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A lytic transglycosylase connects bacterial focal adhesion complexes to the peptidoglycan cell wall

Carlos A Ramírez Carbó, Olalekan G Faromiki, Beiyan Nan

Department of Biology, Texas A&M University, College Station, TX 77843, USA • The Genetics and Genomics Interdisciplinary Program, Texas A&M University, College Station, TX 77843, USA

Abstract

The Gram-negative bacterium *Myxococcus xanthus* glides on solid surfaces. Dynamic bacterial focal adhesion complexes (bFACs) convert proton motive force from the inner membrane into mechanical propulsion on the cell surface. It is unclear how the mechanical force transmits across the rigid peptidoglycan (PG) cell wall. Here we show that AgmT, a highly abundant lytic PG transglycosylase homologous to *Escherichia coli* MltG, couples bFACs to PG. Coprecipitation assay and single-particle microscopy reveal that the gliding motors fail to connect to PG and thus are unable to assemble into bFACs in the absence of an active AgmT. Heterologous expression of *E. coli* MltG restores the connection between PG and bFACs and thus rescues gliding motility in the *M. xanthus* cells that lack AgmT. Our results indicate that bFACs anchor to AgmT-modified PG to transmit mechanical force across the PG cell wall.

eLife assessment

The manuscript by Carbo et al. reports a novel role for the MltG homolog AgmT in gliding motility in M. xanthus. The authors provide **convincing** data to demonstrate that AgmT is a cell wall lytic enzyme (likely a lytic transglycosylase), its lytic activity is required for gliding motility, and that its activity is required for proper binding of a component of the motility apparatus to the cell wall. The findings are **valuable** as they contribute to our understanding of the molecular mechanisms underlying the interaction between gliding motility and the bacterial cell wall.

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Introduction

In natural ecosystems, the majority of bacteria attach to surfaces[1]. Surface-associated motility is critical for many bacteria to navigate and populate their environments[2]. The Gram-negative bacterium *Myxococcus xanthus* moves on solid surfaces using two independent mechanisms: social (S-) motility and adventurous (A-) motility. S-motility, analogous to the twitching motility in *Pseudomonas* and *Neisseria*, is powered by the extension and retraction of type IV pili[3,4]. A-motility is a form of gliding motility that does not depend on conventional motility-related cell

surface appendages, such as flagella or pili[2,5]. AglR, AglQ, and AglS form a membrane channel that functions as the gliding motor by harvesting proton motive force[6,7]. Motor units associate with at least fourteen gliding-related proteins that reside in the cytoplasm, inner membrane, periplasm, and outer membrane[8,9].

In each gliding cell, two distinct populations of gliding complexes coexist: dynamic complexes move along helical paths and static complexes remain fixed relative to the substrate[10–13]. Motors transport incomplete gliding complexes along helical tracks but form complete, force-generating complexes at the ventral side of cells that appear fixed to the substrate (**Fig. 1**)[10,13]. Based on their functional analogy with eukaryotic focal adhesion sites, these complete gliding machineries are called bacterial focal adhesion complexes (bFACs). Despite their static appearance, bFACs are dynamic under single-molecule microscopy. Individual motors frequently display move-stall-move patterns, in which they move rapidly along helical trajectories, join a bFAC where they remain stationary briefly, then move rapidly again toward the next bFAC or reverse their moving directions[11,14,15]. bFACs adhere to the gliding substrate through an outer membrane adhesin[12]. As motors transport bFACs toward lagging cell poles, cells move forward but bFACs remain static relative to the gliding substrate.

bFAC assembly can be quantified at nanometer resolution using the single-particle dynamics of gliding motors[11,16]. Single particles of a fully functional, photoactivatable mCherry (PAmCherry)-labeled AglR display three dynamic patterns, stationary, directed motion, and diffusion[11,16]. The stationary population consists of the motors in fully assembled bFACs, which do not move before photobleach. In contrast, the motors moving in a directed manner carry incomplete gliding complexes along helical tracks, whereas the diffusing motors assemble to even less completeness[10,11,13,16]. As motors only generate force in static bFACs[10], the population of nonmotile motors indicates the overall status of fully assembled bFACs.

However, how bFACs transmit force across the peptidoglycan (PG) cell wall is unclear. PG is a mesh-like single molecule of crosslinked glycan strands that surrounds the entire cytoplasmic membrane. The rigidity of PG defines cell shape and protects cells from osmotic lysis[17,18]. If bFACs physically penetrate PG, their transportation toward lagging cell poles would tear PG and trigger cell lysis. To avoid breaching PG, an updated model proposes that rather than forming stable and rigid complexes, gliding-related proteins only assemble into force-generating machineries in bFACs.

Outside of bFACs, these proteins could localize diffusively or move along with unengaged motor units[10,19]. To simplify this model, we can artificially divide each gliding machinery into two subcomplexes, one on each side of the PG layer. The inner subcomplexes move freely and only assembly with the outer subcomplexes in bFACs, which transform proton motive force into mechanical propulsion on cell surfaces (**Fig. 1**).

The inner subcomplexes, containing the motors, are the force-generating units in bFACs. As motors reside in the fluid inner membrane, to transmit force to the cell surface, the inner subcomplexes must push against two relatively rigid structures, one on each side of the membrane, in opposite directions[19] (**Fig. 1**). MreB is a bacterial actin homolog that supports rod shape in many bacteria[16,17,20]. In *M. xanthus*, MreB also connects to bFACs on the cytoplasmic side and thus plays essential roles in gliding[2,5,6,12,16,21,22]. The inner subcomplexes could push against MreB filaments and PG in the cytoplasm and periplasm, respectively[10,16,19,23]. The interaction between the inner subcomplexes and PG not only satisfies the physical requirement for force generation but also supports bFAC stability. Without this interaction, the inner and outer subcomplexes can only form transient, “slippery” association, which is predicted to produce short and aberrant cell movements[19]. How the inner complex interacts with PG remains unknown. It was speculated that gliding motors in the inner complex could bind PG directly[10]. However, such binding has not been confirmed by experiments.

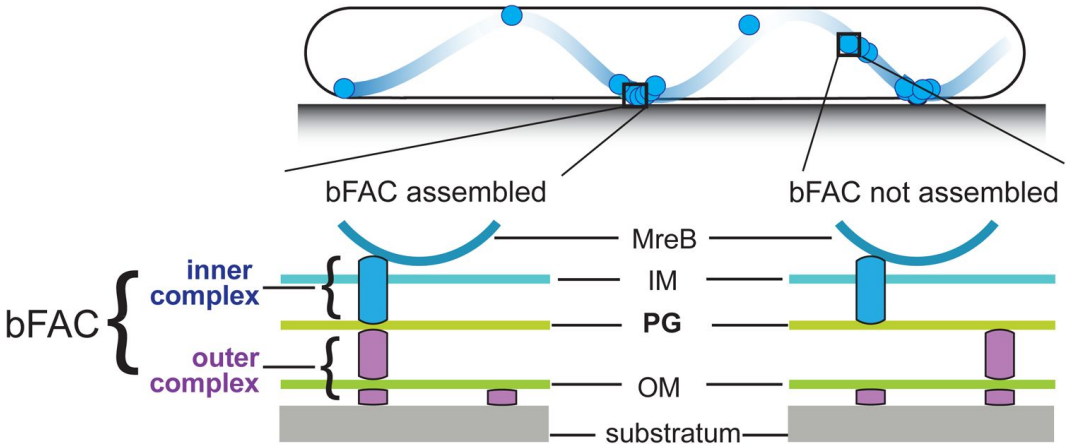

Fig. 1.

Stationary bFACs drive *M. xanthus* gliding.

Motors carrying incomplete gliding complexes either diffuse or move rapidly along helical paths but do not generate propulsion. Motors stall and become nearly static relative to the substrate when they assemble into complete bFACs with other motor-associated proteins at the ventral side of the cell. Stalled motors push MreB and bFACs in opposite directions and thus exert force against outer membrane adhesins. Overall, as motors transport bFACs toward lagging cell poles, cells move forward but bFACs remain static relative to the substrate. IM, inner membrane; OM, outer membrane.

In this study, we found that AgmT, a lytic transglycosylase (LTG) for PG, is required for *M. xanthus* gliding motility. Whereas AgmT only regulates cell morphology moderately during vegetative growth, it is essential for maintaining PG integrity under the stress from the antibiotic mecillinam. Using single-particle tracking microscopy and coprecipitation assays, we found that AgmT is essential for the inner subcomplexes to connect to PG and stall in bFACs. Importantly, expressing *Escherichia coli* MltG heterologously rescues the connection between PG and bFACs and thus restores gliding motility. Hence, the LTG activity of AgmT anchors bFACs to PG, potentially by modifying PG structure. Our findings reveal the long-sought connection between PG and bFACs that allows mechanical force to transmit across the PG cell wall.

Results

AgmT, a putative LTG, is required for gliding motility

To elucidate how bFACs interact with PG, we searched for potential PG-binding domains among the proteins that are required for gliding motility. A previous report identified 35 gliding-related genes in *M. xanthus* through transposon-mediated random mutagenesis[24]. Among these genes, *agmT* (ORF K1515_04910[25]) was predicted to encode an inner membrane protein with a single transmembrane helix (residues 4 – 25) followed by a large "periplasmic solute-binding" domain[24]. No other motility-related genes are found in the vicinity of *agmT*. After careful analysis, we found that AgmT showed significant similarity to the widely conserved YceG/MltG family LTGs. The putative active site, Glu223 (corresponding to E218 in *E. coli* MltG)[26], is conserved in AgmT (**Fig. 2A**).

To confirm the function of AgmT in gliding, we constructed an *agmT* in-frame deletion mutant. We further knocked out the *pilA* gene that encodes pilin for type IV pilus to eliminate S-motility. On a 1.5% agar surface, the *pilA*$^-$ cells moved away from colony edges both as individuals and in "flare-like" cell groups, indicating that they were still motile with gliding motility. In contrast, the ***Δ****aglR pilA*$^-$ cells that lack an essential component in the gliding motor were unable to move outward from the colony edge and thus formed sharp colony edges. Similarly, the ***Δ****agmT pilA*$^-$ cells also formed sharp colony edges, indicating that they could not move efficiently with gliding (**Fig. 2B**).

We then imaged individual ***Δ****agmT pilA*$^-$ cells on 1.5% agar surface at 10-s intervals using bright-field microscopy. To our surprise, instead of being static, individual ***Δ****agmT pilA*$^-$ cells displayed slow movements, with frequent pauses and reversals (**Video 1**). To quantify the effects of AgmT, we measured the velocity and gliding persistency (the distances cells traveled before pauses and reversals) of individual cells. Compared to the *pilA*$^-$ cells that moved at 2.30 ± 1.33 μm/min (n = 46) and high persistency (**Video 2** and **Fig. 2C, D**), ***Δ****agmT pilA*$^-$ cells moved significantly slower (0.88 ± 0.62 μm/min, n = 59) and less persistent (**Video 1** and **Figure. 2C, D**). Such aberrant gliding motility is distinct from the "hyper reversal" phenotype due to the polarity regulators constitutively switching the moving direction of cells, because hyper reversing cells usually maintain gliding velocity at the wild-type level[27]. Instead, such phenotypes match the prediction that when the inner complexes of bFACs lose connection with PG, bFACs can only generate short, and inefficient movements[19]. Our data indicate that AgmT is not essential component in the bFACs. Thus, AgmT is likely to regulate the assembly and stability of bFACs, especially their connection with PG.

AgmT is an LTG

As AgmT is a putative LTG for PG, we then tested if its predicted active site, Glu223, is required for gliding. Because the amino acid following Glu223 is also a glutamate (Glu224), we replaced both glutamate residues with alanine (AgmT^{EAEA}) using site-directed mutagenesis to alter their codons

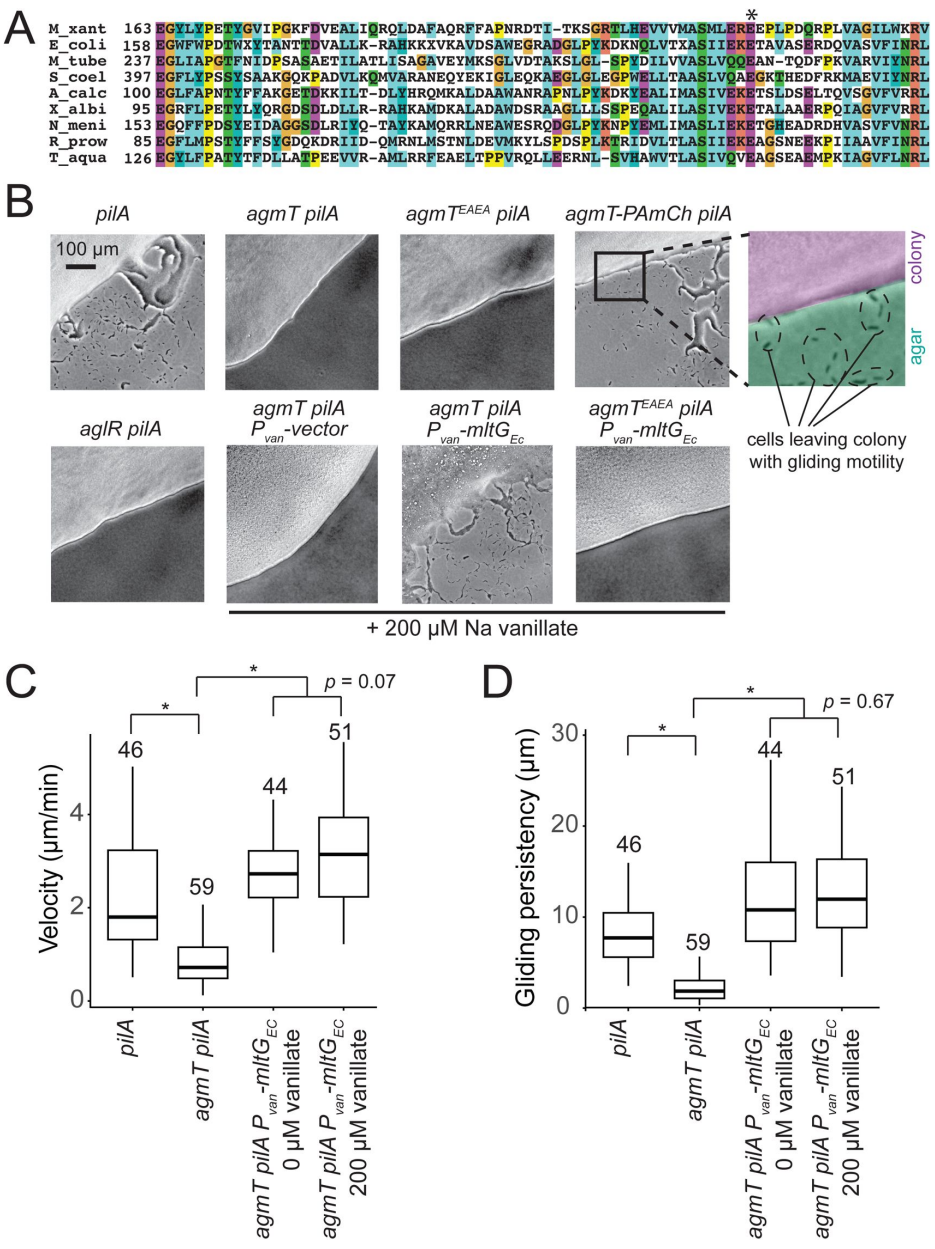

Fig. 2.

AgmT, a putative lytic transglycosylase, is required *for M. xanthus* gliding.

A) AgmT shows significant similarity to a widely conserved PG transglycosylase in YceG/MltG family. The conserved glutamine residue is marked by an asterisk. M_xant, *M. xanthus*; E_coli, *E. coli*; M_tube, *Mycobacterium tuberculosis*; S_coel, *Streptomyces coelicolor*; A_calc, *Acinetobacter calcoaceticus*; X_albi, *Xanthomonas albilineans*; N_meni, *Neisseria menningitidis*; R_prow, *Rickettsia prowazekii*; T_aqua, *Thermus aquaticus*. **B)** AgmT is required for *M. xanthus* gliding. Colony edges were imaged after incubating cells on 1.5% agar surface for 24 h. To eliminate S-motility, we further knocked out the *pilA* gene that encodes pilin for type IV pilus. Cells that move by gliding are able to move away from colony edges. Deleting *agmT* or disabling the active site of AgmT abolish gliding but fusing an PAmCherry (PAmCh) to its C-terminus does not. Heterologous Expression of *E. coli* MltG (MltGEc) restores gliding of *agmT* cells but not the cells that express AgmT^{EAEA}. **C, D)** While cells lacking AgmT moved slower **(C)** and less persistently (**D**, measured by the distances cells traveled before pauses and reversals), the expression of MltGEc restores both the velocity and persistence of gliding in the *agmT* cells. Data were pooled from three independent experiments and *p* values were calculated using a one-way ANOVA test between two unweighted, independent samples. Boxes indicate the 25th - 75th percentiles and bars the median. The total number of cells analyzed is shown on top of each plot. *, p <0.001.

on the chromosome. *agmT*^{EAEA} *pilA*$^{-}$ cells formed sharp colony edges on agar surface (**Fig. 2B**). Thus, the putative LTG activity of AgmT is required for *M. xanthus* gliding motility.

To determine if AgmT is an LTG, we expressed the periplasmic domain (amino acids 25 – 339) of wild-type AgmT and AgmT^{EAEA} in *E. coli*. We purified PG from wild-type *M. xanthus* cells, labeled it with Remazol brilliant blue (RBB), and tested if the purified AgmT variants hydrolyze labeled PG *in vitro* and release the dye[28,29]. Similar to lysozyme that specifically cleaves the β-1,4-glycosidic bonds in PG, wild-type AgmT solubilized dye-labeled *M. xanthus* PG that absorbed light at 595 nm. In contrast, the AgmT^{EAEA} variant failed to release the dye (**Fig. 3A**). Hence, AgmT displays LTG activity *in vitro*.

Whereas AgmT does not affect growth rate (**Fig. S2**), cells that lacked AgmT or expressed AgmT^{EAEA} maintained rod shape but were slightly shorter and wider than the wild-type ones (**Fig. 3B**). Nevertheless, altered morphology alone does not likely account for abolished gliding. In fact, a previously reported mutant that lacks all three class A penicillin-binding proteins (*Δ3*) displays similarly shortened and widened morphology[30] but is still motile by gliding (**Fig. S1**).

A recent report revealed that *Vibrio cholerae* MltG degrades un-crosslinked PG turnover products and prevents their detrimental accumulation in the periplasm[31]. To test if AgmT plays a similar role in *M. xanthus*, we used mecillinam to induce cell envelope stress. Mecillinam is a β-lactam that induces the production of toxic, un-crosslinked PG strands[32]. Rather than collapsing the rod shape, mecillinam only causes bulging near the centers of wild-type *M. xanthus* cells, whereas large-scale cell lysis does not occur, and cell poles still maintain rod shape even after prolonged (20 h) treatment (**Fig. 3C**)[30]. Thus, wild-type *M. xanthus* can largely mitigate mecillinam stress. In contrast, mecillinam-treated *agmT* cells increased their width drastically, displayed significant cell surface irregularity along their entire cell bodies, and lysed frequently (**Fig. 3C**). Cells expressing AgmT^{EAEA} as the sole source of AgmT displayed similar, but even stronger phenotypes under mecillinam stress, growing into elongated, twisted filaments that formed multiple cell poles and lysed frequently (**Fig. 3C**). These results confirm that similar to *V. cholerae* MltG, *M. xanthus* AgmT is important for maintaining cell integrity under mecillinam stress. The different responses from *agmT* and *agmT*^{EAEA} cells suggest that other LTGs could partially substitute AgmT while AgmT^{EAEA} blocks these enzymes from accessing un-crosslinked PG strands.

AgmT is the only LTG *in M. xanthus* that belongs to the YceG/MltG family. Besides *agmT*, the genome of *M. xanthus* contains 13 genes that encode putative LTGs (**Table S1**). To test if these proteins also contribute to gliding, we knocked out each of these 13 genes in the *pilA*$^{-}$ background. The resulting mutants all retained gliding motility, indicating that AgmT is the only LTG that is required for *M. xanthus* gliding (**Fig. S1**).

AgmT is essential for proper bFACs assembly

How does AgmT support gliding? We first tested if AgmT regulates the function of the gliding motor. For this purpose, we quantified the motor dynamics by tracking single particles of AglR, an essential component of the motor. We spotted the cells that express a fully functional, photo-activatable mCherry (PAmCherry)-labeled AglR[11] on 1.5% agar surface. We used a 405-nm excitation laser to activate the fluorescence of a few labeled AglR particles randomly in each cell and quantified their localization using a 561-nm laser at 10 Hz using single particle tracking photo-activated localization microscopy (sptPALM) under highly inclined and laminated optical sheet (HILO) illumination[11,16,33]. Using this setting, only a thin section of each cell surface that was close to the coverslip was illuminated. To analyze the data, we only chose the fluorescent particles that remained in focus for 4 - 12 frames (0.4 - 1.2 s). As free PAmCherry particles diffuse extremely fast in the cytoplasm, entering and exiting the focal plane frequently, they usually appear as blurry objects that cannot be followed at 10-Hz close to the membrane[16]. For this reason, the noise from any potential degradation of AglR- PAmCherry was negligible. Consistent with our previous results[11,14], 32.1% (n = 2,700) of AglR-PAmCherry particles remained within

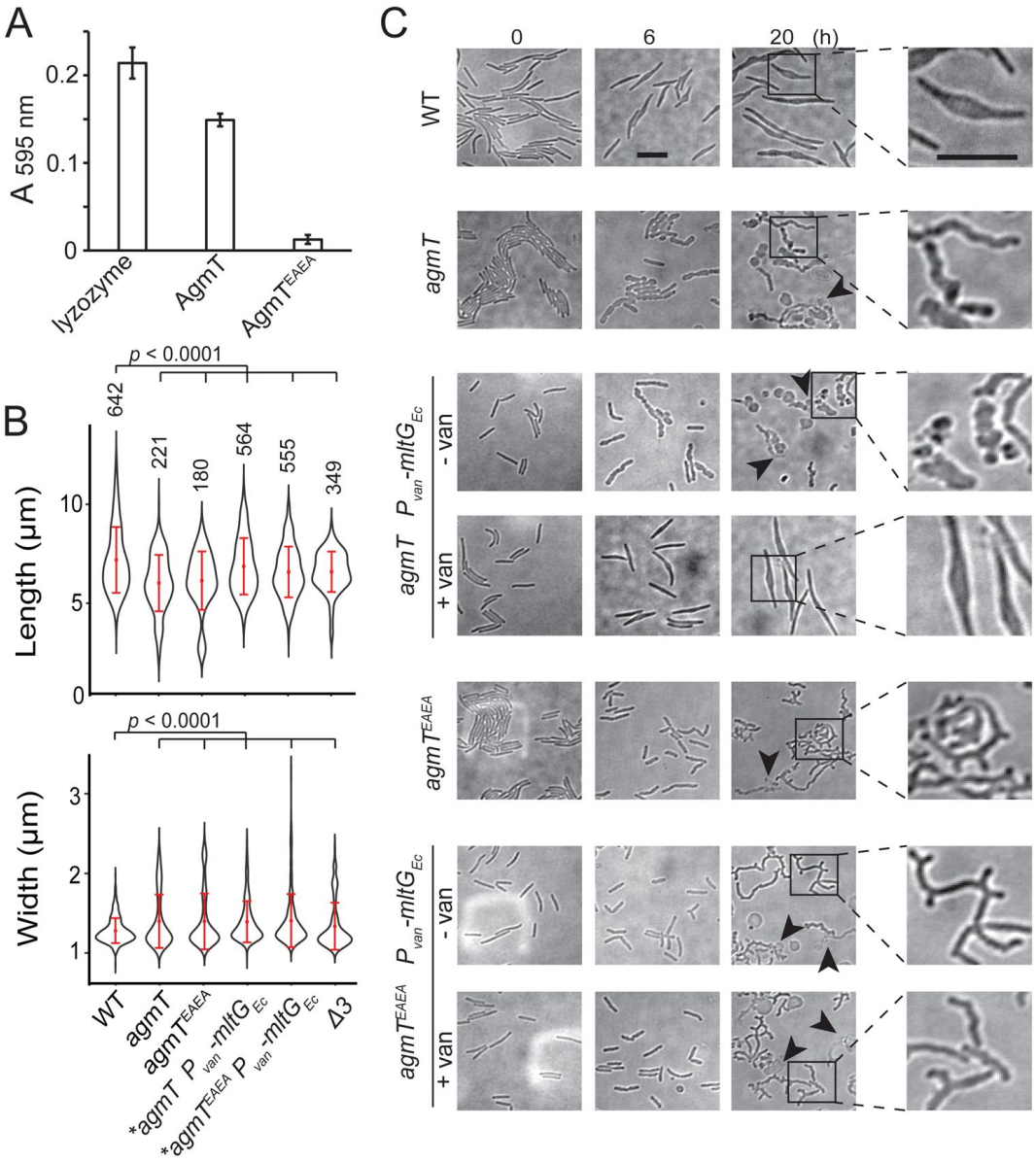

Fig. 3.

AgmT regulates cell morphology and integrity under antibiotic stress.

A) Purified AgmT solubilizes dye-labeled PG sacculi, but AgmT^{EAEA} does not. Lysozyme serves as a positive control. Absorption at 595 nm was measured after 18 h incubation at 25 C. Data are presented as mean values ± SD from three replicates. **B)** AgmT regulates cell morphology. Compared to wild-type cells, cells that lack AgmT and express AgmT^{EAEA} are significantly shorter and wider. A previously reported mutant that lacks all three class A penicillin-binding proteins (*Δ3*) displays similarly shortened and widened morphology but is still motile by gliding (**Fig. S1**). Heterologous expression of *E. coli* MltG partially restores cell length but not cell width in *agmT* and *agmT^{EAEA}* backgrounds. Asterisks, 200 M sodium vanillate added. *p* values were calculated using a one-way ANOVA test between two unweighted, independent samples. Whiskers indicate the 25th - 75th percentiles and red dots the median. The total number of cells analyzed is shown on top of each plot. **C)** AgmT regulates cell morphology and integrity under mecillinam stress (100 µg/ml). Expressing *E. coli* MltG by a vanillate-inducible promoter (*Pvan*) restores resistance against mecillinam in *agmT* cells but not in the cells that express AgmT^{EAEA}. van, 200 M sodium vanillate. Arrows point to newly lysed cells. Scale bars, 5 µm.

one pixel (160 nm × 160 nm) before photobleach, indicating that they were immotile. The remaining 67.9% AglR-PAmCherry particles were motile, leaving trajectories of various lengths (**Fig. 4A**, **4B**).

bFAC assembly is sensitive to mechanical cues. As the agar concentration increases in gliding substrate, more motor molecules engage in bFACs, where they appear immotile[9,11]. We exposed *aglR-PAmCherry* cells to 405-nm excitation (0.2 kW/cm^2) for 2 s, where most PAmCherry molecules were photoactivated and used epifluorescence to display the overall localization of AglR[11,16,30]. As the agar concentration increased, bFACs increased significantly in size (**Fig. 4C**). Accordingly, the immotile AglR particles detected by sptPALM increased from 10.7% (n = 1112) on 0.5% agar to 45.2% (n = 726) on 5.0% agar surface (**Fig. 4B**). Thus, the stationary population of AglR-PAmCherry particles reflects the AglR molecules in fully assembled bFACs.

In contrast to the cells that expressed wild-type AgmT (*agmT*$^+$), AglR particles were hyper motile in the cells that lacked AgmT or expressed AgmT^{EAEA}. On 1.5% agar surfaces, the immotile populations of AglR particles decreased to 10.5% (n = 2,989) and 11.7% (n = 1,408), respectively. Consistently, AglR-containing bFACs were rarely detectable in these cells, even on 5% agar surfaces (**Fig. 4A-C**).

To test if other components in the gliding machinery also depend on AgmT to assemble into bFACs, we tested the localization of AglZ, a cytoplasmic protein that is commonly used for assessing bFAC assembly[10,34]. In the cells that expressed wild-type AgmT, yellow fluorescent protein (YFP)-labeled AglZ formed a bright cluster at the leading cell pole and multiple static, near-evenly spaced clusters along the cell body, indicating the assembly of bFACs (**Fig. 4D**). In contrast, AglZ-YFP only formed single clusters at cell poles in the cells that lacked AgmT or expressed AgmT^{EAEA} (**Fig. 4D**). Taken together, the LTG activity of AgmT is essential for proper bFACs assembly.

AgmT does not assemble into bFACs

We first hypothesized that AgmT could assemble into bFACs, where it could generate pores in PG specifically at bFAC loci that allow the inner and outer gliding complexes interact directly. Alternatively, it could bind to PG and recruit other components to bFACs through protein-protein interactions. Regardless, if AgmT assembles into bFACs, it should localize in bFACs. To test this possibility, we labeled AgmT with PAmCherry on its C-terminus and expressed the fusion protein as the sole source of AgmT using the native promoter and locus of *agmT*. *agmT-PAmCherry pilA*$^-$ cells displayed gliding motility that was indistinguishable from *pilA*$^-$ cells (**Fig. 2B**), indicating that the fusion protein is functional. Similar to many membrane proteins that resistant to dissociation by SDS[35], immunoblot using an anti-mCherry antibody showed that AgmT-PAmCherry accumulated in two bands in SDS-PAGE that corresponded to monomers and dimers of the full-length fusion protein, respectively (**Fig. 5A**). This result is consistent with the structure of *E. coli* MltG that functions as homodimers (PDB: 2r1f). Importantly, AgmT-PAmCherry was about 50 times more abundant than AglR-PAmCherry (**Fig. 5A**). To test if AgmT assembles into bFACs, we first expressed AgmT-PAmCherry and AglZ-YFP together using their respective loci and promoter. We exposed *agmT-PAmCherry* cells to 405-nm excitation (0.2 kW/cm^2) for 2 s to visualize the overall localization of AgmT. We found that on a 1.5% agar surface that favors gliding motility, AglZ formed bright clusters at cell poles and aggregated in near evenly-spaced bFACs along the cell body. In contrast, AgmT localized near evenly along cell bodies without forming protein clusters (**Fig. 5B**). Thus, AgmT does not localize into bFACs. These results echo the fact that despite its abundance, AgmT has not been identified as a component in bFACs despite extensive pull-down experiments using various bFAC components as baits[6,9,36].

As an additional test, we used sptPALM to track the movements of AgmT-PAmCherry single particles on 1.5 agar surfaces at 10 Hz. We reasoned that if AgmT assembles into bFACs, AgmT and gliding motors should display similar dynamic patterns. Distinct from the AglR particles of which 32.1% remained stationary, AgmT moved in a diffusive manner, showing no significant immotile

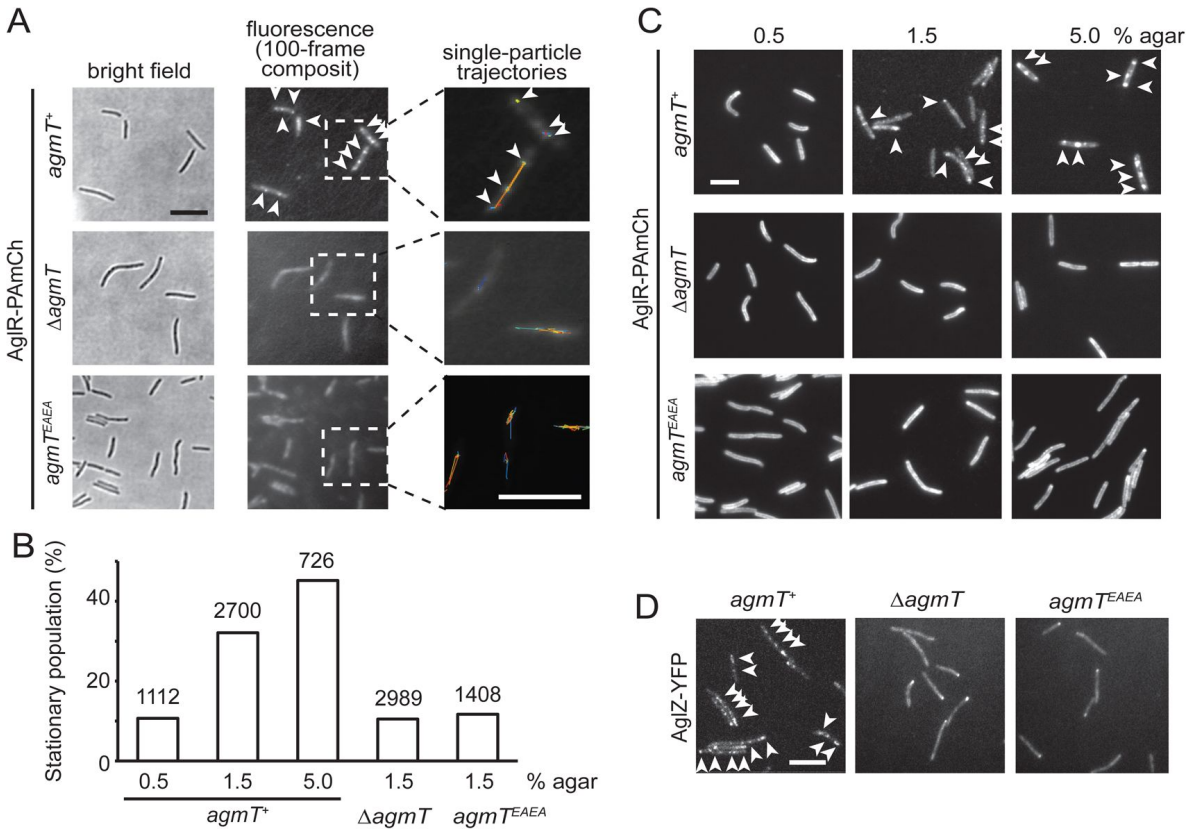

Fig. 4.

AgmT and its LTG activity are essential for proper bFAC assembly.

A) Overall distribution of AglR-PAmCherry particles on 1.5% agar surface is displayed using the composite of 100 consecutive frames taken at 100-ms intervals. Single-particle trajectories of AglR-PAmCherry (AglR-PAmCh) were generated from the same frames. Individual trajectories are distinguished by colors. Deleting *agmT* or disabling the transglycosylase activity of AgmT (AgmT^{EAEA}) decrease the stationary population of AglR particles. **B)** The stationary population of AglR-PAmCherry particles, which reflects the AglR molecules in bFACs, changes in response to substrate hardness (controlled by agar concentration) and the presence and function of AgmT. The total number of particles analyzed is shown on top of each plot. **C)** AglR fails to assemble into bFACs in the absence of active AgmT, even on 5.0% agar surfaces. **D)** AgmT and its LTG activity also support the assembly of AglZ into bFACs. White arrows point to bFACs. Scale bars, 5 μm.

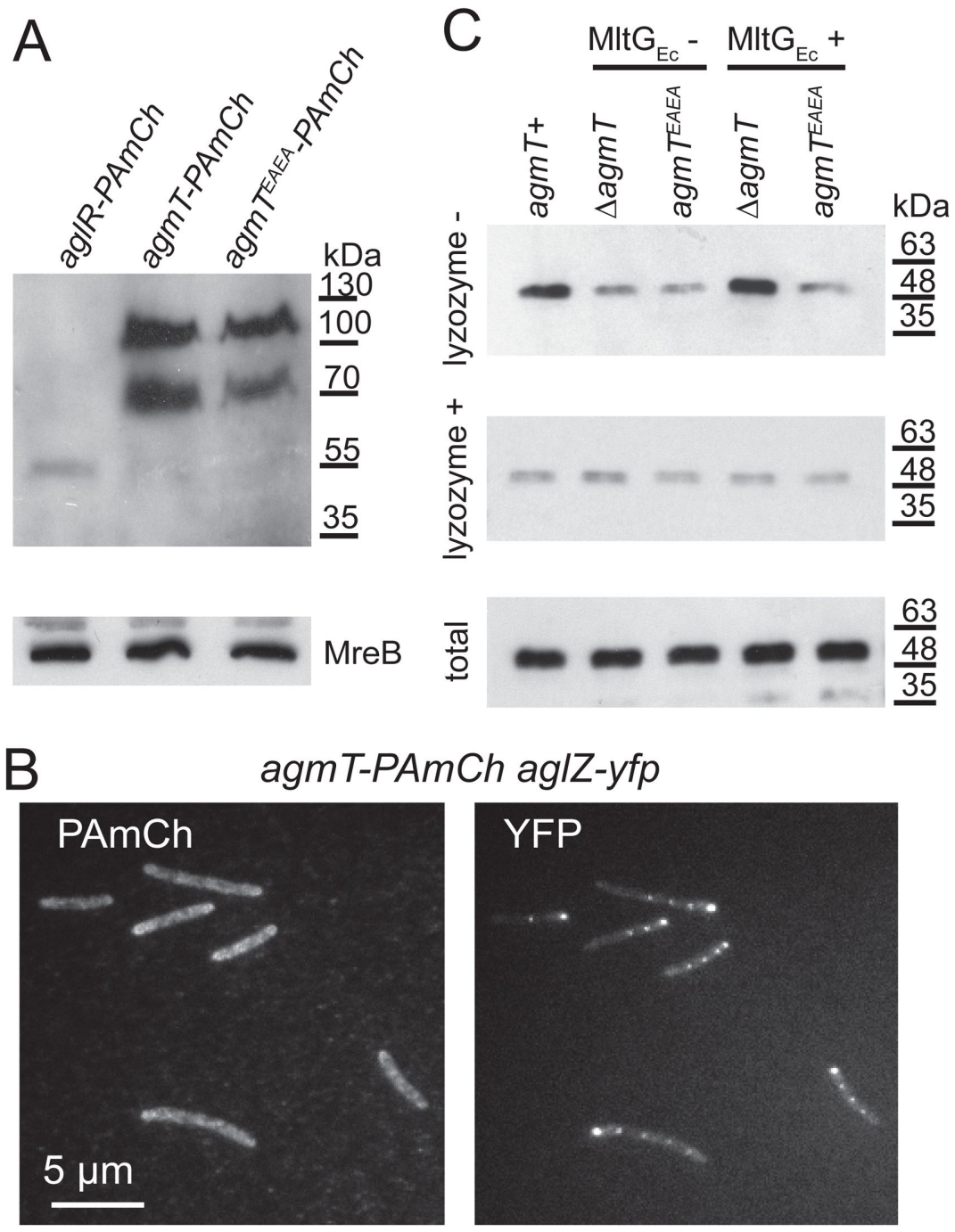

Fig. 5.

AgmT does not assemble into bFACs but connects bFACs to PG.

A) Immunoblotting using *M. xanthus* cell lysates and an anti-mCherry antibody shows that PAmCherry (PAmCh)-labeled AgmT and AgmT^{EAEA} (592 amino acids) accumulate as full-length proteins. AgmT is significantly more abundant than the PAmCherry-labeled motor protein AglR (498 amino acids). The bacterial actin homolog MreB visualized using an MreB antibody is shown as a loading control. **B)** AgmT does not aggregate into bFACs. **C)** The LTG activity of AgmT is required for connecting bFACs to PG and expressing the *E. coli* LTG MltG (MltGEc) restores PG binding by bFACs in cells that lack AgmT but not in the ones that express an inactive AgmT variant (AgmT^{EAEA}). AglR-PAmCherry was detected using an anti-mCherry antibody to mark the presence of bFACs that co-precipitate with PG-containing (lysozyme-) pellets. Lysates from the cells that express AglR-PAmCherry in different genetic backgrounds were pelleted by centrifugation in the presence and absence of lysozyme. The loading control of AglR-PAmCherry in the whole cell is shown as “total”.

population. Importantly, compared to the diffusion coefficients (D) of motile AglR particles (1.8×10^{-2} ± 3.6×10^{-3} μm^2/s (n = 1,833)), AgmT particles diffused much faster ($D = 2.9 \times 10^{-2} \pm 5.3 \times 10^{-3}$ μm^2/s (n = 8,548)). Taken together, AgmT and the gliding motor did not display significant correlation in either their localization or dynamics. We thus conclude that AgmT does not assemble into bFACs.

AgmT connects bFACs to PG through its LTG activity

We then explored the possibility that AgmT could modify PG through its LTG activity and thus generate the anchor sites for certain components in bFACs. If this hypothesis is true, heterologous expression of a non-native LTG could rescue gliding motility in the *ΔagmT* strain. To test this hypothesis, we fused the *E. coli mltG* gene ($mltG_{Ec}$) to a vanillate-inducible promoter and inserted the resulting construct to the *cuoA* locus on *M. xanthus* chromosome that does not interfere gliding[37]. We subjected the *ΔagmT* and $agmT^{EAEA}$ cells that expressed $MltG_{Ec}$ to mecillinam (100 μg/ml) stress. Induced by 200 μM sodium vanillate, $MltG_{Ec}$ restored cell morphology and integrity of the *ΔagmT* strain to the wild-type level (**Fig. 3C**). This result confirmed that $MltG_{Ec}$ was expressed and enzymatically active in *M. xanthus*. In contrast, $MltG_{Ec}$ failed to confer mecillinam resistance to the $agmT^{EAEA}$ cells (**Fig. 3C**). A potential explanation is that $AgmT^{EAEA}$ could still bind to PG and thus block $MltG_{Ec}$ from accessing *M. xanthus* PG.

Consistent with its LTG activity, the expression of $MltG_{Ec}$ restored gliding motility of the *ΔagmT* $pilA^-$ cells on both the colony (**Fig. 2B**) and single-cell (**Fig. 2C, D**) levels. Interestingly, in the absence of sodium vanillate, the leakage expression of $MltG_{Ec}$ using the vanillate-inducible promoter was sufficient to compensate the loss of AgmT. A plausible explanation of this observation is that as *E. coli* grows much faster (generation time 20 - 30 min) than *M. xanthus* (generation time ~4 h), $MltG_{Ec}$ could possess significantly higher LTG activity than AgmT. Induced by 200 μM sodium vanillate, the expression of $MltG_{Ec}$ further but non significantly increased the velocity and gliding persistency (**Fig. 2B-D**). Importantly, the expression of $MltG_{Ec}$ failed to restore gliding motility in the $agmT^{EAEA}$ *pilA* cells, even in the presence of 200 μM sodium vanillate (**Fig. 2B**). Consistent with the mecillinam resistance assay (**Fig. 3C**), this result suggests that $AgmT^{EAEA}$ still binds to PG and that in the absence of its LTG activity, AgmT does not anchor bFACs to PG. As $MltG_{Ec}$ substitutes AgmT in gliding motility, it is the LTG activity, rather than specific interactions between AgmT and bFACs, that is required for gliding.

It is challenging to visualize how AgmT facilitates bFAC assembly through its enzymatic activity *in vitro*. First, the inner complex in a bFAC spans the cytoplasm, inner membrane, and periplasm and contains multiple proteins whose activities are interdependent[8–10]. It is hence difficult to purify the entire inner complex in its functional state. Second, Faure *et al*. hypothesized that AglQ and AglS in the gliding motor could bind PG directly[10]. However, such binding has not been proved experimentally due to the difficulty in purifying the membrane integral AglRQS complex. To overcome these difficulties, we used AglR-PAmCherry to represent the inner complex of bFAC and investigated how bFACs bind PG in their native environment. To do so, we expressed AglR-PAmCherry in different genetic backgrounds as the sole source of AglR using the native *aglR* locus and promoter. We lysed these cells using sonication, subjected their lysates to centrifugation, isolated the pellets that contained PG, and detected the presence of AglR-PAmCherry in these pellets by immunoblots using an mCherry antibody. Eliminating AgmT and disabling its active site significantly reduced the amounts of AglR in the pellets (**Fig. 5C**). Because such pellets contain both the PG and membrane fractions, we further eliminated PG in cell lysates using lysozyme before centrifugation and determined the amount of AglR-PAmCherry in the pellets that only contained membrane fractions. Regardless of the presence and activity of AgmT, comparable amounts of AglR precipitated in centrifugation pellets after lysozyme treatment, indicating that AgmT does not affect the expression or stability of AglR (**Fig. 5C**). Thus, active AgmT facilitates the association between bFACs and PG. Strikingly, the heterologous expression of $MltG_{Ec}$ enriched AglR-PAmCherry in the PG-containing pellets from the *ΔagmT* cells but not the $agmT^{EAEA}$ ones (**Fig. 5C**). These results indicate that AgmT connects bFACs to PG through its LTG activity.

The assembly of bFACs produces wave-like deformation on cell surface[6,38], suggesting that their assembly may require a flexible PG layer[2,6,11,12]. As a major contributor to cell stiffness, PG flexibility affects the overall stiffness of cells[39]. To test the possibility that AgmT and $MltG_{Ec}$ facilitate bFAC assembly by reducing PG stiffness, we adopted the GRABS assay[39] to quantify if the lack of AgmT and the expression of $MltG_{Ec}$ affects cell stiffness. To quantify changes in cell stiffness, we simultaneously measured the growth of the *pilA*$^-$, *ΔagmT pilA*$^-$, and *ΔagmT* P_{van}-$MltG_{Ec}$ *pilA*$^-$ (with 200 μM sodium vanillate) cells in a 1% agarose gel infused with CYE and liquid CYE and calculated the GRABS scores of the *ΔagmT pilA*$^-$, and *ΔagmT* P_{van}-$MltG_{Ec}$ *pilA*$^-$ cells using the *pilA*$^-$ cells as the reference, where positive and negative GRABS scores indicate increased and decreased stiffness, respectively (see Materials and Methods and Ref[39]). The GRABS scores of the *ΔagmT pilA*$^-$, and *ΔagmT* P_{van}-$MltG_{Ec}$ *pilA*$^-$ (with 200 μM sodium vanillate) cells were -0.06 ± 0.04 and -0.10 ± 0.07 (n = 4), respectively, indicating that neither AgmT nor $MltG_{Ec}$ affects cell stiffness significantly. Whereas PG flexibility could still be essential for gliding, AgmT and $MltG_{Ec}$ do not regulate bFAC assembly by modulating PG stiffness. Instead, these LTGs could connect bFACs to PG by generating structural features that are irrelevant to PG stiffness.

Discussion

Gliding bacteria adopt a broad spectrum of nanomachineries. Compared to the trans-envelope secretion system that drives gliding in *Flavobacterium johnsoniae* and *Capnocytophaga gingivalis*, the gliding machinery of *M. xanthus* appears to lack a stable structure that transverses the cell envelope, especially across the PG layer[2,40,41]. In order to generate mechanical force from the fluid *M. xanthus* gliding machinery, the inner complex must establish solid contact with PG and push the outer complex to slide[10,19]. In this work, we discovered AgmT as the long-sought factor that facilitates persistent gliding by connecting bFACs to PG.

It is surprising that AgmT itself does not assemble into bFACs and that $MltG_{Ec}$ substitutes AgmT in gliding. Thus, rather than interacting with bFAC components directly and specifically, AgmT facilitates proper bFAC assembly indirectly through its LTG activity. LTGs usually break glycan strands and produce unique anhydro caps on their ends[42–46]. However, because AgmT is the only LTGs that is required for gliding, it is not likely to facilitate bFAC assembly by generating such modification on glycan strands. *E. coli* MltG is a glycan terminase that controls the length of newly synthesized PG glycans[26]. Likewise, AgmT could generate short glycan strands and thus uniquely modify the overall structure of *M. xanthus* PG, such as producing small pores that retard and retain the inner subcomplexes of bFACs (**Fig. 6**). On the contrary, the *M. xanthus* mutants that lack active AgmT could produce PG with increased strain length, which blocks bFACs from binding to the cell wall and precludes stable bFAC assembly. However, it would be very difficult to demonstrate how glycan length affects the connection between bFACs and PG.

Then how does AgmT, a protein localized diffusively, facilitate bFAC assembly into near-evenly spaced foci? The actin homolog MreB is the only protein in bFACs that displays quasiperiodic localization independent of other components[16,21]. *M. xanthus* MreB assembles into bFACs and positions the latter near evenly along the cell body[6,11,16,21–23]. MreB filaments change their orientation in accordance with local membrane curvatures and could hence respond to mechanical cues[47–49]. Strikingly, *M. xanthus* does assemble bFACs in response to substrate hardness (**Fig. 4C**) and assembled bFACs could distort the cell envelope, generating undulations on the cell surface[6,9,11,38,50]. Taken together, whereas AgmT potentially modifies the entire PG layer, it is still the quasiperiodic MreB filaments that determine the loci for bFAC assembly in response to the mechanical cues from the gliding substrate (**Fig. 6**).

Most bacteria encode multiple LTGs that function in PG growth, remodeling, and recycling[43,46]. This work provides an example that macro bacterial machineries domesticate non-specialized LTGs for specialized functions. Other examples include MltD in the *Helicobacter*

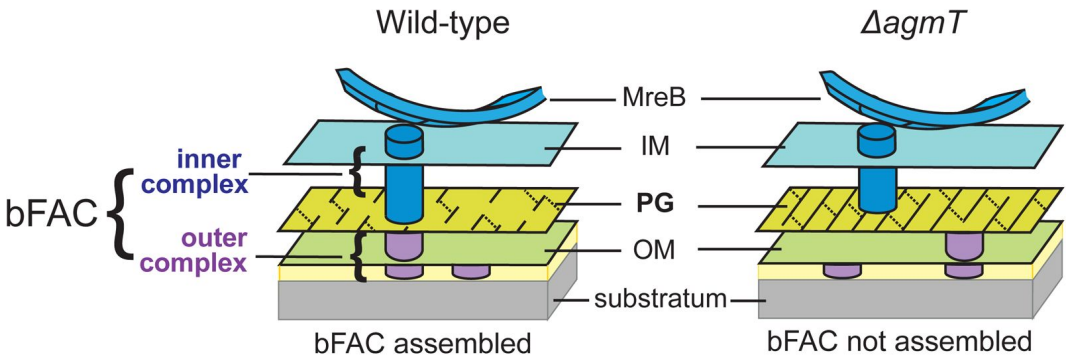

Fig. 6.

A possible mechanism by which AgmT connect bFACs to PG.

AgmT could generate short glycan strands through its LTG activity and thus uniquely modify the overall structure of *M. xanthus* PG, such as producing small pores that retard and retain the inner subcomplexes of bFACs. Likewise, the *M. xanthus* mutants that lack active AgmT could produce PG with increased strain length, which precludes bFACs from binding to the cell wall.

pylori flagellum and MltE in the *E. coli* type VI secretion system[51,52]. Based on their catalytic folds and domain arrangements, LTGs can be categorized inti six distinct families[43]. Both *M. xanthus* AgmT and *E. coli* MltG belong to the YceG/MltG family, which is the first identified LTG family that is conserved in both Gram-negative and positive bacteria[26,43]. About 70% of bacterial genomes, including firmicutes, proteobacteria, and actinobacteria, encode YceG/MltG domains[26]. The unique inner membrane localization of this family and the fact that AgmT is the only *M. xanthus* LTG that belongs to this family (**Table S2**) could partially explain why it is the only LTG that contributes to gliding motility. It will be interesting to investigate if other LTGs, once anchored to the inner membrane, could also facilitate force-generation by bFACs.

Methods

Strains and growth conditions

M. xanthus strains used in this study are listed in **Table S2**. Vegetative *M. xanthus* cells were grown in liquid CYE medium (10 mM MOPS pH 7.6, 1% (w/v) Bacto™ casitone (BD Biosciences), 0.5% yeast extract and 8 mM $MgSO_4$) at 32 °C, in 125-ml flasks with vigorous shaking, or on CYE plates that contains 1.5% agar. All genetic modifications on *M. xanthus* were made on the chromosome. Deletion and insertion mutants were constructed by electroporating *M. xanthus* cells with 4 μg of plasmid DNA. Transformed cells were plated on CYE plates supplemented with 100 μg/ml sodium kanamycin sulfate and 10 μg/ml tetracycline hydrochloride when needed. AgmT and $AgmT^{EAEA}$ were labeled with PAmCherry at their C-termini by fusing their gene to a DNA sequence that encodes PAmCherry through a KESGSVSSEQLAQFRSLD (AAGGAGTCCGGCTCCGTGTCCTCCGAGCAGCTGGCCCAGTTCCGCTCCCTGGA C) linker. All constructs were confirmed by PCR and DNA sequencing.

Immunoblotting

The expression and stability of PAmCherry-labeled proteins were determined by immunoblotting following SDS-PAGE using an anti-mCherry antibody (Rockland Immunochemicals, Inc., Lot 46705) and a goat anti-Rabbit IgG (H+L) secondary antibody, HRP (Thermo Fisher Scientific, catalog # 31460). MreB was detected as the loading control using an anti-MreB serum[21] and the same secondary antibody. The blots were developed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific REF 32109) and a MINI-MED 90 processor (AFP Manufacturing).

Gliding assay

Five microliters of cells from overnight culture were spotted on CYE plates containing 1.5% agar at 4×10^9 colony formation units (cfu)/ml and incubated at 32 °C for 48 h. Colony edges were photographed using a Nikon Eclipse™ e600 phase-contrast microscope with a 10× 0.30 NA objective and an OMAX™ A3590U camera.

Protein expression and purification

DNA sequences encoding amino acids 25 – 339 of AgmT and $AgmT^{EAEA}$ were amplified by polymerase chain reaction (PCR) and inserted into the pET28a vector (Novogen) between the restriction sites of *Eco*RI and *Hind*III and used to transform *E. coli* strain BL21(DE3). Transformed cells were cultured in 20 ml LB (Luria-Bertani) broth at 37 °C overnight and used to inoculate 1 L LB medium supplemented with 1.0% glucose. Protein expression was induced by 0.1 mM IPTG (isopropyl-h-d-thiogalactopyranoside) when the culture reached an OD_{600} of 0.8. Cultivation was continued at 16 °C for 10 h before the cells were harvested by centrifugation at 6,000 × g for 20 min. Proteins were purified using a NGC™ Chromatography System (BIO-RAD) and 5-ml HisTrap™ columns (Cytiva)[15,53]. Purified proteins were concentrated using Amicon™ Ultra centrifugal filter units (Millipore Sigma) with a 10-kDa molecular weight cutoff and stored at -80 °C.

LTG activity (RBB) assay

PG was purified following the published protocol[30,54]. In brief, *M. xanthus* cells were grown until mid-stationary phase and harvested by centrifugation (6,000 × g, 20 min, 25 °C). Supernatant was discarded and the pellet was resuspended and boiled in 1× PBS with 5% SDS for 2 h. SDS was removed by repetitive wash with water and centrifugation (21,000 × g, 10 min, 25 °C). Purified PG from 100 ml culture was suspended into 1 ml 1× PBS and stored at -20 °C. RBB labelling of PG was performed essentially as previously described[28,29]. Purified sacculi were incubated with 20 mM RBB in 0.25 M NaOH overnight at 37 °C. Reactions were neutralized by adding equal volumes of 0.25 M HCl and RBB-labeled PG was collected by centrifugation at 21,000 × g for 15 min. Pellets were washed repeatedly with water until the supernatants became colorless. RBB-labelled sacculi were incubated with purified AgmT and $AgmT^{EAEA}$ (1 mg/ml) at 25 °C for 12 h. lysozyme (1 mg/ml) was used as a positive control. Dye release was quantified by the absorption at 595 nm from the supernatants after centrifugation (21,000 × g, 10 min, 25 °C).

Cell stiffness (GRABS) assay

Cell stiffness was quantified using the GRABS assay[55]. Briefly, overnight cultures of *pilA*$^-$, *ΔagmT pilA*$^-$, and *ΔagmT* P_{van}-$MltG_{Ec}$ *pilA*$^-$ (with 200 μM sodium vanillate) cells were inoculated to liquid CYE medium or embedded in solid CYE medium with 1% agarose to OD_{600} 0.5 in 2-ml optical spectrometer cuvettes and incubated at 32 °C. Growth data were collected over 24 hr, and the GRABS scores were calculated as ($OD_{mutant,\ agarose}/OD_{pilA,\ agarose}$) - ($OD_{mutant,\ liquid}/OD_{pilA,\ liquid}$).

Co-precipitation assay

M. xanthus cells expressing AglR-PAmCherry were grown in liquid CYE to OD_{600} ~1, harvested by centrifugation (6,000 × g, 20 min, 25 °C), washed by 1× PBS, and resuspended into 1× PBS to OD_{600} 6. Cells (1 ml) were lysed using a Cole-Parmer 4710 Ultrasonic Homogenizer. Unbroken cells and large debris were eliminated by centrifugation (6,000 × g, 10 min, 25 °C). Supernatants were subjected to centrifugation at 21,000 × g for 15 min. Pellets that contain both the PG and membrane fractions were resuspended to 1 ml in 1× PBS. To collect the pellets that do not contain PG, supernatants from sonication lysates were incubated with 5 mg/ml lysozyme at 25 °C for 5 h before centrifugation. Five microliters of each resuspended pellet were mixed with 5 μl 2× loading buffer and applied to SDS-PAGE. AglR-PAmCherry was detected using immunoblotting.

Microscopy Analysis

For all imaging experiments, we spotted 5 μl of cells grown in liquid CYE medium to OD_{600} ~1 on agar (1.5%) pads. For the treatments with inhibitors, inhibitors were added into both the cell suspension and agar pads. The length and width of cells were determined from differential interference contrast (DIC) images using a MATLAB (MathWorks) script[16,30,56]. DIC and fluorescence images of cells were captured using an Andor iXon Ultra 897 EMCCD camera (effective pixel size 160 nm) on an inverted Nikon Eclipse-Ti™ microscope with a 100× 1.49 NA TIRF objective. For sptPALM, *M. xanthus* cells were grown in CYE to 4 ×10^8 cfu/ml and PAmCherry was activated using a 405-nm laser (0.3 kW/cm^2), excited and imaged using a 561-nm laser (0.2 kW/cm^2). Images were acquired at 10 Hz. For each sptPALM experiment, single PAmCherry particles were localized in at least 100 individual cells from three biological replicates. sptPALM data were analyzed using a MATLAB (MathWorks) script[16,56]. Briefly, cells were identified using differential interference contrast images. Single PAmCherry particles inside cells were fit by a symmetric 2D Gaussian function, whose center was assumed to be the particle's position[16]. Particles that explored areas smaller than 160 nm × 160 nm (within one pixel) in 0.4 - 1.2 s were considered immotile[16,30,56]. Sample trajectories were generated using the TrackMate[57] plugin in the ImageJ suite (*https://imagej.net*).

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Author Contributions

C. R. C., O. G. F., and B. N. designed the study, performed experiments and data analysis, and wrote the manuscript. All authors read and approved the manuscript.

Competing Interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting files.

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Editors

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Michael Laub
Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, United States of America

Senior Editor
Bavesh Kana
University of the Witwatersrand, Johannesburg, South Africa

Reviewer #1 (Public review):

Summary:

This manuscript nicely outlines a conceptual problem with the bFAC model in A-motility, namely, how the energy derived from the inner membrane AglRQS motor transduced through the cell wall into mechanical force on the cell surface to drive motility? To address this, the authors make a significant contribution by identifying and characterizing a lytic transglycosylase (LTG) called AgmT. This work thus provides clues and a future framework work to address mechanical force transmission from the cytoplasm through the cell envelope to the cell surface.

Strengths:

(i) Convincing evidence shows AgmT functions as a LTG and, surprisingly, that mltG from E. coli complements the swarming defect of an agmT mutant.

(ii) Show 13 other LTGs found in M. xanthus are not required for A-motility.

(iii) Authors show agmT mutants develop morphological changes in response to treatment with a beta-lactam antibiotic, mecillinam.

(iv) The use of single molecule tracking to monitor the assembly and dynamics of bFACs in WT and mutant backgrounds.

(v) The authors understand the limitations of their work and do not overinterpret their data.

Weaknesses:

The authors provided more experiments and clearly addressed my prior concerns in their revised manuscript.

https://doi.org/10.7554/eLife.99273.2.sa2

Reviewer #2 (Public review):

The manuscript by Carbo et al. reports a novel role for the MltG homolog AgmT in gliding motility in M. xanthus. The authors conclusively show that AgmT is a cell wall lytic enzyme (likely a lytic transglycosylase), its lytic activity is required for gliding motility, and that its activity is required for proper binding of a component of the motility apparatus to the cell

wall. The data are generally well-controlled. The marked strength of the manuscript includes the detailed characterization of AgmT as a cell wall lytic enzyme, and the careful dissection of its role in motility. Using multiple lines of evidence, the authors conclusively show that AgmT does not directly associate with the motility complexes, but that instead its absence (or the overexpression of its active site mutant) results in failure of focal adhesion complexes to properly interact with the cell wall.

https://doi.org/10.7554/eLife.99273.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *This manuscript nicely outlines a conceptual problem with the bFAC model in A-motility, namely, how is the energy produced by the inner membrane AglRQS motor transduced through the cell wall into mechanical force on the cell surface to drive motility? To address this, the authors make a significant contribution by identifying and characterizing a lytic transglycosylase (LTG) called AgmT. This work thus provides clues and a future framework work for addressing mechanical force transmission between the cytoplasm and the cell surface.*
>
> *Strengths:*
>
> *(1) Convincing evidence shows AgmT functions as an LTG and, surprisingly, that mltG from E. coli complements the swarming defect of an agmT mutant.*
>
> *(2) Authors show agmT mutants develop morphological changes in response to treatment with a b-lactam antibiotic, mecillinam.*
>
> *(3) The use of single-molecule tracking to monitor the assembly and dynamics of bFACs in WT and mutant backgrounds.*
>
> *(4) The authors understand the limitations of their work and do not overinterpret their data.*
>
> *Weaknesses:*
>
> *(1) A clear model of AgmT's role in gliding motility or interactions with other A-motility proteins is not provided. Instead, speculative roles for how AgmT enzymatic activity could facilitate bFAC function in A-motility are discussed.*

We appreciate the reviewer for this comment. We have added a new figure, Fig. 6, and updated the Discussion to propose a mechanism, "rather than interacting with bFAC components directly and specifically, AgmT facilitates proper bFAC assembly indirectly through its LTG activity. LTGs usually break glycan strands and produce unique anhydro caps on their ends40-44. However, because AgmT is the only LTGs that is required for gliding, it is not likely to facilitate bFAC assembly by generating such modification on glycan strands. *E. coli* MltG is a glycan terminase that controls the length of newly synthesized PG glycans25. Likewise, AgmT could generate short glycan strands and thus uniquely modify the overall structure of *M. xanthus* PG, such as producing small pores that retard and retain the inner subcomplexes of bFACs (Fig. 6). On the contrary, the *M. xanthus* mutants that lack active

AgmT could produce PG with increased strain length, which blocks bFACs from binding to the cell wall and precludes stable bFAC assembly. However, it would be very difficult to demonstrate how glycan length affects the connection between bFACs and PG".

> *(2) Although agmT mutants do not swarm, in-depth phenotypic analysis is lacking. In particular, do individual agmT mutant cells move, as found with other swarming defective mutants, or are agmT mutants completely nonmotile, as are motor mutants?*

We appreciate the reviewer for bringing up an important question. Prompted by this question, we analyzed the gliding phenotype of the *ΔagmT pilA* mutant on the single cell level. We found that the *ΔagmT pilA* cells are not completely static. Instead, they move for less than half cell length before pauses and reversal. We moved on to quantify the velocity and gliding persistency and found that the gliding phenotype of the *ΔagmT pilA* cells matches the prediction on the bFACs that loses the connection between the inner subcomplexes and PG.

We then imaged individual *ΔagmT pilA*- cells on 1.5% agar surface at 10-s intervals using bright-field microscopy. To our surprise, instead of being static, individual *ΔagmT pilA*- cells displayed slow movements, with frequent pauses and reversals (Video 1). To quantify the effects of AgmT, we measured the velocity and gliding persistency (the distances cells traveled before pauses and reversals) of individual cells. Compared to the *pilA*- cells that moved at 2.30 ± 1.33 μm/min (n = 46) and high persistency (Video 2 and Fig. 2C, D), *ΔagmT pilA*- cells moved significantly slower (0.88 ± 0.62 μm/min, n = 59) and less persistent (Video 1 and Figure. 2C, D). Such aberrant gliding motility is distinct from the "hyper reversal" phenotype. Although the hyper reversing cells constitutively switching their moving directions, they usually maintain gliding velocity at the wild-type level27. due to the polarity regulators Instead, the slow and "slippery" gliding of the *ΔagmT pilA*- cells matches the prediction that when the inner complexes of bFACs lose connection with PG, bFACs can only generate short, and inefficient movements19. Our data indicate that AgmT is not essential component in the bFACs. Thus, AgmT is likely to regulate the assembly and stability of bFACs, especially their connection with PG.

> *(3) The bioinformatic and comparative genomics analysis of agmT is incomplete. For example, the sequence relationships between AgmT, MltG, and the 13 other LTG proteins in M. xanthus are not clear. Is E. coli MltG the closest homology to AgmT? Their relationships could be addressed with a phylogenetic tree and/or sequence alignments. Furthermore, are there other A-motility genes in proximity to agmT? Similarly, does agmT show specific co-occurrences with the other A-motility genes across genera/species?*

We answered the first question in the Discussion (it was in the first Results section in the previous version), "Both *M. xanthus* AgmT and *E. coli* MltG belong to the YceG/MltG family, which is the first identified LTG family that is conserved in both Gram-negative and positive bacteria25,41. About 70% of bacterial genomes, including firmicutes, proteobacteria, and actinobacteria, encode YceG/MltG domains25. The unique inner membrane localization of this family and the fact that AgmT is the only *M. xanthus* LTG that belongs to this family (Table S2) could partially explain why it is the only LTG that contributes to gliding motility".

For the second, we added one sentence in the Results, "No other motility-related genes are found in the vicinity of *agmT*".

For the third question, we do not believe a co-occurrence analysis is necessary. Because *M. xanthus* gliding is very unique but "about 70% of bacterial genomes, including firmicutes, proteobacteria, and actinobacteria, encode YceG/MltG domains25", gliding should show no co-occurrence with the YceG/MltG family LTGs.

> *(4) Related to iii, what about the functional relationship of the endogenous 13 LTG genes? Although knockout mutants were shown to be motile, presumably because AgmT is present, can overexpression of them, similar to E. coli MltG, complement an agmT mutant? In other words, why does MltG complement and the endogenous LTG proteins appear not to be relevant?*

We appreciate the reviewer for this question, which prompted us to think the uniqueness of AgmT more carefully. AgmT is unique for its inner-membrane localization, rather than activity. We answered this question in the discussion, "LTGs usually break glycan strands and produce unique anhydro caps on their ends40-44. However, because AgmT is the only LTGs that is required for gliding, it is not likely to facilitate bFAC assembly by generating such modification on glycan strands". We then moved on to propose a possible mechanism, "*E. coli* MltG is a glycan terminase that controls the length of newly synthesized PG glycans25. Likewise, AgmT could generate short glycan strands and thus uniquely modify the overall structure of *M. xanthus* PG, such as producing small pores that retard and retain the inner subcomplexes of bFACs (Fig. 6). On the contrary, the *M. xanthus* mutants that lack active AgmT could produce PG with increased strain length, which blocks bFACs from binding to the cell wall and precludes stable bFAC assembly. However, it would be very difficult to demonstrate how glycan length affects the connection between bFACs and PG".

> *(5) Based on Figure 2B, overexpression of MltG enhances A-motility compared to the parent strain and the agmT-PAmCh complemented strain, is this actually true? Showing expanded swarming colony phenotypes would help address this question.*

We appreciate the reviewer for bringing up an important question. Prompted by this question, we analyzed the effects of MltG expression at the single-cell level. We found that "Consistent with its LTG activity, the expression of MltGEc restored gliding motility of the *ΔagmT pilA*- cells on both the colony (Fig. 2B) and single-cell (Fig. 2C, D) levels. Interestingly, in the absence of sodium vanillate, the leakage expression of MltGEc using the vanillate-inducible promoter was sufficient to compensate the loss of AgmT. A plausible explanation of this observation is that as *E. coli* grows much faster (generation time 20 - 30 min) than *M. xanthus* (generation time ~4 h), MltGEc could possess significantly higher LTG activity than AgmT. Induced by 200 μM sodium vanillate, the expression of MltGEc further but non significantly increased the velocity and gliding persistency (Fig. 2B-D). Importantly, the expression of MltGEc failed to restore gliding motility in the *agmTEAEA pilA* cells, even in the presence of 200 μM sodium vanillate (Fig. 2B). Consistent with the mecillinam resistance assay (Fig. 3C), this result suggests that AgmTEAEA still binds to PG and that in the absence of its LTG activity, AgmT does not anchor bFACs to PG". These results are shown in the new panels C and D in Figure 2.

> *(6) Cell flexibility is correlated with gliding motility function in M. xanthus. Since AgmT has LTG activity, are agmT mutants less flexible than WT cells and is this the cause of their motility defect?*

We appreciate the reviewer for bringing up an important question. We saw cells that lack AgmT making S-turns and U-turns frequently under microscope. We used a GRABS assay to quantify cell stiffness and found that neither the absence of AgmT nor the expression of MltGEc affect cell stiffness. We added this result in the manuscript, "The assembly of bFACs produces wave-like deformation on cell surface6,37, suggesting that their assembly may require a flexible PG layer2,6,11,12. As a major contributor to cell stiffness, PG flexibility affects the overall stiffness of cells38. To test the possibility that AgmT and MltGEc facilitate bFAC assembly by reducing PG stiffness, we adopted the GRABS assay38 to quantify if the lack of AgmT and the expression of MltGEc affects cell stiffness. To quantify changes in cell

stiffness, we simultaneously measured the growth of the *pilA*-, *ΔagmT pilA*-, and *ΔagmT Pvan-MltGEc pilA*- (with 200 μM sodium vanillate) cells in a 1% agarose gel infused with CYE and liquid CYE and calculated the GRABS scores of the *ΔagmT pilA*-, and *ΔagmT Pvan-MltGEc pilA*- cells using the *pilA*- cells as the reference, where positive and negative GRABS scores indicate increased and decreased stiffness, respectively (see Materials and Methods and Ref38). The GRABS scores of the *ΔagmT pilA*-, and *ΔagmT Pvan-MltGEc pilA*- (with 200 μM sodium vanillate) cells were -0.06 ± 0.04 and -0.10 ± 0.07 (n = 4), respectively, indicating that neither AgmT nor MltGEc affects cell stiffness significantly. Whereas PG flexibility could still be essential for gliding, AgmT and MltGEc do not regulate bFAC assembly by modulating PG stiffness. Instead, these LTGs could connect bFACs to PG by generating structural features that are irrelevant to PG stiffness".

> ***Reviewer #2 (Public Review):***
>
> *The manuscript by Carbo et al. reports a novel role for the MltG homolog AgmT in gliding motility in M. xanthus. The authors conclusively show that AgmT is a cell wall lytic enzyme (likely a lytic transglycosylase), its lytic activity is required for gliding motility, and that its activity is required for proper binding of a component of the motility apparatus to the cell wall. The data are generally well-controlled. The marked strength of the manuscript includes the detailed characterization of AgmT as a cell wall lytic enzyme, and the careful dissection of its role in motility. Using multiple lines of evidence, the authors conclusively show that AgmT does not directly associate with the motility complexes, but that instead its absence (or the overexpression of its active site mutant) results in the failure of focal adhesion complexes to properly interact with the cell wall.*
>
> *An interpretive weakness is the rather direct role attributed to AgmT in focal adhesion assembly. While their data clearly show that AgmT is important, it is unclear whether this is the direct consequence of AgmT somehow promoting bFAC binding to PG or just an indirect consequence of changed cell wall architecture without AgmT. In E. coli, an MltG mutant has increased PG strain length, suggesting that M. xanthus's PG architecture may likewise be compromised in a way that precludes AglR binding to the cell wall. However, this distinction would be very difficult to establish experimentally. MltG has been shown to associate with active cell wall synthesis in E. coli in the absence of protein-protein interactions, and one could envision a similar model in M. xanthus, where active cell wall synthesis is required for focal adhesion assembly, and MltG makes an important contribution to this process.*

Based on the data that AgmT does not assemble into bFACs and that heterologous MltGEc substitutes *M. xanthus* AgmT in gliding, we believe that AgmT facilitates the proper assembly of bFACs indirectly. At the end of Introduction, we pointed out, "Hence, the LTG activity of AgmT anchors bFAC to PG, potentially by modifying PG structure". Following the reviewer's recommendation, we revised the Discussion to emphasize that AgmT facilitates proper bFAC assembly indirectly through its LTG activity. For the reviewer's convenience, the revised paragraph is pasted here, with the changes highlighted in blue:

"It is surprising that AgmT itself does not assemble into bFACs and that MltGEc substitutes AgmT in gliding. Thus, rather than interacting with bFAC components directly and specifically, AgmT facilitates proper bFAC assembly indirectly through its LTG activity. LTGs usually break glycan strands and produce unique anhydro caps on their ends40-44. However, because AgmT is the only LTGs that is required for gliding, it is not likely to facilitate bFAC assembly by generating such modification on glycan strands. *E. coli* MltG is a glycan terminase that controls the length of newly synthesized PG glycans25. Likewise, AgmT could generate short glycan strands and thus uniquely modify the overall structure of *M. xanthus* PG, such as producing small pores that retard and retain the inner subcomplexes of bFACs (Fig. 6). On the contrary, the *M. xanthus* mutants that lack active AgmT could produce PG with

increased strain length, which blocks bFACs from binding to the cell wall and precludes stable bFAC assembly. However, it would be very difficult to demonstrate how glycan length affects the connection between bFACs and PG".

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *The last sentence of the Discussion implies that anchoring LTG (AgmT) in the inner membrane is important. I did not see this mentioned about AgmT. Does it contain an inner membrane anchoring domain? Along these lines, the AgmT and MltG proteins appear to be of different sizes (Figure 1A). Please clarify, perhaps including full-length sequence alignment and/or domain architecture for these proteins.*

We revised the first paragraph in the Results and clarified, "Among these genes, agmT (ORF K1515_0491023) was predicted to encode an inner membrane protein with a single N-terminal transmembrane helix (residues 4 – 25) and a large "periplasmic solute-binding" domain22."

We appreciate the reviewer for spotting the mistake in Fig. 2A. The *E. coli* MltG sequence shown in the alignment starts from residue 158, instead of 88. We have corrected this mistake in the figure. *M. xanthus* AgmT and *E. coli* MltG are of similar sizes, with 239 and 240 amino acids, respectively.

> *In Figure 3 legend, define D3.*

The definition of D_3_ was added into the figure legend.

> *Figure 4A shows 100-frame composite micrographs, but no time interval between frames is given.*

The imaging frequency, 10 Hz, was stated in the text. We also added this information into the figure legend.

> *Line 98, the term "Especially" does not flow well, change to "This includes the characteristic..." or similar.*

We deleted "especially" from the sentence.

> *Line 179, "not" is not accurate, replace with "rarely."*

Changed.

> *Line 188, add a qualifier, "proper" before "bFACs assembly."*

Added.

> *Lines 196 and 202, provide the sizes of each protein in these fusion constructs.*

We added these numbers to the figure legend.

> *In Figure 5A add arrows to identify each band. State in legend whether this is a denaturing gel, if so, why are AgmT-PAmCherry homodimers present?*

Protein electrophoresis was done using SDS-PAGE. It is not unusual that some proteins, especially membrane proteins, are resistant to dissociation by SDS and appear as multimers in SDS-PAGE. The authors have seen this phenomenon repeatedly in both our experiments and the literature. Nevertheless, we clarified our experimental condition in the text, “Similar to many membrane proteins that resistant to dissociation by SDS34, immunoblot using an anti-mCherry antibody showed that AgmTPAmCherry accumulated in two bands in SDS-PAGE that corresponded to monomers and dimers of the full-length fusion protein, respectively (Fig. 5A)”.

A few examples for membrane proteins remaining as oligomers are listed in below:

Rath et al., 2009, PNAS 106: 1760-1765

Sulistijo *et al.*, 2003, J Biol Chem 278: 51950-51956

Sukharev 2002, Biophy J 83: 290-298

Neumann *et al.*, 1998, J Bacteriol 180: 3312-3316

Blakey *et al.*, 2002, Biochem J 364: 527-535

Wegner and Jones, 1984, J Biol Chem 259: 1834-1841

Jiang *et al.*, 2002, Nature 417: 515-522

Heginbotham and Miller, 1997, Biochem 36: 10335-10342

Gentile et al., 2002, J Biol Chem 277: 44050-44060

> *Line 207, "near evenly along cell bodies" does not seem consistent with Figure 5B as there looks to be an enrichment of AgmT at cell poles.*

We have replaced panel 5B with more typical images. Due to the shape difference between cell poles and the cylindrical nonpolar regions, many surface-associated proteins could appear “enriched” at cell poles. This effect was very obvious in Fig. 5B, possibly due to the unevenness of the agar surface. We examined our data carefully and did not find significant polar enrichment. Compared to AglZ that significantly enriches at poles and forms evenly-spaced clusters along the cell body, the localization of AgmT is completely different.

> *Lines 252 and 260, change "Fig. 5B" to "Fig. 5C."*

We apologize for these mistakes. They have been corrected.

> *Line 266, insert "the" before "cell envelope."*

Added.

> *Line 278, insert "presumably" between "AgmT generates (small openings)"*

Corrected.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *- Major comment: I would rephrase conclusions regarding a direct role of AgmT in focal adhesion assembly since these data are indirect (AglR binding to the cell wall is reduced in the absence of AgmT - this could also be interpreted as the absence of AgmT causing altered cell wall architecture that precludes AglR binding). Example: I don't think the data*

> *support line 222 "AgmT connects bFACs to PG", perhaps rephrased to accommodate more agnostic explanations. Likewise, line 308 states that MltG has been "adopted" by the gliding motility machinery. This conclusion cannot be drawn from the data presented.*

We agree with the reviewer that the conclusions should be stated precisely. At the end of Introduction, we pointed out, “Hence, the LTG activity of AgmT anchors bFAC to PG, potentially by modifying PG structure”. Following the reviewer’s recommendation, we revised the Discussion to emphasize that AgmT facilitates bFAC assembly indirectly through its LTG activity. For the reviewer’s convenience, the revised paragraph is pasted here, with the changes highlighted in blue:

“It is surprising that AgmT itself does not assemble into bFACs and that MltGEc substitutes AgmT in gliding. Thus, rather than interacting with bFAC components directly and specifically, AgmT facilitates proper bFAC assembly indirectly through its LTG activity. LTGs usually break glycan strands and produce unique anhydro caps on their ends40-44. However, because AgmT is the only LTGs that is required for gliding, it is not likely to facilitate bFAC assembly by generating such modification on glycan strands. *E. coli* MltG is a glycan terminase that controls the length of newly synthesized PG glycans25. Likewise, AgmT could generate short glycan strands and thus uniquely modify the overall structure of *M. xanthus* PG, such as producing small pores that retard and retain the inner subcomplexes of bFACs (Fig. 6). On the contrary, the *M. xanthus* mutants that lack active AgmT could produce PG with increased strain length, which blocks bFACs from binding to the cell wall and precludes stable bFAC assembly. However, it would be very difficult to demonstrate how glycan length affects the connection between bFACs and PG”.

However, we believe that the conclusion that “AgmT connects bFACs to PG" still stands true. Although AgmT is not likely to interact with the gliding machinery directly, its activity does increase the binding between bFACs and PG.

We agree with the reviewer that “adopt” may not be the best word to describe AgmT’s function in gliding. In the revised manuscript, we changed the phrase to “contributes to gliding motility”.

> *- Line 35: define "bFAC" at first use.*

Fixed.

> *- Figure 2: Mention in the caption why the pilA mutation is significant. Also, make more clear what one is supposed to see. You could include an arrow showing motile cells extruding from the colony edge, and mark + label the edge of the colony.*

Following the reviewer’s recommendations, we described the motility phenotypes in detail in the main text, “On a 1.5% agar surface, the *pilA*- cells moved away from colony edges both as individuals and in “flare-like” cell groups, indicating that they were still motile with gliding motility. In contrast, the *ΔaglR pilA*- cells that lack an essential component in the gliding motor, were unable to move outward from the colony edge and thus formed sharp colony edges. Similarly, the *ΔagmT pilA*- cells also formed sharp colony edges, indicating that they could not move efficiently with gliding (Fig. 2B)”.

We also added a schematic block into panel B and two sentences into the legend, “To eliminate S-motility, we further knocked out the *pilA* gene that encodes pilin for type IV pilus. Cells that move by gliding are able to move away from colony edges.”

> *- Figure 3 caption. Mecillinam concentration should presumably be µg/mL, not g/mL?*

Also, remove the ".van,." in the second to last line.

We apologize for these mistakes. We have corrected them in the figure legend.

> *- Line 212 - at this point in the manuscript, the fact that AgmT likely does not assemble into bFACs is quite well established, so I would start this paragraph with something like "As an additional test, we...".*

Revised as the reviewer recommended.

> *- Figure 5C - this assay needs a protein loading control. How about whole-cell AglR before pelleting PG?*

We do have a whole-cell loading control, which we have added into the revised figure.

> *- Figure 5A - how are the dimers visible? Is this a native gel? If so, please add to the Methods section (I would find information on Western Blot there, but not on gel electrophoresis).*

Protein electrophoresis was done using SDS-PAGE. It is not unusual that some proteins, especially membrane proteins, are resistant to dissociation by SDS and appear as multimers in SDS-PAGE. The authors have seen this phenomenon repeatedly in both our experiments and the literature. Nevertheless, we clarified our experimental condition in the text, “Similar to many membrane proteins that resistant to dissociation by SDS34, immunoblot using an anti-mCherry antibody showed that AgmTPAmCherry accumulated in two bands in SDS-PAGE that corresponded to monomers and dimers of the full-length fusion protein, respectively (Fig. 5A)”.

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