The polarity of myxobacterial gliding is regulated by direct interactions between the gliding motors and the Ras homolog MglA

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Gliding motility in \textit{Myxococcus xanthus} is powered by flagella stator homologs that move in helical trajectories using proton motive force. The Frz chemosensory pathway regulates the cell polarity axis through MglA, a Ras family GTPase; however, little is known about how MglA establishes the polarity of gliding, because the gliding motors move simultaneously in opposite directions. Here we examined the localization and dynamics of MglA and gliding motors in high spatial and time resolution. We determined that MglA localizes not only at the cell poles, but also along the cell bodies, forming a decreasing concentration gradient toward the lagging cell pole. MglA directly interacts with the motor protein AglR, and the spatial distribution of AglR reversals is positively correlated with the MglA gradient. Thus, the motors moving toward lagging cell poles are less likely to reverse, generating stronger forward propulsion. MglB, the GTPase-activating protein of MglA, regulates motor reversal by maintaining the MglA gradient. Our results suggest a mechanism whereby bacteria use Ras family proteins to modulate cellular polarity.

Generating and maintaining polarity is fundamental to the proper functioning of cells. Eukaryotic cells generate polarity for migration and the accurate positioning of macromolecules and organelles (1, 2). For bacteria, polarity is important for motility, division, signal transduction, and pathogenesis (3, 4). The Gram-negative soil bacterium \textit{Myxococcus xanthus} is a model organism for use in the study of cell polarity for its directed surface motilities. \textit{M. xanthus} cells move on solid surfaces using two distinct mechanisms. The first mechanism, social motility (S-motility), is powered by the extension and retraction of type IV pili from the cell poles (5, 6). In contrast, the second mechanism, gliding motility (adventurous or A-motility), uses proton motive force to power the movement of motor complexes containing flagella stator homologs (7–11). Gliding \textit{M. xanthus} cells on 1.5% agar plates typically reverse their polarity approximately every 8–12 min (12). The Frz chemosensory pathway regulates the reversal frequency and thus the direction of cell movements of both motility systems (12–16). MglA, a Ras family GTPase, has been identified as the central regulator of cell polarity and the principal responder to Frz pathway signaling (13–15). It has been reported that MglA is connected to the Frz pathway by the response regulator RomR (17–19). Importantly, MglA switches between an active GTP-bound form and an inactive GDP-bound form, which is regulated by MglB, the cognate GTPase-activating protein (GAP) of MglA, providing another layer of regulation (13, 14).

The importance of cell polarity in S-motility is obvious, because the S-motility motors localize to cell poles in an MglA-dependent manner (5, 20). In contrast, cell polarity for gliding motility is enigmatic, because the gliding motor complexes, as represented by the MotA homolog AglR and motor-associated proteins, such as AgmU (GltD), localize in blurry patches that move simultaneously in opposite directions along a helical track (7, 8, 10, 11).

The gliding complexes consist of the motor proteins AglR, AglQ, and AglS, along with numerous motor-associated proteins that localize in the cytoplasm, inner membrane, and periplasm (21). Genomic analysis has shown that the \textit{M. xanthus} motor complexes, unlike the MotAB complexes of enteric bacteria, lack peptidoglycan-binding domains and are free to move within the membrane (7). Consistent with this idea, the motor protein AglR and the motor-associated protein AgmU (GltD) have been observed to decorate a helical macrostructure that rotates as cells move forward (7, 8). In addition, tracking the movements of single AglIR molecules using single-particle photoactivatable localization microscopy (sptPalm) (22) revealed that the gliding motors containing AglIR molecules move in helical trajectories. A subpopulation of motors slow down and accumulate into evenly distributed “traffic jam” clusters at the ventral sides of cells, where they contact surfaces. The traffic jam clusters appear to be stationary in relation to the substratum when cells move forward (7). These clusters were originally called “focal adhesion sites” because of some similarities with eukaryotic motility complexes (9, 23).

Based on the results of our high-resolution experiments, we proposed a revised model of bacterial gliding (the helical rotor model) that envisions the distance between two adjacent traffic jam sites as corresponding to the period of the helical track (11). According to this model, motors at these sites push against the

Significance

The bacterium \textit{Myxococcus xanthus} moves on surfaces by directed gliding motility. MglA, a Ras family GTPase, regulates cell polarity in \textit{M. xanthus}; however, little is known about how MglA establishes cell polarity during gliding, because gliding motors move simultaneously in opposite directions. We found that MglA interacts with the gliding motors directly and is localized in a decreasing intracellular gradient. Furthermore, the motors tend to reverse their moving direction at locations where the activity of MglA is high. Our data suggest that biased reversals along the MglA gradient make the motors moving toward the lagging cell poles less likely to reverse, generating stronger forward propulsion. Thus, bacterial cells, like eukaryotic cells, can use Ras homolog localization to establish cellular polarity.


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gliding surface, deform the cell membrane, and exert force against the surface slime (Fig. 1A) (7). This model explains evenly distributed traffic jam sites in gliding cells, without invoking that force is transmitted to the surface by breaching the peptidoglycan barrier (21, 23); however, how the bidirectional motion of gliding motors generates unidirectional cell movements remains unknown.

In the present study, we found that MglA directly interacts with AglR. Using a combination of sptPALM and conventional fluorescence microscopy, we showed that MglA localizes not only at the cell poles, but also along the cell bodies, forming a gradient toward the lagging cell poles. We investigated the role of MglA in regulating gliding motility by tracking the movements of motors in various genetic backgrounds. We found that the probability of AglR reversal is positively correlated with the local MglA gradient. Our observations suggest that the MglA gradient dictates the polarity of gliding by triggering the reversal of gliding motors asymmetrically.

**Results**

**Single Gliding Motors Frequently Reverse Their Direction of Movement Along Cell Bodies.** Previous work using various microscopy techniques, including deconvolution, sptPALM, and structured illumination microscopy, showed that the gliding motor protein AglR decorates and moves along helical tracks (7, 8). We were interested in exploring how the movement of the motors can determine the direction of cell movement, because motor complexes potentially can move in opposite directions (7, 8) (Fig. 1A). To investigate the polarity in the movements of single motors, we constructed *M. xanthus* strains that were WT for gliding motility and that expressed pamCherry-labeled AglR (a MotA homolog) or AglQ (a MotB homolog) as the sole AglR or AglQ protein under their native regulatory control (7). We then used sptPALM to follow the movements of these single motor molecules. We found that single molecules of both AglR-pamCherry and AglQ-pamCherry move along helical trajectories (7) (Fig. 1B, Fig. S1, and Movies S1 and S2). Given that the movements of AglR and AglQ appeared to be indistinguishable (Fig. S1), we chose to follow the movements of AglR as representative of intact AglRQS motors. The motor molecules showed displacement along both cell lengths and cell widths; however, we were interested in analyzing their displacement only along the long cell axes, to simplify the analysis. Thus, we imaged only the bottom portion of the cells using near total internal reflection (TIR) illumination, which allows imaging of cell structures located near the surface.

The kymographs in Fig. 1B show that fluorescent AglR spots move in either direction (7). We tracked 786 single AglR molecules from 24 cells and found that 70% of molecules showed obvious displacements with respect to the substratum, whereas other molecules remained stationary before bleaching. We suggest that these “stalled” AglR molecules correspond to the motors in the putative traffic jam sites near cell surfaces (7) (Fig. 1B, blue dots). We further analyzed the motile molecules and found that 52% reversed their moving directions before bleaching (Fig. 1B, arrows). Most importantly, AglR molecules were observed reversing their moving directions both at cell poles and along cell bodies (Fig. 1B, arrows). Because AglR-pamCherry is fully functional and the fluorescence of pamCherry molecules is randomly activated (7), we suggest that the AglR motors that we observed represent the overall behavior of the assembled motors in each cell. Taken together, these data show that in a gliding cell, instead of showing unidirectional movements from pole to pole, the gliding motor complexes frequently change their direction of movement.

**The Gliding Motors Interact Directly with MglA.** MglA plays a central regulatory role in establishing the polar axis for gliding motility. Mutations in *mglA* cause dramatic changes in the reversal frequency of gliding cells (13, 14). We tested whether MglA directly interacts with the gliding motor protein AglR in the absence of the other gliding-associated proteins present in *M. xanthus* using a bacterial adenylate cyclase two-hybrid (BACTH) assay. We fused *mglA* and *aglR* to the 5′ ends of the gene fragments that encode complementary fragments of adenylate cyclase (CyaA) and express the chimeric genes. Protein interactions can be determined by the blue color of colonies associated with β-galactosidase activity (24). As shown in Fig. 2, MglA directly interacted with AglR. In addition, the motor protein showed strong self-interaction. In contrast, MglA did not appear to form oligomers, because the *Escherichia coli* strain carrying pUT18-*mglA* and pKT25-*mglA* did not turn blue. This observation is consistent with the deduced crystal structure.
not detected in the cross-linked samples from the cells expressing only AglR or MglA. This interaction between AglR and MglA is specific because even though AglR and MglA were overexpressed in *E. coli*, the addition of formaldehyde did not result in any non-specific cross-linked bands between AglR, MglA, and other *E. coli* proteins (Fig. 2C). The cross-linking data did not provide sufficient information on the stoichiometry of the AglR–MglA interaction. In *E. coli* and *Vibrio alginolyticus*, MotA homologs form four-protein bundles (26, 27). If this is the case in *M. xanthus* as well, then the molecular weight of the putative complex formed by four GST-AglR molecules and one His-MglA molecule is >200 kDa, consistent with the molecular weight of the cross-linked bands in our experiments (Fig. 2C).

**MglA Forms a Gradient in Nonpolar Regions.** MglA has been observed to localize primarily at the cell poles (13, 14); however, most of the components in the gliding machinery, including AglR, localize along the whole cell body (8, 9, 21), creating a spatial dilemma for the interaction between MglA and the motors. We confirmed the observation that cells expressing MglA-GFP showed bright fluorescence spots primarily at the cell poles; however, we also found blury GFP fluorescence at non-polar regions along the cell length (Fig. 3A). To distinguish MglA localization at these regions from background illumination and autofluorescence, we labeled MglA with a photoactivatable fluorophore, mEos2 (28). MglA-mEos2 was expressed under the control of the native mglA promoter, and its fluorescence was activated using sptPALM. As shown in Fig. 3A, large protein clusters were activated at both cell poles, consistent with a high polar concentration; however, clear fluorescence spots were also activated at nonpolar regions along the cell body, confirming the observation at lower resolution that MglA localized along the cell body as well (Fig. 3A). The MglA-mEos2 clusters showed random movements that did not result in obvious displacement (Movie S3).

Because the polar clusters of MglA are asymmetric in WT cells, with bright clusters at leading cell poles and dimmer clusters at lagging poles (13, 14), we wondered whether the distribution of MglA molecules along cell bodies is also asymmetric. We scanned the fluorescence signals of MglA-GFP along long cell axes and found that as the distance from the leading cell pole increased, fluorescence intensities of MglA-GFP decreased gradually (Fig. S2). The average MglA-GFP of 20 cells, with lengths normalized to 1, showed that MglA in nonpolar regions is localized in a gradient along the cell bodies, with the highest concentrations nearest the leading cell poles (Fig. 3B, curve).

**MglA Gradients Regulate the Probability and Spatial Distribution of Gliding Motor Reversals.** To directly investigate how MglA gradients establish cell polarity, we mapped the reversal events of single AglR molecules along the cell bodies. We recorded 197 motor reversals in 20 cells with WT gliding motility, normalized the length of each cell to 1, and analyzed the spatial distribution of these reversals. We defined the regions 0–0.1 and 0.9–1.0 as the leading and lagging cell poles, respectively. We found that the motors mostly reversed their direction of movement at locations where MglA was most highly concentrated. The highest numbers of reversals were recorded at the cell poles. Surprisingly, we found that the spatial distribution of AglR reversals was also asymmetric at nonpolar regions (Fig. 3B, bars), showing a strong correlation with the MglA gradients ($r = 0.81$; $df = 14$; two-tailed $P = 0.43$) (Fig. 3B, Inset). Significantly, approximately 70% of AglR reversals in the nonpolar regions were observed in the front one-half of cells, where the local MglA concentration is higher (Fig. 3B).

We conclude that in WT cells, gliding motors are more likely to reverse in the regions with higher MglA concentrations. As the result of the asymmetric distribution of MglA, the motors...
moving toward lagging cell poles are less likely to reverse their moving direction, because they travel toward locations with lower MglA activity. In contrast, the motors moving toward leading cell poles are more likely to reverse direction, because they travel against the MglA gradient.

To further investigate the correlation between motor reversals and MglA concentration, we mapped the distribution of AglR reversals in the ΔmglA strain. AglR molecules still moved actively in the absence of MglA (Fig. 3C, stars and Movie S4); however, most of the AglR molecules were restricted to a few regions, including the cell poles. Multiple molecules were repeatedly activated around those loci, especially near the cell poles, leaving nearly continuous tracks in the kymograph (Fig. 3D). Strikingly, in the 20 cells analyzed, only 18 reversals were observed (arrow). (D) The random distribution of AglR reversals in the 20 cells analyzed.

Enhanced MglA Activity Causes Symmetric MglA Localization and Symmetric Motor Reversals. To determine whether enhanced MglA activity might affect MglA localization and the probability of motor reversals, we overexpressed MglA-GFP as a merodiploid (Fig. S3). As shown in Fig. 4A and C (curve), overexpression of MglA caused the asymmetric gradient of MglA to disappear. Instead, MglA appeared to be distributed uniformly at the nonpolar regions. The concentration of MglA at the cell poles was only slightly (<20%) difference higher than the average concentration at nonpolar regions (Fig. 4A and C, curve).

Similarly, MglA-mEos2 showed bright fluorescence clusters localized at random along the cell bodies (Fig. 4B). Under these overexpression conditions, AglR molecules changed their moving directions within seconds, appearing as zigzag tracks in kymographs (Fig. 4B and Movie S5). We recorded 300 reversals in the 20 cells analyzed, a 52.3% increase compared with the WT control. The reversals were distributed randomly along the cell length (Fig. 4C, columns). The even spatial distribution of reversals observed matches the plot of averaged fluorescence intensity of MglA-GFP (Fig. 4C, curve).

If the frequency of motor reversals were positively correlated with the MglA gradient, then the MglA overexpression phenotype could be mimicked by increasing the GTP-bound form of MglA while keeping the total MglA concentration at WT levels. To test this hypothesis, we expressed MglA

mglA

overexpression (Fig. 4E and Movie S6). We recorded 346 AglR reversals in the 20 cells studied (a 75.6% increase compared with WT cells) and found that the motor reversals were distributed almost equally along the long cell axes, with higher numbers at the cell poles (28% and 17% more reversals at the leading and lagging poles, respectively; Fig. 4F, columns). Taken together, our data suggest that the reversal probability of motors is positively correlated with MglA activity. In WT cells, MglA forms concentration gradients to maintain polarity by reversing motors asymmetrically. This asymmetry is disturbed when either the expression level or the activity state of MglA is altered.
Enhanced MglA Activity Changes the Dynamics and Stability of Traffic Jam Sites. As shown in Fig. 4, along with causing symmetric distribution of motor reversals, overexpressing WT MglA or expressing the hyperactive MglA<sup>Q82L</sup> also increased the reversal frequency of gliding motors. To further understand the effect of hyper-MglA activity on cell gliding, we analyzed motor behavior in the WT control, MglA overexpression strain, and the hyper-MglA activity on cell gliding, we analyzed motor behavior in different backgrounds using four parameters. (i) Time that AglR moved before reversing; (ii) distance that AglR traveled along the cell length before reversing; (iii) percentage of AglR molecules that stopped moving (stalled population); and (iv) time that AglR molecules remained stalled at the traffic jam sites (stalling time). In WT cells, AglR molecules traveled for 1.6 ± 0.5 μm (mean ± SD) before reversing, resulting in displacement of 1.3 ± 0.4 μm (Fig. 4 G and H), and 30.7% of AglR molecules stalled, for an average of 2.3 ± 0.7 s (Fig. 4 I and J). In contrast, AglR molecules in the presence of excess MglA or the hyperactive MglA<sup>Q82L</sup> traveled for significantly shorter times before reversing (0.9 ± 0.4 s and 0.8 ± 0.3 s, respectively), resulting in shorter displacements (0.6 ± 0.2 μm and 0.5 ± 0.2 μm, respectively) (Fig. 4 G and H). In the two strains with increased MglA activity, a smaller population of AglR stalled (16.7% and 7.1%, respectively), with a slightly shorter stalling time (1.6 ± 0.5 s and 1.5 ± 0.3 s, respectively; Fig. 4 I and J). As a result of the less stable traffic jam sites, the gliding efficiency of both the MglA overexpression strain and the mglA<sup>Q82L</sup> hyperactive strain were reduced (Fig. S4 and Discussion).

MglB Regulates Motor Reversal by Maintaining the MglA Gradients. MglB is the cognate GAP of MglA that catalyzes the switch of MglA from the GTP-bound active state to the GDP-bound inactive state (13, 14). We found that both deletion and overexpression of MglB reduced gliding efficiency (Fig. S4). To
investigate the regulatory roles of MglB, we mapped MglA localization and AglR movements in the MglB deletion and overexpression backgrounds. In the ΔmglB cells, MglA-GFP and MglA-mEos2 formed bright clusters at both cell ends (13, 14) (Fig. 5A). In the absence of MglB, AglR-pamCherry molecules frequently switched their travel directions at various locations (Movie S7). (C) In the 20 cells analyzed, reversals (columns) were distributed nearly evenly along cell bodies, with slightly higher frequency at cell poles, where the MglA-GFP concentration (curve) is higher. (D) Overexpression of MglB (MglB OE) delocalized MglA. MglA was distributed nearly evenly along cell bodies, with slightly higher concentrations at cell poles. (E) The phenotype of the MglB OE strain partially mimicked the strain carrying mglA deletion. AglR molecules were still able to move, but reversed less frequently and stalled for longer periods (Movie S8). (F) The 20 cells analyzed, reversal frequency of AglR (columns) was reduced, and reversal occurred randomly along cell bodies, consistent with the localization of MglA (curve). A smaller population of the molecules that stalled in traffic jam sites as a fraction the whole AglR population. (G) Time that AglR moved before reversal. (H) Distance that AglR traveled before reversal. (I) Population of the molecules that stalled in traffic jam sites as a fraction of the whole AglR population. (J) Time that AglR molecules remained in traffic jam sites.

The phenotype of the ΔmglB strain mimics the strains expressing MglAQ82L or overexpressing WT MglA. In this strain, AglR-pamCherry molecules frequently switched their travel directions at various locations (Movie S7). (C) In the 20 cells analyzed, reversals (columns) were distributed nearly evenly along cell bodies, with slightly higher frequency at cell poles, where the MglA-GFP concentration (curve) is higher. (D) Overexpression of MglB (MglB OE) delocalized MglA. MglA was distributed nearly evenly along cell bodies, with slightly higher concentrations at cell poles. (E) The phenotype of the MglB OE strain partially mimicked the strain carrying mglA deletion. AglR molecules were still able to move, but reversed less frequently and stalled for longer periods (Movie S8). (F) The 20 cells analyzed, reversal frequency of AglR (columns) was reduced, and reversal occurred randomly along cell bodies, consistent with the localization of MglA (curve). (G–J) Summary of AglR-pamCherry behavior in different backgrounds using four parameters. (G) Time that AglR moved before reversal. (H) Distance that AglR traveled before reversal. (I) Population of the molecules that stalled in traffic jam sites as a fraction of the whole AglR population. (J) Time that AglR molecules remained in traffic jam sites.

Statistical analysis showed that deletion of mglB increased the reversal frequency of motors. Motors traveled for 0.9 ± 0.4 s and 0.6 ± 0.2 μm before reversing, significantly shorter compared with WT cells (Fig. 5G and H). As a result, a smaller population of AglR (13%) was able to reach the traffic jam sites before reversing (Fig. 5I). Although motors stalled in those sites for 1.9 ± 0.6 s, comparable to the stalling time in WT cells (Fig. 5I), the gliding efficiency of cells was still reduced (Fig. S4). This phenotype is strikingly similar to that of the mglA<sup>Q82L</sup> and MglA overexpression strains (Fig. 4). We conclude that the hyper-reversing behavior of AglR in ΔmglB cells is most likely the result of elevated MglA activity, given that the absence of MglB reduces the GTPase activity of MglA, locking more MglA molecules in the GTP-bound active form (13, 14).
propulsion. However, a substantial population of AglR still may be able to join the traffic jam sites and exert force (Fig. 5 I and J), consistent with the subtle reduction of gliding efficiency in the MglB overexpression strain (Fig. S4).

Discussion

The generation and maintenance of cell polarity are fundamental for the migration of cells. In this study, we found that the polarity of M. xanthus gliding motility is regulated by MglA through its direct interaction with the gliding motors. We found that MglA not only localizes to the cell poles, but also forms a decreasing gradient toward the lagging cell pole in the nonpolar localization of MglA, with the highest concentration closest to the leading cell pole. Single motor complexes reverse their moving directions at various locations along the cell bodies (Fig. 1). Surprisingly, we found that the distribution of AglR reversals along the cell length is also asymmetric, showing a positive correlation with the MglA gradient (Fig. 3). Manipulating the activity of MglA resulted in loss of the MglA gradient and symmetric reversals of AglR (Figs. 4 and 5).

Our findings address two major questions regarding the generation and regulation of polarity in M. xanthus gliding.

i) How can the bidirectional movements of individual motors generate cellular polarity in gliding? Cell polarity for gliding is enigmatic, because the gliding motors and their associated proteins, as represented by AglR, AglQ, and AgmU (GltD), move simultaneously in opposite directions along a helical track (8). In M. xanthus cells, the probability of reversal of motors is regulated by the localized activity of MglA. At cell poles where MglA is most concentrated, almost all of the motors reverse. At nonpolar locations, MglA is less concentrated, forming a gradient that decreases toward the lagging cell pole. According to our model, motors moving toward the lagging cell pole are less likely to reverse due to the decreasing MglA concentration. In contrast, motors moving toward the leading cell pole are more likely to reverse due to the increasing localized MglA concentration. Taken together, the net effect of the MglA gradient is stronger forward propulsion generated by motors moving toward the lagging cell pole, which propels cells forward (Fig. 6A). This polarity is maintained until an upstream regulator, possibly a Frz pathway-dependent protein, signals an inversion of the MglA gradient (13, 14). These movements of individual M. xanthus gliding motors are analogous to the directional switching of flagella motors during swimming of E. coli cells in a chemotactic gradient (Fig. 6B). E. coli cells swimming in the appropriate direction (toward attractants or away from repellents) will increase the probability that cells rotate their flagella counterclockwise, maintaining forward swimming (a run) for a longer time before tumbling. If cells are swimming in the inappropriate direction, then the flagella motors rotate clockwise, causing cells to increase their tumble frequency before embarking in a new direction (Fig. 6B).

ii) How does MglA regulate the gliding of cells? According to our model, the change in cell reversal frequency is determined by the sum of the reversals of individual motors (Fig. 6A). Deleting mglA reduces the reversal frequency of motors to near zero. In that case, the motors pile up at traffic jam sites, especially at the cell poles, and, unable to escape by reversing, disrupt cellular motility (Fig. 3). Hyperactivity of MglA reportedly increases the reversal frequency of cells (13, 14). In the present study, we found that hyperactivity of MglA elevated the reversal probability of single motors. Hyperreversal of motors reduced both the number of motors stalled at traffic jam sites and the time that they remained there (Figs. 4 and 5). According to the helical rotor model (7, 8, 11), the distance between two adjacent traffic jam sites corresponds to the period of the helical track. In WT cells, AglR traveled 1.3 ± 0.4 μm along the cell length before reversing (Fig. 4H), matching the period of the helical tracks, which is also the distance between two adjacent traffic jam sites (1.3 ± 0.5 μm, measured from AglR) (7, 8). Motors in WT cells were able to escape from one traffic jam site and reach the next site before reversing. In the presence of excess MglA activity, motors escaping from one traffic jam site will move for short distances and reverse before reaching the next site, owing to the increased probability of a reversal. As a result, significantly smaller populations of motors can move into traffic jam sites (Fig. 4F). Thus, in this case, the traffic jam sites become more dynamic and less persistent, resulting in hyperreversal of cells.

The functions of MglA provide a mechanism by which a simple bacterium establishes a polarity axis by internal protein localization. How MglA establishes and maintains this gradient and how MglA triggers motor reversals still remain to be investigated, however. In eukaryotic cells, Ras family proteins assemble motility complexes at the front of migrating cells (2, 29). The reversals of M. xanthus gliding motors also may involve the reassembly of gliding complexes. In this case, MglA might reverse the moving direction of gliding motors by disassembling and reassembling motor complexes.
Expression of AgIgR and MglA. AgIgR-pamCherry was expressed under the control of the agIgR promoter in its original chromosomal locus as reported previously (7). For studying the localization of MglA and MglA°COT, the plasmids containing the cognate mglA and mglA°COT were constructed by Christian Kaimer from pMR3562 (17, 30), and the expression of GFP-labeled MglA was induced by 10 μM vanillate. mglA, mglA°COT, and mglB were amplified with the following primers: vanMglAF (5′-GGATTCCATTTCAATTACATCTACCGG-3′), vanMglAR, 5′-AGATCTACACACCTTGTGGCTGGTA-3′, vanMgBF, 5′-GGATCCGACCTGGCGAGCCGCCGTGG-3′, and mglBFR (5′-GGATCCGACCTGGCGAGCCGCCGTGG-3′). The genes were then cloned into the KpnI and BgII sites of plasmid pMR3679 (30). The overexpression of MglA and MglB was induced by 100 μM vanillate. Gilding motility was assayed as described previously (21). For sptPALM studies, the mEos2 gene was amplified with the primers mEosF (5′-AGATCTCTGTCGCGAGCCGCCGTGG-3′) and mEosR (5′-GAATCTTACCACCCTTCTTGAGCTCGGTGA-3′); inserted into the vectors pUT18 and pKNT25 using the BamHI and EcoRI sites; cotransformed into the E. coli host strain DHM1; and grown overnight in LB medium. Then 5 μL of each culture was spotted onto LB plates containing ampicillin (100 μg/mL), kanamycin (100 μg/mL), 5-bromo-4-chloro-3-indolyl-β-d-galactoside activity was assayed as described previously (21), and the intensity of fluorescence was scanned after 100 ms time. Fluorescence micrographs were acquired with “stacks-reslice” command in the ImageJ software suite, and reversals of motors were identified in kymographs.

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Supporting Information

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Fig. S1. AglQ-pamCherry shows the same helical motion as AglR. (A) The 2D trajectory of a single AglQ-pamCherry was recorded at 100-ms intervals. The cell boundaries were marked with blue lines. The overlay shows the AglR position in consecutive frames (also see Movie S1). (B) The 2D projection of a 3D helical trajectory. $\theta$ is defined as the angle between the long cell axis and the trajectories in each 100-ms interval. (C) The angle distribution histogram is similar to that of AglR (Inset), with a peak at 45–50°, consistent with the helicity of the trajectories. (D) The distribution of the projected velocities of AglQ-pamCherry ($V_{2\theta}$) plotted as a function of $\theta$. $\theta$ is small at the cell boundaries and large near the cell center. $V_{2\theta}$ is positively correlated with $\theta$. Green and yellow lines denote the distribution patterns of 90% molecules of AglQ and AglR, respectively.

Fig. S2. Gallery of MglA-GFP localization. Analysis of 20 cells showed MglA-GFP forming gradients along cell bodies, which positively correlate with the spatial distribution of AglR reversals (also see Fig. 3).

Fig. S3. MglA and MglB protein expression determined by immunoblotting.
Fig. S4. The gliding motility phenotypes of the strains with altered MglA or MglB activity. (A) The colony edges. (B) Colony diameters at 0 h and after 48 h incubation in 37 °C on CYE plates containing 1.5% (wt/vol) agar. To exclude social motility, the pilA gene, which encodes pilin subunits, was disrupted in each strain. P values were calculated using the Student paired t test against the data of the pilA colonies, with a two-tailed distribution.

Movie S1. Movements of AglQ-pamCherry single molecules in a WT cell. Images were taken at 100-ms intervals and are played at a speed of 10 frames/s (real time).
Movie S2. Movements of AglR-pamCherry single molecules in a WT cell. Images were taken at 100-ms intervals and are played at a speed of 30 frames/s (3x real time).

Movie S3. Movements of MglA-mEos2 single molecules and molecule clusters in a WT cell. Images were taken at 100-ms intervals and are played at a speed of 30 frames/s (3x real time).

Movie S4. Movements of AglR-pamCherry single molecules in a ΔmglA cell. Images were taken at 100-ms intervals and are played at a speed of 30 frames/s (3x real time).
Movie S5. Movements of AgIR-pamCherry single molecules in a cell overexpressing MglA using the vanilate-inducible promoter in the presence of 100 μM sodium vanilate. Images were taken at 100-ms intervals and are played at a speed of 30 frames/s (3× real time).

Movie S6. Movements of AgIR-pamCherry single molecules in a cell expressing MglA<sub>Q82L</sub>, the constitutively active mutant of MglA. Images were taken at 100-ms intervals and are played at a speed of 30 frames/s (3× real time).
**Movie S7.** Movements of AglR-pamCherry single molecules in a ΔmglB cell. Images were taken at 100-ms intervals and are played at a speed of 30 frames/s (3× real time).

**Movie S8.** Movements of AglR-pamCherry single molecules in a cell overexpressing MglB using the vanilate-inducible promoter in the presence of 100 μM sodium vanilate. Images were taken at 100-ms intervals and are played at a speed of 30 frames/s (3× real time).