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

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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.

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, also known as gliding motility, does not depend on conventional motility-related cell surface appendages, such as flagella or pili[2,5]. In *M. xanthus*, AglR, AglQ, and AglS form a membrane channel that functions as the gliding motor by harvesting proton motive force[6,7]. The motor unit associates with at least fourteen gliding-related proteins that reside in the cytoplasm, inner membrane, periplasm, and outer membrane[8-10].

Recently, the mechanisms for bFAC assembly and the coupling between bFACs and substrate begin to emerge[8,11,12]. 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[11,13-15]. 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**)[11,15]. 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[13,16,17]. bFACs adhere to the gliding substrate through an outer membrane adhesin[12]. As motors transport bFACs

48 toward lagging cell poles, cells move forward but bFACs remain static relative to the
49 gliding substrate.

50 bFAC assembly can be quantified at nanometer resolution using the single-particle
51 dynamics of gliding motors[13,18]. Single particles of a fully functional, photoactivatable
52 mCherry (PAmCherry)-labeled AglR display three dynamic patterns, stationary,
53 directed motion, and diffusion[13,18]. The stationary population consists of the motors in
54 fully assembled bFACs, which do not move before photobleach. In contrast, the motors
55 moving in a directed manner carry incomplete gliding complexes along helical tracks,
56 whereas the diffusing motors assemble to even less completeness[11,13,15,18]. As motors only
57 generate force in static bFACs[11], the population of nonmotile motors indicates the
58 overall status of fully assembled bFACs.

59 However, how bFACs transmit force across the peptidoglycan (PG) cell wall is
60 unclear. PG is a mesh-like single molecule of crosslinked glycan strands that surrounds
61 the entire cytoplasmic membrane. The rigidity of PG defines cell shape and protects
62 cells from osmotic lysis[19,20]. If bFACs physically penetrate PG, their transportation
63 toward lagging cell poles would tear PG and trigger cell lysis. To avoid breaching PG,
64 an updated model proposes that there are two subcomplexes in each bFAC, one on each
65 side of the PG layer. Rather than forming stable and rigid interactions, these two
66 subcomplexes only associate transiently to form a bFAC[11,21] (**Fig. 1**).

67 As motors reside in the fluid inner membrane, to transmit force to the cell surface,
68 they must push against two relatively rigid structures, one on each side of the
69 membrane, in opposite directions[21] (**Fig. 1**). MreB is a bacterial actin homolog that
70 associates with the motors and plays essential roles in gliding[2,5,6,14,18,22]. The motors could
71 push against MreB filaments in the cytoplasm, whereas PG could be the structure that

motors push against in the periplasm[11,21]. The interaction between the inner complex and PG not only satisfies the physical requirement for force generation but also supports efficient bFACs assembly. Without this interaction, once the inner subcomplex disassociates from the outer one, it will quickly escape bFACs and no longer contribute to force generation[21]. In this case, the fraction of time each motor generates force (remains in bFACs) will be extremely low[21]. How the inner complex interacts with PG remains unknown. It was speculated that gliding motors in the inner complex could bind PG directly[11]. However, such binding has not been confirmed by experiments.

In this study, we found that AgmT, a lytic transglycosylase (LTG) for PG, is essential for *M. xanthus* gliding motility. AgmT is required for maintaining PG integrity under the stress from the antibiotic mecillinam. Using single-particle tracking microscopy and coprecipitation assays, we found that AgmT is required for the gliding motors 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 bFAC to PG, potentially by modifying PG structure. Our findings reveal the long-sought connection between PG and bFAC that allows mechanical force to transmit across the PG cell wall.

Results

AgmT, a putative LTG, is essential 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[23]. Among these genes, *agmT* (ORF K1515_04910[24]) was predicted to encode a protein with a large "periplasmic solute-binding" domain[23]. After careful analysis, we found that AgmT showed significant similarity to the widely conserved YceG/MltG family LTGs. About 70% of bacterial genomes, including firmicutes, proteobacteria, and actinobacteria, encode YceG/MltG domains[25]. Especially, the putative active site, Glu223 (corresponding to E218 in *E. coli* MltG)[25], is conserved in AgmT (**Fig. 2A**). To confirm the function of AgmT in gliding, we constructed an *agmT* in-frame deletion mutant. To eliminate S-motility, we further knocked out the *pilA* gene that encodes pilin for type IV pilus. In contrast to the *pilA*$^-$ cells that were still motile with gliding motility, the Δ*aglR pilA*$^-$ cells that lack an essential component in the gliding motor, were unable to move outward from the colony edge. Similarly, the Δ*agmT pilA*$^-$ cells also formed sharp colony edges, indicating that they were nonmotile with gliding (**Fig. 2B**). 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 on the chromosome. *agmT*^{EAEA} *pilA*$^-$ cells were nonmotile (**Fig. 2B**). Thus, the putative LTG activity of AgmT is essential for *M. xanthus* gliding motility.

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

115 gliding motility, indicating that AgmT is the only LTG that is required for *M. xanthus*
116 gliding (**Fig. S1**).

117 **AgmT is an LTG.** To determine if AgmT is an LTG, we expressed the periplasmic
118 domain (amino acids 25 – 339) of wild-type AgmT and AgmT^{EAEA} in *E. coli*. We purified
119 PG from wild-type *M. xanthus* cells, labeled it with Remazol brilliant blue (RBB), and
120 tested if the purified AgmT variants hydrolyze labeled PG *in vitro* and release the
121 dye[26,27]. Similar to lysozyme that specifically cleaves the ®-1,4-glycosidic bonds in PG,
122 wild-type AgmT solubilized dye-labeled *M. xanthus* PG that absorbs light at 595 nm. In
123 contrast, the AgmT^{EAEA} variant failed to release the dye (**Fig. 3A**). Hence, AgmT displays
124 LTG activity *in vitro*.

125 Whereas AgmT does not affect growth rate (**Fig. S2**), cells that lacked AgmT or
126 expressed AgmT^{EAEA} maintained rod shape but were slightly shorter and wider than the
127 wild-type ones (**Fig. 3B**). Nevertheless, altered morphology alone does not likely
128 account for abolished gliding. In fact, a previously reported mutant that lacks all three
129 class A penicillin-binding proteins (⊗3) displays similarly shortened and widened
130 morphology[28], but is still motile by gliding (**Fig. S1**).

131 A recent report revealed that *Vibrio cholerae* MltG degrades un-crosslinked PG
132 turnover products and prevents their detrimental accumulation in the periplasm[29]. To
133 test if AgmT plays a similar role in *M. xanthus*, we used mecillinam to induce cell
134 envelope stress. Mecillinam is a ®-lactam that induces the production of toxic, un-
135 crosslinked PG strands[30]. Rather than collapsing the rod shape, mecillinam only causes
136 bulging near the centers of wild-type *M. xanthus* cells, whereas large-scale cell lysis does
137 not occur and cell poles still maintain rod shape even after prolonged (20 h) treatment
138 (**Fig. 3C**)[28]. Thus, wild-type *M. xanthus* can largely mitigate mecillinam stress. In

139 contrast, mecillinam-treated *agmT* cells increased their width drastically, displayed
140 significant cell surface irregularity along their entire cell bodies, and lysed frequently
141 (**Fig. 3C**). Cells expressing AgmT^{EAEA} as the sole source of AgmT displayed similar, but
142 even stronger phenotypes under mecillinam stress, growing into elongated, twisted
143 filaments that formed multiple cell poles and lysed frequently (**Fig. 3C**). These results
144 confirm that similar to *V. cholerae* MltG, *M. xanthus* AgmT is important for maintaining
145 cell integrity under mecillinam stress. The different responses from *agmT* and *agmT^{EAEA}*
146 cells suggest that AgmT could interact with other PG-related enzymes and that the
147 inactive AgmT variant might still modulate PG through these enzymes. Alternatively,
148 other LTGs could partially substitute AgmT while AgmT^{EAEA} blocks these enzymes
149 from accessing un-crosslinked PG strands.

150 **AgmT is essential for bFACs assembly.** How does AgmT support gliding? We first
151 tested if AgmT regulates the function of the gliding motor. For this purpose, we
152 quantified the motor dynamics by tracking single particles of AglR, an essential
153 component of the motor. We spotted the cells that express a fully functional, photo-
154 activatable mCherry (PAmCherry)-labeled AglR[13] on 1.5% agar surface. We used a 405-
155 nm excitation laser to activate the fluorescence of a few labeled AglR particles randomly
156 in each cell and quantified their localization using a 561-nm laser at 10 Hz using single
157 particle tracking photo-activated localization microscopy (sptPALM) under highly
158 inclined and laminated optical sheet (HILO) illumination[13,18,31]. Using this setting, only a
159 thin section of each cell surface that was close to the coverslip was illuminated. To
160 analyze the data, we only chose the fluorescent particles that remained in focus for 4 -
161 12 frames (0.4 - 1.2 s). As free PAmCherry particles diffuse extremely fast in the
162 cytoplasm, entering and exiting the focal plane frequently, they usually appear as
163 blurry objects that cannot be followed at 10-Hz close to the membrane[18]. For this reason,
164 the noise from any potential degradation of AglR-PAmCherry was negligible.

165 Consistent with our previous results[13,16], 32.1% (n = 2,700) of AglR-PAmCherry particles
166 remained within one pixel (160 nm · 160 nm) before photobleach, indicating that they
167 were nonmotile. The remaining 67.9% AglR-PAmCherry particles were motile, leaving
168 trajectories of various lengths (**Fig. 4A, 4B**).

169 bFAC assembly is sensitive to mechanical cues. As the agar concentration increases
170 in gliding substrate, more motor molecules engage in bFACs, where they appear
171 immobile[10,15]. We exposed *aglR-PAmCherry* cells to 405-nm excitation (0.2 kW/cm^2) for 2
172 s, where most PAmCherry molecules were photoactivated and used epifluorescence to
173 display the overall localization of AglR[13,18,32]. As the agar concentration increased bFACs
174 increased significantly in size (**Fig. 4C**). Accordingly, the immobile AglR particles
175 detected by sptPALM increased from 10.7% (n = 1112) on 0.5% agar to 45.2% (n = 726)
176 on 5.0% agar surface (**Fig. 4B**). Thus, the stationary population of AglR-PAmCherry
177 particles reflects the AglR molecules in fully assembled bFACs.

178 In contrast to the cells that expressed wild-type AgmT (*agmT*$^+$), AglR particles were
179 hyper motile in the cells that lacked AgmT or expressed AgmT^{EAEA}. On 1.5% agar
180 surfaces, the immobile populations of AglR particles decreased to 10.5% (n = 2,989) and
181 11.7% (n = 1,408), respectively. Consistently, AglR-containing bFACs were not
182 detectable in these cells, even on 5% agar surfaces (**Fig. 4A-C**).

183 To test if other components in the gliding machinery also depend on AgmT to
184 assemble into bFACs, we tested the localization of AglZ, a cytoplasmic protein that is
185 commonly used for assessing bFAC assembly[11,33]. In the cells that expressed wild-type
186 AgmT, yellow fluorescent protein (YFP)-labeled AglZ formed a bright cluster at the
187 leading cell pole and multiple stationary, near-evenly spaced clusters along the cell
188 body, indicating the assembly of bFACs (**Fig. 4D**). In contrast, AglZ-YFP only formed

189 single clusters at cell poles in the cells that lacked AgmT or expressed AgmT^{EAEA} (**Fig.**
190 **4D**). Taken together, the LTG activity of AgmT is essential for bFACs assembly.

191 **AgmT does not assemble into bFACs.** We first hypothesized that AgmT itself could
192 assemble into bFACs, where it could either generate pores in PG that allow the inner
193 and outer gliding complexes interact directly or bind to PG and recruit other
194 components to bFACs through protein-protein interactions. If this is the case, AgmT
195 should localize in bFACs. To test this possibility, we labeled AgmT with PAmCherry on
196 its C-terminus and expressed the fusion protein as the sole source of AgmT using the
197 native promoter and locus of *agmT*. *agmT-PAmCherry pilA*$^{-}$ cells displayed gliding
198 motility that was indistinguishable from *pilA*$^{-}$ cells (**Fig. 2B**), indicating that the fusion
199 protein is functional. Immunoblot using an anti-mCherry antibody showed that AgmT-
200 PAmCherry accumulated in two bands that corresponded to monomers and dimers of
201 the full-length fusion protein, respectively (**Fig. 5A**). This result is consistent with the
202 structure of *E. coli* MltG that functions as homodimers (PDB: 2r1f). Importantly, AgmT-
203 PAmCherry was about 50 times more abundant than AglR-PAmCherry (**Fig. 5A**). To
204 test if AgmT itself assembles into bFACs, we first expressed AgmT-PAmCherry and
205 AglZ-YFP together using their respective loci and promoter. We exposed *agmT-*
206 *PAmCherry* cells to 405-nm excitation (0.2 kW/cm^2) for 2 s to visualize the overall
207 localization of AgmT. We found that on a 1.5% agar surface that favors gliding motility,
208 AglZ aggregated into bFACs whereas AgmT localized near evenly along cell bodies
209 without forming protein clusters (**Fig. 5B**). Thus, AgmT does not localize into bFACs.
210 These results echo the fact that despite its abundance, AgmT has not been identified as
211 a component in bFACs despite extensive pull-down experiments using various bFAC
212 components as baits[6,10,34].

213 We reasoned that if AgmT assembles into bFACs, AgmT and gliding motors should
214 display similar dynamic patterns. To test this possibility further, we used sptPALM to
215 track the movements of AgmT-PAmCherry single particles on 1.5 agar surfaces at 10
216 Hz. Distinct from the AglR particles of which 32.1% remained stationary, AgmT moved
217 in a diffusive manner, showing no significant immobile population. Importantly,
218 compared to the diffusion coefficients (D) of mobile AglR particles ($1.8 \cdot 10^{-2} \pm 3.6 \cdot 10^{-3}$
219 ⌈m^2/s (n = 1,833)), AgmT particles diffused much faster ($D = 2.9 \cdot 10^{-2} \pm 5.3 \cdot 10^{-3}$ ⌈m^2/s (n
220 = 8,548)). Taken together, neither the localization nor dynamics of AgmT displayed
221 significant correlation with bFAC components. We thus conclude that AgmT does not
222 assemble into bFACs

223 **AgmT connects bFACs to PG through its LTG activity.** We then explored the
224 possibility that AgmT could modify PG through its LTG activity and thus generate the
225 anchor sites for certain components in bFACs. If this hypothesis is true, heterologous
226 expression of a non-native LTG could rescue gliding motility in the ⊗*agmT* strain. To
227 test this hypothesis, we fused the *E. coli mltG* gene ($mltG_{Ec}$) to a vanillate-inducible
228 promoter and inserted the resulting construct to the *cuoA* locus on *M. xanthus*
229 chromosome that does not interfere gliding[35]. We subjected the ⊗*agmT* and $agmT^{EAEA}$
230 cells that expressed $MltG_{Ec}$ to mecillinam (100 ⌈g/ml) stress. Induced by 100 ⌈M sodium
231 vanillate, $MltG_{Ec}$ restored cell morphology and integrity of the ⊗*agmT* strain to the wild-
232 type level (**Fig. 3C**). This result confirmed that $MltG_{Ec}$ was expressed and enzymatically
233 active in *M. xanthus*. In contrast, $MltG_{Ec}$ failed to confer mecillinam resistance to the
234 $agmT^{EAEA}$ cells (**Fig. 3C**). A potential explanation is that $AgmT^{EAEA}$ could still bind to PG
235 and thus block $MltG_{Ec}$ from accessing *M. xanthus* PG. Consistent with its activity, $MltG_{Ec}$
236 restored gliding motility in the *agmT pilA* cells but not in the $agmT^{EAEA}$ *pilA* ones (**Fig.
237 2B**). As $MltG_{Ec}$ substitutes AgmT in gliding motility, it is the LTG activity, rather than
238 specific interactions between AgmT and bFACs, that is required for gliding.

239 It is challenging to visualize how AgmT facilitates bFAC assembly through its
240 enzymatic activity *in vitro*. First, the inner complex in a bFAC spans the cytoplasm,
241 inner membrane, and periplasm and contains multiple proteins whose activities are
242 interdependent[8,10,11]. It is hence difficult to purify the entire inner complex in its
243 functional state. Second, Faure *et al*. hypothesized that AglQ and AglS in the gliding
244 motor could bind PG directly[11]. However, such binding has not been proved
245 experimentally due to the difficulty in purifying the membrane integral AglRQS
246 complex. To overcome these difficulties, we used AglR-PAmCherry to represent the
247 inner complex of bFAC and investigated how bFACs bind PG in their native
248 environment. To do so, we expressed AglR-PAmCherry in different genetic
249 backgrounds as the sole source of AglR using the native *aglR* locus and promoter. We
250 lysed these cells using sonication, subjected their lysates to centrifugation, isolated the
251 pellets that contained PG, and detected the presence of AglR-PAmCherry in these
252 pellets by immunoblots using a mCherry antibody. Eliminating AgmT and disabling its
253 active site significantly reduced the amounts of AglR in the pellets (**Fig. 5B**). Because
254 such pellets contain both the PG and membrane fractions, we further eliminated PG in
255 cell lysates using lysozyme before centrifugation and determined the amount of AglR-
256 PAmCherry in the pellets that only contained membrane fractions. Regardless of the
257 presence and activity of AgmT, comparable amounts of AglR precipitated in
258 centrifugation pellets after lysozyme treatment, indicating that AgmT does not affect
259 the expression or stability of AglR (**Fig. 5B**). Thus, active AgmT facilitates the
260 association between bFACs and PG. Strikingly, the heterologous expression of $MltG_{Ec}$
261 enriched AglR-PAmCherry in the PG-containing pellets from the ⊗*agmT* cells but not
262 the $agmT^{EAEA}$ ones (**Fig. 5B**). These results indicate that AgmT connects bFACs to PG
263 through its LTG activity.

264 **Discussion**

265 Gliding bacteria adopt a broad spectrum of nanomachineries. Compared to the trans-
266 envelope secretion system that drives gliding in *Flavobacterium johnsoniae* and
267 *Capnocytophaga gingivalis* that both contain PG, the gliding machinery of *M. xanthus*
268 appears to lack a stable structure that transverses cell envelope, especially across the PG
269 layer[2,36,37]. In order to generate mechanical force from the fluid *M. xanthus* gliding
270 machinery, the inner complex must establish solid contact with PG and push the outer
271 complex to slide[11,21]. In this work, we discovered AgmT as the long-sought factor that
272 connects bFACs to PG. Such connection not only satisfies the requirement for force
273 generation but is also essential for bFAC assembly.

274 It is rather unexpected that AgmT itself does not assemble into bFACs. Because
275 $MltG_{Ec}$ substitutes AgmT in gliding, rather than interacting with bFAC components
276 specifically, AgmT facilitates bFAC assembly through its LTG activity. A few
277 possibilities could explain the function of AgmT. First, LTGs usually break glycan
278 strands and produce unique anhydro caps on their ends[38-41]. Certain components in
279 bFACs could specifically bind to such modified glycan strands in PG and further recruit
280 other components. Second, AgmT could generate small openings all over the PG
281 meshwork that allow the inner and outer subcomplexes in bFACs to interact locally.
282 Third, AgmT could reduce the overall rigidity of *M. xanthus* PG, allow the bulky inner
283 subcomplexes to deform the PG layer, and thus establish a viscous coupling between
284 bFACs and PG[6,21].

285 Then how does AgmT, a protein localized diffusively, facilitate bFAC assembly
286 into near-evenly spaced foci? The actin homolog MreB is the only protein in bFACs that
287 displays quasiperiodic localization independent of other components[18,22]. *M. xanthus*
288 MreB assembles into bFACs and positions the latter near evenly along the cell
289 body[6,13,18,22,42]. MreB filaments change their orientation in accordance with local

290 membrane curvatures and could hence respond to mechanical cues[43-46]. Strikingly, *M.*
291 *xanthus* does assemble bFACs in response to substrate hardness (**Fig. 4C**) and
292 assembled bFACs could distort the cell envelope, generating undulations on the cell
293 surface[6,10,13,47,48]. Taken together, whereas AgmT potentially modifies the entire PG layer,
294 it is still the quasiperiodic MreB filaments that determine the loci for bFAC assembly in
295 response to the mechanical cues from the gliding substrate.

296 Most bacteria encode multiple LTGs that function in PG growth, remodeling, and
297 recycling. Trans-envelope structures often require highly specialized LTGs to penetrate
298 PG[49]. This work provides an example that macro bacterial machineries domesticate non-
299 specialized LTGs for specialized functions. Other examples include MltD in the
300 *Helicobacter pylori* flagellum and MltE in the *E. coli* type VI secretion system[50,51]. Both *M.*
301 *xanthus* AgmT and *E. coli* MltG belong to the YceG family, which is the first identified
302 LTG family that is conserved in both Gram-negative and positive bacteria[39]. LTGs in
303 this family localize to the inner membrane and feature periplasmic transglycosylase
304 domains[39]. Due to their unique localization, the YceG family LTGs are important for
305 determining the length of nascent glycan strands and for degrading the toxic un-
306 crosslinked strands from futile PG synthesis[29,52]. Due to the wide distribution of YceG
307 family LTGs, it is challenging to study how the unique *M. xanthus* gliding machinery
308 adopts AgmT in evolution. Nevertheless, the fact that AgmT is the only *M. xanthus* LTG
309 that belongs to this family (**Table S2**) could partially explain why it is the only LTG that
310 has been adopted by the gliding machinery. It will be interesting to investigate if other
311 LTGs, once anchored to the inner membrane, could also facilitate bFAC assembly.

312 **Methods**

313 **Strains and growth conditions**

314 *M. xanthus* strains used in this study are listed in **Table S2**. Vegetative *M. xanthus* cells
315 were grown in liquid CYE medium (10 mM MOPS pH 7.6, 1% (w/v) Bacto™ casitone
316 (BD Biosciences), 0.5% yeast extract and 8 mM $MgSO_4$) at 32 °C, in 125-ml flasks with
317 vigorous shaking, or on CYE plates that contains 1.5% agar. All genetic modifications
318 on *M. xanthus* were made on the chromosome. Deletion and insertion mutants were
319 constructed by electroporating *M. xanthus* cells with 4 μg of plasmid DNA.
320 Transformed cells were plated on CYE plates supplemented with 100 μg/ml sodium
321 kanamycin sulfate and 10 μg/ml tetracycline hydrochloride when needed. AgmT and
322 $AgmT^{EAEA}$ were labeled with PAmCherry at their C-termini by fusing their gene to a
323 DNA sequence that encodes PAmCherry through a KESGSVSSEQLAQFRSLD
324 (AAGGAGTCCGGCTCCGTGTCCTCCGAGCAGCTGGCCCAGTTCCGCTCCCTGGA
325 C) linker. All constructs were confirmed by PCR and DNA sequencing.

326 **Immunoblotting**

327 The expression and stability of PAmCherry-labeled proteins were determined by
328 immunoblotting using an anti-mCherry antibody (Rockland Immunochemicals, Inc.,
329 Lot 46705) and a goat anti-Rabbit IgG (H+L) secondary antibody, HRP (Thermo Fisher
330 Scientific, catalog # 31460). MreB was detected using an anti-MreB serum[22] and the same
331 secondary antibody. The blots were developed with Pierce™ ECL Western Blotting
332 Substrate (Thermo Fisher Scientific REF 32109) and MINI-MED 90 processor (AFP
333 Manufacturing).

334 **Gliding assay**

335 Cells at 4×10^9 colony formation units (cfu)/ml were spotted on CYE plates (5 μl/spot)
336 containing 1.5% agar, incubated at 32 °C for 48 h. Colony edges were photographed

337 using a Nikon Eclipse™ e600 microscope with a 10× 0.30 NA objective and an OMAX™
338 A3590U camera.

339 **Protein expression and purification**

340 DNA sequences encoding amino acids 25 – 339 of AgmT and AgmT^{EAEA} were amplified
341 by polymerase chain reaction (PCR) and inserted into the pET28a vector (Novogen)
342 between the restriction sites of *Eco*RI and *Hind*III. The resulting plasmids were
343 transformed into *E. coli* strain BL21(DE3). Transformed cells were cultured in 20 ml LB
344 (Luria-Bertani) broth at 37 °C overnight and used to inoculate 1 L LB medium
345 supplemented with 1.0% glucose. Protein expression was induced by 0.1 mM IPTG
346 (isopropyl-h-d-thiogalactopyranoside) when the culture reached an OD_{600} of 0.8.
347 Cultivation was continued at 16 °C for 10 h before the cells were harvested by
348 centrifugation at 6,000 × g for 20 min. Proteins were purified using the NGC™
349 Chromatography System (BIO-RAD) and a 5-ml HisTrap™ (Cytiva)[17,53]. Purified
350 proteins were concentrated using Amicon™ Ultra centrifugal filter units (Millipore
351 Sigma) with a molecular cutoff of 10 kDa and stored at -80 °C.

352 **RBB assay**

353 PG was purified following the published protocol[28,54]. In brief, *M. xanthus* cells were
354 grown until mid-stationary phase and harvested by centrifugation (6,000 × g, 20 min, 25
355 °C). Supernatant was discarded and the pellet was resuspended and boiled in 1× PBS
356 with 5% SDS for 2 h. SDS was removed by repetitive wash with MilliQ water and
357 centrifugation (21,000 × g, 10 min, 25 °C). Purified PG from 100 ml culture was
358 suspended into 1 ml 1× PBS and stored at -20 °C. RBB labelling of PG was performed
359 essentially as previously described[26,27]. Purified sacculi were incubated with 20 mM RBB
360 in 0.25 M NaOH overnight at 37 °C. Reactions were neutralized by adding equal
361 volumes of 0.25 M HCl and RBB-labeled PG was collected by centrifugation at 21,000 ×

362 g for 15 min. Pellets were washed repeatedly with MilliQ water until the supernatants
363 became colorless. RBB-labelled sacculi were incubated with purified AgmT and
364 $AgmT^{EAEA}$ (1 mg/ml) at 25 °C for 12 h. lysozyme (1 mg/ml) was used as a positive
365 control. Dye release was quantified by the absorption at 595 nm from the supernatants
366 after centrifugation (21,000 × g, 10 min, 25 °C).

367 **PG binding assay**

368 *M. xanthus* cells expressing AglR-PAmCherry were grown in liquid CYE to OD_{600} ~1,
369 harvested by centrifugation (6,000 × g, 20 min, 25 °C), washed by 1× PBS, and
370 resuspended into 1× PBS to OD_{600} 6. Cells (1 ml) were lysed using a Cole-Parmer 4710
371 Ultrasonic Homogenizer (40% output). Unbroken cells and large debris were eliminated
372 by centrifugation (6,000 × g, 10 min, 25 °C). Supernatants were subjected to
373 centrifugation at 21,000 × g for 15 min. Pellets that contain the PG and membrane
374 fractions were resuspended to 1 ml in 1× PBS. To collect the pellets that do not contain
375 PG, supernatants from sonication lysates were incubated with 5 mg/ml lysozyme at 25
376 °C for 5 h before centrifugation. Resuspended pellets (5 ⌈l each) were mixed with equal
377 volumes of 2× loading buffer and applied to SDS PAGE and immunoblotting.

378 **Microscopy Analysis**

379 For all imaging experiments, 5 ⌈l cells were spotted on an agarose pad. For the
380 treatments with inhibitors, inhibitors were added into both the cell suspension and
381 agarose pads. The length and width of cells were determined from differential
382 interference contrast (DIC) images using a MATLAB (MathWorks) script[18,28,55]. DIC and
383 fluorescence images of cells were captured using a Hamamatsu ImagEM X2™ EM-CCD
384 camera C9100-23B (effective pixel size 160 nm) on an inverted Nikon Eclipse-Ti™
385 microscope with a 100× 1.49 NA TIRF objective. For sptPALM, *M. xanthus* cells were
386 grown in CYE to 4 ×10^8 cfu/ml and PAmCherry was activated using a 405-nm laser (0.3

387 kW/cm^2), excited and imaged using a 561-nm laser (0.2 kW/cm^2). Images were acquired
388 at 10 Hz. For each sptPALM experiment, single PAmCherry particles were localized in
389 at least 100 individual cells from three biological replicates. sptPALM data were
390 analyzed using a MATLAB (MathWorks) script[18,28,55]. Briefly, cells were identified using
391 differential interference contrast images. Single PAmCherry particles inside cells were
392 fit by a symmetric 2D Gaussian function, whose center was assumed to be the particle's
393 position[18]. Particles that explored areas smaller than 160 nm × 160 nm (within one pixel)
394 in 0.4 - 1.2 s were considered immobile. Sample trajectories were generated using the
395 TrackMate[56] plugin in the ImageJ suite (https://imagej.net).

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546

Figure Legends

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.

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. Deleting or Disabling the active site of AgmT abolishes gliding but fusing an PAmCherry (PAmCh) to its C-terminus does not. Heterologous Expression of *E. coli* MltG restores gliding of ⊗*agmT* cells but not the cells that express $AgmT^{EAEA}$.

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. 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 ⌠g/ml). Expressing *E. coli* MltG by a vanillate-inducible promoter (P_{van}) restores resistance against mecillinam in *agmT* cells but not in the cells that express $AgmT^{EAEA}$. van,. Arrows point to newly lysed cells. Scale bars, 5 µm.

Fig. 4. AgmT and its LTG activity are essential for bFAC assembly. A) Overall distribution of AglR-PAmCherry particles on 1.5% agar surface is displayed using the composite of 100 consecutive frames. Single-particle trajectories of AglR-PAmCherry 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-labeled AgmT and $AgmT^{EAEA}$ accumulate as full-length proteins. AgmT is significantly more abundant than the motor protein AglR. 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 ($MltG_{Ec}$) 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.

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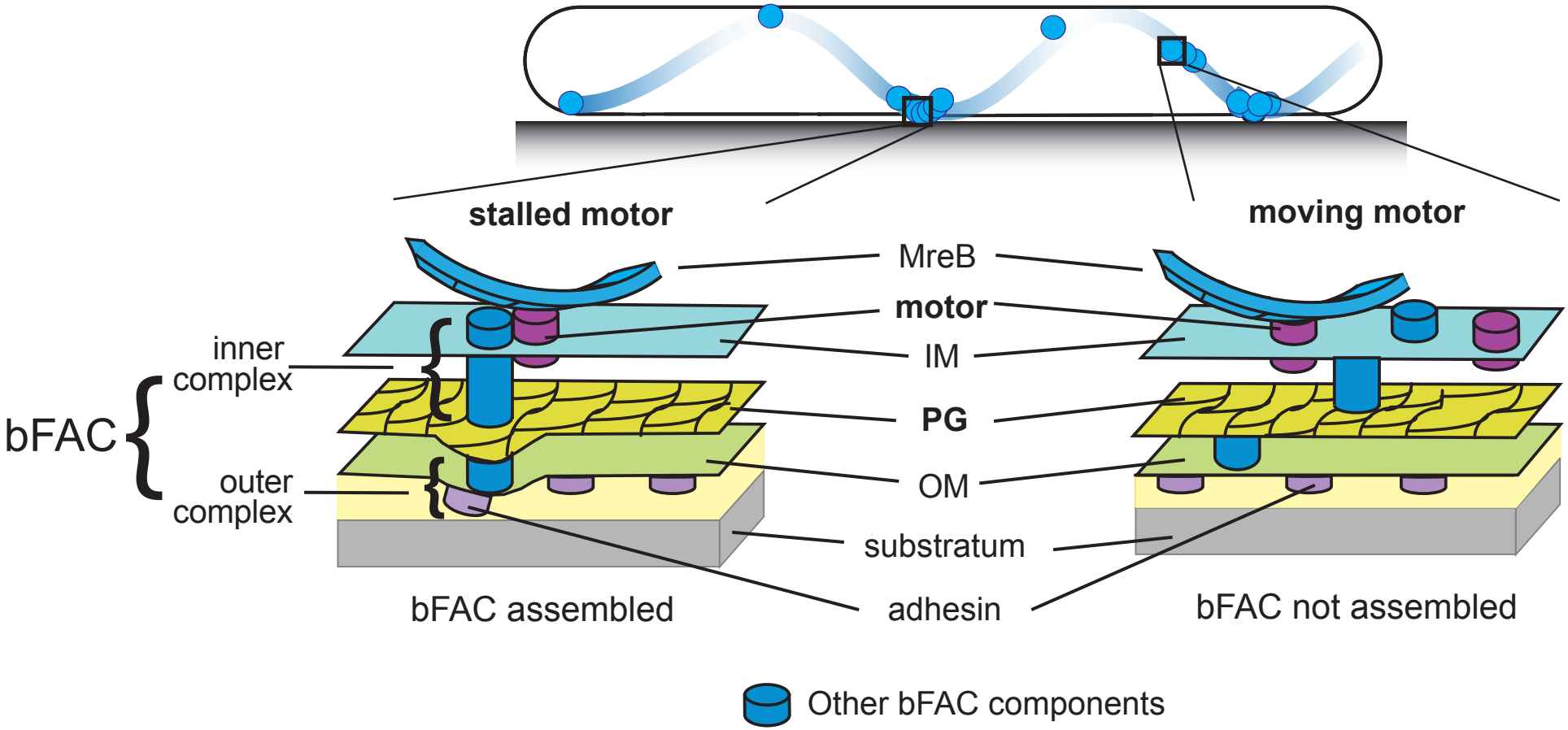
Author Contributions

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.


stalled motor
moving motor
MreB
motor
IM
PG
OM
substratum
adhesin
inner complex
bFAC
outer complex
bFAC assembled
bFAC not assembled
Other bFAC components

A

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                                                                                                                                  *
M_xant  163 EGYLYPETYGVIPGKFDVEALIQRQLDAFAQRFFAPNRDTI-TKSGRTLHEVVVMASMLEREEPLPDQRPLVAGILWKRV
E_coli   88 EGWFWPDTWXYTANTTDVALLK-RAHKKXVKAVDSAWEGRADGLPYKDKNQLVTXASIIEKETAVASERDQVASVFINRL
M_tube  237 EGLIAPGTFNIDPSASAETILATLISAGAVEYMKSGLVDTAKSLGL-SPYDILVVASLVQQEAN-TQDFPKVARVIYNRL
S_coel  397 EGFLYPSSYSAAKGQKPADVLKQMVARANEQYEKIGLEQKAEGLGLEGPWELLTAASLVQAEGKTHEDFRKMAEVIYNRL
A_calc  100 EGLFAPNTYFFAKGETDKKILT-DLYHRQMKALDAAWANRAPNLPYKDKYEALIMASIVEKETSLDSELTQVSGVFVRRL
X_albi   95 EGRFLPETYLYQRGDSDLDLLR-RAHKAMDKALADAWDSRAAGLLLSSPEQALILASIVEKETALAAERPQIAGVFVRRL
N_meni  153 EGQFFPDSYEIDAGGSDLRIYQ-TAYKAMQRRLNEAWESRQDGLPYKNPYEMLIMASLIEKETGHEADRDHVASVFVNRL
R_prow   85 EGFLMPSTYFFSYGDQKDRIID-QMRNLMSTNLDEVMKYLSPDSPLKTRIDVLTLASIIEKEAGSNEEKPIIAAVFINRL
T_aqua  126 EGYLFPATYTFDLLATPEEVVR-AMLRRFEAELTPPVRQLLEERNL-SVHAWVTLASIVQVEAGSEAEMPKIAGVFLNRL
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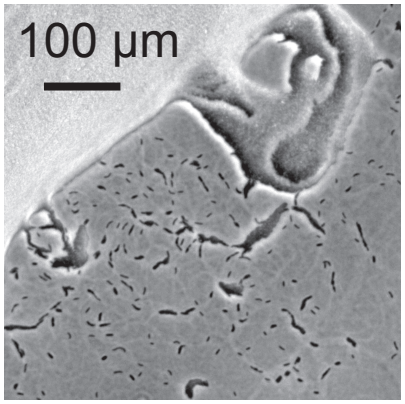
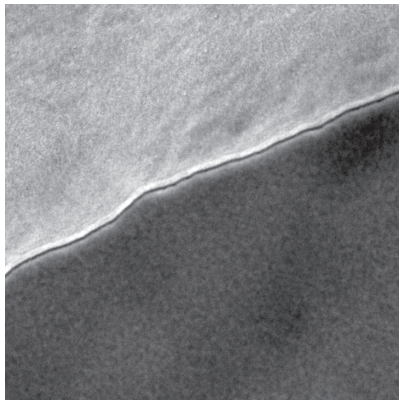
B

pilA



aglR pilA



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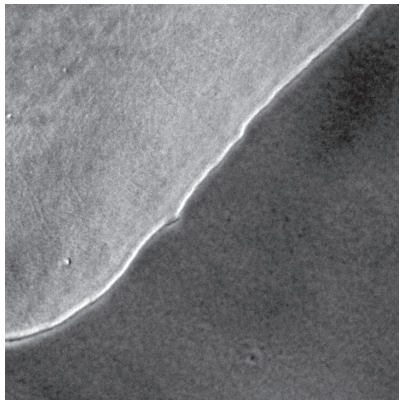
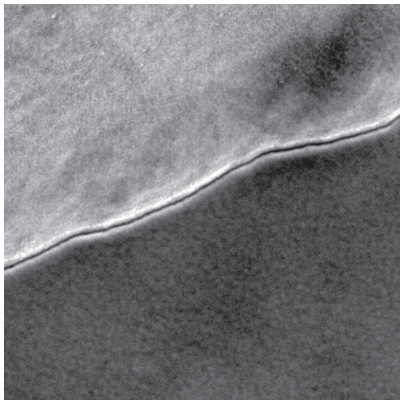
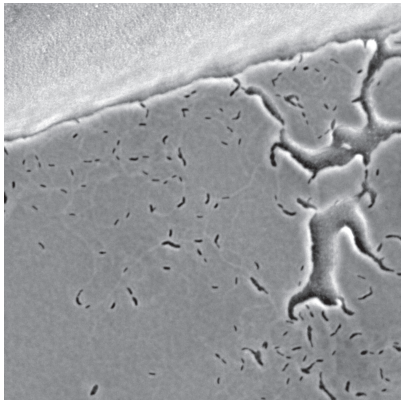


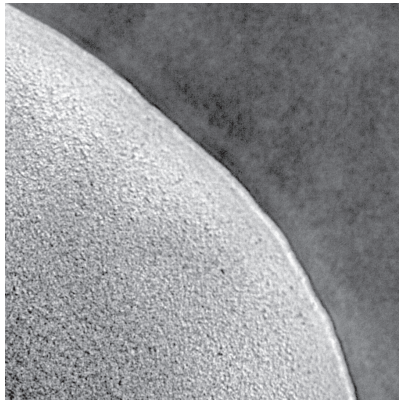
agmT^{EAEA} *pilA*



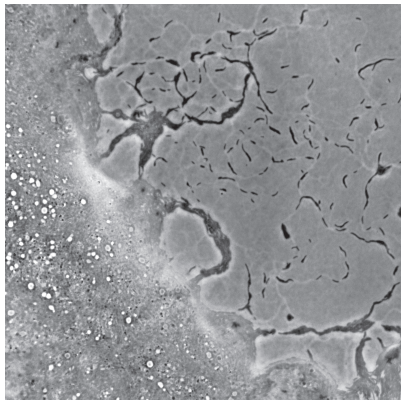
agmT-PAmCh pilA



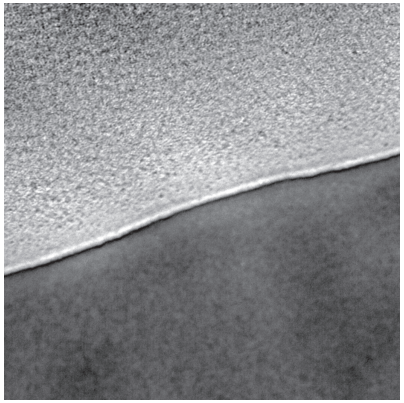
agmT pilA P_{van}*-vector*



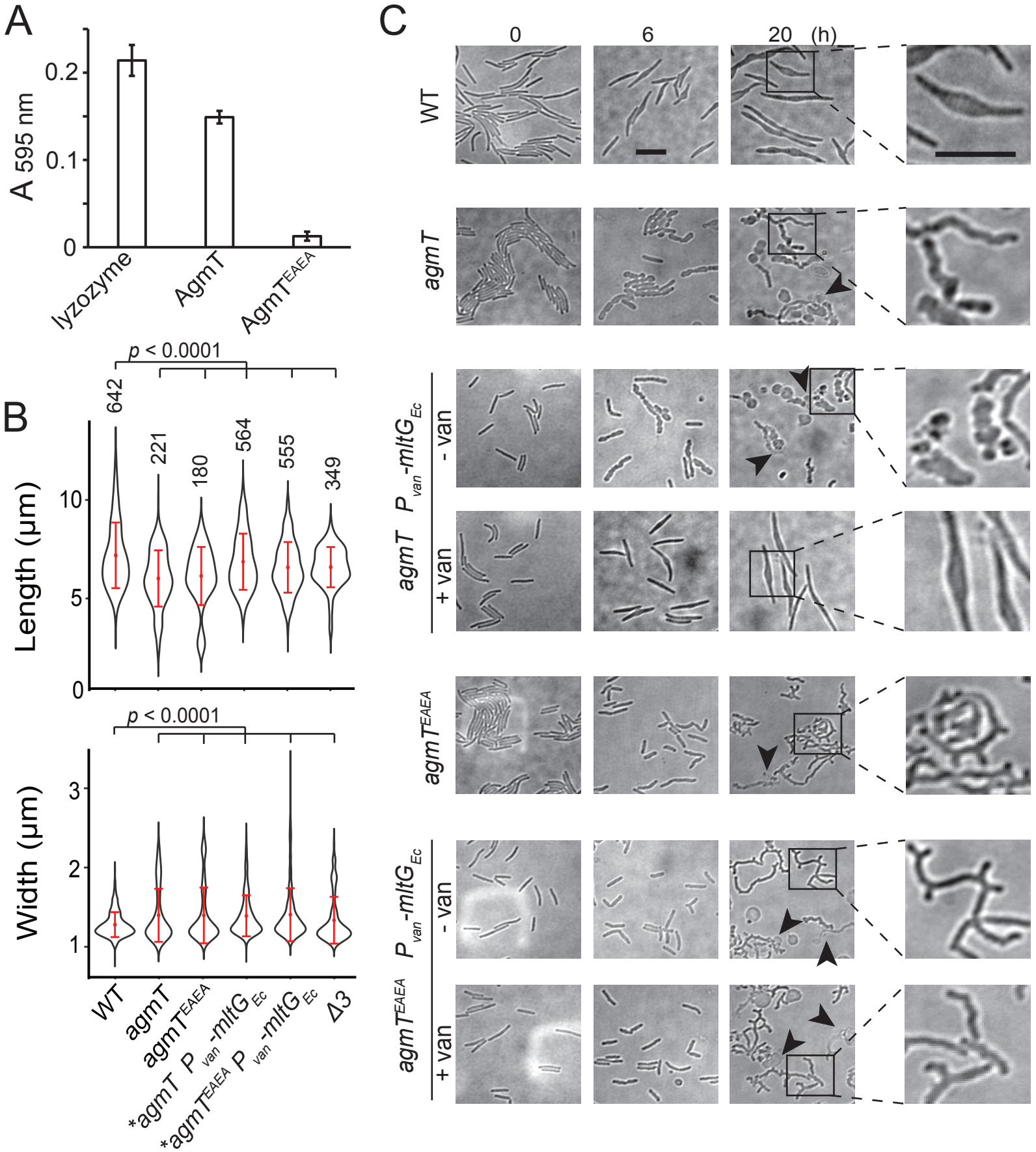
agmT pilA P_{van}*-mltG*$_{Ec}$

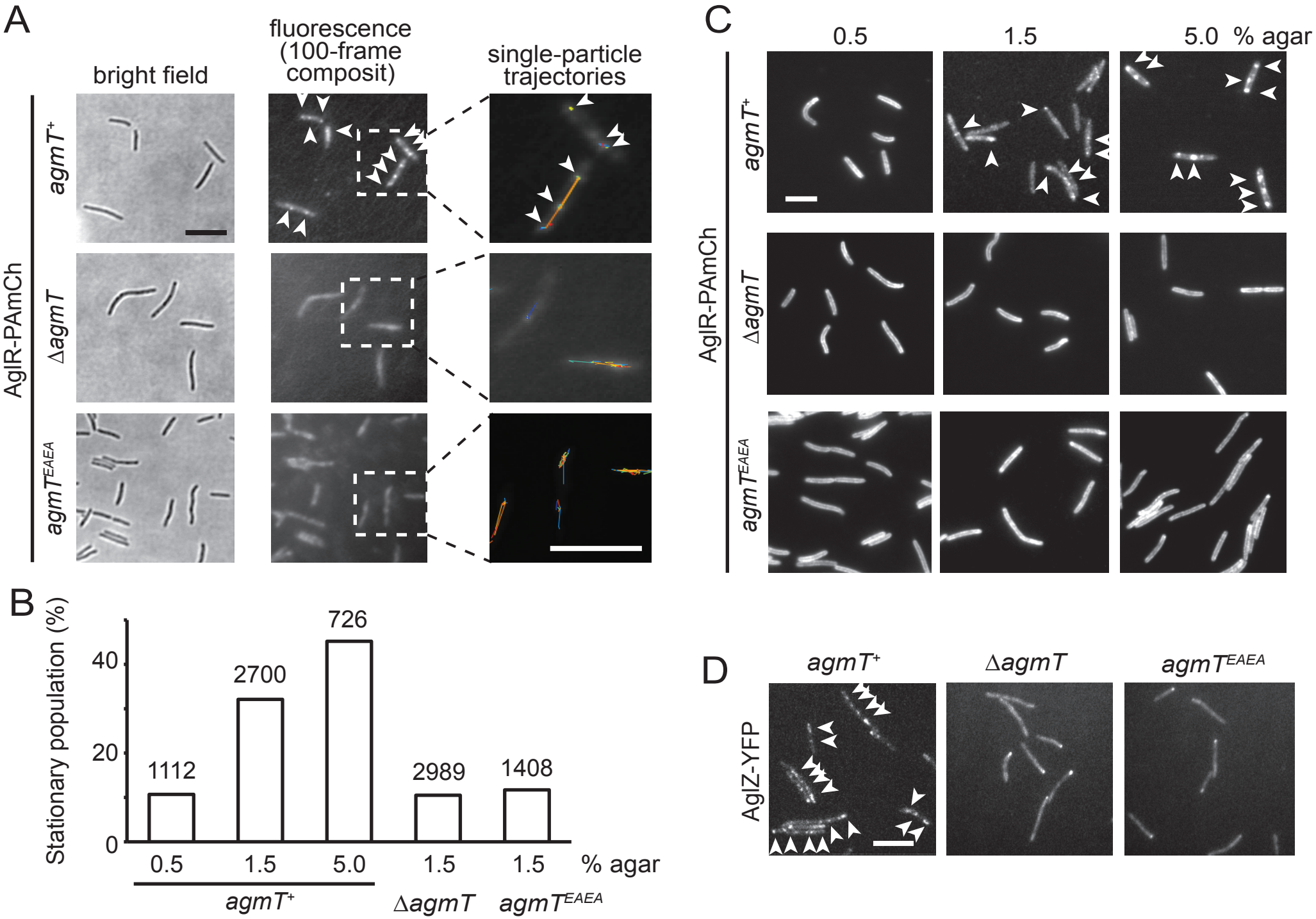


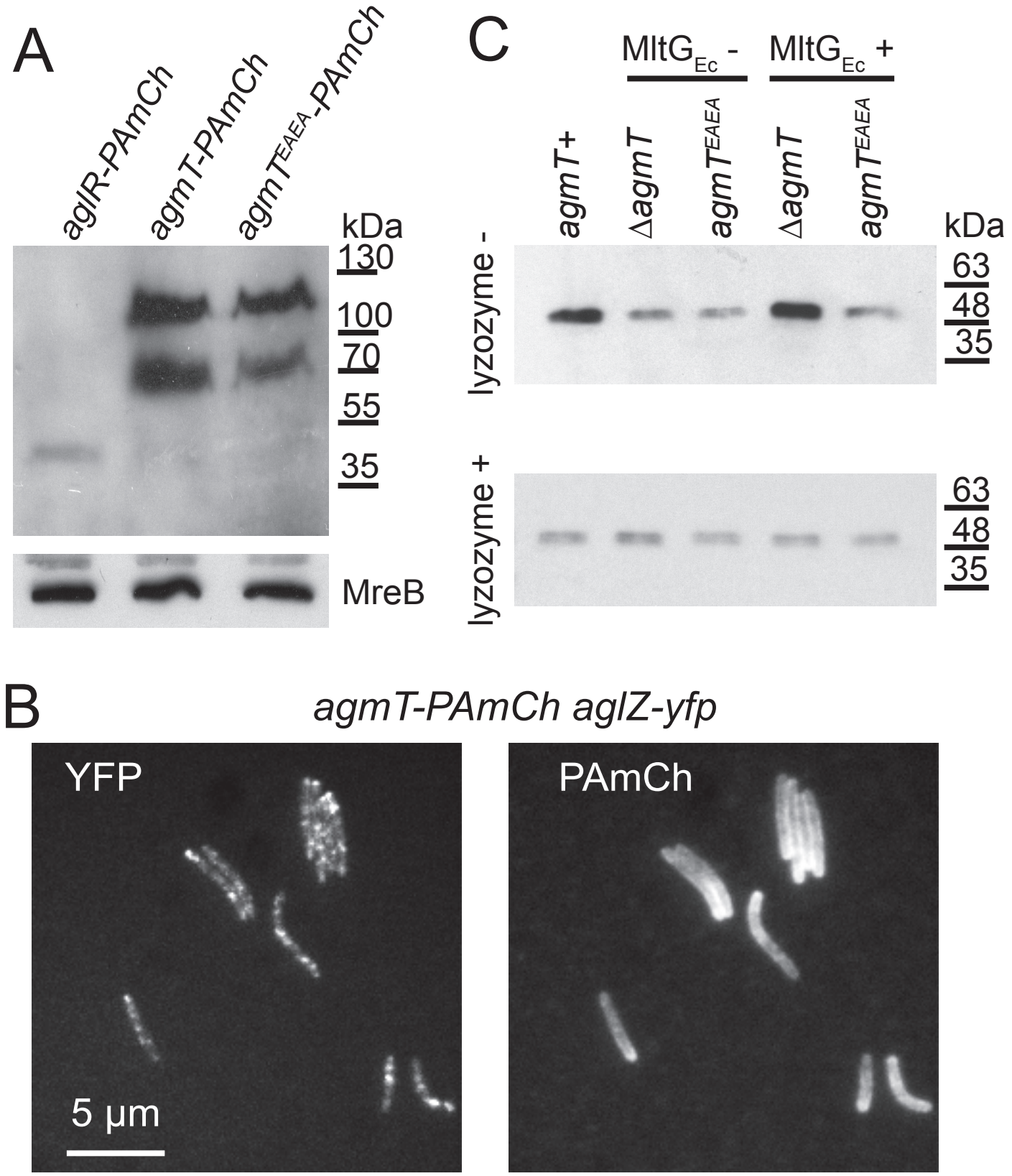
agmT^{EAEA} *pilA* P_{van}*-mltG*$_{Ec}$



+ 200 µM Na vanillate


A
A 595 nm
0.2
0.1
0
lyzozyme
AgmT
AgmT^EAEA
B
p < 0.0001
642
221
180
564
555
349
Length (µm)
10
5
0
p < 0.0001
Width (µm)
3
2
1
WT
agmT
agmT^EAEA
*agmT P_van-mltG_Ec
*agmT^EAEA P_van-mltG_Ec
Δ3
C
0
6
20 (h)
WT
agmT
agmT P_van-mltG_Ec
- van
+ van
agmT^EAEA
agmT^EAEA P_van-mltG_Ec
- van
+ van


A
bright field
fluorescence (100-frame composit)
single-particle trajectories
AglR-PAmCh
agmT+
ΔagmT
agmTEAEA
B
Stationary population (%)
0
20
40
1112
2700
726
2989
1408
0.5
1.5
5.0
1.5
1.5
% agar
agmT+
ΔagmT
agmTEAEA
C
0.5
1.5
5.0 % agar
AglR-PAmCh
agmT+
ΔagmT
agmTEAEA
D
agmT+
ΔagmT
agmTEAEA
AglZ-YFP


A
aglR-PAmCh
agmT-PAmCh
agmTEAEA-PAmCh
kDa
130
100
70
55
35
MreB
C
MltGEc -
MltGEc +
agmT+
ΔagmT
agmTEAEA
ΔagmT
agmTEAEA
kDa
lyzozyme -
lyzozyme +
63
48
35
63
48
35
B
agmT-PAmCh aglZ-yfp
YFP
5 µm
PAmCh