Kaiser 1991a), to yield plasmid pPLH434. Plasmid pPLH434 was integrated into the *M. xanthus* genome to create a mutant derivative of *M. xanthus* that expresses this extended version of *mglA* in place of the WT *mglA* gene. Plasmid DNA was electroporated into *M. xanthus* host DK6204 ($\Delta mglBA$), as described (Kashefi and Hartzell 1995). Km^R colonies, which arise by homologous recombination between the plasmid DNA and the *mglBA* promoter, were detected after 5 days.

M. xanthus DK6204 contains remnants of *mglB* and *mglA* (deletion of a *BalI* fragment removed 57% of *mglB* and 84% of *mglA*; see ref (Hartzell and Kaiser 1991b)). To avoid potential problems from residual *mgl* DNA that might arise after recombination, a markerless *M. xanthus* strain, MxH2370, which carries a complete deletion of the *mglB* and *mglA* genes, was engineered as the host for the *mglA-yfp* fusions. To construct MxH2370, plasmid pKD111, which contains DNA upstream and downstream of *mglBA*, was electroporated into DK1622 and Km^R transformants were grown on medium lacking kanamycin to enrich for cells that had experienced excision of the vector with loss of the WT genes. Aliquots were removed and plated on CTPM agar and Km^S colonies were screened visually for the nonmotile phenotype. PCR and Southern blot analysis were used to confirm that the Km^S mutants lacked the *mglB* and *mglA* genes. The phenotype is identical to DK6204, but MxH2370 is lacking all of *mglBA* whereas DK6204 retains a portion of *mglB* and *mglA*.

Plasmids pJP20 and pJP25 carry *mglA-yfp* and *yfp-mglA* fusions, respectively, that were constructed by the PCR overlap method (Ho et al. 1989) using reagents described in Table 1. Plasmid pJP20 carries a 285 bp region upstream of the start codon of *mglB*, *mglB*, *mglA-yfp* and 220 bp of DNA downstream of the *mgl* operon. The otherwise native *mglA* and *yfp* genes were joined by a linker that added 12 additional amino acids introduced to minimize steric interference between the two proteins (Fig 3). Plasmid pJP25 contains identical upstream and downstream portions of the *mgl* operon but differs in that *yfp* is fused with the 5' end of *mglA* with a six amino acid linker (GGGGA) between Yfp and MglA to help facilitate folding and flexion. pJP20 was electroporated into DK1622 (WT) and MxH2370 ($\Delta mglBA$) as described (Kashefi and Hartzell 1995). pJP25 was electroporated into MxH2370 to produce MxH2519.

Protein purification

The MglA-His6 and Ha-Ras-His6 proteins were expressed from the tightly regulated phage T7 early promoter in *E. coli* host BL21 (DE3). The *mglA* gene expressed from plasmid pAGS120 is predicted to encode a fusion protein that has an additional 14 amino acids (MASSTGGQQMGRDP) at the N-terminus, and an additional six His residues at the C-terminus. To express proteins from these plasmids, single colonies were inoculated into Superbroth (SB) medium supplemented with Km⁴⁰ and 1% (w/v) glucose, and grown at 37°C, until they reached A₆₀₀ = 0.4. Then, cells were diluted 1:100 into 500 ml of fresh SB medium with Km and grown at 32°C with shaking to A₆₀₀ = 0.6. To induce expression of the fusion proteins, IPTG was added to a final concentration of 0.2 mM and the cultures were shifted to 30°C. After 3 h, cells were chilled rapidly on ice, harvested by centrifugation at 10,000xg for 10 min at 4°C and stored at -20°C.

The purification of MglA-His was carried out at 4°C, unless otherwise noted. Ha-Ras-His was purified in parallel using the same buffers. The cell pellet from the host with plasmid pAGS120 (for MglA) and pRJS2 (for HaRas-His) was suspended in 20 ml of extraction buffer (EB) [50 mM Hepes (pH 7.6), 300 mM NaCl] and centrifuged at 10,000 xg for 10 min. Cells were suspended in 20 ml of EB and lysed by two passages through a French pressure cell at 20,000 psi. Lysates were centrifuged at 10,000 x g for 10 min to pellet inclusion bodies containing MglA. The Ha-Ras-His samples did not contain inclusion bodies and the EB sample was used directly for subsequent steps. Pellets were resuspended in 20 ml MglA solubilization buffer (MSB) [6 M Urea, 50 mM Hepes (pH 7.6), 300 mM NaCl], gently mixed on a rotating platform for 1 h, then centrifuged at 10,000 x g for 10 min. The soluble material was mixed with 15 ml of homogenously suspended Talon metal affinity resin (BD Biosciences-Clontech) equilibrated with MSB. Mixtures were rocked gently at 25°C for 20 min and allowed to settle in 60 ml columns. To reduce nonspecific binding, the resin was washed with 150 ml of MSB and with 50 ml of MglA stringent wash buffer [50 mM Hepes (pH 7.6), 300 mM NaCl, 200 mM arginine, 20 mM imidazole]. To elute MglA-His6 from the resin, 50 ml of MglA elution buffer [50 mM Hepes (pH 7.6), 150 mM imidazole, 300 mM NaCl, 200 mM arginine, 5 mM MgCl₂, 1 mM guanosine, 5% (v/v) glycerol] was passed through the column. The eluate was reduced to 10 ml using a 350 ml stirred ultrafiltration cell (Amicon). After concentration, samples were dialyzed against 500 ml of 6 M Urea for 6 h, then against MglA refolding buffer [50 mM Hepes (pH 7.6), 1 M arginine, 50 mM NaCl, 5 mM MgCl₂, 1mM guanosine, 1mM DTT, and 5% (v/v) glycerol] for 12 h. An equal volume of MglA freezing buffer [50 mM Hepes (pH 7.6), 1 M arginine, 85% (v/v) glycerol, 1 mM guanosine, 1 mM DTT] was added to the samples, and protein were stored at -20°C. To guard against potential problems caused by endogenous GTPases that might be trapped in the inclusion body material, we performed a control experiment in parallel. Inclusion material from *E. coli* cells overproducing the *M. xanthus* protein AgIU-His (White and Hartzell 2000)(N. Burke and P. Hartzell, unpublished), which does not bind

or hydrolyze GTP, was processed with reagents identical to those used for purification of MglA-His. AglU-His material eluted from the column did not have any activity in the GTPase assays described below (data not shown).

GTP hydrolysis and binding

Hydrolysis of GTP was measured using the fluorescent-labeled GTP and GDP analogues, mantGTP (*N*-methylantraniloyl-GTP, 5 mM trisodium salt) and mantGDP (5 mM disodium salt from Molecular Probes). Reactions were performed at 25°C in MglA dilution buffer [1 M arginine, 20 mM HEPES (pH 7.6), 5 mM MgCl₂ (except where noted), 5% (v/v) glycerol, 1 mM DTT]. Measurements were taken using a SPEX FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon Inc). The amount of MglA-His capable of binding nucleotide after renaturation was determined by incubating defined amounts of refolded apoprotein with excess mantGDP for 2 hr and removing unbound mantGDP by dialysis. MglA-His was denatured by heating to release mantGDP and protein was removed by filtration. The amount of mantGDP in the filtrate was determined by fluorescence intensity and by TLC and was compared against a standard curve made from serial dilutions of mantGDP (2 mM stock from Molecular Probes). The fraction of active MglA was determined as mol mantGDP (mol MglA)⁻¹. To measure rates of GTP hydrolysis, 5 μM GTPase (Ha-Ras-His or MglA-His) and 0.1 μM mantGTP were mixed, and fluorescence intensity was monitored over time (excitation 370 nm, emission 435 nm). The decrease in fluorescence was fit to single exponential decay curves, from which rates of hydrolysis rate were calculated using Prism software.

Equilibrium binding constants were determined by plotting the amplitude of fluorescence intensity versus the concentration of mant-nucleotide. MglA-His (1 μM) was mixed with amounts of mantGTP or mantGDP ranging from 0.5 μM to 10 μM, and the amplitudes of fluorescence intensity were recorded at various times. The resulting curves were fit to quadratic hyperbolic functions characteristic of one-site binding equilibria. To monitor nucleotide exchange, 3 μM of MglA-His was mixed with 0.3 μM mantGDP for 5 min at 25°C. Unlabeled GTP, GDP or ATP was added to 100 μM, and the decrease in fluorescence intensity was recorded versus time, as the mantGDP was exchanged for unlabeled nucleotide.

Immunoblot Analysis

Samples (recombinant MglA, Ras, or whole cell extracts) and Magic Mark (Invitrogen) standards were separated by SDS-PAGE with a 12.5% (w/v) Tris-glycine gel and transferred to a Polyscreen PVDF membrane (Perkin-Elmer). The membrane was blocked with commercial Odyssey Blocking Buffer (LI-COR) overnight at 4°C and developed with primary antibody (monoclonal anti-His6 C-terminal (Invitrogen), rabbit polyclonal anti-MglA (1:1000), or monoclonal mouse anti-GFP primary antibodies (1:2500 dilution) for Yfp fusions (Clontech). Secondary antibodies were IR800-labeled goat anti-mouse (Rockland; 1:5000 dilution) for anti-His tagged protein and goat anti-rabbit (conjugated with alkaline phosphatase) for the MglA antibody (1:2000 dilution). Blots developed with IR800 were scanned using the 800 nm channel of a LiCor Odyssey Infrared Imaging System (Li-Cor Biosciences). Blots developed with alkaline phosphatase conjugated antibody were assayed with 70 μl of nitro blue tetrazolium (65 mg/ml in 70% (v/v) dimethylformamide (DMF)) and 70 μl of 5-Bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (50 mg/ml in 70% (v/v) DMF) in alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 10 mM MgCl₂ and 100 mM NaCl).

Microscopy

To observe motile single cells of MglA-Yfp fluorescent strains, we placed cells on CTPM 1.5% (w/v) agar pads or in a solution of 0.5% (v/v) methylcellulose and CTPM broth. Agar pads were prepared as described elsewhere (Higgs and Merlie 2008) with the following modifications. Solidified agar was covered with polyvinyl film and stored at 4° C. Small volumes of the cells were scraped from the edge of the plate colonies, suspended in 15 µl of TPM buffer (10 mM Tris, 1 mM potassium phosphate, 5 mM MgSO₄), spotted on the agar pad and immediately covered with cover glass. Slides were wrapped loosely with polyvinyl film and incubated at 33°C in the dark for at least 30 min. Images were captured with a Nikon i80 Eclipse fluorescence microscope equipped with Lambda LS unit (Shutter Instruments). Fluorescence images were captured at 15 sec intervals for 15 min with a Photometrix Cool SNAP ES digital camera and analyzed using NIS Elements AR 3.0 imaging software (Nikon). Phase video (for quantifying the rate of motility) images were captured at 30 sec intervals for 30 min using a Nikon Microphot FXA microscope. Individual cells were tracked with MetaMorph 6.3r7 software. Changes in position were used as the input data for MotilityMacro (kindly provided by D. Astling and D. Zusman) to calculate cell reversals and number of pauses. Quantitative analysis of the MglA-Yfp distribution was done with NIS-Elements Advanced Research software and MetaMorph.

Fluorescence images of static cells were captured with a Zeiss PASCAL confocal microscope with a 25 mW Argon 488 nm laser and BP505–530 filter. Z-plane images were taken at 0.2 µm steps through non-moving cells attached to glass and deconvolved to construct a three-dimensional image of MglA-Yfp. The acquisition software was AIM Version 4.0 SP2.

Immunofluorescence

Log phase ($\approx 4.5 \times 10^8$) *M. xanthus* cells were harvested from CTPM broth and suspended in PBS. Samples were fixed with 1.6% (v/v) formaldehyde and 0.1% (v/v) glutaraldehyde for one hr in room temp ($\approx 21^\circ$ C) with gentle shaking. Fixed cells were washed with PBS-T (1 $\times$ PBS pH 7.4, 0.02% (w/v) Tween) and Tris-glucose-EDTA buffer (50 mM glucose, 20 mM Tris pH 7.5, 10 mM EDTA) and subsequently permeabilized with lysozyme (final concentration 10 µg/ml) for 10 min. After washing with PBS-T, cells suspension was spotted on poly L-lysine coated microscopic slides and incubated for 1 hr at room temp. Slides were washed with PBS-T and blocked overnight at 4° C in PBS-T with 2% (w/v) BSA. Samples were incubated with primary rabbit polyclonal anti-MglA antibodies (adsorbed with $\Delta mglBA$ extract and diluted 1:200 in PBS-T) for at least two hrs at room temp with gentle shaking. After washing step (4 times for 15 min with PBS-T) slides were treated with secondary Alexa Fluor® 488 nm (1:400 dil) for at least one hr at room temp with gentle shaking and washed 4 $\times$ 15 min with PBS-T.

Motility and development assays

Motility phenotypes of mutants were compared with that of the WT strain using swarming assays after 4 days of growth on CTPM with 0.3% (w/v) and 1.5% (w/v) agar surfaces (Shi and Zusman 1993), by microscopic examinations of colony edges, and by time-lapse microscopy on 1.5% (w/v) agar pads for A-motility and in 0.5% (v/v) methylcellulose in CTPM broth for S-motility. Concentrated cells were plated onto TPM starvation medium as described (Kroos et al. 1986) to monitor development and spore production. Plates were incubated at 32°C for 5 days, during which developing cells were examined visually for aggregation, rippling, and fruiting body morphogenesis, using a Nikon SMZ-U stereomicroscope. Assays for heat-resistant spores were performed as described previously (White and Hartzell 2000). Strains DK1622 (WT) and MxH2370 ($\Delta mglBA$) were used as controls.

RESULTS

MglA has modest GTPase activity *in vitro*

To demonstrate that MglA is a small GTPase, we expressed the *mglA* gene in *Escherichia coli* to facilitate the purification of MglA protein. We were able to subclone the WT *mglA* gene into plasmid vector pET24 to produce pAGS120, which placed *mglA* under control of the T7 promoter. To ensure that the modifications used to express MglA in *E. coli* did not alter its activity, we constructed a derivative of *M. xanthus* that expresses the *mglA-his* gene in place of the WT *mglA* gene. Homologous recombination between the *mgl* promoter on pPLH434 (*pmgl mglB mglA-his*) with the *mgl* promoter region on the chromosome of *M. xanthus* strain DK6204 ($\Delta mglBA$) yielded Km^R recombinants that were fully motile and exhibited normal multicellular development (data not shown). This result confirmed that the additional N- and C-terminal residues in this fusion protein did not alter the function of MglA *in vivo*.

Efforts to express MglA as a soluble protein in *E. coli* were unsuccessful. Even with low temperature and modest induction, MglA-His was associated exclusively with the inclusion body material and had to be refolded for biochemical assays. In contrast, a significant portion of the Ha-Ras-His was present in the soluble fraction. MglA-His and Ha-Ras-His were purified to apparent homogeneity over a metal-affinity column, as shown in Fig. 1A. Purified proteins reacted specifically with monoclonal anti-His6 antibodies (data not shown). MglA-His reacted with polyclonal anti-MglA (Fig 1B). GTP hydrolysis was assayed using 2'-/3'-O-(N'-Methylanthraniloyl)-GTP (mantGTP), a fluorescent analog of GTP (Lenzen et al. 1995). Because the purification of MglA-His involved its renaturation from inclusion bodies, we took care to quantify the specific activity of renatured protein capable of binding nucleotide, and found that >90% of the renatured fusion protein could bind this substrate. This result showed that the MglA-His protein can be denatured and renatured without significant loss of activity.

To determine the rates of hydrolysis of GTP by MglA-His and Ras-His, we measured the decrease in fluorescence emission at 435 nm concurrent with the conversion of mantGTP to mantGDP *versus* time at 25°C, as shown in Fig. 2A. The program Graphpad Prism 4.0 was used to fit a single exponential decay curve of the form $F = A_0 + Ae^{-kt}$ to the data. We determined a single turnover hydrolysis rate for the purified Ha-Ras-His protein to be $3.6 \times 10^{-4} \text{ sec}^{-1}$ in the presence of 5 mM $MgCl_2$, similar to the published value of $3.4 \times 10^{-4} \text{ sec}^{-1}$ (Neal et al. 1988). The single turnover hydrolysis rate for the MglA-His protein was found to be $3.4 \times 10^{-4} \text{ sec}^{-1}$ in the presence of 5 mM $MgCl_2$ and $2.1 \times 10^{-4} \text{ sec}^{-1}$ in the presence of 5 mM $MnCl_2$. After the addition of EDTA to chelate Mg^{2+} bound to MglA-His and subsequent dialysis, the single turnover rate for MglA-His was found to be reduced to $9.9 \times 10^{-5} \text{ sec}^{-1}$, demonstrating that substrate turnover is dependent on the presence of divalent cation. As a negative control, we showed that the activity we measured was not due to contamination by *E. coli* GTPases in the inclusion body material from *E. coli* cells expressing AgIU, a non-MglA containing sample (data not shown). The equilibrium binding constants for MglA-His with mant-GTP and mant-GDP were calculated from the data presented in Fig. 2B and C to be 11.8 μM , and 9.6 μM , respectively. The addition of excess ATP did not affect the equilibrium binding of mantGTP or mantGDP. The rates of nucleotide exchange of mantGTP or mantGDP were found to be too rapid to be measured accurately after the addition of excess GTP (or GDP), even using stop-flow fluorescence spectroscopy.

MglA associates with a helical element in the cell

The subcellular localization of several known gliding proteins has yielded important insight into the coordination and operation of gliding. At least three gliding proteins, RomR, FrzS, and AglZ are dependent upon MglA for correct localization (Leonardy et al. 2007; Zusman et al. 2007). To determine how MglA is organized, WT and mutant strains were probed with anti-MglA antibody and examined by fluorescence microscopy. As shown in Fig 3B–F, the pattern of antibody staining showed that MglA associates with a helical track reminiscent of the tracks produced by MreB from *Bacillus subtilis* and *Escherichia coli* (Defeu Soufo and Graumann 2005; Graumann 2007; Jones et al. 2001; Kruse et al. 2003). The helix was wrapped along the long axis with helix angle between 50°–60° and had a criss-cross (or ‘figure eight’) pattern (cell tracing in Fig 3D) similar to that described for MreB from *E. coli* (Kruse et al. 2003) and *B. subtilis* (Jones et al. 2001). The helical pitch was estimated to be $\sim 1.32 \pm 0.2 \mu\text{m}$ ($n = 43$ cells), larger than the helical pitch of $0.73 \mu\text{m}$ reported for MreB from *Bacillus* (Jones et al. 2001) or $0.46 \mu\text{m}$ reported for *E. coli* (Kruse et al. 2003). These results suggest that MglA can associate with a cytoskeletal component such as *M. xanthus* MXAN6789, which encodes a protein that shares 63% identity (80% positives) with MreB from *E. coli* (AP_00379).

No helical pattern was detected in MxH2370 (ΔmglBA) (Fig 3A), which does not produce MglA, but nearly identical helical patterns were detected in the WT strain (Fig 3B) and the strains expressing MglA-Yfp (Fig 3C, D). Significantly, the IF staining pattern was the same in two gliding mutants, ΔaglZ (Fig 3E) and ΔpilA (Fig 3F), as in the WT strain (Fig 3B). These data suggest that MglA may associate with a structure, such as the cytoskeleton, that is independent of these A- and S-motility components.

The MglA-Yfp fusion protein is functional *in vivo*

MglA interacts with AglZ, a dynamic protein that forms focal adhesion clusters (Mignot et al. 2006). To determine if the distribution of MglA, like AglZ, changes in gliding cells, we engineered strains to express N- and C-terminal Yfp fusions with MglA and characterized the phenotypes of the Km^R recombinants with these fusions. Comparing the rates of gliding as well as reversal frequencies, swarming, and colony edge morphologies determined gliding function. DK1622 is a yellow, A^+S^+ strain that we use as the wild type. MxH2370, is a yellow strain that grows at a rate similar to DK1622 in liquid and on plates, but is defective in swarming, A- and S-motility, fruiting body development (dev^-), and sporogenesis (spo^-).

Integration of plasmid pJP25, which carries *mglB yfp-mglA*, by a single recombination event into MxH2370 yielded a strain that was nonmotile by microscopic analysis and nonswarming on 0.3 and 1.5% (w/v) agar. Hence, Yfp-MglA was unable to complement the ΔmglBA mutant. Based on this negative result with a 5' fusion we turned to construction of 3' fusions. Several 3' fusions were constructed and assayed for the ability to complement the *mgl* mutant. Among these, pJP20, which carries *mglB mglA-yfp*, yielded a yellow, motile derivative named MxH2424 when integrated into MxH2370. As shown in Fig 4C and inset (black and white), MxH2424 ($\Delta\text{mglBA} + \text{mglB mglA-yfp}$) formed swarming proficient colonies with characteristic A- and S-motile cells at the colony edge, indicating that the *mglA-yfp* fusion gene complemented the motility defects of the nonmotile parent (Fig 4B). The average rate of gliding for MxH2424 was $1.8 \mu\text{m min}^{-1}$, 75% the rate of the WT ($2.3 \mu\text{m min}^{-1}$). Videomicroscopy was used to track the movements of cells gliding on 1.5% (w/v) agarose slabs for 20 min and the cumulative tracks are shown as tracings in Fig 4 panels D–F. The tracings show that the ΔmglBA strain did not move (Fig 4E). In contrast, the time-lapse tracings of MxH2424 cells (Fig 4F) yielded long gentle curves that were nearly identical to those of the WT cells (Fig 4D) showing that MglA-Yfp complemented the

gliding defect of the $\Delta mglBA$ mutant. Greater than 90% of the MxH2424 cells were motile whereas fewer than 10% of the $\Delta mglBA$ parent cells showed any movement (data not shown). Although cell motility was near WT levels, swarming functions were not fully restored. Swarming of MxH2424 on 0.3% CTPM (w/v) agar was 45% that of the control and 59% that of the control on 1.5% (w/v) agar after 5 days at 32°C. Recent simulations of swarm expansion predicted accurately that cell flux decreases as the number of non-reversing cells increases (Wu et al. 2009). To determine if the reduction in swarm area for MxH2424 was related to cell reversals, the reversal frequency and pause time were measured. The MxH2424 cells reversed direction more often (reversal period 14.8 min) and paused more often (8.2 hr^{-1}) than did the control cells (reversal period 17.1 min; 7.3 hr^{-1}), a combination that could account for the reduced swarming capacity. The reversal period was longer than reported elsewhere (Blackhart and Zusman 1985; Spormann and Kaiser 1999), which we attribute, in part, to differences in composition of the medium (CYE vs CTT vs CTPM with lower $[\text{Mg}^{2+}]$).

MglA-Yfp complemented the developmental defect of the $\Delta mglBA$ mutant. The $\Delta mglBA$ mutant is unable to aggregate or form mounds during development (Fig 4H) and sporulation efficiency is less than 0.01% that of the control. In contrast, the complemented derivative, MxH2424, was able to aggregate and form dark fruiting bodies as shown in Fig 4I. Sporulation was increased 3000 fold over the $\Delta mglBA$ parent to a final sporulation efficiency of 32% compared to the control. Based on these multiple phenotypic parameters, the MglA-Yfp fusion expressed from pJP20 was selected for further analysis. Although MglA-Yfp from pJP20 complemented the gliding and developmental defects of the $\Delta mglBA$ mutant, the fusion protein was not a perfect match for the native protein. Fusion of Yfp to MglA might perturb the dynamics of interactions with other partners, such as AglZ.

Immunoblot analysis, shown in Fig 5, confirmed the presence of the MglA-Yfp fusion protein. The merodiploid strains produced the 22 kDa MglA and the 49 kDa MglA-Yfp whereas MxH2424 produced only the 49 kDa MglA-Yfp. Anti-MglA and anti-Gfp antibodies recognized MglA-Yfp and there was no evidence for proteolysis of MglA-Yfp using either antibody probe. Approximately the same amount of MglA-Yfp protein was produced in the WT and mutant backgrounds, but the relative abundance of MglA-Yfp appeared to be lower than that of the native MglA. If the concentration of MglA is critical for full motility, then the amount of MglA-Yfp in MxH2424 might not be sufficient for WT swarm.

MglA-Yfp forms polar and lateral clusters as the cell glides on a hard surface

Fluorescence microscopy was used to monitor the activity of MglA fusions in cells that were gliding on rich medium (CTPM) with 1.5% (w/v) agarose. To capture fluorescence images over a 20 min time frame without quenching MglA-Yfp, it was necessary to limit the exposure time of each image by collecting data for a single focal plane. As shown in Fig 6, cells on a solid surface produced both polar and lateral clusters of MglA-Yfp. Only faint polar fluorescence was observed in cells that lacked MglA-Yfp. Images showing the sequence of cluster formation for MglA-Yfp are presented in Fig 6 and in supplemental material (MxH2423.mov, MxH2424.mov). The original set of images of a cell moving from right to left at 15 sec intervals for 150 sec is shown in Panel A; Panels B and C show inverted images and fluorescence intensity profiles, respectively, of the images in panel A. The arrows in panel A and dashed lines in panel B each align with a specific cluster that is fixed relative to the solid surface.

Examination of MglA-Yfp clusters in over 200 cells revealed several trends. *First*, the intensity of an MglA-Yfp cluster at the leading pole typically was greater than the lagging pole cluster and the amount of MglA-Yfp at the leading pole decreased as the cell moved

forward. There was a decrease in fluorescence intensity at the leading pole concomitant with the appearance of a lateral cluster of MglA-Yfp, hinting that some MglA-Yfp was released from the leading pole.

Second, lateral clusters formed as the cell moved forward. Each cluster remained fixed relative to the substratum as illustrated by the two types of arrows in Fig 6A and the three dashed lines (one for each of three clusters in order of appearance) in Fig 6B. On average 55% of the cells tracked produced lateral clusters that appeared to remain in fixed positions with regard to the agar surface (range 48% to 60%; n=89 for MxH2423 and n=142 for MxH2424). This pattern is reminiscent of focal adhesion clusters described for AglZ-Yfp (Mignot et al. 2006), which interacts with MglA (Yang et al. 2004). With respect to the cell body, new clusters appeared to emerge near the leading pole (time course in Fig 6A, untreated image and B, inverted image) and the intensity of fluorescence of an individual lateral cluster decreased as the cell moved (Fig 6, column C arrows and Supplemental material SupFigtracking.pdf). The decreased intensity might be due to degradation of MglA-Yfp or diffusion of MglA-Yfp from the cluster (cluster collapse) resulting in a concentration of MglA-Yfp that was below the limit of detection by epifluorescence.

Third, lateral clusters appeared to originate from the leading pole. Upon cell reversal, MglA-Yfp emerged from the new leading pole (an example is seen in the center of MxH2424.mov in supplemental material).

Bending has been proposed to occur at focal adhesion sites (Mignot et al. 2006) because the cell body can flex around an anchor point. Consistent with this report, we found instances (about 30% of bends) where fluorescence intensity increased at positions in the cell where a bend occurred and clusters appeared to become ‘stuck’ at bend sites (an example is the cell bending in MxH2424bending.mov).

Confocal microscopy was used to create a three-dimensional image of MglA-Yfp in living cells to compare with the uniform helical patterns of MglA from fixed, liquid-grown cultures probed with antibody. As shown in Fig 7, a helical pattern was detected in non-fixed cells stuck to a glass surface. Panels A–C in Fig 7 show a Z-series through *M. xanthus* MxH2424 cells. The fluorescence patterns for one cell, outlined in green, are depicted in the images to the right of each photo. The reconstructed image is shown in panel D and a model representing the proposed MglA-Yfp-associated structure is shown in Fig 7E. While a single helix was evident in >90% of the cells (n=150), the dual (figure-eight) helical pattern seen in fixed cells (Fig 3) was present in some of the live cells expressing MglA-Yfp as shown in Fig 7F and G. These data suggest that although MglA can associate with the Mbl-like helical structure (seen in fixed cells), only one helix is used at a time in a living cell attached to a surface.

Distribution of MglA-Yfp is affected by changes in surface matrix

As mentioned earlier, MglA is essential for A and S motility, each of which function optimally on different surfaces. The time-lapse videomicroscopy described above was performed on CTPM with 1.5% (w/v) agar, a surface that favors A-motility. To explore the requirements for placement of MglA-Yfp as a function of A or S motility, we compared the distribution patterns of MglA-Yfp as a function of the gliding matrix. As shown in Fig 8A and B, lateral clusters were found in most (>80%; sum of the first four patterns that include lateral clusters) of the MglA-Yfp cells gliding on 1.5% (w/v) agar. We were unable to detect a steady pattern of lateral clusters in a subset of cells (~20%) on 1.5% (w/v) agar. This may be attributed to irregularities in the agar surface, which caused these cells to be out of the focal plane needed to capture MglA-Yfp clusters or may indicate that a portion of cells are not using A-motility. Methylcellulose, which is proposed to stimulate retraction of pili (Li et

al. 2003), has been used as a medium to observe isolated cells moving by S-motility (Higgs and Merlie 2008). When the distribution of MglA-Yfp was examined in strains gliding in methylcellulose, MglA was found at the cell poles and diffuse in the cytosol (Fig 8A, C and MxH2424MC.mov in supplemental material). Lateral clusters were detected only in cells that were not moving (data not shown). This result shows that the pattern of MglA-Yfp localization changes significantly depending on the substratum, which likely correlates with the motility system operating at the time that the observations were made. Hence the pattern of MglA may be indicative of a switch between the A and S-motility systems.

DISCUSSION

Our previous genetic work has shown that both the adventurous and social motility phenotypes of *M. xanthus* depend on MglA. In this study, we focused on the mechanistic role of MglA to further dissect these unique modes of cell motility. First, using purified MglA, we confirmed that this protein has Mg^{2+} -dependent GTPase activity. Secondly, because the homology of MglA is most similar to the Sar and Arf GTPases, both of which are known to function in vesicular transport and cytoskeleton assembly, we used antibody against MglA and MglA-Yfp fusions to determine the localization of MglA in fixed cells and live cells, respectively. The localization studies showed that MglA is distributed along helical axes and that its distribution is both dynamic and distinct with regard to the substratum. The pattern for MglA in cells gliding on a hard agar surface was similar to those reported for other components of *M. xanthus* gliding machinery (Mauriello et al. 2009a; Mignot et al. 2006). We discuss the implications of these results on various models describing gliding motility.

Although claims of MglA as a monomeric GTPase have been made, this report documents the biochemical activity of MglA. Attempts to recover MglA as a soluble, folded protein from *E. coli* hosts were unsuccessful, even when co-expressed with MglB, which appears to stabilize MglA ((Hartzell and Kaiser 1991b) and unpublished results). It is encouraging that a recent report shows that it is possible to produce soluble MglA; differences in the *E. coli* expression strain may account for the ability to recover MglA as a soluble, folded protein (Mauriello et al.).

GTP hydrolysis by refolded MglA was slow, similar to that of typical Ras-like GTPases. In eukaryotes, the *in vivo* rates of hydrolysis and nucleotide exchange are stimulated several orders of magnitude by proteins such as GAPs and GEFs. However, we found no evidence for a GAP protein in *M. xanthus*. Addition of cell extract to a GTPase assay did not stimulate GTPase activity of MglA nor were we able to identify a GAP protein from the annotated genome or from BLAST searches (data not shown). The intrinsic rate of hydrolysis may reflect the rate *in vivo* or it may be accelerated there by as yet unidentified chemical or protein factors. The rate of GTP hydrolysis by soluble MglA was significantly higher (Mauriello et al.) than the intrinsic rate of GTP hydrolysis in our study. This hints that an unknown factor might co-purify with soluble MglA that is able to accelerate its GTPase activity.

Our immunofluorescence studies suggest that MglA associates with the *M. xanthus* cytoskeleton and was independent of functioning A- and S-motility systems. Our findings agree with a concurrent study showing that A22, a compound known to disrupt MreB, perturbed the MglA pattern (Mauriello et al 2010). The anti-MglA antibody staining pattern appeared to be distributed along two helical tracks that spiraled right and left, which generated a criss-cross pattern similar to that seen with MreB in other rod-shaped cells.

While the pattern showed that MglA likely associates with the cytoskeleton, it did not indicate whether this association was static or dynamic. To address this question, an MglA-Yfp fusion that complemented the motility and development defects of an *mgl* null mutant was constructed. Z-series reconstructions taken from live, static cells expressing the MglA-Yfp fusion provided independent confirmation that MglA was associated with a helical structure in the cell. Surprisingly, in contrast with the criss-cross helical pattern observed in fixed cells stained with antibody, only one MglA-Yfp helical track was detected in most of the live cells. We think it unlikely that the criss-cross helical pattern is an artifact of antibody staining because the same pattern was observed occasionally by fluorescence microscopy of MglA-Yfp in unfixed cells. Rather, the results suggest that MglA can associate with two helices, but do not use both helices at the same time in an actively gliding cell. Two simple models are consistent with our understanding of MglA. One model assumes that the MglA-associated cytoskeletal component has directional polarity. Hence, each helical track might be used to carry MglA when the cell is moving in different directions, similar to intraflagellar transport rafts, which use separate tracks during assembly of the flagella in *Chlamydomonas* (Qin et al. 2004). Alternatively tracks might be used to transport different forms of MglA from pole to pole; one track would be specific for MglA-GTP while the other would be specific for MglA-GDP.

The distribution of MglA was matrix-specific: polar clusters predominated when cells were gliding in methylcellulose, a matrix that favors S-motility but both lateral and polar clusters formed in cells gliding on 1.5% (w/v) agar, a matrix that favors A-motility. This suggests that lateral clusters are highly associated with A-motility. This observation agrees with previous results showing that A and S-motility are matrix-specific (Shi and Zusman 1993) and suggests that MglA may be part of a matrix switch that cells use to regulate motility in response to the substratum as has been shown in other microbes (Blair et al. 2008; McCarter et al. 1988). We propose that when cells are on dry, hard agar surfaces, release of MglA from the leading pole stimulates formation of lateral clusters, which are correlated highly with A-motility. In contrast, when cells are on a soft surface, MglA remains concentrated at the poles and the formation of lateral clusters is inhibited. Under these conditions, S-motility predominates. There are a number of proteins that might participate with MglA in the proposed matrix switch by tethering MglA to a pole or regulating its GTPase activity. Candidates include motility proteins such as RomR, FrzS, MasK, and MglB that localize to a cell pole and are known to interact directly or indirectly with MglA (Leonardy et al. 2008; Leonardy et al. 2007; Mignot et al. 2005; Mignot et al. 2007)(J. Patryn, S. Fremgen, P. Hartzell, unpublished results). The correct form of MglA, in turn, may help to establish the polarity of some of these proteins (Leonardy et al. 2008).

The behavior of MglA-Yfp clusters in cells gliding on 1.5% (w/v) agar was similar to the focal adhesion complexes described for AglZ (Mignot et al. 2006), a filament-forming coiled-coil protein that interacts with MglA and is required for A-motility (Yang et al. 2004). Although the mechanism of A-motility is still unknown, it has been proposed that focal adhesion complexes provide propulsion for A-motility (Mignot et al. 2006). Focal adhesion complexes appear to form at sites where gliding proteins coalesce at a point of contact between the substratum and the cytoskeleton (Lapidus and Berg 1982; Mignot 2007; Mignot and Kirby 2008). AglZ-Yfp is thought to associate with helical cytoskeletal elements and that forward movement is accompanied by rotation of the cell along a path that mirrors the internal helical structure. Focal adhesion complexes may comprise part of the ring elements detected by electron microscopy that have been proposed to generate energy for gliding (Freese et al. 1997). If the model is correct, A-motility might share functional similarities with movement of the apicomplexan *Toxoplasma gondii*, which involves formation of a transmembrane adhesion complex, the glideosome, coupled with spiral rotation (Opitz and Soldati 2002). Alternatively, focal adhesion complexes may be sites

where glycocalyx (slime), whose secretion has been proposed to provide force for A-motility (Yu and Kaiser 2007), is released.

Why are lateral clusters visible on 1.5% agar but not in cells gliding in methylcellulose? One possibility, which is consistent with the behavior of some eukaryotic GTPases, is that clusters large enough to be detected by epifluorescence would develop with one form of MglA, such as the GTP-bound form, but not the other form. In our model, release of MglA•GTP from the leading pole would stimulate the formation of visible clusters (focal adhesions) that are associated with A-motility. Hydrolysis of GTP in the clusters would yield the less stable MglA•GDP form, which would be released from the clusters and returned to the leading pole along the retrograde track where it could interact with an exchange factor to replenish the active GTP bound form of MglA. Work is in progress to distinguish these possibilities.

CONCLUSIONS

The results give us new insight into the role of MglA, a cytoplasmic GTPase that is essential for gliding motility. MglA associated with the cytoskeleton and may act upstream of AglZ in the formation of focal adhesion clusters that are important for A-motility. There was a strong correlation between the localization of MglA and the motility system being used. These results and the requirement of MglA for correct placement of other motility proteins suggest that MglA is a master regulator that may be responsible for activating A and S-gliding systems in response to matrix sensing. Our findings support the growing body of evidence that proteins essential for gliding motility and chemotaxis may rely on interactions with components of the cytoskeleton for their proper functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Purification of MglA

(A) SDS-PAGE showing Ha-Ras and MglA purified from *E. coli* as described in Methods. Lane 1) Benchmark Protein Std 10–220 kDa (Invitrogen); 2) Ha-Ras-His fusion from pRJS2; 3) MglA-His from pAGS120. (B) Immunoblot analysis with antibodies directed against MglA. Lane 1) Magic Mark Western Std (Invitrogen) 20–120 kDa; 2) MglA-His from pAGS120.

Fig 2. MglA catalyzes hydrolysis of GTP

(A) Fluorescence decay was monitored at 435 nm and the rate of hydrolysis was determined from the single turnover rate constant using Prism Graphpad. The time-dependent change in fluorescence for mantGTP was monitored in the absence (mant GTP; solid grey line) and presence of 5 μM of MglA (dashed line) or Ha-Ras (solid black line) protein. The mean values for four independent assays are presented; the standard deviations for these measurements were $<10\%$. (B) (C) Equilibrium binding of mantGTP and mantGDP to MglA-His. The K_d values of MglA for mantGTP (panel B) and mantGDP (panel C) were measured by plotting the amplitude of fluorescence intensity *versus* the concentration of mant nucleotide. MglA (1 μM) was mixed with concentrations of mantGTP varying from

0.5 μM to 10 μM . Results shown represent the average values from three independent series of measurements.

Fig 3. MglA associates with a helical structure in fixed *M. xanthus* cells

Epifluorescence was used to observe the pattern of MglA in fixed cells. (A) MxH2370 (no MglA control), (B) DK1622, (C) MxH2423 (DK1622 + *mglBA*), (D) MxH2424 ($\Delta mglBA$ + *mglBA-yfp*), (E) MxH2265 ($\Delta aglZ$), and (F) DK10409 ($\Delta pilA$). IF was performed as described in Methods. Probe= anti-MglA antibody. Bars=10 μ m

Fig 4. MglA-Yfp complements the $\Delta mglBA$ mutant

Colony morphology and microscopic motility assays show that MglA-Yfp can complement MxH2370, the $\Delta mglBA$ mutant. All images were taken at the same magnification after 5 d incubation on CTPM 1.5% (w/v) agar. (A) Colony morphology of DK1622 (WT); diameter 21 mm (B) $\Delta mglBA$ (MxH2370); diameter 4 mm, and (C) MxH2424 (MxH2370::pJP20 (*mglBA-yfp*)); diameter 12mm. Inset near (C) shows the colony edge of MxH2424. (D–F) Gliding patterns for 10 cells were generated from time-lapse video images of each strain on 1.5% (w/v) agarose pads using the Metamorph tracking software. (D)= DK1622, (E)= MxH2370, and (F)=MxH2424. (G–I) MglA-Yfp complements the development defect of the $\Delta mglBA$ mutant. (G)= DK1622, H= MxH2370, and I= MxH2424. Images were taken after 96 hr on TPM agar. Bar = 50 μ m.

Fig 5. Production of MglA-Yfp in *M. xanthus*

(A) Plasmid pJP20 contains a 2.34 kb *mglB* and *mglA-yfp* fragment under control of the *mgl* promoter. (B) Immunoblot analysis with antibodies directed against Gfp. Lane 1) MW standards; 2) DK1622; 3) MxH2423 (DK1622::pJP20); 4) MxH2424 (MxH2370 pJP20). The arrow indicates the position of MglA-Yfp (49 kDa). (C) Immunoblot analysis with antibodies directed against MglA. Lane 1) MW standards; 2) MxH2370 ($\Delta mglBA$); 3) MxH2424 ($\Delta mglBA$ pJP20); 4) DK1622; 5) MxH2423 (DK1622 pJP20). Arrows indicate the position of MglA (22 kDa) and MglA-Yfp (49 kDa).

Fig 6. Clusters of MglA-Yfp form in actively gliding cells

Time lapse images of a cell gliding on 1.5% (w/v) agar show the formation of lateral clusters of MglA-Yfp. Images in column A (inverted images in column B) show the fluorescence distribution over the time (15 s intervals) of a typical MxH2424 cell moving right to left (arrow with asterisk (*) in $t=0$ image). Two sets of arrows in (A) show the positions of lateral clusters, which remain fixed relative to the substratum. The large arrowhead in the center of $t=0$ image does not move in subsequent images even though the cell has moved. The dashed line over the inverted image in panel aligns with this cluster. A second set of arrowheads (small arrowhead) shows the appearance of a new cluster at $t=45$ s. Column C shows a pseudo 3D projection showing the relative intensity of fluorescence particles in the

cell. The X and Y axes units are scaled in pixels and the Z axis represents the intensity pixel values (from 0 to 65535) for 24-bit images. The intensity of the cluster at the leading pole diminishes as new lateral clusters appear. Arrowheads in (C) correspond to the same clusters indicated in (A). Additional examples are shown in Supplemental material (SupFigtracking.pdf)

Fig 7. Image reconstruction of MglA-Yfp generated from sections taken through live cells (A–C) Confocal microscopy was used to acquire a Z-series of fluorescence images of *M. xanthus* cells producing MglA-Yfp (MxH2424); (D) Images from the Z-series were reconstituted to produce a composite image; (E) Cartoon depicting the arrangement of Z-section images to form a helix; (F–G) Left and right-handed helices were detected in some cells.

Fig 8. The distribution pattern of MglA-Yfp depends on the gliding matrix

The location of MglA-Yfp clusters was determined by analysis of fluorescence microscopy time-lapse images. (A) Seven distribution patterns for MglA-Yfp are shown in the left column. The percentage of cells that displayed a particular pattern is shown for WT cells on 1.5% CTPM (w/v) agar and CTPM plus 0.5% (v/v) methylcellulose. Only motile cells were included in the analysis; n=100 cells for 1.5% (w/v) agar and n=60 cells for MC. (B) Confocal image of MxH2424 cells on solid surface, bar = 10 μ m; (C) Fluorescence image of MxH2424 cells in methylcellulose (taken from video file), bar = 10 μ m.

Table 1

Strains and molecular reagents

Name	Genotype	Features	Construction [‡]	References
DK10409	$\Delta pilA$	Km ^S , A ⁺ S ⁻		(Wu et al. 1997)
DK1622	Wild type (WT)	Km ^S , A ⁺ S ⁺		(Wall et al. 1999)
DK6204	$\Delta mglBA$	Km ^S , A ⁻ S ⁻		(Hartzell and Kaiser 1991b)
MxH1855	$\Delta mglBA + mglBAhis$	Km ^R , A ⁺ S ⁺	pPLH434 into DK6204	This study
MxH2265	$\Delta aglZ$	Km ^S , A ⁻ S ⁺		(Yang et al. 2004)
MxH2370	$\Delta mglBA$	Km ^S , A ⁻ S ⁻	pKD111 into DK1622, grown without selection, pick Km ^S nonmotile colonies	This study
MxH2376	$\Delta mglBA + mglBA$	Km ^R , A ⁺ S ⁺	pKD100 into MxH2370	This study
MxH2423	WT + $mglB^+mglA-yfp$	Km ^R , A ⁺ S ⁺	pJP20 into DK1622	This study
MxH2424	$\Delta mglBA + mglB^+mglA-yfp$	Km ^R , A ⁺ S ⁺	pJP20 into MxH2370	This study
MxH2519	$\Delta mglBA + mglB^+yfp-mglA$	Km ^R , A ⁻ S ⁻	pJP25 into MxH2370	This study
<i>Escherichia coli</i> TOP10	F- <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80lacZ\Delta M15$ $\Delta lacX74$ <i>recA1</i> <i>araD139</i> $\Delta(ara-leu)7697$ <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>			Invitrogen
<i>Escherichia coli</i> BLD21 (DE3)	F ⁻ <i>ompT</i> <i>gal</i> [<i>dcm</i>][<i>lon</i>] <i>hsdS^h</i> (r _B ⁻ m _B ⁻)			Invitrogen

Plasmids		
pAGS120	<i>Bam</i> HI- <i>Hind</i> III PCR157 ligated with <i>Bam</i> HI- <i>Hind</i> III pET24b, Km ^R	This study
pBR322-c-Ha-Ras	c-Ha-Ras, Ap ^R	gift of F. Tamanai
pCR-Blunt II-TOPO	Cloning vector, Km ^R	Invitrogen
pET24	Expression vector, Km ^R	Novagen
pJC58	<i>eyfp</i> sequence cloned into pDSW204	(Chen and Beckwith 2001)
pJP20	<i>P_{mgl}mglB mglA-yfp</i> ; PCR15 in pCR-Blunt II-TOPO	This study
pJP25	<i>P_{mgl}mglB mglA-yfp</i> ; PCR174 in pCR-Blunt II-TOPO	This study
pKD100	<i>P_{mgl}mglB mglA-yfp</i> ; PCR10 in pCR-Blunt II-TOPO	This study
pKD111	DNA upstream and downstream of <i>mgl</i> ; 4764bp <i>Dra</i> I- <i>Sac</i> I fragment of pKD112 ligated with 3194bp <i>Dra</i> I- <i>Sac</i> I fragment of pKD113	This study
pKD112	DNA upstream of <i>mgl</i> ; PCR7 in pCR-Blunt II-TOPO	This study
pKD113	DNA downstream of <i>mgl</i> ; PCR8 in pCR-Blunt II-TOPO	This study
pPLH325	2.2kb <i>mglBA</i> operon	(Hartzell and Kaiser 1991a)
pPLH434	<i>mglBA-his</i>	This study
pRJS2	c-Ha-Ras; <i>Bam</i> HI- <i>Xho</i> I digested PCR-RS1 ligated with <i>Bam</i> HI- <i>Xho</i> I pET24b	This study

PCR Products			
PCR#	Oligo pairs	Template	Product
RS1	304×305	pBR322-c-Ha-Ras	595 bp <i>Hras</i>

PCR Products			
PCR#	Oligo pairs	Template	Product
5	49×617	pJP19	2147 bp <i>mglBmglA-yfp</i>
7	461×462	<i>M. xanthus</i> DK1622 chromosomal DNA	2402 bp region upstream of <i>mgl</i>
8	463×464	<i>M. xanthus</i> DK1622 chromosomal DNA	1958 bp region downstream of <i>mgl</i>
10	49×93	pPLH325	1536 bp <i>mgl</i> operon
12	616×93	pPLH325	247 bp 3' <i>mgl</i>
15	49×93	PCR5 and PCR12	2340 bp <i>mglBmglA-yfp-mgl3'</i>
32	49×450	pPLH325	1316 bp <i>mglBmglA</i>
34	451×452	pJC58	716 bp <i>yfp</i> for 3' <i>mglA</i> fusion
40	49×452	PCR32 and PCR34	2120 bp <i>mglBmglA-yfp</i> or 3' <i>mglA</i> fusion
116	701×704	pJP20	746 bp <i>yfp</i> for 5' <i>mglA</i> fusion
122	703×93	pKD100	823 bp <i>mglA</i> for 5' <i>mglA</i> fusion
138	546×702	pKD100	806 bp <i>mglB</i> for 5' <i>mglA</i> fusion
157	150×151	pPLH325	595bp <i>mglA</i> with 5' <i>Bam</i> HI and 3' <i>Hind</i> III
160	701×93	PCR116 and PCR122	1551 bp <i>yfp-mglA</i> for 5' <i>mglA</i> fusion
170	546×704	PCR116 and PCR138	1525 bp <i>mglB yfp</i> for 5' <i>mglA</i> fusion
174	546×93	PCR160 and PCR170	2330 bp <i>mglB yfp-mglA</i> for 5' <i>mglA</i> fusion

Oligo #	Purpose	Sequence
49	Upstream <i>mgl</i> for	5' ATC ACA AGC TTA AGA GTC AGG CCC C 3'
93	Downstream	5' ATC GAA TTC AGC GGA TGC GGC GGG CT 3'
150	<i>mglA</i> rev	5' CGGGATCCCATGTCCTTCATCAA
151	<i>mglA</i> for	5' CCCAAGCTTACCACCTTCTTGAG
304	<i>mglA</i> rev	5' CCG GGA TCC GAT GAC CGA ATA CAA ACT GGT TG 3'
305	Ha Ras for	5' CCG CTC GAG AGA CAG AAC GCA TTT GCA GGA C 3'
450	Ha Ras rev	5' GCA GCC GGG GCA GCA CCC CGC GCC CCC ACC ACC
451	3' <i>mglA</i> overlap	CTT CTT GAG CTC GTT 3'
452	5' <i>yfp</i> overlap	5' GGG TGC TGC CCC GGC TGC TGC GGG GCG GGG ATG
461	3' <i>yfp</i> end	GTG AGC AAG GGC GAG GA 3'
462	<i>mgl</i> upstream for	5' TTA CTT GTA CAG CTC GTC CAT GC 3'
463	<i>mgl</i> upstream rev	5' GGG CGT ATG CGC TCC TCA GCG TTC GCT 3'
464	<i>mgl</i> downstream for	5' CTT CGG ACG CGC GCA GCG TCT TGC CAT CGC GG 3'
546	<i>mgl</i> downstream rev	5' GCG CAG CTT CTG GAG CAG GTT GCC CTG GAG G 3'
616	<i>mgl</i> upstream	5' GCA AGC GCC CAG GCG GGC GAC CCC GGT CCC CGC CC 3'
617	<i>mglA</i> downstream	5' AAG CTT GCG GAG TAT AGA GAA AGC CGG GAA GGC CCC 3'
701	3' <i>yfp</i>	5' ACG AGC TGT ACA AGT AAA GCA AGC GCC CAG GCG GG 3'
702	<i>mglB</i> 3' overlap	5' CCG CCT GGG CGC TTG CTT TAC TTG TAC AGC TCG TC 3'
703	<i>yfp</i> 5' overlap	5' GGG AAG CCA TGA TGG TGA GCA AGG GCG AGG AGC TGT TCA CC 3'
704	<i>mglA</i> 5' overlap	5' CGC CCT TGC TCA CCA TCA TGG CTT CCC GGG TTA CTC GCT G 3'

[‡] Plasmids were introduced by electroporation (Kashefi and Hartzell 1995).