1   # CglB adhesins secreted at bacterial focal adhesions mediate gliding motility

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36 **ABSTRACT:**

37   The predatory deltaproteobacterium *Myxococcus xanthus* uses a helically-trafficked

38 motor at bacterial focal adhesion (bFA) sites to power gliding motility. Using TIRF and force

39 microscopy, we herein identify the integrin αI-domain-like outer-membrane (OM) lipoprotein

40 CglB as an essential substratum-coupling protein of the gliding motility complex. Similar to

41 most known OM lipoproteins, CglB is anchored on the periplasmic side of the OM and thus a

42 mechanism must exist to secrete it to the cell surface in order for it to interact with the

43 underlying substratum. We reveal this process to be mediated by a predicted OM β-barrel

44 structure of the gliding complex. This OM platform was found to regulate the conformational

45 activation and secretion of CglB across the OM. These data suggest that the gliding complex

46 promotes surface exposure of CglB at bFAs, thus explaining the manner by which forces exerted

47 by inner-membrane motors are transduced across the cell envelope to the substratum; they also

48 uncover a novel protein secretion mechanism, highlighting the ubiquitous connection between

49 secretion and bacterial motility.

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53 **Keywords:**

54 bacterial motility, integrins, protein secretion systems, molecular motor, cell-surface lipoprotein,

55 protein folding, disulphide bonds, von Willebrand Factor A, TonB-dependent transporter, outer-

56 membrane exchange

INTRODUCTION:

Directed surface motility of cells from all biological kingdoms involves highly-dynamic cell–substratum interactions. In eukaryotic cells, this process involves the engagement and activation of surface-exposed integrin(-like) adhesins, directionally transported by molecular motors (myosin) via integrin coupling to the internal cytoskeleton (actin)[1]. For metazoan organisms, nascent integrin adhesions to the extracellular matrix (ECM) lead to integrin nucleation and the formation of large eukaryotic focal adhesion (eFA) sites; these assemblies remain fixed-in-space relative to a translocating cell, promoting local traction, transduction of motor forces, and cell translocation[2]. Such surface motility is not restricted to eukaryotic cells. Long known to move on surfaces in the absence of outward appendages (such as flagella or type IV pili) via a process termed "gliding motility"[3], individual cells of the Gram-negative predatory deltaproteobacterium *Myxococcus xanthus* were shown to utilize a trans-envelope multi-protein Agl–Gliding transducer (Glt) complex (**Fig. 1A**) to power gliding motility[4,5]. In motile cells, these complexes associate at the leading pole and move directionally in the bacterial inner membrane (IM) toward the lagging cell pole, following a right-handed helical trajectory[6-8] (**Fig. 1A**). These rotational movements likely probe the substratum beneath gliding cells, leading to immobilization of the Agl–Glt complex at fixed bacterial focal adhesion (bFA) sites as well as cell translocation via left-handed rotation of the bacterium around its long axis[8] (**Fig. 1A**).

At the molecular scale, the mechanisms that promote force transmission and adhesion at bFAs are not known. The IM components of the motility complex assemble at the pole on a cytoplasmic scaffold formed by bacterial actin MreB, the Ras-like protein MglA and the coiled-coil AglZ protein[9,10]. The IM complex contains the molecular motor AglRQS, a TolQR/ExbBD/MotAB-like channel that uses the proton gradient formed across the bacterial IM to energize long-range movements of the IM complex in the bacterial envelope[11]. However, the manner in which these intracellular motions are coupled to the substratum in order to propel the cell is unknown. One hypothesis states that trafficking motor units deform the peptidoglycan meshwork in the periplasm, propagating surface wave deformations and viscous interactions between the outer membrane (OM) and the substratum (**Fig. 1A, panel i**). However, observations and mechanical modelling of cell–cell collision events suggest that interactions between a gliding cell and the substratum are elastic in nature, consistent with localized adhesion points and the existence of an anchored adhesin[12] (**Fig. 1A, panel i**). In the cell envelope, the IM

88   motor moves by establishing transient contacts with a complex of Glt proteins localized in the

89   OM (herein called the OM platform, see below), linked via periplasmic domains of retractile

90   proteins that traverse the peptidoglycan meshwork[8] (**Fig. 1A**). When in contact with the

91   substratum, these motions tether the OM platform at bFAs, which is proposed to create local

92   adhesions and movement of the cell (**Fig. 1A**). Consistent with localized adhesion at bFA sites,

93   gliding cells abandoned patches of OM motility-complex proteins on the substratum at positions

94   formerly occupied by bFA sites[8]. However, given that the OM platform is required for motor

95   movements, it could not be assessed whether the OM platform is directly responsible for

96   adhesion to the substratum[3,8] and thus, the manner of adhesion, a core aspect of the motility

97   mechanism, remained unaddressed.

98   The proteins GltA, GltB, GltH, and GltK constitute the proposed OM platform (**Fig. 1A,**

99   **panel ii**). While the exact structure of this platform is not known, GltA, GltB, and GltH are all

100   localized in the OM and are predicted to form integral OM β-barrels[3,4,8,13,14] (**Fig. 1**,

101   **Supplementary Fig. S1A-C**). GltA and GltB interact with each other, as well as with GltC

102   present in the periplasmic leaflet of the OM[13] (the function of GltC is not addressed herein).

103   GltK and GltH interactions have yet to be defined but data presented in our investigation suggest

104   that these proteins are functionally associated with the OM platform. Herein, we also identify the

105   OM lipoprotein CglB[15] as a main substratum-coupling adhesin of the Agl–Glt gliding motility

106   apparatus associated with the OM platform. Specifically, the results support the presence of a

107   protein assembly formed by GltA, B, H, and K that regulates CglB surface exposure, secreting it

108   to the cell surface upon stimulation of the motility complex at bFAs.

109 **RESULTS:**

110 ***GltA, B, and H form a putative integral OM protein complex***

111   The Glt OM platform contains predicted OM-spanning β-barrel proteins (**Fig. 1**,

112 **Supplementary Fig. S1A-C**) and thus is unlikely to mediate direct substratum-anchoring of the

113 motility complex. To probe the integral OM nature of these proteins, we tested the sensitivity of

114 GltA, GltB, and GltH in the OM to digestion by Proteinase K. In this assay, proteins that are

115 largely surface-exposed are rapidly digested by addition of exogenous protease, unlike proteins

116 that are protected by the membrane environment[13,16]. As expected for predicted integral OM

117 proteins, GltA, GltB, and GltH were not readily susceptible to digestion by Proteinase K at the

118 surface in WT cells (**Fig. 1B,C,D**). To probe the presence of an OM-platform protein complex,

119 the Proteinase K susceptibility of a given constituent was tested in the absence of a different

120 OM-platform protein; the rationale was that cell-surface topology for various OM-platform

121 proteins could be altered due to a disrupted interaction network resulting from the missing

122 platform component. As previously detected[13], neither GltA nor GltB was stable in a mutant

123 background lacking the other, as reflected by the lower levels of GltA in Δ*gltB* cells and GltB in

124 Δ*gltA* cells (compared to WT) (**Fig. 1B,C, Supplementary Fig. S1D**). This is consistent with

125 the demonstrated interactions between these integral OM proteins[13]. Absence of GltK did not

126 alter Proteinase K susceptibility of GltA, GltB, or GltH, indicating that this periplasm-facing OM

127 lipoprotein[13] does not affect the topology of the OM-platform proteins (**Fig. 1B,C,D**). However,

128 the absence of GltH rendered GltA and GltB more Proteinase K-sensitive (**Fig. 1B,C**).

129 Similarly, GltH was more sensitive to Proteinase K digestion in the absence of GltA or GltB

130 (**Fig. 1D**). The potential for these Proteinase K-susceptibility changes to be due to general OM

131 defects in the respective mutants is unlikely as none of the mutant strains demonstrate obvious

132 membrane and/or cell fitness defects[13]. These (and previous[13]) data thus support the existence of

133 an integral OM complex formed by GltABH proteins (**Fig. 1A, panel ii**). Functional analysis of

134 the role of the OM platform in regulating gliding-complex adhesion to the substratum was

135 subsequently evaluated below.

CglB, a predicted α-integrin-like protein, is a candidate motility adhesin

We next searched for a candidate adhesin that might interact with the OM platform. The CglB protein is an ideal candidate because it is essential for efficient gliding motility[4,15,17,18] (**Supplementary Fig. S2A**), localizes to the bacterial OM[14], and is pulled down as prey with various gliding complex members via GST chromatography using Glt-protein bait[5]. The *cglB* gene also co-occurs with those encoding the Agl–Glt machinery in bacterial genomes, supporting a functional link (**Supplementary Fig. S3**). Along with all Agl–Glt gliding apparatus components, we also found CglB to be required for "polymertropism" (**Supplementary Fig. S2D**), as determined by the ability of *M. xanthus* to preferentially spread in the direction of aligned substratum polymers[19-21]. This suggests that these proteins may play a role in probing the physical properties of the substratum at bFAs, similar to eukaryotic integrins that interact with the substratum and sense its physical properties at eFAs[22-24].

Fold-recognition analysis of CglB indicated structural analogies with numerous metazoan α-integrins and structurally-similar Apicomplexan parasite gliding motility adhesins MIC2 and TRAP (from *Toxoplasma* and *Plasmodium*, respectively)[25,26] (**Supplementary Table S2**). The top bacterial match was the αI/αA-like GBS104 adhesive tip pilin[27] from *Streptococcus agalactiae* (**Supplementary Table S2**). Integrins typically possess an α subunit in which a C-terminal domain of seven repeating beta blades constitutes a beta-propeller domain[28]. Of the 18 human α-subunit variants, half possess an additional module (termed αI or αA) in-between the 2nd and 3rd propeller blades containing a VWA domain[29], characterized by a Rossmann fold with multiple α-helices shielding an interior β-sheet[30]. VWA domains typically function in cell adhesion and can be found in numerous proteins of the eukaryotic ECM. Generation of a CglB tertiary structure model, combined with analysis of evolutionarily-coupled amino acid positions within the protein, confirmed that CglB likely adopts the above-described αI/αA structure containing a VWA domain (**Fig. 2A, Supplementary Fig. S1E**). In particular, the VWA domain contains a predicted MIDAS (metal ion-dependent adhesion site) motif, a discontinuous structural feature (Asp-x-Ser-x-Ser…Thr…Asp). At this site, the coordination of a divalent metal ion (e.g. $Mg^{2+}/Mn^{2+}/Ca^{2+}$) induces structural changes in VWA domains upon ligand binding that stabilize this adhesive domain in a high-affinity state for the ligand[31]. Incidentally, CglB-dependent gliding by individual cells on a hard substratum (**Fig. 2C**) was also found to require the presence of divalent cations (**Supplementary Figure S2G**). Highly-conserved

167   potential MIDAS residues in CglB map to D56, S58, S60, T182 and D211 (**Fig. 2A, inset**). We

168   tested the functional significance of the putative CglB MIDAS motif in a complementation

169   assay, ectopically expressing CglB$_{WT}$, CglB$_{D56A}$, and CglB$_{S58A}$ from the native *cglB* promoter.

170   Contrary to Δ*cglB* cells in which CglB$_{WT}$ expression restored motility, CglB$_{D56A}$ was stably

171   expressed (unlike CglB$_{S58A}$) but unable to complement gliding deficiency (**Supplementary Fig.**

172   **S2E, F**).

173   An intact VWA domain thus appears essential for CglB stability and single-cell gliding

174   motility. Direct demonstration of metal binding by the MIDAS motif of purified CglB was not

175   possible due to insurmountable purification challenges attributed to the high CglB Cys content

176   (17 out of 416 aa = 4.1%) (**Supplementary Fig. S2B**), with the majority of these residues

177   predicted to localize in flexible loops (**Fig. 2A**). These Cys residues likely form structure-

178   determining intra-protein disulphide bonds, as titration of the reducing agent DTT resulted in a

179   migration shift from faster- to slower-moving CglB-specific bands via SDS-PAGE and α-CglB

180   Western immunoblot (**Fig. 2B**). These data are also consistent with the high sensitivity of

181   single-cell gliding motility to low substratum concentrations of DTT (**Supplementary Fig.**

182   **S2C**). Taken together, the VWA domain MIDAS motif and intra-protein disulphide bonds are

183   likely important structural determinants of CglB.

184   *CglB is essential for substratum-coupling of the Agl–Glt machinery*

185   We subsequently investigated the contribution of CglB to surface coupling of the Agl–Glt

186   complex. To probe the role of CglB in bFA formation, we analyzed the dynamics of AglZ-YFP

187   clusters in cells on hard agar for the Δ*cglB* mutant (which stably expresses AglZ-YFP

188   [**Supplementary Fig. S4A**]). AglZ-YFP clusters still appeared in Δ*cglB* cells; however, in

189   marked contrast to WT cells, AglZ-YFP clusters in Δ*cglB* cells were not stationary relative to the

190   substratum but rather moved directionally from one pole to the other (**Fig. 2C,D** and

191   **Supplementary Fig. S4B**). This behaviour was similar to that observed in non-adhered motility

192   complexes[8]. CglB is therefore required to assemble bFAs on hard agar surfaces. The function of

193   CglB is clearly distinct from the OM-platform β-barrel proteins because trafficking AglZ-YFP

194   clusters are not formed in any of the Δ*gltA*/*B*/*H* mutant backgrounds[8].

195   In Δ*cglB* cells, trafficking AglZ-YFP clusters move in-and-out of the epifluorescence

196   focal plane as they rotate counter-clockwise around the cell envelope (**Fig. 1A, Supplementary**

197   **Fig. S4B**), making them difficult to precisely track and thus accurately study their dynamic

198   properties. To resolve these difficulties, we recently developed a TIRFM assay in which *M.*

199   *xanthus* cells glide in chitosan-coated microfluidic chambers (**Supplementary Fig. S4C**); in this

200   system, trafficking AglZ-YFP clusters are also observable in WT cells due to the suboptimal

201   nature of the chitosan surface for bFA adhesion[8,32]. Since the depth-of-field in TIRFM is

202   restricted to near the cell–substratum interface, photobleaching is reduced and thus tracking of

203   the trafficking AglZ-YFP clusters near the ventral face of the cell can be performed at high

204   spatio-temporal resolution[8]. On chitosan, Δ*cglB* cells were also non-motile and again,

205   immobilized AglZ-YFP clusters could not be detected (**Supplementary Fig. S4C**). Trafficking

206   AglZ-YFP clusters in Δ*cglB* cells behaved similarly to those in WT cells; although slight effects

207   were observed via TIRFM on the trafficking frequency of AglZ-YFP clusters (from the leading

208   to the lagging cell poles), the trafficking speed and lifetime of AglZ-YFP clusters were

209   unchanged in the absence of CglB (**Fig. 2E-H**). Since AglZ-YFP trafficking reflects the activity

210   of the motility engine, we conclude that CglB does not affect motor activity, but rather its

211   adhesion to the underlying substratum at bFAs.

212   To test the contribution of adhesive properties by CglB to the tip of the motility complex,

213   we adopted a force microscopy approach; herein, force generation by the motility complex can

214   be directly monitored in live *M. xanthus* cells immobilized atop a semi-solid 0.7% agarose

215   matrix deposited on glass slides[11]. Within this setting, the motility complex cannot propel cells

216   (likely because it cannot adhere) but its activity can transport polystyrene beads deposited by an

217   optical trap directly on the cell surface (**Fig. 2I**). Trafficking gliding-machinery units that collide

218   and recruit such beads move them directionally over long distances[11,12] (**Supplementary Fig.**

219   **S4D**). We therefore tested whether bead transport requires the CglB adhesin. Whereas beads

220   were transported multiple times, at lengths up to ~8 μm, along the surface of WT cells, such

221   events were absent in ΔcglB cells (**Fig. 2J**). This demonstrates that bead recruitment and

222   trafficking requires CglB, consistent with adhesive and force transduction functions for CglB.

223   Taken together, we conclude that CglB is required for tethering the gliding motility

224   complex to an engaged extracellular motif, be it a solid surface for cell gliding or cargo for

225   transport in immobilized cells. Contrary to the OM-platform proteins GltA, B, and H[8] (see

226   below), CglB is not required for Agl–Glt-complex assembly and trafficking suggesting that it

227   functions to couple trafficking units to the substratum, as would be expected for an adhesin.

The Glt OM platform regulates CglB exposure and retention at the cell surface

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229   Our various attempts to localize CglB in gliding cells were unsuccessful due to loss of

230   protein functionality when fused to fluorescent protein or epitope tags (the folding of CglB in the

231   periplasmic space, as well as its transit across the OM, are likely limiting steps, see below).

232   Localizing CglB to the cell surface is nevertheless essential because CglB is an OM

233   lipoprotein[14,15]; as such it would canonically be thought to insert into the periplasmic leaflet of

234   the OM, though the prevalence of surface-exposed lipoproteins is becoming more widely

235   acknowledged[33-35]. Indeed, Proteinase K shaving of liquid-grown cells (in which the motility

236   complex is not active) did not affect CglB stability, suggesting that CglB is periplasmically-

237   oriented under these conditions (**Supplementary Fig. S5A**). One possibility is that CglB surface

238   exposure is directly regulated by the Glt OM platform. To explore this possibility, we first

239   compared CglB levels in whole-cell samples of each respective *glt* mutant strain. While present

240   at comparable levels in Δ*gltC, D, E, F, G, H, I,* and *J* backgrounds, cell-associated CglB was

241   severely depleted in OM-platform mutants Δ*gltA*, Δ*gltB*, and Δ*gltK* (but not Δ*gltH*) (**Fig. 3A**).

242   Fractionation analysis revealed that CglB was still produced by the Δ*gltA*/*B*/*K* mutants (**Fig. 3B**);

243   however, unlike in WT cells — where CglB was detected in whole-cell and outer-membrane

244   vesicle (OMV) fractions — CglB in these three mutant backgrounds was only recovered in

245   culture supernatants (**Fig. 3B, Supplementary Fig. S5B**). In the Δ*gltA* and Δ*gltB* mutants, such

246   shedding to the supernatant was not observed for GltK (**Fig. 3B**), another OM lipoprotein

247   required for gliding motility which remains OMV-associated, nor for the intracellular protein

248   MglA, which was detected at levels comparable to the WT strain (**Fig. 3B**); together, these data

249   indicate that cell integrity is not affected when components of the OM platform are disrupted.

250   Therefore, cell-association (and thus OM localization) of CglB depends on GltA, GltB, and

251   GltK.

252   Supernatant-localized CglB migrates faster than cell-associated CglB via SDS-PAGE (in

253   both whole-cell and OMV samples) under equivalent denaturing conditions (**Fig. 3B**), suggesting

254   that supernatant CglB is of reduced molecular weight and may have been proteolytically cleaved.

255   Further support for proteolytic processing of CglB was provided via mass spectrometry analysis

256   of tryptic peptides obtained from immunoprecipitated supernatant CglB, which revealed that the

257   first 76 N-terminal residues were unaccounted for (**Supplementary Fig. S5C**). Our efforts at N-

258   terminal sequencing of supernatant CglB were inconclusive, and as such we were unable to

259   determine the specific identity of the amino acids at the new N-terminus of the truncated protein.

260   Thus CglB may be cleaved by a protease prior to its release into the supernatant. To test this

261   hypothesis, we screened the effect of various protease inhibitors for their capacity at restoring

262   CglB localization to the cell envelope. Growth in the presence of EDTA restored cell-associated

263   CglB in this background (**Fig. 3C**). Similarly, EDTA also restored cell-associated CglB in the

264   Δ*gltK* and Δ*gltA* mutants (**Fig. 3D**; see below for discussion of doublet band). Resuspension of

265   EDTA-grown Δ*gltB* cells in EDTA-free buffer resulted in the resumption of CglB release from

266   the cells, indicating that CglB restoration is not permanent, consistent with a protease inhibition

267   effect (**Supplementary Fig. S5D**). Since EDTA chelates divalent cations, CglB release from

268   Δ*gltK*/*B*/*A* cells would be consistent with the activity of a metalloprotease. While the

269   involvement of a putative protease in CglB surface shedding awaits further clarification, the

270   results suggest that interactions between CglB, GltA, GltB, and GltK regulate CglB cell-surface

271   association.

272   To determine the subcellular site of CglB proteolytic processing in the Δ*gltA*, *B* and *K*

273   mutants, we took advantage of the fact that EDTA blocks proteolysis and probed surface-

274   exposure of CglB via the Proteinase K shaving assay. In marked contrast to WT cells and in the

275   presence of EDTA, cell-associated CglB (**Fig. 3D**) was immediately digested by Proteinase K in

276   the Δ*gltK*, Δ*gltB*, and Δ*gltA* backgrounds (**Fig. 3E**, see below for discussion of doublet band).

277   Interestingly, upon digestion of full-length CglB in Δ*gltA* cells, there was an immediate

278   appearance of a ~34 kDa CglB degradation product that persisted throughout the time course,

279   suggesting that it was protected from further digestion. This protection required GltK and GltB

280   as the ~34 kDa product was almost undetectable in these backgrounds (discussed further below).

281   GltH is not as central as GltA and GltB with respect to surface retention of CglB because CglB

282   remains cell-associated in the *gltH* mutant (**Fig. 3A**). Nevertheless, CglB also shows increased

283   sensitivity to Proteinase K in the absence of GltH (with the appearance and steady accumulation

284   of an ~34 kDa Proteinase K-resistant band), suggesting that a fraction of the CglB population is

285   also surface exposed (**Fig. 3E**). These results thus indicate that CglB becomes surface-exposed

286   in the absence of individual OM-platform components GltA, B, K, and to a lesser extent GltH

287   (see Discussion).

288

The Glt OM proteins are required for CglB exposure at the cell surface

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290   Two hypotheses could explain the cell-surface protease sensitivity of CglB in

291 Δ*gltA/B/K/H* cells: (i) CglB accesses the cell surface via an as-yet unknown system and interacts

292 with the Glt OM platform proteins, which shield it from the action of the putative surface

293 metalloprotease. Alternatively, (ii) the Glt OM platform proteins are directly responsible for

294 CglB cell-surface exposure through a regulated process that becomes constitutive as soon as one

295 of its components (i.e. GltA, B, K, and to a lesser extent H) is removed.

296   To discriminate between these hypotheses, we probed cell-association of CglB in

297 Δ*gltABH* triple-mutant cells lacking any of the β-barrel components of the OM platform. The

298 level of cell-associated CglB in these triple-mutant cells was markedly restored (although not

299 fully to WT levels, possibly due to stabilization of CglB by interactions with the OM gliding

300 proteins) (**Fig. 3F**). This rules out the first hypothesis. Using various combinations of double β-

301 barrel component knockouts, CglB restoration was found to minimally require the simultaneous

302 deletion of *gltA* and *gltH* (**Fig. 3F**). To examine whether the restoration of cell-associated CglB

303 was due to a lack of surface exposure of the adhesin, we performed Proteinase K shaving of

304 Δ*gltABH* cells. In this background, CglB was fully protected from digestion by Proteinase K,

305 illustrating that CglB is no longer surface-exposed (**Fig. 3G**). Taken together, the above results

306 indicate that interactions between GltA, B, K and H regulate CglB exposure at the cell surface.

307 Importantly, full Proteinase K-protection of CglB in the Δ*gltABH* triple mutant (i) is a strong

308 indication that these proteins play a central role in modulating CglB surface exposure, and (ii)

309 rules out non-specific OM defects in the absence of OM platform components.

GltK is required for CglB function in vivo

310   While analysing CglB restoration in EDTA-grown cells, CglB was observed to migrate

311 as two bands in WT cells: a major slower-migrating band and a minor faster-migrating band

312 (**Fig. 3D**). In similar conditions, only the faster-moving band was observed in the Δ*gltK*

313 background. Thus, GltK could be involved in the formation of the slower-migrating species, a

314 process in which it may be assisted by GltB and to a lesser extent by GltA, because the slower-

315 migrating form accumulated to various ratios in these mutants, with almost full efficiency in the

316 Δ*gltA* mutant (**Fig. 3D**). It is not currently clear if the two CglB forms result from a stabilization

317 or maturation effect of GltK on CglB, but the slower-migrating CglB band constitutes the most

318 abundant species in WT extracts (**Fig. 3D**) and thus probably represents the functional form of

319 CglB, which we subsequently confirmed (see below).

320   GltK could potentially interact with CglB via Cys residues as it also possesses a high Cys

321 ratio (9 out of 184 aa = 4.9%) relative to other Glt proteins (**Supplementary Fig. S2B**) and

322 displays redox-dependent folding (**Fig. 4A**); but in the absence of an *in vitro* assay with purified

323 proteins, this could not be directly tested. Nevertheless, the potential for GltK to promote CglB

324 function *in vivo* was examined via two further experiments. First, TIRFM-analyzed clusters in

325 Δ*gltK* cells revealed cluster behaviour analogous to that observed in Δ*cglB* cells (**Fig. 2C-G**);

326 namely, clusters travelled directionally within Δ*gltK* cells with lifetimes equivalent to those in

327 WT cells, but with higher trafficking frequency (**Fig. 4B, C, D**). The trafficking speed of

328 clusters was found to be statistically faster in Δ*gltK* cells compared to WT (**Supplementary Fig.**

329 **S4E**), but within the same tight range already described for Δ*cglB* cells (**Fig. 2F**). In contrast, no

330 trafficking clusters were observed in Δ*gltA* cells (**Fig. 4B**), consistent with previous results[8].

331 This demonstrates that although GltA is required for cluster formation and trafficking, GltK is

332 not (similar to CglB). Combined with the Proteinase K-susceptibility data (**Fig. 1B, C**), these

333 results suggest that in the absence of GltK, the OM platform is assembled and conducive to

334 AglZ-YFP trafficking but that CglB-mediated adhesion is not functional.

335   We next sought to genetically demonstrate that GltK permits CglB to function *in vivo*.

336 We thus exploited the known ability of *M. xanthus* to transfer OM lipoproteins between

337 compatible cells by a process known as OM exchange (OME)[36]. During OME, direct

338 interactions of TraA OM receptors lead to transient OM fusion synapses between adjacent cells

339 and efficient exchange of OM lipids and lipoproteins between contacting cells[37,38]. In this

341   manner, OME of CglB can efficiently rescue gliding motility when donor CglB^{+} cells are mixed

342   with recipient CglB^{−} cells[15]. GltK (previously CglC) is also a transferable OM lipoprotein[13,17].

343   Therefore, we reasoned that if ΔgltK cells were mixed with ΔcglB cells, motility rescue of the

344   ΔcglB cells would only occur if bi-directional OME allows GltK transfer (from the ΔcglB donor

345   cells) to activate CglB in the ΔgltK cells (**Fig. 4E**). To ensure that gliding motility rescue could

346   only arise from the *trans*-complementation of the ΔcglB cells, an *aglQ* mutation was added to the

347   CglB[+] donor, thus deleting an essential component of the Agl gliding motor[11]. This motor

348   protein cannot be transferred via OME as it is an integral IM protein. Introduction of a

349   fluorescent mCherry marker into the CglB[−] recipient strain allowed for (i) direct observation of

350   motility rescue (**Fig. 4F**) and (ii) separation of mCherry^{+} cells via fluorescence-activated cell

351   sorting (FACS) (**Fig. 4G**) for testing of CglB transfer by Western immunoblot. The ΔaglQ

352   donor strain functioned as an effective donor for both CglB and GltK, ensuring that the

353   experiment could be performed (**Fig. 4F**, Mix 1 & Mix 2). Upon mixing ΔaglQ ΔgltK double-

354   mutant cells (GltK[−] CglB[+]) with mCherry[+] ΔcglB (GltK[+] CglB^{−}) cells, gliding motility of the

355   mCherry^{+} cells was restored (**Fig. 4F**, Mix 3). Western immunoblotting of FACS-separated

356   mCherry^{+} cells (**Fig. 4G**) confirmed that this gliding restoration was due to CglB transfer (**Fig.**

357   **4H**).

358   The prediction that GltK transfer from GltK[+] CglB^{−} cells to GltK[−] CglB^{+} cells is essential

359   for CglB transfer and stimulation of GltK[+] CglB[−] cell motility was tested by mixing ΔaglQ

360   ΔgltK cells (GltK[−] CglB[+]) with an mCherry[+] ΔcglB ΔgltK (CglB[−] GltK[−]) strain. In this case, no

361   motility stimulation was observed (**Fig. 4E,F**, Mix 4) nor was any CglB transferred via OME

362   (**Fig. 4H**). The lack of CglB transfer is likely due to a defect in CglB maturation/stabilization

363   that occurs in the absence of GltK because mixing a ΔgltA mutant — where CglB is also secreted

364   but where the slower-migrating CglB form accumulates to near-WT levels (**Fig. 3D**) — with a

365   ΔcglB mutant still resulted in efficient motility stimulation (GltA itself is not transferable

366   [**Supplementary Fig. S6**]). GltK is thus required for CglB function *in vivo* by promoting the

367   accumulation of the slower-migrating CglB band, which either occurs as GltK protects CglB

368   from proteolysis or promotes a maturation step.

369 **DISCUSSION:**

370   Previously, we demonstrated that on hard surfaces *Myxococcus* cells are propelled by

371 directionally-transported Agl–Glt complexes that become tethered at bFAs where they exert

372 traction forces against the underlying substratum[8]. However, while we demonstrated that the

373 external OM Glt proteins are effectively anchored to the surface, the specific protein(s)

374 conferring the adhesive properties, as well as the mechanism by which such adhesion is

375 regulated to promote forward movements, remained unknown. The characterization of CglB, a

376 protein first studied >40 years ago[17], and its interactions with the OM Glt proteins provides a

377 solution to these questions.

378   Together, the data described in our current investigation indicate that CglB exposure at

379 the cell surface and its proper functioning is a complex process requiring the concerted actions of

380 a host of additional proteins such as GltK, GltB, GltA, and GltH. Combining these data with

381 previously-published findings, we propose the following model for CglB-mediated surface

382 coupling of the Agl–Glt complex at bFAs:

383 (i) Following its assembly at the leading cell pole, the cytoplasmic–IM–periplasmic Agl–Glt

384   complex contacts the periplasmic face of OM gliding machinery modules, constituted by

385   GltKBAH "loaded" with CglB, through the peptidoglycan meshwork; this initiates a

386   right-handed helical movement of the machinery toward the lagging cell pole. No bFA is

387   formed until the complex reaches the "ventral side" of the cell that lies in contact with the

388   underlying substratum (**Fig. 5, *Step 1***).

389 (ii) Once the cytoplasmic–IM–periplasmic module contacts a substratum-associated OM

390   module, the contractile force/action of the cytoplasmic–IM–periplasmic module on the

391   OM complex "fires" CglB into activated contact with the substratum (**Fig. 5, *Step 2***).

392   Substratum-anchored CglB is thus connected across the OM by GltK and the associated

393   GltABH OM proteins, interacting with other periplasmic Glt proteins[8]. This physically

394   links the substratum and the IM motor, allowing for force transduction (**Fig. 5, *Step 3***, see

395   below).

396 (iii) After the power stroke of the IM constituents of the Agl–Glt machinery, the substratum-

397   adhered OM module is displaced, resulting in displacement of the OM and peptidoglycan

398   sacculus (**Fig. 5, *Step 4***). Displacement requires substratum-bound CglB to be turned

399   over to allow new OM modules (containing CglB not yet bound to the substratum) to

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400   interact with the IM motor (**Fig. 5, *Step 4***) and to propel the screw-like movement of the

401   cell (**Fig. 5, *Step 5***). Such turnover could occur by site-specific proteolysis of CglB,

402   regulated by the GltABHK complex (**Fig. 5, *Step 3***). This hypothesis is attractive

403   because proteolytic turnover also occurs for integrin adhesions in metazoans[39] and de-

404   regulation of CglB secretion triggers cell-surface cleavage of CglB at its N-terminus.

405   However, this proteolytic event could also be a natural consequence of unregulated

406   surface exposure of the adhesin in liquid-grown cells, with other potential adhesin-

407   cycling mechanisms functioning instead on solid surfaces.

408

409   Our findings assign an adhesion function to the CglB lipoprotein and provide a potential

410   mechanism explaining the manner in which this protein accesses the cell surface. While OM

411   lipoproteins are generally thought to be exposed on the periplasmic leaflet of the OM, surface-

412   exposed lipoproteins have recently come to the fore in bacterial cell biology[34]. Although several

413   scenarios have been proposed, the surface-exposure mechanism has not been solved for most of

414   these lipoproteins[33]. Interestingly in *Escherichia coli*, RcsF, an OM lipoprotein sensor of cell-

415   envelope stress, becomes exposed at the cell surface as it interacts with the β-barrel porin

416   OmpA[40,41]. For CglB, our results suggest that OM translocation could be more complex and

417   driven by a dedicated system formed by the GltA, B, H, and K proteins. On the periplasmic

418   leaflet of the OM, GltK promotes CglB function via a maturation or stabilization process, which

419   is also somewhat assisted by GltA and GltB. It is not currently clear whether GltA, B, and H

420   form a complex that secretes CglB directly or whether it regulates the secretion activity of

421   another system. While the latter cannot be formally ruled out, the data suggests that GltH and

422   GltA could form pores that secrete CglB redundantly, with GltH functioning as the major pore:

423   (i) GltH is responsible for constitutive CglB secretion in the Δ*gltA, B,* and *K* mutants, and (ii) the

424   simultaneous deletion of *gltA* and *gltH* abolishes CglB secretion. The CglB adhesin is partially

425   accessible to Proteinase K in the absence of GltH (whereas it is fully protected in WT cells) also

426   suggesting the existence of a minor GltH-independent route to the cell surface. Solving the

427   actual structure and exact interactions within the OM platform is an ambitious endeavour, but it

428   will be ultimately necessary to understand the manner in which interactions between three

429   predicted porin-like proteins regulate CglB surface exposure.

430

431 CglB secretion in liquid-grown cells is not observed in WT cells and is likely a by-

432 product of the sensitized genetic backgrounds. Thus in WT cells CglB surface exposure appears

433 tightly controlled. We propose that CglB is only exposed at bFAs following dynamic

434 interactions between the IM and OM complexes[8]. The exact protein interaction cascade has yet

435 to be defined, but the proton-motor-associated IM-spanning proteins GltG/J have been proposed

436 to undergo motor-driven extension/retraction cycles in a retractile mechanism that would allow

437 cyclic interaction with the OM complex through the peptidoglycan[8]. Both proteins carry

438 predicted TolA/TonB-like flexible periplasmic domains and globular periplasmic TonB_C

439 domains[3,8]. In ExbBD-TonB systems, these domains interact with TonB-box motifs in OM

440 receptor proteins. Both OM β-barrel proteins GltB and GltA contain extended, unstructured N-

441 terminal domains, with a potential TonB-box consensus sequence in the former[8]. Thus, the

442 motorized IM proteins could mechanically act on the OM platform loaded with CglB, resulting

443 in its export past the OM (through the OM platform or as-yet unidentified proteins) and

444 activation of the adhesin upon engagement of the substratum. Conceptually, this mechanism

445 could be compared to the firing of a gunlock cannon in which an arm (GltG/J) exerts mechanical

446 force on a lanyard (TonB box on the Glt OM module), resulting in firing of a projectile (CglB)

447 through the barrel of the cannon (GltABH).

448 We therefore propose that the gliding motility complex functions akin to a secretion

449 apparatus, in which a trafficking unit permits the surface exposure and cycling of an adhesin

450 when assembled at bFAs. Energy for the secretion process would come from the H^+ gradient[11].

451 In bacteria, TonB-dependent transporters (TBDTs) are generally viewed as macromolecular

452 import systems for siderophores and carbohydrates, but recently, the *Myxococcus* β-barrel TBDT

453 Oar was found to be required for secretion of a developmental protease across the OM[42]. Thus,

454 protein secretion may be an overlooked function of TBDTs, which should now be taken into

455 account when studying these systems. In particular, the secretory function of the Agl–Glt system

456 may shed light on its evolutionary origin. Previously, we demonstrated that the Agl–Glt system

457 potentially evolved from a general class of envelope systems with functions as diverse as cellular

458 motility and spore coat assembly[43,44]. Bacterial genome-mining analyses indicate that

459 deltaproteobacterial Agl–Glt-like systems may have evolved following acquisition and a

460 dramatic expansion of a widely-conserved gammaproteobacterial "core" complex[43,44].

461 Importantly, this proposed core system is constituted by seven conserved genes (encoding AglQ,

462   R, S, GltC, D, E, and G homologues) including one encoding a putative TBDT (AglRQS–GltG)

463   and OM proteins in the periplasmic leaflet (GltD and E). The original function of the core

464   complex is not known but its composition could be sufficient for protein secretion. This scenario

465   is hypothetical but the results reported herein demonstrate that *Myxococcus* gliding motility and

466   protein secretion are intertwined; this appears to be a common theme across diverse bacterial

467   motility mechanisms as exemplified by flagellar motility (Type-III secretion), twitching motility

468   (Type-IV pili/Type-II secretion), and Bacteroidetes gliding motility[45,46]. Bacteroidetes gliding

469   presents an interesting parallel: a Type-IX secretion system secretes a gliding motility adhesin

470   (SprB) which becomes propelled along the cell surface by the gliding machinery[47-50]. In fact, the

471   Type-IX secretion system may be directly embedded into the Bacteroidetes gliding machinery;

472   thus although they are evolutionarily distant, Bacteroidetes and myxobacterial gliding motility

473   may follow similar operational principles.

474   Finally, the discovery that CglB acts as an essential adhesin of the gliding motility

475   complex further highlights functional parallels between bFA and eFA mechanisms. In

476   eukaryotic cells, the migration of surface-adhered cells via eFA-based locomotion involves the

477   coordinated actions of a trans-envelope suite of proteins to transduce integrin-mediated cell–

478   substratum adhesion to mechanical force and movement to propel the cell forward. Adhesion to

479   the substratum at sites of activated integrins is regulated in multiple ways, including via cellular

480   chaperones[51] and surface proteases[39,52]. Similar to eFAs, bFAs also interact with a secreted

481   ECM[53]; herein the CglB VWA domain could potentially probe the physical properties of the

482   substratum, akin to the scenario in eukaryotic integrins[22-24]. Thus, the fundamental requirements

483   for integrin-coupled focal adhesion-based motility may be similar in metazoan, protozoan, and

484   bacterial cells. These parallels could be important to further elucidate the mechanisms by which

485   motility promotes multicellular behaviors[54]: similar to eukaryotic integrins, CglB could act as a

486   sensor, regulating cell-cell interactions during development and predation.

512   **AUTHOR CONTRIBUTIONS:**

513   STI and TM conceived of and planned the study. STI performed bright field microscopy

514   and stereoscopy. STI and BJB carried out epifluorescence microscopy, with fluorescent cluster

515   analysis by TM. JBF completed TIRFM, with analysis by LMF. STI, NYJ, and FS quantified

516   cell motility. STI and AMB performed force microscopy, with analysis by AMB and JWS. LM

517   and GB carried out FACS experiments. GS generated phylogenetic data. STI performed protein

518   modelling and evolutionary coupling analysis. STI, LM, and BF generated mutant constructs.

519   DJL tested polymertropism responses, with analysis by STI and DJL. STI, LM, and NYJ

520   performed all Proteinase K digestions and processed all Western blots. LM performed

521   fractionation assays. BF generated anti-CglB pAb. STI and TM wrote the manuscript. STI and

522   TM generated figures. STI, MN, MS, AGG, JWS, and TM contributed personnel and/or funding

523   support.

524 **DECLARATION OF INTERESTS:**

525 The authors declare no competing interests.

REFERENCES:

526

527   1   Sun Z, Guo SS, & Fässler R, Integrin-mediated mechanotransduction. *J Cell Biol* **215**, 445-
528   456 (2016).

529   2   Kanchanawong P, Shtengel G, Pasapera AM, Ramko EB, Davidson MW, Hess HF, &
530   Waterman CM, Nanoscale architecture of integrin-based cell adhesions. *Nature* **468**, 580-
531   584 (2010).

532   3   Islam ST & Mignot T, The mysterious nature of bacterial surface (gliding) motility: a focal
533   adhesion-based mechanism in *Myxococcus xanthus*. *Semin Cell Dev Biol* **46**, 143-154
534   (2015).

535   4   Luciano J, Agrebi R, Le Gall AV, Wartel M, Fiegna F, Ducret A, Brochier-Armanet C, &
536   Mignot T, Emergence and modular evolution of a novel motility machinery in bacteria.
537   *PLOS Genet* **7**, e1002268 (2011).

538   5   Nan B, Mauriello EMF, Sun I-H, Wong A, & Zusman DR, A multi-protein complex from
539   *Myxococcus xanthus* required for bacterial gliding motility. *Mol Microbiol* **76**, 1539-1554
540   (2010).

541   6   Nan B, Bandaria JN, Moghtaderi A, Sun I-H, Yildiz A, & Zusman DR, Flagella stator
542   homologs function as motors for myxobacterial gliding motility by moving in helical
543   trajectories. *Proc Natl Acad Sci USA* **110**, E1508-E1513 (2013).

544   7   Fu G, Bandaria JN, Le Gall AV, Fan X, Yildiz A, Mignot T, Zusman DR, & Nan B,
545   MotAB-like machinery drives the movement of MreB filaments during bacterial gliding
546   motility. *Proc Natl Acad Sci U S A* **115**, 2484-2489 (2018).

547   8   Faure LM, Fiche J-B, Espinosa L, Ducret A, Anantharaman V, Luciano J, Lhospice S, Islam
548   ST, Tréguier J, Sotes M, Kuru E, Van Nieuwenhze MS, Brun Y, Théodoly O, L A,
549   Nollmann M, & Mignot T, The mechanism of force transmission at bacterial focal adhesion
550   complexes. *Nature* **539**, 530-535 (2016).

551   9   Mauriello EMF, Mouhamar F, Nan B, Ducret A, Dai D, Zusman DR, & Mignot T, Bacterial
552   motility complexes require the actin-like protein, MreB and the Ras homologue, MglA.
553   *EMBO J* **29**, 315-326 (2010).

554   10   Treuner-Lange A, Macia E, Guzzo M, Hot E, Faure L, Jakobczak B, Espinosa L, Alcor D,
555   Ducret A, Keilberg D, Castaing JP, Gervais SL, Franco M, Søgaard-Andersen L, & Mignot
556   T, The small G-protein MglA connects to the MreB actin cytoskeleton at bacterial focal
557   adhesions. *J Cell Biol* **210**, 243-256 (2015).

558   11   Sun M, Wartel M, Cascales E, Shaevitz JW, & Mignot T, Motor-driven intracellular
559   transport powers bacterial gliding motility. *Proc Natl Acad Sci USA* **108**, 7559-7564 (2011).

560   12   Balagam R, Litwin DB, Czerwinski F, Sun M, Kaplan HB, Shaevitz JW, & Igoshin OA,
561   Myxococcus xanthus gliding motors are elastically coupled to the substrate as predicted by
562   the focal adhesion model of gliding motility. *PLoS Comput Biol* **10**, e1003619 (2014).

13   Jakobczak B, Keilberg D, Wuichet K, & Søgaard-Andersen L, Contact- and protein transfer-dependent stimulation of assembly of the gliding motility machinery in *Myxococcus xanthus*. *PLOS Genet* **11**, e1005341 (2015).

14   Kahnt Jr, Aguiluz K, Koch Jr, Treuner-Lange A, Konovalova A, Huntley S, Hoppert M, Søgaard-Andersen L, & Hedderich R, Profiling the outer membrane proteome during growth and development of the social bacterium *Myxococcus xanthus* by selective biotinylation and analyses of outer membrane vesicles. *J Proteome Res* **9**, 5197-5208 (2010).

15   Rodriguez AM & Spormann AM, Genetic and molecular analysis of *cglB*, a gene essential for single-cell gliding in *Myxococcus xanthus*. *J Bacteriol* **181**, 4381-4390 (1999).

16   Selkrig J *et al.*, Discovery of an archetypal protein transport system in bacterial outer membranes. *Nat Struct Mol Biol* **19**, 506-510 (2012).

17   Hodgkin J & Kaiser D, Cell-to-cell stimulation of movement in nonmotile mutants of *Myxococcus*. *Proc Natl Acad Sci USA* **74**, 2938-2942 (1977).

18   Youderian P, Burke N, White DJ, & Hartzell PL, Identification of genes required for adventurous gliding motility in *Myxococcus xanthus* with the transposable element *mariner*. *Mol Microbiol* **49**, 555-570 (2003).

19   Lemon DJ, Yang X, Srivastava P, Luk Y-Y, & Garza AG, Polymertropism of rod-shaped bacteria: movement along aligned polysaccharide fibers. *Sci Rep* **7**, 7643 (2017).

20   Lemon DJ, Schutzman DA, & Garza AG, Bacterial surface spreading is more efficient on nematically aligned polysaccharide substrates. *J Bacteriol* **200**, e00610-00617 (2018).

21   Fontes M & Kaiser D, *Myxococcus* cells respond to elastic forces in their substrate. *Proc Natl Acad Sci USA* **96**, 8052-8057 (1999).

22   Lazopoulos KA & Stamenović D, Durotaxis as an elastic stability phenomenon. *J Biomech* **41**, 1289-1294 (2008).

23   Vincent LG, Choi YS, Alonso-Latorre B, del Álamo JC, & Engler AJ, Mesenchymal stem cell durotaxis depends on substrate stiffness gradient strength. *Biotechnol J* **8**, 472-484 (2013).

24   Lo C-M, Wang H-B, Dembo M, & Wang Y-l, Cell movement is guided by the rigidity of the substrate. *Biophys J* **79**, 144-152 (2000).

25   Song G, Koksal AC, Lu C, & Springer TA, Shape change in the receptor for gliding motility in *Plasmodium* sporozoites. *Proc Natl Acad Sci USA* **109**, 21420-21425 (2012).

26   Song G & Springer TA, Structures of the *Toxoplasma* gliding motility adhesin. *Proc Natl Acad Sci USA* **111**, 4862-4867 (2014).

27   Krishnan V, Dwivedi P, Kim BJ, Samal A, Macon K, Ma X, Mishra A, Doran KS, Ton-That H, & Narayana SVL, Structure of *Streptococcus agalactiae* tip pilin GBS104: a model for GBS pili assembly and host interactions. *Acta Crystallogr D Biol Crystallogr* **69**, 1073-1089 (2013).

28   Chouhan B, Denesyuk A, Heino J, Johnson MS, & Denessiouk K, Conservation of the human integrin-type beta-propeller domain in bacteria. *PLOS ONE* **6**, e25069 (2011).

29   Shimaoka M & Springer TA, Therapeutic antagonists and conformational regulation of integrin function. *Nat Rev Drug Discov* **2**, 703-716 (2003).

30   Whittaker CA & Hynes RO, Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. *Mol Biol Cell* **13**, 3369-3387 (2002).

31   Shimaoka M, Takagi J, & Springer TA, Conformational regulation of integrin structure and function. *Annu Rev Biophys Biomol Struct* **31**, 485-516 (2002).

32   Tréguier J, Bugnicourt L, Gay G, Diallo M, Islam ST, Toro A, David L, Théodoly O, Sudre G, & Mignot T, Chitosan films for microfluidic studies of single bacteria and perspectives for antibiotic susceptibility testing. *mBio* **10**, e01375-01319 (2019).

33   Wilson MM & Bernstein HD, Surface-exposed lipoproteins: an emerging secretion phenomenon in Gram-negative bacteria. *Trends Microbiol* **24**, 198-208 (2016).

34   Konovalova A & Silhavy TJ, Outer membrane lipoprotein biogenesis: Lol is not the end. *Philos Trans R Soc Lond B Biol Sci* **370** (2015).

35   Hooda Y & Moraes TF, Translocation of lipoproteins to the surface of gram negative bacteria. *Curr Opin Struct Biol* **51**, 73-79 (2018).

36   Cao P, Dey A, Vassallo CN, & Wall D, How myxobacteria cooperate. *J Mol Biol* **427**, 3709-3721 (2015).

37   Cao P & Wall D, Direct visualization of a molecular handshake that governs kin recognition and tissue formation in myxobacteria. *Nat Commun* **10**, 3073 (2019).

38   Ducret A, Fleuchot B, Bergam P, & Mignot T, Direct live imaging of cell–cell protein transfer by transient outer membrane fusion in *Myxococcus xanthus*. *eLife* **2**, e00868 (2013).

39   Giebeler N & Zigrino P, A disintegrin and metalloprotease (ADAM): historical overview of their functions. *Toxins* **8**, 122 (2016).

40   Cho S-H, Szewczyk J, Pesavento C, Zietek M, Banzhaf M, Roszczenko P, Asmar A, Laloux G, Hov A-K, Leverrier P, Van der Henst C, Vertommen D, Typas A, & Collet J-F, Detecting envelope stress by monitoring β-barrel assembly. *Cell* **159**, 1652-1664 (2014).

629 41 Konovalova A, Perlman DH, Cowles CE, & Silhavy TJ, Transmembrane domain of surface-
630   exposed outer membrane lipoprotein RcsF is threaded through the lumen of β-barrel
631   proteins. *Proc Natl Acad Sci USA* **111**, E4350-E4358 (2014).

632 42 Gómez-Santos N, Glatter T, Koebnik R, Świątek-Połatyńska MA, & Søgaard-Andersen L, A
633   TonB-dependent transporter is required for secretion of protease PopC across the bacterial
634   outer membrane. *Nat Commun* **10**, 1360 (2019).

635 43 Wartel M, Ducret A, Thutupalli S, Czerwinski F, Le Gall A-V, Mauriello EMF, Bergam P,
636   Brun YV, Shaevitz J, & Mignot T, A versatile class of cell surface directional motors gives
637   rise to gliding motility and sporulation in *Myxococcus xanthus*. *PLoS Biol* **11**, e1001728
638   (2013).

639 44 Agrebi R, Wartel M, Brochier-Armanet C, & Mignot T, An evolutionary link between
640   capsular biogenesis and surface motility in bacteria. *Nat Rev Microbiol* **13**, 318-326 (2015).

641 45 Costa TRD, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, &
642   Waksman G, Secretion systems in Gram-negative bacteria: structural and mechanistic
643   insights. *Nat Rev Microbiol* **13**, 343-359 (2015).

644 46 McBride MJ, Bacteroidetes gliding motility and the Type IX secretion system. *Microbiol
645   Spectr* **7** (2019).

646 47 Nelson SS, Bollampalli S, & McBride MJ, SprB is a cell surface component of the
647   *Flavobacterium johnsoniae* gliding motility machinery. *J Bacteriol* **190**, 2851-2857 (2008).

648 48 Nakane D, Sato K, Wada H, McBride MJ, & Nakayama K, Helical flow of surface protein
649   required for bacterial gliding motility. *Proc Natl Acad Sci U S A* **110**, 11145-11150 (2013).

650 49 Shrivastava A, Roland T, & Berg Howard C, The screw-like movement of a gliding
651   bacterium is powered by spiral motion of cell-surface adhesins. *Biophys J* **111**, 1008-1013
652   (2016).

653 50 Shrivastava A & Berg HC, A molecular rack and pinion actuates a cell-surface adhesin and
654   enables bacterial gliding motility. *Sci Adv* **6**, eaay6616 (2020).

655 51 Murphy-Ullrich JE & Sage EH, Revisiting the matricellular concept. *Matrix Biol* **37**, 1-14
656   (2014).

657 52 Kelwick R, Desanlis I, Wheeler GN, & Edwards DR, The ADAMTS (A Disintegrin and
658   Metalloproteinase with Thrombospondin motifs) family. *Genome Biol* **16**, 1-16 (2015).

659 53 Ducret A, Valignat M-P, Mouhamar F, Mignot T, & Theodoly O, Wet-surface-enhanced
660   ellipsometric contrast microscopy identifies slime as a major adhesion factor during
661   bacterial surface motility. *Proc Natl Acad Sci USA* **109**, 10036-10041 (2012).

662 54 Islam ST, Vergara Alvarez I, Saïdi F, Guiseppi A, Vinogradov E, Sharma G, Espinosa L,
663   Morrone C, Brasseur G, Guillemot J-F, Benarouche A, Bridot J-L, Ravicoularamin G,

664   Cagna A, Gauthier C, Singer M, Fierobe H-P, Mignot T, & Mauriello EMF, Modulation of
665   bacterial multicellularity via spatio-specific polysaccharide secretion. *PLOS Biol* **18**,
666   e3000728 (2020).

667   [55]   Reese MG, Application of a time-delay neural network to promoter annotation in the
668   *Drosophila melanogaster* genome. *Comput Chem* **26**, 51-56 (2001).

669   [56]   Goldman BS *et al.*, Evolution of sensory complexity recorded in a myxobacterial genome.
670   *Proc Natl Acad Sci USA* **103**, 15200-15205 (2006).

671   [57]   Han K, Li ZF, Peng R, Zhu LP, Zhou T, Wang LG, Li SG, Zhang XB, Hu W, Wu ZH, Qin
672   N, & Li YZ, Extraordinary expansion of a *Sorangium cellulosum* genome from an alkaline
673   milieu. *Sci Rep* **3**, 2101 (2013).

674   [58]   Huntley S, Hamann N, Wegener-Feldbrugge S, Treuner-Lange A, Kube M, Reinhardt R,
675   Klages S, Muller R, Ronning CM, Nierman WC, & Sogaard-Andersen L, Comparative
676   genomic analysis of fruiting body formation in Myxococcales. *Mol Biol Evol* **28**, 1083-1097
677   (2011).

678   [59]   Huntley S, Kneip S, Treuner-Lange A, & Søgaard-Andersen L, Complete genome sequence
679   of *Myxococcus stipitatus* strain DSM 14675, a fruiting myxobacterium. *Genome Announc* **1**,
680   e00100-00113 (2013).

681   [60]   Huntley S, Zhang Y, Treuner-Lange A, Kneip S, Sensen CW, & Sogaard-Andersen L,
682   Complete genome sequence of the fruiting myxobacterium *Corallococcus coralloides* DSM
683   2259. *J Bacteriol* **194**, 3012-3013 (2012).

684   [61]   Ivanova N *et al.*, Complete genome sequence of Haliangium ochraceum type strain (SMP-
685   2T). *Stand Genomic Sci* **2**, 96-106 (2010).

686   [62]   Li Z-F, Li X, Liu H, Liu X, Han K, Wu Z-H, Hu W, Li F-f, & Li Y-Z, Genome sequence of
687   the halotolerant marine bacterium *Myxococcus fulvus* HW-1. *J Bacteriol* **193**, 5015-5016
688   (2011).

689   [63]   Müller S, Willett JW, Bahr SM, Darnell CL, Hummels KR, Dong CK, Vlamakis HC, &
690   Kirby JR, Draft genome sequence of *Myxococcus xanthus* wild-type strain DZ2, a model
691   organism for predation and development. *Genome Announc* **1**, e00217-00213 (2013).

692   [64]   Schneiker S *et al.*, Complete genome sequence of the myxobacterium *Sorangium*
693   *cellulosum*. *Nat Biotech* **25**, 1281-1289 (2007).

694   [65]   Sharma G, Khatri I, & Subramanian S, Complete genome of the starch-degrading
695   myxobacteria *Sandaracinus amylolyticus* DSM 53668T. *Genome Biol Evol* **8**, 2520-2529
696   (2016).

697   [66]   Sharma G, Narwani T, & Subramanian S, Complete genome sequence and comparative
698   genomics of a novel myxobacterium *Myxococcus hansupus*. *PLOS ONE* **11**, e0148593
699   (2016).

67   Stevens DC, Young J, Carmichael R, Tan J, & Taylor RE, Draft genome sequence of gephyronic acid producer *Cystobacter violaceus* strain Cb vi76. *Genome Announc* **2**, e01299-01214 (2014).

68   Sharma G & Subramanian S, Unravelling the complete genome of *Archangium gephyra* DSM 2261T and evolutionary insights into myxobacterial chitinases. *Genome Biol Evol* **9**, 1304-1311 (2017).

69   Sharma G, Khatri I, & Subramanian S, Comparative genomics of myxobacterial chemosensory systems. *J Bacteriol* **200**, e00620-00617 (2018).

70   Wu M & Eisen JA, A simple, fast, and accurate method of phylogenomic inference. *Genome Biol* **9**, R151 (2008).

71   Stamatakis A, RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688-2690 (2006).

72   Nudleman E, Wall D, & Kaiser D, Cell-to-cell transfer of bacterial outer membrane lipoproteins. *Science* **309**, 125-127 (2005).

73   Pathak DT & Wall D, Identification of the *cglC*, *cglD*, *cglE*, and *cglF* genes and their role in cell contact-dependent gliding motility in *Myxococcus xanthus*. *J Bacteriol* **194**, 1940-1949 (2012).

74   Johnson LS, Eddy SR, & Portugaly E, Hidden Markov model speed heuristic and iterative HMM search procedure. *BMC Bioinformatics* **11**, 431 (2010).

75   Letunic I & Bork P, Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res* **44**, W242-W245 (2016).

76   Rice P, Longden I, & Bleasby A, EMBOSS: The European molecular biology open software suite. *Trends Genet* **16**, 276-277 (2000).

77   Drozdetskiy A, Cole C, Procter J, & Barton GJ, JPred4: a protein secondary structure prediction server. *Nucl Acids Res* (2015).

78   Letunic I, Doerks T, & Bork P, SMART: recent updates, new developments and status in 2015. *Nucl Acids Res* **43**, D257-D260 (2015).

79   Söding J, Biegert A, & Lupas AN, The HHpred interactive server for protein homology detection and structure prediction. *Nucl Acids Res* **33**, W244-W248 (2005).

80   Källberg M, Wang H, Wang S, Peng J, Wang Z, Lu H, & Xu J, Template-based protein structure modeling using the RaptorX web server. *Nat Protoc* **7**, 1511-1522 (2012).

81   Barbato A, Benkert P, Schwede T, Tramontano A, & Kosinski J, Improving your target-template alignment with MODalign. *Bioinformatics* **28**, 1038-1039 (2012).

733   82   Šali A & Blundell TL, Comparative protein modelling by satisfaction of spatial restraints. *J*
734   *Mol Biol* **234**, 779-815 (1993).

735   83   Vehlow C, Stehr H, Winkelmann M, Duarte JM, Petzold L, Dinse J, & Lappe M, CMView:
736   interactive contact map visualization and analysis. *Bioinformatics* **27**, 1573-1574 (2011).

737   84   Wang S, Li W, Zhang R, Liu S, & Xu J, CoinFold: a web server for protein contact
738   prediction and contact-assisted protein folding. *Nucleic Acids Res* **44**, W361-W366 (2016).

739   85   Ducret A, Quardokus EM, & Brun YV, MicrobeJ, a tool for high throughput bacterial cell
740   detection and quantitative analysis. *Nat Microbiol* **1**, 16077 (2016).

741   86   Wang S, Arellano-Santoyo H, Combs PA, & Shaevitz JW, Measuring the bending stiffness
742   of bacterial cells using an optical trap. *J Vis Exp*, e2012 (2010).
743

744 **FIGURE TITLES AND LEGENDS:**

745

746 **Figure 1: Evidence of an Integral OM Complex Formed by GltA, B, and H.**

747 **A)** Gliding motility mediated by bFAs in *M. xanthus*. Following their assembly at the leading

748 pole, motility complexes move toward the lagging cell pole in a counter clockwise (CCW)

749 rotational trajectory. Clockwise (CW) and CCW directionalities are defined by observing the cell

750 cylinder from the leading pole in the *y,z* plane. When the complexes interact with the substratum,

751 they form bacterial focal adhesion (bFA, concentric circles) sites and propel rotational

752 movements of the cell. *Panel (i)*: bFAs are formed according to two possible mechanisms: In the

753 viscous interaction model, the periplasmic complex accumulates at bFAs and pushes against the

754 elastic peptidoglycan (PG) to create cell envelope deformations at bFAs and thus create viscous

755 interactions with the substratum. The function of the outer-membrane (OM) complex is not

756 accounted for in this model. In the elastic model, the periplasmic complex establishes transient

757 interactions through the PG, contacting the OM complex which itself interacts with the

758 substratum via an unknown adhesive molecule (*pink circle*). *Panel (ii):* Proposed Glt OM

759 platform based on previous reports and this study. The OM localization of GltA, GltB, GltH,

760 GltC and GltK is based on structural bioinformatic as well as fractionation analyses presented

761 here and elsewhere[4,13,14]. The integral association of GltA, B and H is based on bioinformatic

762 and Proteinase K accessibility assays in this study and another report[13]. Direct GltA–GltB,

763 GltA–GltC, and GltB–GltC interactions were already biochemically demonstrated by pull-down

764 assays[13]. The connection with GltH is further indicated from results reported in this study. The

765 periplasmic leaflet (*orange*) and outer leaflet (*yellow*) of the OM are indicated. Legend: Peri,

766 periplasm.

767 Western immunoblot of **B)** GltA, **C)** GltB, and **D)** GltH susceptibility to digestion by Proteinase

768 K in OM-module Agl–Glt mutant strains. Digestion aliquots were removed at 15-min intervals

769 and TCA-precipitated to stop digestion. Legend for Panels B, C, and D: ◄, full-length protein;

770 ○, loading control (non-specific protein band labelled by the respective α-GltA/α-GltB/α-GltH
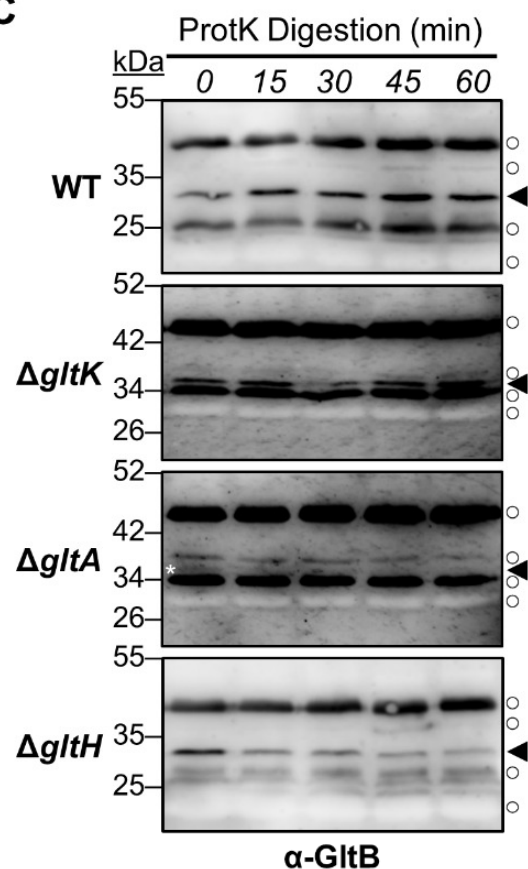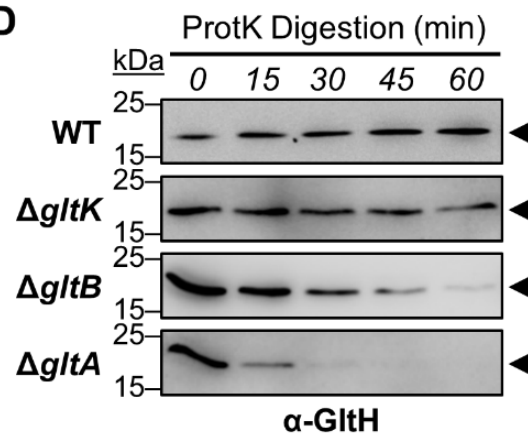
771 pAb).
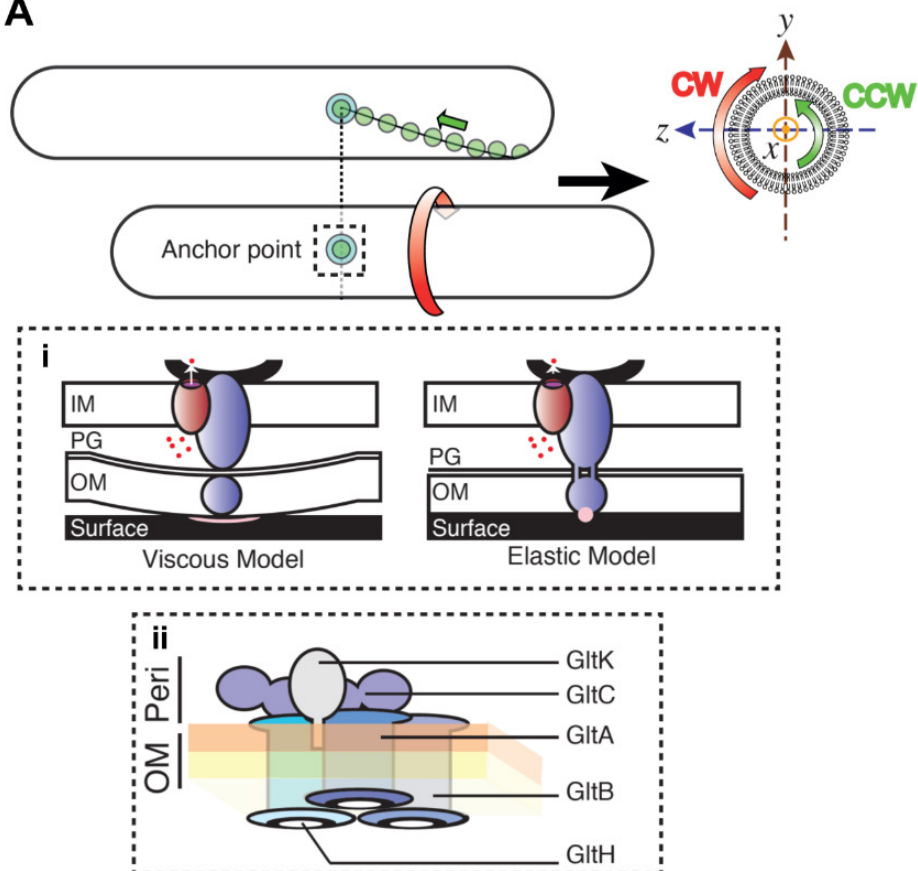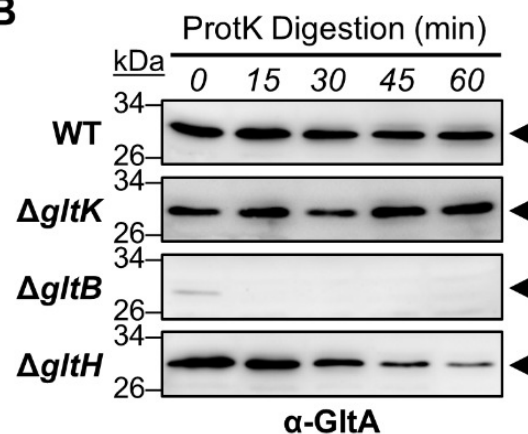
FIGURE 1

Figure 2: CglB, a protein with a potential α-integrin-like fold, is essential for motility complex adhesion to the substratum

A) Tertiary structure homology model of CglB. The N- and C-terminus of the protein are indicated. Colour code: *yellow*, β-strands; *red*, α-helices, *black spheres*, Cys residues. Dotted line denotes a magnified view of the MIDAS motif containing residues D56, S58, S60, T182, D211. See also **Supplementary Figs. S1E, S2E, F** and **Supplementary Table S2**.

B) α-CglB Western blot of WT whole-cell extracts treated with increasing concentrations of DTT to break disulphide bonds. Legend: ◄, full-length CglB; ○, loading control (non-specific protein band labelled by α-CglB pAb). See also **Supplementary Fig. S2B**.

C) Kymograph in WT vs. Δ*cglB* cells indicating AglZ-YFP cluster position over time (hashed lines). Scale bar: 2 μm. Legend: white arrowheads, AglZ-YFP clusters followed for their entire lifetime; black arrowheads, AglZ-YFP clusters followed for an incomplete lifetime. See also **Supplementary Fig. S4B**.

D) Mean-squared displacement (MSD) analysis of AglZ-YFP cluster position tracking in WT (n = 48 clusters) and Δ*cglB* (n = 23 clusters) *M. xanthus* cells. The mean of MSD at each time interval is displayed ± SEM, with a second-order polynomial line fit to each dataset.

E) Frequency of trafficking Agl–Glt complexes via TIRFM (of AglZ-YFP) on chitosan-coated glass surfaces in PDMS microfluidic chambers for WT (n = 44 cells) and Δ*cglB* (n = 41 cells) strains. The distribution of the two datasets are significantly different (*), as calculated via unpaired two-tailed Mann-Whitney U-test ($p < 0.05$). See also **Supplementary Fig. S4C**.

F) Speed of Agl–Glt complex trafficking via TIRFM (of AglZ-YFP) on chitosan-coated glass surfaces in PDMS microfluidic chambers for WT (n = 260 clusters) and Δ*cglB* (n = 371 clusters) strains. The distribution of the two datasets are not significantly different, as calculated via unpaired two-tailed Mann-Whitney U-test ($p > 0.05$). See also **Supplementary Fig. S4C**.

G) Stability of trafficking Agl–Glt complexes via TIRFM (of AglZ-YFP) on chitosan-coated glass surfaces in PDMS microfluidic chambers for WT (n = 333 clusters) and Δ*cglB* (n = 409 clusters) strains. The distribution of the two datasets are not significantly different, as calculated via unpaired two-tailed Mann-Whitney U-test ($p > 0.05$). See also **Supplementary Fig. S4C**.

H) Directionality of trafficked Agl–Glt complexes via TIRFM (of AglZ-YFP) on chitosan-coated glass surfaces for WT (n = 44 cells) and Δ*cglB* (n = 41 cells) strains. "Front" and "back" are defined as cell poles with high and low AglZ-YFP fluorescence intensity, respectively.

804   Relative to the respective reference WT cluster type, only the distribution of front-to-back

805   clusters were significantly different in the $\Delta cglB$ cells ($p < 0.05$); all other cluster types did not

806   display distributions different from WT ($p > 0.05$), as calculated via unpaired two-tailed Mann-

807   Whitney U-test. See also **Supplementary Fig. S4C**.

808   **I)** Trafficking of surface-deposited polystyrene beads on *M. xanthus* cells. Images were acquired

809   at 10 s intervals. See also **Supplementary Fig. S4D**.

810   **J)** Length of tracked bead runs > 0.1 µm in *M. xanthus* cells. Images from 10 s intervals were

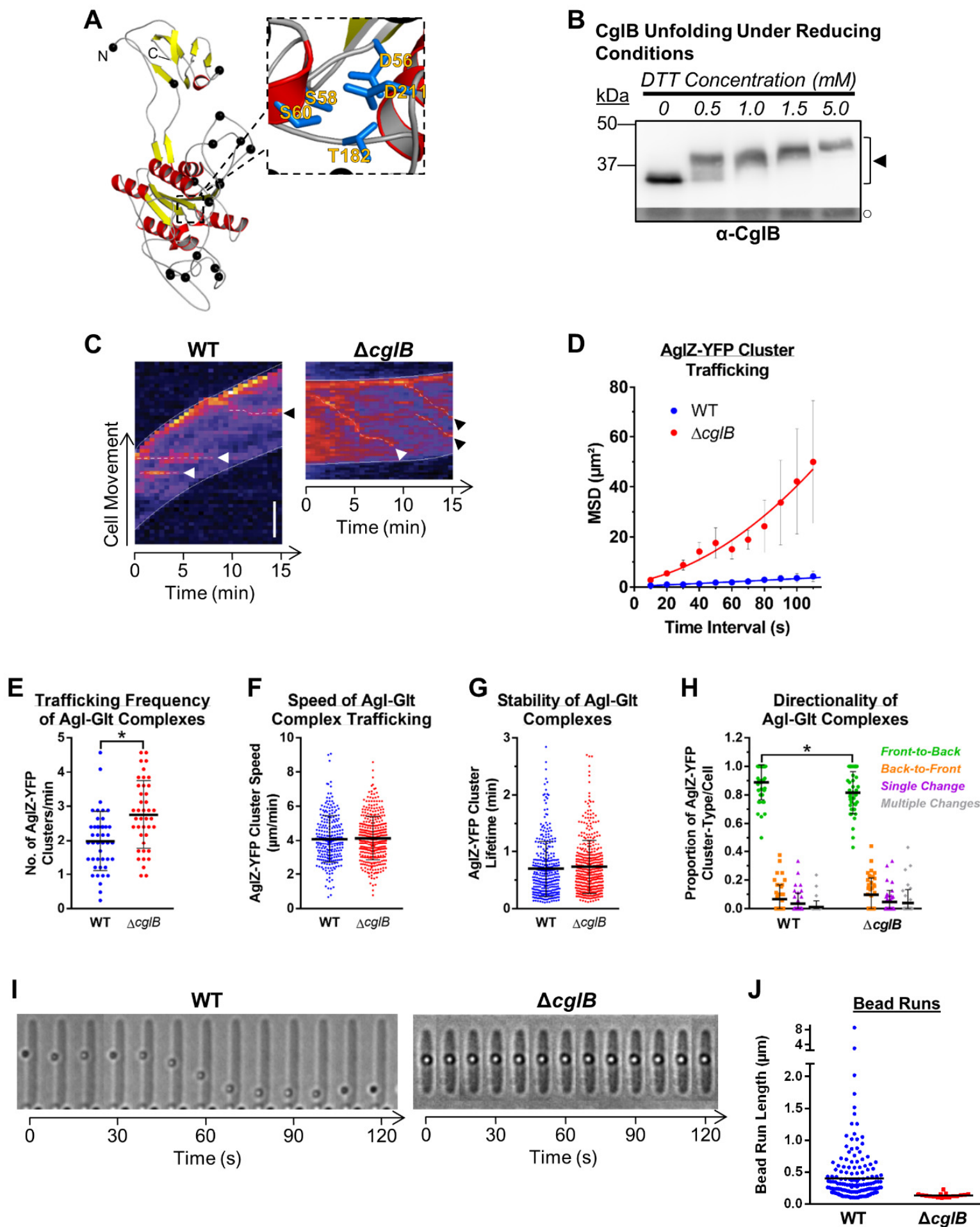811   analyzed. See also **Supplementary Fig. S4D**.

FIGURE 2

813 **Figure 3: CglB Secretion is Mediated by the Glt OM Platform**

814 α-CglB Western blots from:

815 **A)** Whole-cell extracts from different Δ*glt* mutants. Non-adjacent lanes on the blot are separated

816 by vertical black lines. White space separates two distinct blots processed at the same time.

817 **B)** Fractionated samples containing whole cells (WC), supernatants (Sup), and outer-membrane

818 vesicles (OMV) from various genetic backgrounds. Detection of the gliding motility OM

819 lipoprotein GltK was added as a control, with the protein only detected in WC and OMV

820 samples, showing that the various mutations do not affect OM integrity, with the supernatant

821 localization in this instance being specific to CglB. MglA is a cytoplasmic protein added as a

822 control to show that cell lysis is negligible and does not account for the presence of CglB in

823 supernatants. Legend: ◄, full-length protein; ○, loading control (non-specific protein band

824 labelled by the respective pAb).

825 **C)** Whole-cell extracts from Δ*gltB* cells grown in the presence of different protease inhibitors.

826 White space separates two distinct blots from the same experiment.

827 **D)** Restoration of cell-associated CglB in EDTA-grown Δ*gltK*/*B*/*A* cells. Different ratios of the

828 slower- and faster-migrating bands were observed depending on the mutation of the specific

829 strain. Lower, darker zones on each blot correspond to sections of the same blot image for which

830 the contrast has been increased to highlight lower-intensity protein bands. Legend: ◄, full-

831 length CglB; ○, loading control (non-specific protein band labelled by α-CglB pAb).

832 **E)** Protein samples from cells resuspended in TPM buffer and digested with exogenous

833 Proteinase K. Aliquots of the digestion mixture were removed at 15-min intervals and TCA-

834 precipitated to stop digestion. "P" and "P$_{EDTA}$" denote lanes containing the untreated parent

835 strain and the parent strain grown in the presence of EDTA, respectively. Lower, darker zones

836 on each blot correspond to sections of the same blot image for which the contrast has been

837 increased to highlight lower-intensity protein bands. Legend: ◄, full-length CglB; ←, CglB

838 degradation band; ○, loading control (non-specific protein band labelled by α-CglB antibody).

839 See also **Supplementary Fig. S5A**.

840 **F)** Whole-cell extracts from different combinations of Δ*glt* OM-module mutations in the same

841 strain. Non-adjacent lanes on the blot are separated by vertical black lines. Legend: ◄, full-

842 length CglB; ○, loading control (non-specific protein band labelled by α-CglB pAb).

843   **G)** Protein samples from cells resuspended in TPM buffer and digested with exogenous

844   Proteinase K. Aliquots of the digestion mixture were removed at 15-min intervals and TCA-

845   precipitated to stop digestion. Legend: ◄, full-length CglB; ○, loading control (non-specific

846   protein band labelled by α-CglB pAb).

FIGURE 3

A Whole Cells

α-CglB

B Differential CglB Subcellular Localization in Δ*gltA/B/K* Mutants

α-CglB
α-GltK
α-MglA

WT   Δ*gltA*   Δ*gltB*   Δ*gltK*

C Protease Inhibition (Whole Cells)

α-CglB

WT   Δ*gltB* (grown in presence of protease inhibitors)

D Restoration of CglB in Whole Cells

α-CglB

Cells grown with EDTA
(TCA, acetone treated)

E Surface Exposure of Restored CglB

ProtK Digestion (min)

WT   Grown with EDTA
Δ*gltK*   Grown with EDTA
Δ*gltB*   Grown with EDTA
Δ*gltA*   Grown with EDTA
Δ*gltH*   Grown without EDTA

α-CglB

F OM-Module Mutations to Restore CglB in Whole Cells

α-CglB

G OM-Module Mutations That Block CglB ProtK Sensitivity

ProtK Digestion (min)

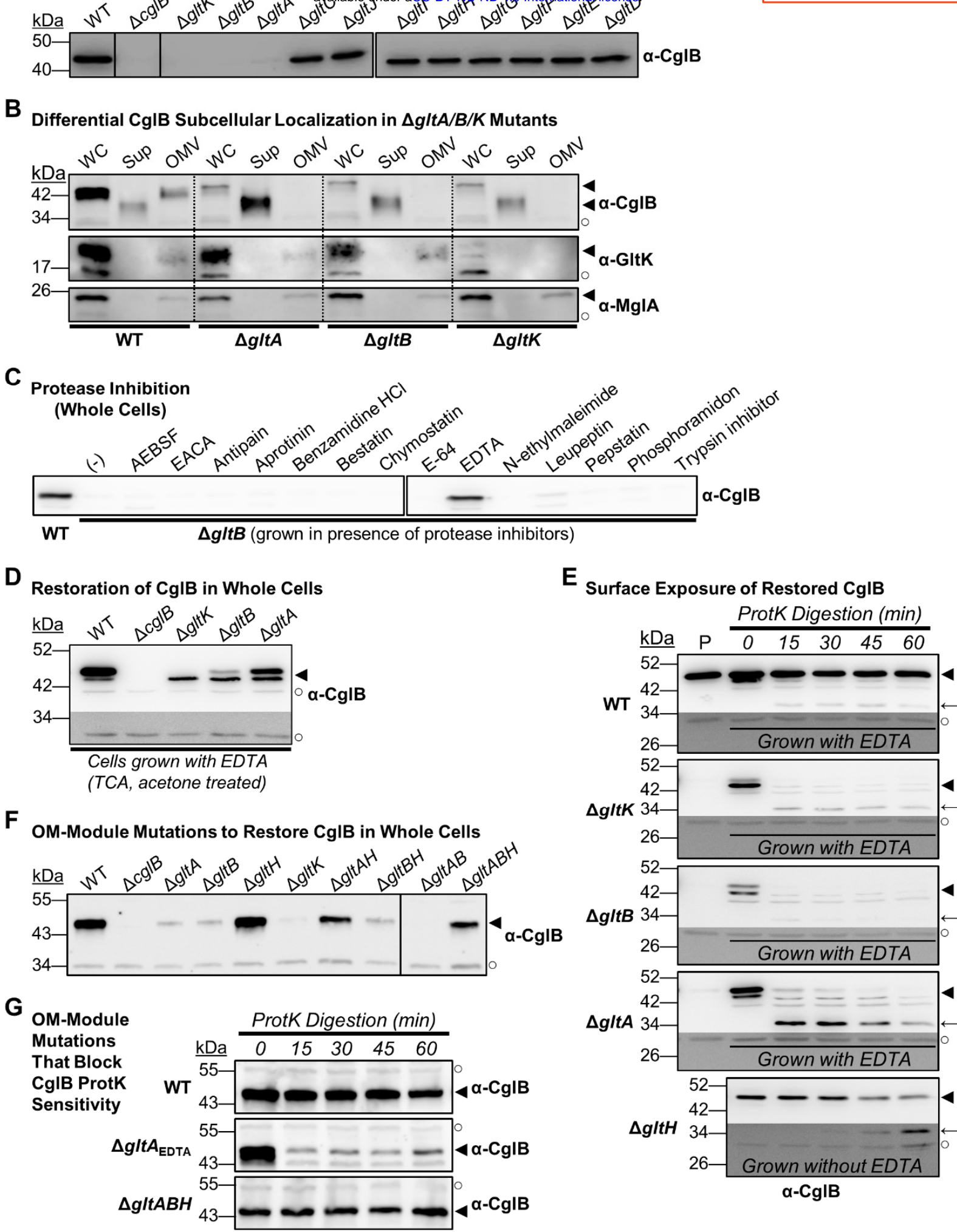WT   α-CglB
Δ*gltA*EDTA   α-CglB
Δ*gltABH*   α-CglB

848   **Figure 4: Role of GltK in CglB Function**

849   **A)** α-GltK Western blot of WT whole-cell extracts treated with increasing concentrations of DTT

850   to break disulphide bonds. Legend: ◄, full-length GltK; ○, loading control (non-specific protein

851   band labelled by α-GltK pAb). See also **Supplementary Fig. S2B**.

852   **B)** Kymograph of Δ*gltA* vs. Δ*gltK* cells showing AglZ-YFP cluster position over time. Scale

853   bar: 2 μm. Legend: white arrowheads, AglZ-YFP clusters followed for their entire lifetime; black

854   arrowheads, AglZ-YFP clusters followed for an incomplete lifetime. See also **Supplementary**

855   **Fig. S4C**.

856   **C)** Frequency of trafficking Agl–Glt complexes via TIRFM (of AglZ-YFP) on chitosan-coated

857   glass surfaces in PDMS microfluidic chambers for WT (n = 37 cells) and Δ*gltK* (n = 44 cells)

858   strains. The distribution of the two datasets are significantly different (*), as calculated via

859   unpaired two-tailed Mann-Whitney U-test ($p < 0.05$). See also **Supplementary Fig. S4C**.

860   **D)** Stability of trafficking Agl–Glt complexes via TIRFM (of AglZ-YFP) on chitosan-coated

861   glass surfaces in PDMS microfluidic chambers for WT (n = 90 clusters) and Δ*gltK* (n = 223

862   clusters) strains. The distribution of the two datasets are not significantly different, as calculated

863   via unpaired two-tailed Mann-Whitney U-test ($p > 0.05$). See also **Supplementary Fig. S4C**.

864   **E)** Schematic of inter-strain mixes to study OME and the interplay between GltK and CglB.

865   **F)** Gliding motility restoration in mCherry^{+} cells via *trans*-complementation following OME.

866   Note that motile single cells, as well as gliding-dependent flares emanating from swarm fronts

867   (which are dependent on collective effects of single-cell motility), are only observable when

868   CglB is efficiently transferred (white arrows). Scale bar: 20 μm.

869   **G)** *M. xanthus* cells from strain-mixing experiments to probe GltK-dependent CglB transfer via

870   OME. *Left panel:* Fluorescence profile of the total cell population after 6 h of cell mixing on an

871   agar substratum, as determined via flow cytometry. *Right panel:* FACS analysis of the mCherry[+]

872   cell population after sorting.

873   **H)** Western immunoblot to detect the presence of CglB and mCherry following OME via various

874   strain–strain mixes. Legend: ◄, full-length protein; ○, loading control (non-specific protein band

875   labelled by respective pAb).
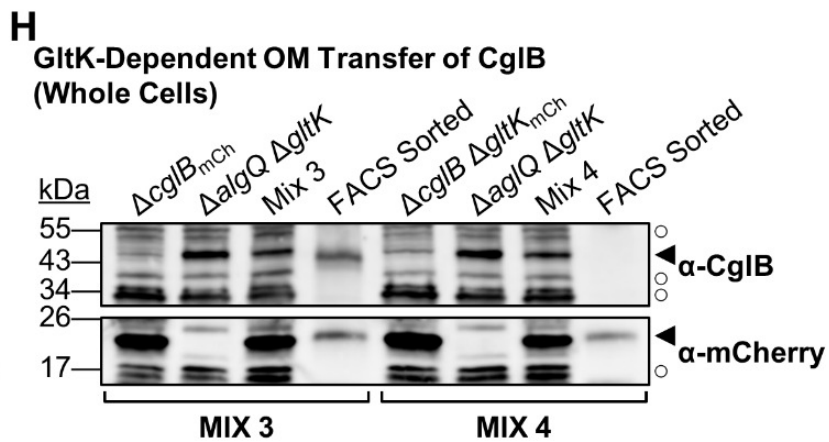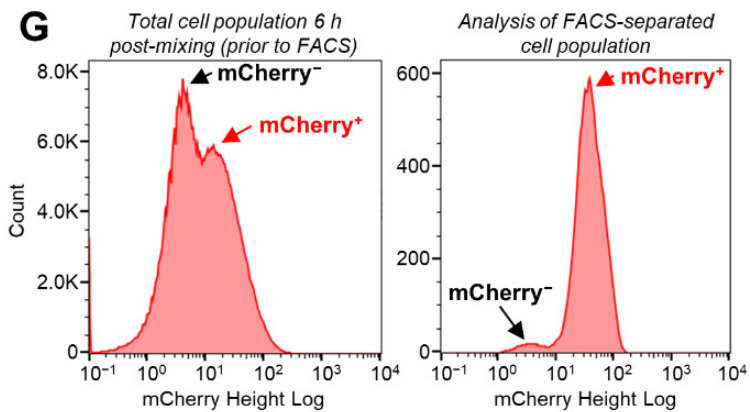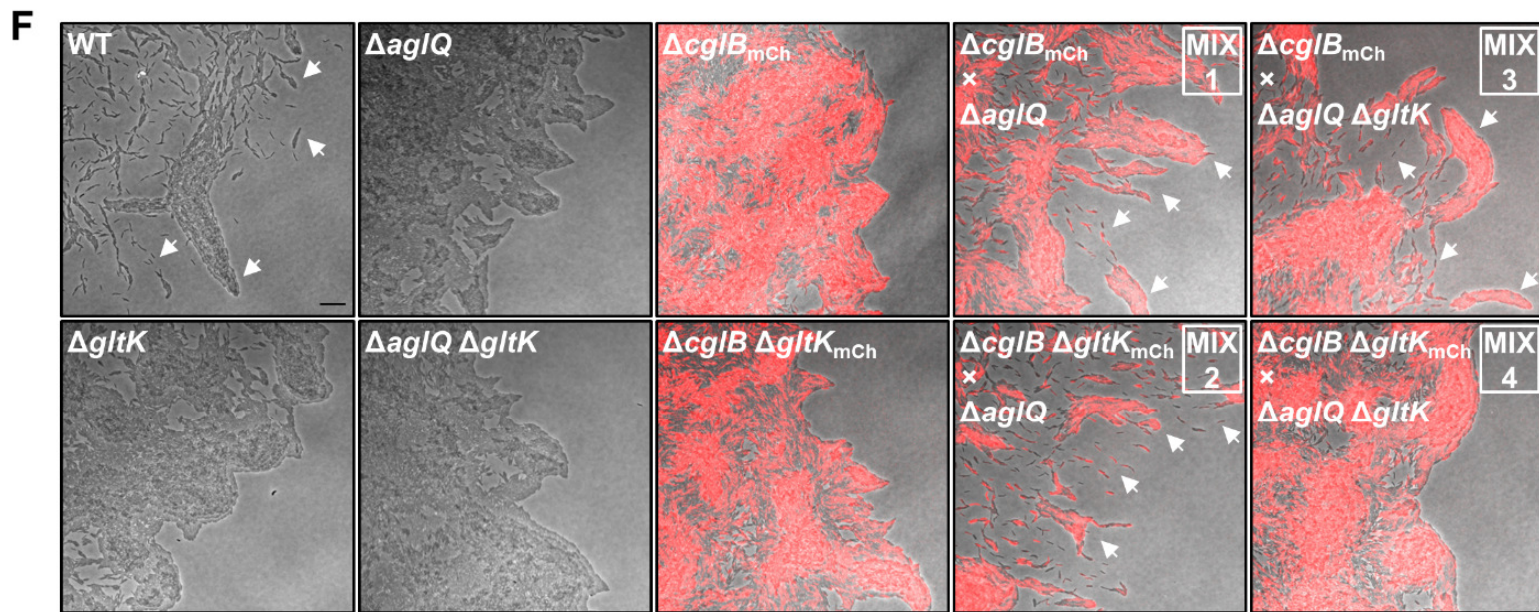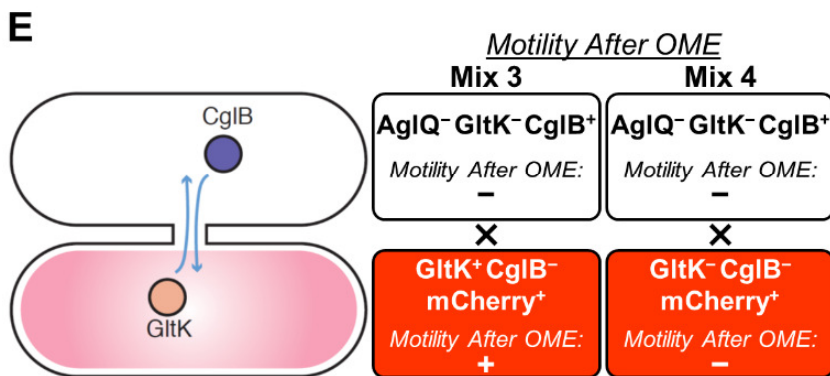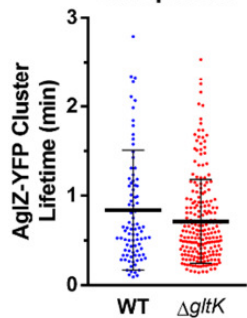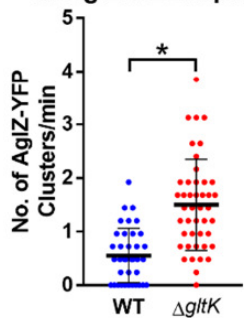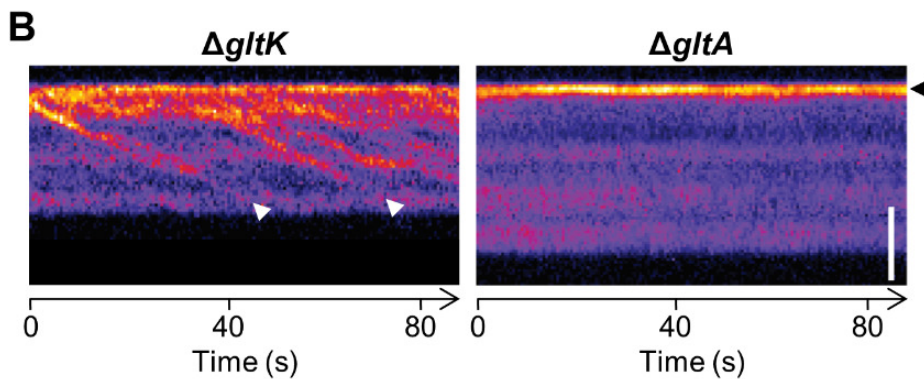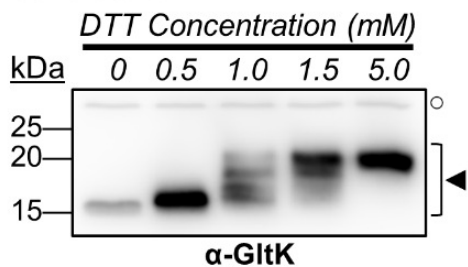
FIGURE 4

877 **Figure 5: Proposed Model for Agl–Glt Complex Substratum-Tethering by CglB at bFAs.**

878 *Step 1:* At bFAs, the PMF-driven (*red dots*) mechanical action of the IM motor complex allows

879 dynamic interactions of the IM complex with the OM platform via flexible protein periplasmic

880 domains. *Step 2:* These domains promote localized CglB activation via GltK and secretion of

881 CglB across the OM, requiring the OM-platform structure. CglB then interacts with the

882 underlying substratum via its integrin-like domain, thus mediating force transduction at bFAs.

883 *Step 3:* After the power stroke of the IM machinery, the "used" OM platform is displaced.

884 Adhesin turnover must occur to allow the motility complex to engage new CglB-loaded OM

885 platform complexes and mediate forward propulsion. The exact turnover mechanism remains to

886 be identified and could involve action of a surface metalloprotease to release CglB from the OM

887 platform. *Step 4:* Displacement of the "used" OM platform, coupled with release of the "used"

888 adhesin, promotes forward propulsion of the cell body, while the IM complex remains spatially

889 fixed relative to the substratum. The IM complex subsequently recruits the adjacent inactive

890 CglB-loaded OM platform and brings it in register. *Step 5:* This again locally activates a new

891 CglB secretion and processing cycle. This creates an OM-platform adhesin flow at bFAs,

892 propelling the cell forward. For simplicity a single Agl–Glt complex is shown at a bFA, but it is

893 possible that bFAs are formed by several co-localized complexes that act in concert.

894

FIGURE 5

bFA

IM

PG

OM

Substratum

❶ IM complex transduces force to OM platform

❹ IM complex recruits next OM platform

❷ CglB secreted across OM, binds substratum

❸ After power stroke, OM platform displaced, CglB adhesin removed by proteolysis

❺ New CglB secretion & processing cycle is initiated

❹ Cell moves, IM complex stays fixed relative to substratum

OM

Substratum

896 **METHODS:**
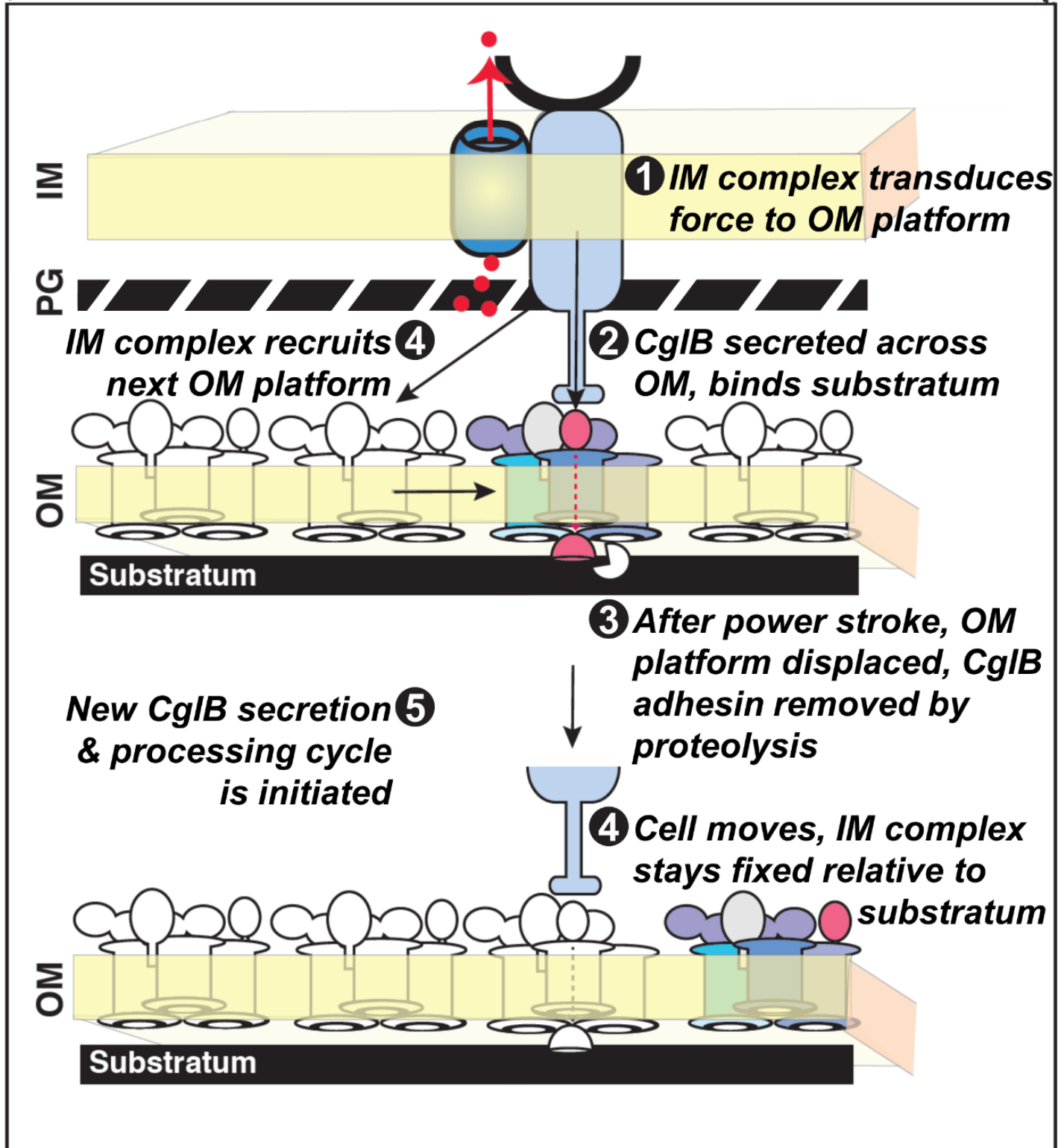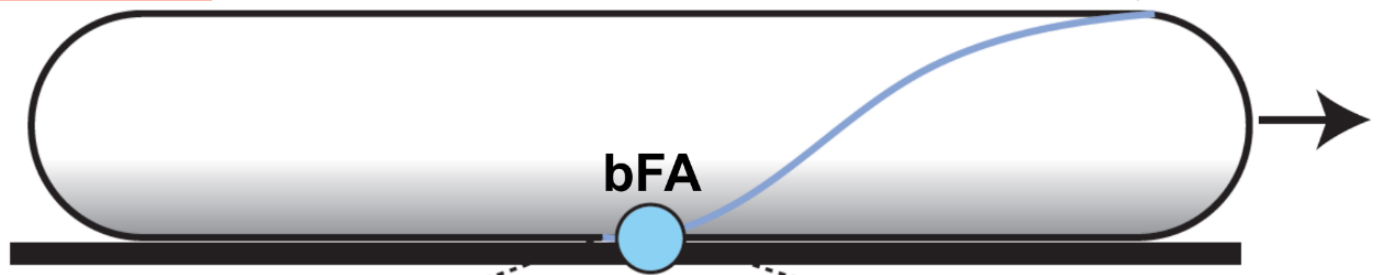
897 ***Bacterial Cell Culture and Phenotypic Analysis***

898 *M. xanthus* strains were cultured in CYE (10% w/v Bacto Casitone peptone, 5% w/v

899 yeast extract, 1% w/v $MgCl_2$, 10 mM MOPS [pH 7.4]) broth with shaking (220 rpm), or on CYE

900 solidified with 1.5% agar, at 32 ºC. To examine the effects of protease inhibition on CglB

901 liberation, cells were grown in the presence of individual protease inhibitor panel constituents

902 (Sigma, Cat.# INHIB1) at the recommended concentration: 4-(2-Aminoethyl benzenesulfonyl

903 fluoride HCl (AEBSF) (1 mM), ε-aminocaproic acid (EACA) (5 mg/mL), antipain HCl (100

904 µM), aprotinin (300 nM), benzamidine HCl hydrate (2 mM), bestatin HCl (40 µM), chymostatin

905 (50 µg/mL), E-64 (10 µM), ethylenediaminetetraacetic acid disodium dihydrate (EDTA) (1

906 mM), N-ethylmaleimide (500 µM), leupeptin hemisulfate (75 µM), pepstatin A (1 µM),

907 phosphoramidon disodium salt (10 µM), and soybean trypsin inhibitor (1 µM). Cell

908 resuspensions were done in TPM buffer (10 mM Tris-HCl, pH 7.6, 8 mM $MgSO_4$, and 1 mM

909 KH_2PO_4). For OME tests, strains were grown to exponential phase in 20 mL CYE, then

910 resuspended in TPM buffer to an OD_{600} of 5.0. For strain-mixing experiments, cell

911 resuspensions were mixed together at a 1:1 ratio. Samples were then spotted (10 µL) on a CTT

912 1.5% agar plate and incubated (32 ºC, 48 h) prior to microscopy analysis and/or harvest. All

913 strains and plasmids used are listed in **Supplementary Table S1**.

914

915 ***Polymertropism Testing***

916 Aspect ratio (AR) vs. time experiments and analyses were adapted from previous work[21]

917 and were conducted as described previously[19]. Briefly, *M. xanthus* cells were grown in CYE

918 broth at 28 °C to approximately 5×10^8 cells/mL, then sedimented (4000 × *g*, 10 min), followed

919 by resuspension in CYE broth to a final concentration of 5×10^9 cells/mL and inoculation (4 µL)

920 of compressed and uncompressed square CYE agar plates. Agar in compressed plates was

921 squeezed against the plate wall and held in position by the insertion of a length of 5.56 mm outer

922 diameter Tygon tubing[21]. Plates were incubated at 30 °C and the colony perimeters marked after

923 24, 48, 72, 96, and 120 h. Upon experiment completion, the AR of each swarm at each time

924 point was calculated by dividing the colony width by the colony height such that an elongated

925 swarm produced an AR greater than one, while a round swarm produced an AR near-or-equal to

926 one. Linear best-fit lines were plotted for each replicate dataset, followed by slope (AR/time)

927   determination. Slope values were averaged for each strain, then normalized as a percentage of

928   the AR/time for the WT strain.

929

930   ***DNA Manipulations***

931   The upstream region of *cglB* (from -213 bp), including a promoter region (from -190 bp

932   to -141 bp) predicted by BDGP[55], as well as *cglB* itself was amplified via PCR using Q5 high-

933   fidelity DNA polymerase, followed by digestion of the product and plasmid pSWU30 with

934   HindIII-HF (5') and SacI-HF (3'), then ligation via T4 DNA ligase (all enzymes from NEB) to

935   yield pCglB$_{WT}$. Oligonucleotide primers for QuikChange site-directed mutagenesis were

936   generated using PrimerX (http://bioinformatics.org/primerx/). Sequencing results were analyzed

937   by Sequencher and/or ApE software.

938

939   ***Generation of α-CglB polyclonal antibodies***

940   CglB (lacking signal peptide) elaborating a C-terminal hexa-histidine tag (CglB$_{21-416}$-

941   His$_6$) was purified under denaturing conditions. Fractions were collected in 50 mM Tris pH 8.0,

942   300 mM NaCl, 250 mM imidazole, 6 M urea and used to immunize rabbits (Eurogentec, 28-day

943   speedy protocol). The α-CglB 1° pAb produced was then tested for specificity by using the wild-

944   type and the Ω*cglB* strains. The α-GltA, α-GltB, and α-GltH 1° pAb were raised previously[4,13].

945

946   ***Phylogeny and gene co-occurrence***

947   This study explored 27 myxobacterial genomes, distributed within 3 suborders and 10

948   families[56-69], in addition to 58 outgroup genomes (members from 32 non-Myxococcales

949   Deltaproteobacteria, 4 α-, 6 β-, 9 γ-, 4 £-proteobacteria, 2 Firmicutes, and 1 Actinobacteria).

950   Highly-conserved gapless concatenated alignment of 25 housekeeping protein sequences[66,70] was

951   subjected to RAxML to build a maximum likelihood phylogenetic tree using JTT Substitution

952   Matrix and 100 bootstrap values[71]. Sequential distribution of gliding motility genes, i.e. *agl*, *glt*

953   (M1, G1 and G2 clusters)[4] and *cglB*[15,72,73] was identified within all 85 genomes under study

954   using two iterations of homology searching via JackHMMER (HMMER 3.1b1 [May 2013])[74]

955   with an E-value cut-off of 1e^{-5}, query coverage of 35%, and 35% sequence similarity. The

956   relative distribution of gliding motility proteins was mapped to the multi-protein phylogeny

957   using iTol v3[75]. The strip right to the phylogeny depicts the taxonomic classes (from top to

958   bottom: Myxococcales, non-Myxococcales δ-proteobacteria, α-, β-, γ-proteobacteria, and

959   fibrobacteres respectively).

960

Tertiary structure homology detection & modelling

962   Pair-wise alignments were performed with EMBOSS Needle software[76]. Protein

963   secondary structure was analyzed via Jpred4[77], with domain detection performed using

964   SMART[78]. Preliminary identification of structural homologues to CglB, GltK, GltB, GltA, and

965   GltH was carried out using fold-recognition searches of the Protein Data Bank using HHpred[79]

966   and RaptorX[80]. To improve template–target alignments, outputs were manually curated in

967   MODalign[81] to minimize breaks in predicted target secondary structure motifs. Curated

968   alignments to well-matched templates were then input into MODELLER[82] to generate a 3º

969   structure homology model.

970   CMView[83] was used to identify intra-protein contacts between Cα positions within model

971   structures with a minimum sequence separation of 10 amino acids. Evolutionarily-coupled

972   amino acid positions within proteins were identified using CoinFold[84], with the highest-

973   stringency L (length of the protein) number of coupled residues extracted. Positions of coupled

974   amino acids as well as intra-protein contacts were individually plotted in GraphPad Prism v.6,

975   followed by tricolour overlay using ImageJ to identify overlapping positions and colour

976   swapping in Photoshop (Adobe) to improve clarity.

977

SDS-PAGE, in-gel fluorescence, and Western immunoblotting

979   For detection of proteins from whole cells via Western immunoblot, TPM-washed cells

980   were sedimented and resuspended at OD_{600} 1.0 in 1× Laemmli sample buffer containing 5% β-

981   mercaptoethanol for reducing SDS-PAGE (unless otherwise indicated). Samples were boiled (10

982   min), loaded (20 μL) on 10-well 1 mm-thick gels, then resolved on 10% acrylamide gels (80 V

983   for 45 min for stacking, 120 V for 75 min for resolving), then electroblotted (100 V for 60 min)

984   to nitrocellulose membranes. Blots were rinsed with Tris-buffered saline (TBS) buffer, blocked

985   for 30 min at room temperature with 5% milk in TBS, then incubated rocking overnight in the 4

986   ºC cold room in 1:10 000 α-CglB, or 1:3000 α-GltH, or 1:1000 α-mCherry pAb mixture in TBS

987   with 0.05% Tween-20 (TBS-T). The next day, blots were rinsed twice (5 min) with TBS-T,

988   incubated with goat α-rabbit 2º antibody conjugated to HRP (1:5000) (Bio-Rad) in TBS-T at

989 room temperature (1 h), then rinsed twice (5 min) again with TBS-T. For all detections using α-

990 GltA (1:5000) and α-GltB (1:5000) pAb, identical processing steps were followed, but using

991 PBS-based buffers (instead of TBS). All immunoblots were developed using the SuperSignal

992 West Pico (Thermo) chemiluminescence substrate, captured on either a GE Imager with

993 ImageQuant software or an Amersham Imager 600 machine.

994 For fractionated whole cell–supernatant–OMV samples in 1× Laemmli buffer, samples

995 were boiled (10 min) and loaded (20 µL) on 15-well 4-20 % acrylamide precast gradient gels

996 (Biorad). Supernatant-alone samples were similarly boiled and loaded on a cast 10% acrylamide

997 gel. Gels were resolved at 120 V, followed by electroblotting to nitrocellulose membranes at

998 100 V. Immunodetection was performed with diluted polyclonal antisera as follows: α-CglB

999 (1:10 000), α-MglA (1:5000), and α-GltK (1:5000). Detection via secondary antibody was done

1000 with goat α-rabbit mAb (1:5000) conjugated to HRP (Biorad). Immunoblots were developed

1001 using the SuperSignal West Femto (Thermo) chemiluminescence substrate, captured on GE

1002 Imager with ImageQuant software.

1003 For analysis of AglZ-YFP in-gel fluorescence, TPM-washed cells were resuspended in

1004 $1\times$ non-reducing Laemmli sample buffer to an OD_{600} of 4.0. Cell resuspensions were heated for

1005 30 min (65 ºC), loaded (20 µL) on an 8% SDS-PAGE gel, and resolved for 45 min at 80 V, then

1006 75 min at 120 V. Cultures, cell resuspensions, and SDS-PAGE gels (before, during, and after

1007 resolution) were all shielded from ambient light to reduce photobleaching of the YFP moiety.

1008 Resolved gels were scanned on a Typhoon FLA9500 flat-bed imager (GE Healthcare). AglZ-

1009 YFP was excited with a 473 nm laser, with fluorescence captured using the BPB1 filter (PMT

1010 800). Pre-stained protein ladder bands were detected via excitation with a 635 nm laser and

1011 capture using the LPR filter (PMT 800). Quantification of band fluorescence intensity was

1012 performed using ImageJ via the "plot lanes" function, followed by determination of the area

1013 under the curve. AglZ-YFP signal for each lane was normalized to the faster-migrating auto-

1014 fluorescent band in the same lane; these values were then expressed as a percentage of the signal

1015 in WT cells for a given biological replicate.

1016

1017 ***Sample Fractionation***

1018 To separate supernatant and outer-membrane vesicle (OMV) fractions, WT, Δ*gltA*, Δ*gltB*

1019 and Δ*gltK* vegetative cells were grown in CYE medium to OD_{600} 0.7. Intact cells were first

1020   eliminated by sedimentation at 7830 rpm (10 min, RT). After addition of 1 mM PMSF,

1021   supernatants were sedimented at 125 000 × g (2 h, 4 °C). The resulting pellets (OMV fraction)

1022   and supernatants (soluble fractions) were then treated separately. The OMV pellets were washed

1023   with TPM, sedimented again at 125 000 × g (2 h, 4 °C), and then resuspended directly in 500 μL

1024   1× Laemmli protein sample buffer. The soluble supernatant fractions were treated with TCA (10

1025   % final concentration) for 30 min on ice and then sedimented at 11 000 rpm (1 h, 4 °C). The

1026   resulting pellets (precipitated proteins) were washed with 100% acetone, sedimented at 7830 rpm

1027   (10 min, 4 °C), and dried overnight at RT. Dried pellets were then resuspended in 1.5 mL TPM,

1028   sedimented at 15 000 rpm (30 min, 4 °C) and finally resuspended in 500 μL 1× Laemmli protein

1029   sample buffer.

1030   For isolation of supernatant-alone samples, 50 mL CYE cultures (inoculated at OD_{600}

1031   0.02) were grown overnight with shaking (220 rpm, 32 ºC) to OD_{600} 0.2-0.3, sedimented (6000 ×

1032 g, 15 min, 20 ºC), followed by passage of supernatants through a 0.22 μm syringe filter to

1033   remove any intact cells and cell debris. Filtered supernatants were each spun through a Vivaspin

1034   20 3-kDa cutoff column with polyethersulfone membrane (8000 × g, 60 min, 22 ºC, fixed-angle

1035   rotor) to concentrate proteins contained therein to 1 mL final volume. Concentrated supernatants

1036   were then sedimented in a tabletop ultracentrifuge (MLA130 rotor, 120 000 × g, 1.25 h, 4 ºC) to

1037   remove any OMVs or cell debris left in the samples. Clarified 1 mL supernatant samples were

1038   transferred to microfuge tubes and treated with 200 μL 100% TCA to precipitate the proteins.

1039   Tubes were heated at 65 ºC for 5 min, then spun (16 300 × g, 20 min, RT) to sediment

1040   precipitate. TCA-precipitated pellets were washed with 1 mL acetone, sedimented (16 300 × g,

1041   20 min, RT), followed by supernatant aspiration. Protein pellets were left uncapped in the

1042   chemical hood overnight to ensure evaporation of acetone. Pellets were resuspended in 500 μL

1043   2× Laemmli sample buffer lacking reducing agent, then diluted to 1× with ddH$_2$O.

1044

Immunoprecipitation & mass spectrometry

1046   Cells of Δ*gltB* from 100 mL CYE cultures were sedimented (4000 × g, 24 ºC, 15 min).

1047   Supernatants were decanted, pooled, and passed through a 0.2 μm syringe filter. Filtered

1048   supernatant was then concentrated using four Vivaspin20 columns (10 kDa cutoff) (Sartorius),

1049   spun at 8000 × g (20 ºC) in a fixed-angle centrifuge, with the supernatant concentrated to the dead

1050   volume limit of each column. Concentrated supernatants (~ 80 μL each) were subsequently

1051 pooled, and diluted 1:2 with filter-sterilized 1× PBS (binding buffer) to equilibrate sample pH.

1052 Separately, a single 1 mL Pierce Protein A column (Thermo) per each pooled supernatant was

1053 equilibrated in filter-sterilized binding buffer at room temperature as per the manufacturer's

1054 instructions. Filtered α-CglB antiserum (1 mL) was sedimented in a microfuge to remove remnant

1055 cells and/or debris (4000 × g, 5 min), diluted 1:1 with binding buffer, then sedimented at 12 000 ×

1056 g to clarify the sample as binding buffer addition may have resulted in lipoprotein precipitation.

1057 The Protein A column was primed by passage of 5 mL binding buffer. To bind antibody to the

1058 column, the 2 mL of diluted antiserum was added to the top of the column and allowed to drip

1059 through, followed by washing with 15 mL of binding buffer to remove unbound pAb. The ~ 960

1060 µL of supernatant concentrate was added to the top of the column and allowed to distribute

1061 throughout the resin bed at room temperature (60 min). The column was then again washed with

1062 15 mL binding buffer. To elute bound pAb (and any associated proteins) from the column, 5 mL

1063 of elution buffer (0.1 M glycine, pH to 2.5 with HCl) was added.

1064 To analyze the protein content of the pull-down, 500 µL of column eluate was concentrated

1065 in a microfuge using a Vivaspin500 column (10 kDa cutoff) to a dead volume of ~20 µL, then

1066 diluted 1:1 with 2× reducing Laemmli sample buffer. Samples (20 µL) were run into the stacking

1067 gel via SDS-PAGE (80 V, 13 min). Gel bands stained with SimplyBlue Safestain were excised

1068 from the stacking portion of the gel and the proteins digested by trypsin or Endoproteinase Glu-C.

1069 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analyses were

1070 performed on a Q-Exactive plus mass spectrometer (ThermoFisher Scientific) by staff at the

1071 Proteomics Platform of the Mediterranean Institute of Microbiology (Marseille). Processing of

1072 the spectra for protein identification was performed with PROTEOME DISCOVERER software

1073 (Thermo Scientific, versions 1.4.0.288 and 2.1.0.81).

1074

1075 ***Proteinase K surface digestion***

1076 Cells were resuspended in TPM at OD_{600} 2.0, followed by addition of 200 µg/mL

1077 Proteinase K and a brief vortex pulse to mix. An aliquot (50 µL) was immediately removed at t

1078 = 0 and placed into a tube containing 5 µL of 100% trichloroacetic acid (TCA). Digestion

1079 mixtures were incubated at room temperature on a rocker platform, with aliquots removed every

1080 15 min and placed into respective pre-aliquoted tubes of TCA. Upon removal of digestion

1081 reaction aliquots, TCA-containing sample tubes were heated at 65 ºC for 5 min, chilled on ice,

1082   then sedimented at 14 000 × g (5 min). Following supernatant removal, precipitated protein

1083   pellets were washed via resuspension in 500 µL of 100% acetone. Samples were then

1084   sedimented as before (14 000 × g, 5 min), followed by careful aspiration of the supernatants.

1085   Tubes were left uncapped overnight in the fume hood to promote evaporation of residual

1086   acetone, followed by storage at -80 ºC until needed. Precipitated protein pellets were

1087   resuspended in 50 µL 1× Laemmli sample buffer (with reducing agent as indicated) and analyzed

1088   via SDS-PAGE and Western immunoblot.

1089

1090   *Motility and fluorescence analysis*

1091   For phase-contrast and fluorescence-microscopy on agar pads, cells from exponentially-

1092   growing cultures were sedimented and resuspended in TPM buffer to OD_{600} 5.0, spotted (5 µL)

1093   on a glass coverslip, then overlaid with a pad of 1.5% agar prepared with TPM. For motility

1094   analysis, cells were left to adhere for 5 min prior to imaging at 32 ºC using a TE2000-E-PFS

1095   microscope (Nikon) with a 40× objective and a CoolSNAP HQ2 camera (Photometrics) with

1096   Metamorph software (Molecular Devices). AglZ-YFP fluorescence was imaged using a

1097   monolithic aluminum microscope (homemade) equipped with a 1.49 NA/100X objective (Nikon

1098   Instruments) and imaged on an iXon DU 897 EMCCD camera (Andor Technology). Illumination

1099   was provided by a 488 nm DPSS laser (Vortran Stradus), and sample positioning was performed

1100   using a P611 three-axis nanopositioner (Physik Instrument). Instrument control was programmed

1101   in LabView (National Instruments) providing integrated control of all components. Cell gliding

1102   speeds were calculated using the MicrobeJ module for FIJI [85]. Gliding cell montages were

1103   generated using FIJI. Kymograph panels were generated using the FIJI Kymograph Builder

1104   function. AglZ-YFP clusters were detected manually and tracked with the MTrackJ FIJI plugin.

1105   Using an R software script, the points of the AglZ-YFP cluster trajectories (x0, x1, ... , xn; y0,

1106   y1, ... , yn) were used to calculate the mean square displacement (MSD) at time

1107 $$t = d^2{}_t = (x_t - x_0)^2 + (y_t - y_0)^2: \qquad MSD(t) = \frac{1}{t}\sum_0^t d_t^2$$

1108

1109   For total internal reflection fluorescence microscopy (TIRFM), imaging of real-time

1110   AglZ-YFP trafficking was performed as previously detailed in chitosan-coated

1111   polydimethylsiloxane (PDMS) microfluidic channels[8]. In brief, cells were injected into the

1112   chamber and left to adhere (30 min) without flow, with unadhered cells then removed via manual

1113   injection with TPM. TIRFM was performed on attached cells with active autofocus using an

1114   inverted microscope with 100× oil-immersion Plan-Achromat objective, atop a closed-loop

1115   piezoelectric stage. AglZ-YFP was excited with a 488-nm laser, with emission collected by the

1116   objective, through a dichroic mirror and bandpass filters, and captured by an emCCD camera.

1117   For imaging of the YFP channel in real time, 500 images were captured at 20 Hz[8].

1118

1119   ***Flow chamber construction and bead assay***

1120   Prior to experiments, 1 mL of *M. xanthus* DZ2 WT+AglZ-YFP and mutant DZ2

1121   Δ*cglB*+AglZ-YFP overnight culture was grown to OD_{600} ~0.6, sedimented (8000 rpm, 5 min),

1122   and resuspended in 400 mL TPM buffer. Flow chambers were made by combining two layers of

1123   double sided tape, a 1 mm-thick glass microscope slide, and a 100 μm-thick glass cover slip

1124   (#1.5) as previously described[86]. The tape was separated to allow a final volume of

1125   approximately 60 μL. Agarose (40 μL at 0.7%) dissolved in 6 M DMSO was injected into the

1126   chamber and allowed to sit at room temperature for 15 min. The chamber was washed with 400

1127   μL TPM then injected with *M. xanthus* cells (60 μL) and left at room temperature to facilitate

1128   cell attachment to the agarose-coated surface for 30 min. Unattached cells were then thoroughly

1129   washed away with a total of 2 mL TPM media containing 10 mM glucose. The flow chamber

1130   was then mounted onto the microscope for imaging. For bead experiments, 1 μL of uncoated

1131   polystyrene beads (diameter 520 nm) (Bangs Laboratories) were washed and diluted in 1 mL

1132   TPM containing 10 mM glucose and injected into the flow chamber. Beads were optically

1133   trapped and placed about a third of the cell length away from the pole of the immobilized cell of

1134   interest.

1135

1136   ***Bead tracking and video analysis***

1137   For a chosen *M. xanthus* cell (WT or mutant), 3-min movies were recorded and analyzed

1138   using a custom MATLAB tracking code. The code uses filtering mechanisms to subtract the

1139   image background from that of the cell-attached bead. Firstly, an internal MATLAB centroid

1140   function identified the *x,y* pixel values of the centre of the bead for each frame in the video,

1141   followed by pixel value conversion to microns. This was then used to compute motor-driven

1142   bead runs and velocities for each cell. The threshold value for a run was previously determined

1143   by disabling molecular motors and decreasing bead motion in WT cells by carefully injecting 20

1144   µM of nigericin, a pH-gradient/proton motive force-inhibitory drug, into the mounted flow

1145   chamber. This drug concentration decreased bead velocity but not motor force production

1146   translated to the beads. In these previous experiments, 40 µM nigericin was used, leading to

1147   negligible bead motion[12].

1148

1149   ***Flow cytometry***

1150   Analysis of the presence of CglB in mCherry^{+} cells within a mixed population was

1151   performed with strains TM1380 (or TM1290) and TM1365, positive and negative for the

1152   mCherry marker, respectively. Fluorescence-activated cell sorting (FACS) was performed with

1153   a Bio-Rad S3E cell sorter. The blue laser (488 nm, 100 mW) was used for forward (FSC) and

1154   side scatter (SSC) signals and the green laser (561 nm, 100 mW) for excitation of mCherry.

1155   Signals were collected using the emission filter FL3 (615/25 nm). Bacterial samples in TPM

1156   buffer (maximum optical density of 0.1) were run in the low-pressure mode at ~10,000 particles

1157   per second. The threshold on FSC was 0.15% and the voltages of the photomultipliers were 403,

1158   299 and 722 volts for FSC, SSC and FL3, respectively. The density plots obtained (small angle

1159   scattering FSC versus wide angle scattering SSC signal) were gated on the population of interest

1160   and filtered to remove multiple events. Populations of 300,000 – 500,000 events were used and

1161   analyzed statistically using Prosort or FlowJo software. For sorting in "purity" mode, up to 60

1162   million events were collected and at least ten tubes of 3.5 mL were used for sorting high-height

1163   peak values of mCherry fluorescence. A total volume of 35 mL was collected and pooled,

1164   followed by addition of 10% TCA to precipitate total protein content in the tube. Samples were

1165   sedimented via ultracentrifuge (164 700 $\times$ g, 1 h, 4 °C), then the pellet was resuspended in 500

1166   µL of TPM, sedimented again (22 065 $\times$ g, 30 min, 4 °C), and later used for Western-blot

1167   analysis.

1168   SUPPLEMENTAL INFORMATION TITLES AND LEGENDS:

1169   **Supplementary Figure S1: Defining the Structures of GltA, B, H, and CglB**

1170   **A)** GltA (i) tertiary structure homology model (based on the OprF β-barrel protein from

1171   *Pseudomonas aeruginosa*), (ii) overlay of intra-protein amino acid contact positions from the

1172   tertiary structure model (*black*) vs. evolutionarily-coupled amino acid positions from the primary

1173   structure (*grey*) (with conserved positions between the two datasets indicated in *red*), (iii)

1174   alignment with PDB 4RLC for MODELLER input.

1175   **B)** GltB (i) tertiary structure homology model (based on the OprF β-barrel protein from

1176   *Pseudomonas aeruginosa*), (ii) overlay of intra-protein amino acid contact positions from the

1177   tertiary structure model (*black*) vs. evolutionarily-coupled amino acid positions from the primary

1178   structure (*grey*) (with conserved positions between the two datasets indicated in *red*), (iii)

1179   alignment with PDB 4RLC for MODELLER input.

1180   **C)** GltH (i) tertiary structure homology model (based on the OmpA β-barrel protein from

1181   *Shigella flexneri*), (ii) overlay of intra-protein amino acid contact positions from the tertiary

1182   structure model (*black*) vs. evolutionarily-coupled amino acid positions from the primary

1183   structure (*grey*) (with conserved positions between the two datasets indicated in *red*), (iii)

1184   alignment with PDB 3NB3 for MODELLER input.

1185   **D)** α-GltA and α-GltB Western immunoblots from whole-cell extracts from different Δ*glt*

1186   mutants resolved via reducing SDS-PAGE.

1187   **E)** CglB (i) overlay of intra-protein amino acid contact positions from the tertiary structure

1188   model (*black*) vs. evolutionarily-coupled amino acid positions from the primary structure (*grey*)

1189   (with conserved positions between the two datasets indicated in *red*), (ii) alignment with PDB
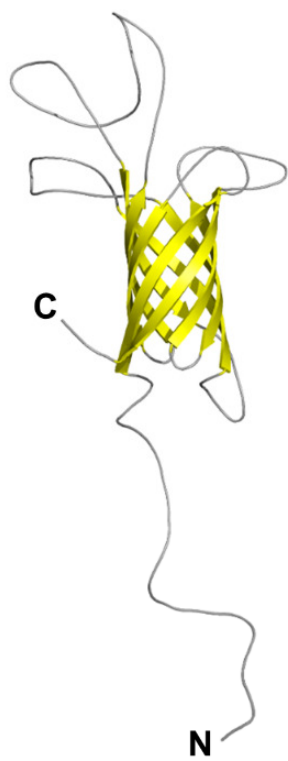
1190   3TXA for MODELLER input. See also Figure 2A. For the template alignments in figure panels

1191   A, B, C, E: residue colouration is based on the conservation score as determined by Jalview:

1192   maroon, 10 (out of 10); red, 9; orange, 8; yellow, 7. Conservation scores ≤ 6 have not been

1193   displayed to enhance the clarity of the figure. Secondary structure properties for each protein are

1194   indicated above/below the aligned sequence, based on the respective PDB entry and GltA/B/H/T

1195   secondary structure prediction via JPred, with β strands (*green arrows*) indicated.
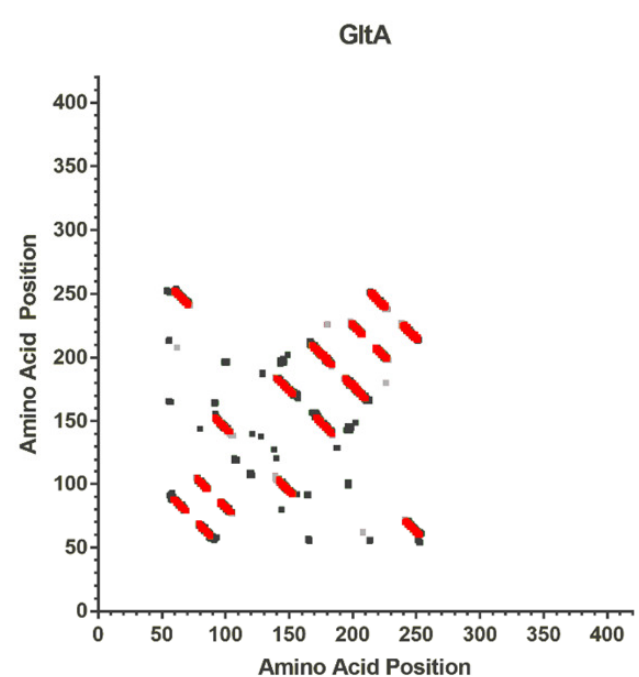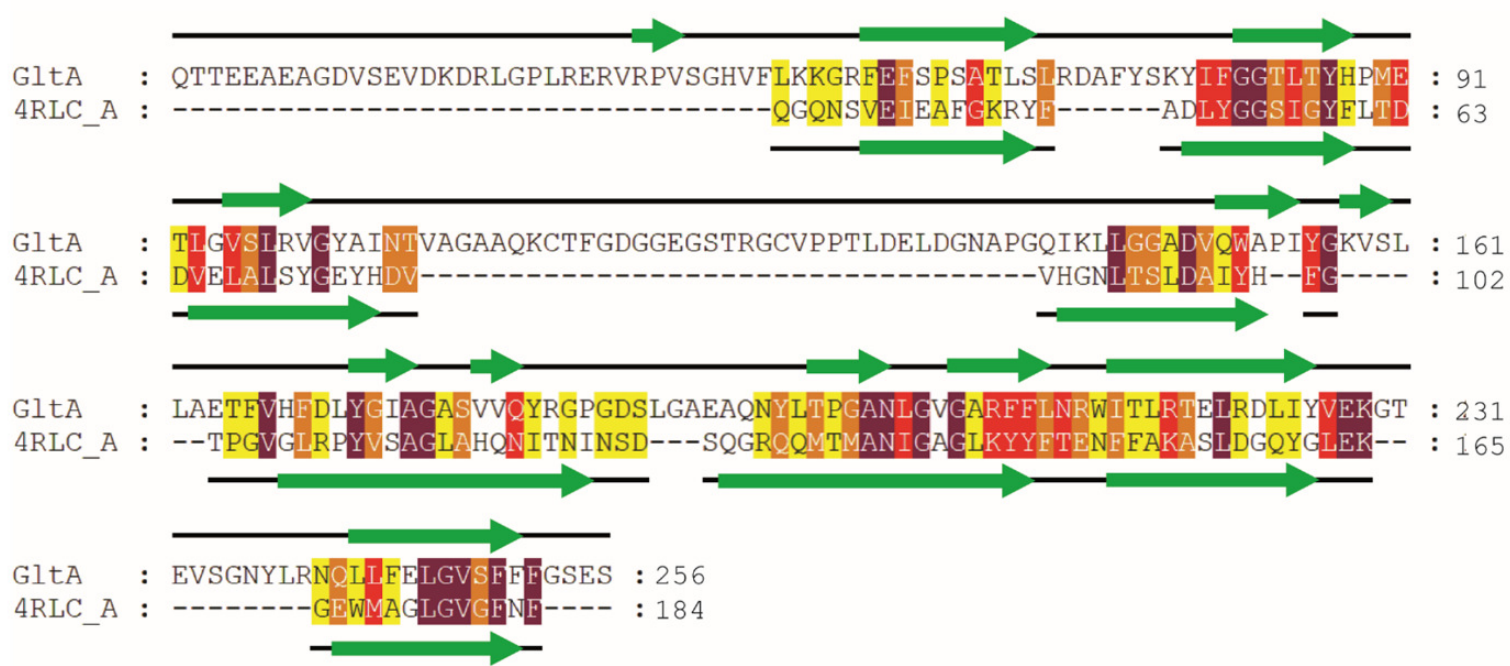
SUPPLEMENTARY FIGURE S1

A

(i)

(ii)

(iii)


```
GltA   : QTTEEAEAGDVSEVDKDRLGPLRERVRPVSGHVFLKKGRFEFSPSATLSIRDAFYSKYIFGGTLTYHPME : 91
4RLC_A : ---------------------------------QGQNSVEIEAFGKRYF------ADLYGGSIGYFLTD : 63

GltA   : TLGVSLRVGYAINTVAGAAQKCTFGDGGEGSTRGCVPPTLDELDGNAPGQIKLLGGADVQWAPIYGKVSL : 161
4RLC_A : DVELAISYGEYHDV----------------------------------VHGNLTSLDAIYH--FG---- : 102

GltA   : LAETFVHFDLYGIAGASVVQYRGPGDSLGAEAQNYLTPGANLGVGARFFLNRWITLRTELRDLIYVEKGT : 231
4RLC_A : --TPGVGLRPYVSAGLAHQNITNINSD---SQGRCQMTMANIGAGLKYYFTENFFAKASLDGQYGLEK-- : 165

GltA   : EVSGNYLRNQLIFELGVSFFFGSES : 256
4RLC_A : --------GEWMAGLGVGFNF---- : 184
```

SUPPLEMENTARY FIGURE S1

B

(i)

C

N

(ii)

GltB

Amino Acid Position (y-axis): 0, 50, 100, 150, 200, 250, 300, 350, 400

Amino Acid Position (x-axis): 0, 50, 100, 150, 200, 250, 300, 350, 400

(iii)

```
GltB    : QSQEGMGLDLTDESQAESSQDAESPAPPPMEEATPAAASRPADEPLEADPQLPLTDITQEDRVKSVQRKV : 89
4RLC_A  : --------------------------------------------------------------------- : -

GltB    : YLKKGRFEITPLVSLSVNDP---FYSKVGLSVRGAYYLADTIAVSARASLMQVVPSDDVRTAKRTFNSKI : 156
4RLC_A  : -QGQNSVEIEAFGKRYF---------ADLYGGSIGYFLTDDVELALSYGEYH-----DV----------- : 77

GltB    : YYSVPQWSAMGDVEWSPIYGKVAFLNSILHFDGYILAGAGVVNTETSAL--PGRGLNPAADLGLGMRFVA : 224
4RLC_A  : ---VHGNLTSLDAIYH-------FGTPGVGLRPYVSAGLAHQNITNINSDSQGRQQMTMANIGAGLKYYF : 147

GltB    : QDYIAVNVALINTSYVDQPLGSSKGAIQNVMTLNAGISLFLPFKSTGRDSE :275
4RLC_A  : TENFFAKASLDGQYGLEK---------GEWMAGLGVGRNF----------- :184
```

SUPPLEMENTARY FIGURE S1

C

(i)

(ii)

GltH

(iii)

Predicted signal peptide

```
GltH   : MKALASLALCASFVLPTVASAQQPTTAQGDRPAVTFDEIERGIYFAVLGGPLFITNPPAAEGTPRP : 66
3NB3_A : -----------------------------------APKDNTWYTGAKLGWSQ------------- : 38

GltH   : FSSGPMAQVEVGVDLGEHVSVGL-FLMGSSVRTGAQYVGNSGGAVSGDFSTLIPGAVLRARLVGFA : 131
3NB3_A : HENKLGAGAFGGYQVNPYVGFEMGYDWLGRMPY---------------AYKAQG-VQLTAKLGYPIT : 89

GltH   : DSQEVKRTWFYLRAGAGYAMFSPQRL----LPDSDILVFAGPGVEYYTRLRHFSVGLEVTGQYLVS : 193
3NB3_A : -----DDLDIYTRLGGMVWRADTYSNVYGKNHDTGVSPVFAGGVEYAI-TPEIATRLEYQWTN--- : 146

GltH   : GGSFGFAVAPNIRYAF : 209
3NB3_A : -----GMLSLGVSYRF : 157
```

SUPPLEMENTARY FIGURE S1

D

SUPPLEMENTARY FIGURE S1

E

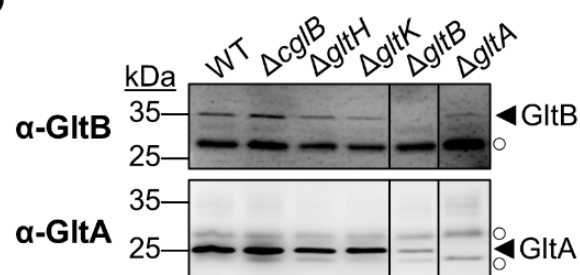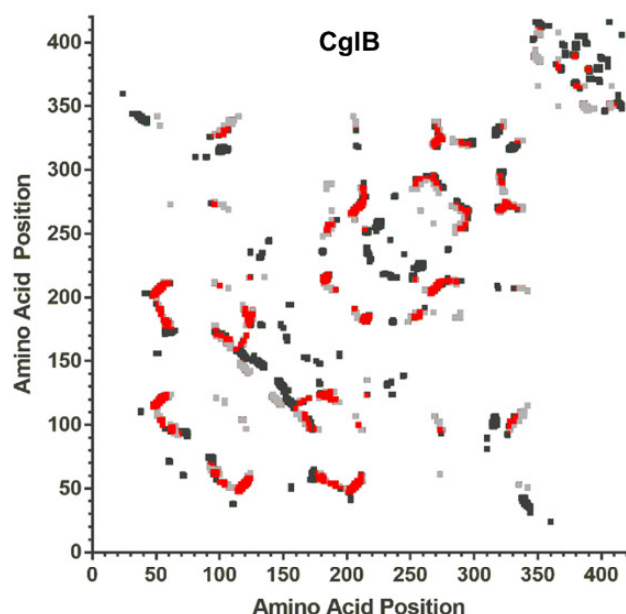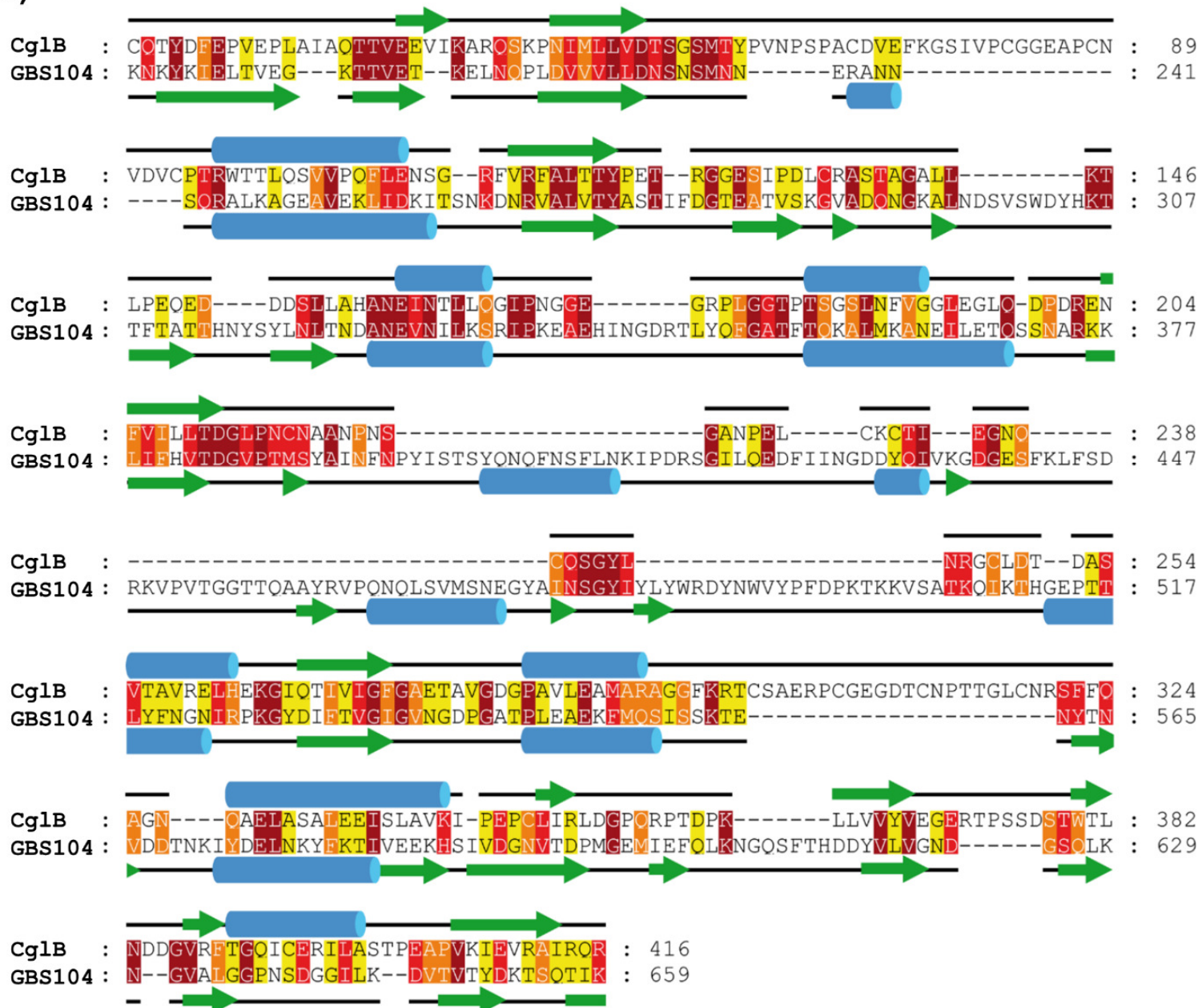**(i)**

(ii)

Supplementary Figure S2: Importance of CglB to Gliding Motility and Linked Behaviours

A) Single-cell gliding speeds for *M. xanthus* OM-module mutant strains (n = 100 cells) on hard (1.5%) agar. The lower and upper boundaries of the boxes correspond to the 25th and 75th percentiles, respectively. The median (line through centre of boxplot) and mean (+) of each dataset are indicated. Lower and upper whiskers represent the 10th and 90th percentiles, respectively; data points above and below the whiskers are drawn as individual points. Asterisks denote datasets displaying statistically significant dataset differences ($p < 0.0001$) relative to WT, as determined via 1-way ANOVA with Tukey's multiple comparisons test.

B) Ratio of number of Cys residues per gliding motility complex protein, divided by the total number of amino acids in that protein.

C) Single-cell gliding speeds for WT *M. xanthus* DZ2 (n = 700 cells) on hard (1.5%) agar supplemented with increasing concentrations of DTT to reduce disulphide bonds of proteins in contact with the substratum. The lower and upper boundaries of the boxes correspond to the 25th and 75th percentiles, respectively. The median (line through centre of boxplot) and mean (+) of each dataset are indicated. Lower and upper whiskers represent the 10th and 90th percentiles, respectively; data points above and below the whiskers are drawn as individual points. Asterisks denote datasets displaying statistically significant dataset differences ($p < 0.0001$) relative to 0 mM DTT treatment, as determined via 1-way ANOVA with Tukey's multiple comparisons test.
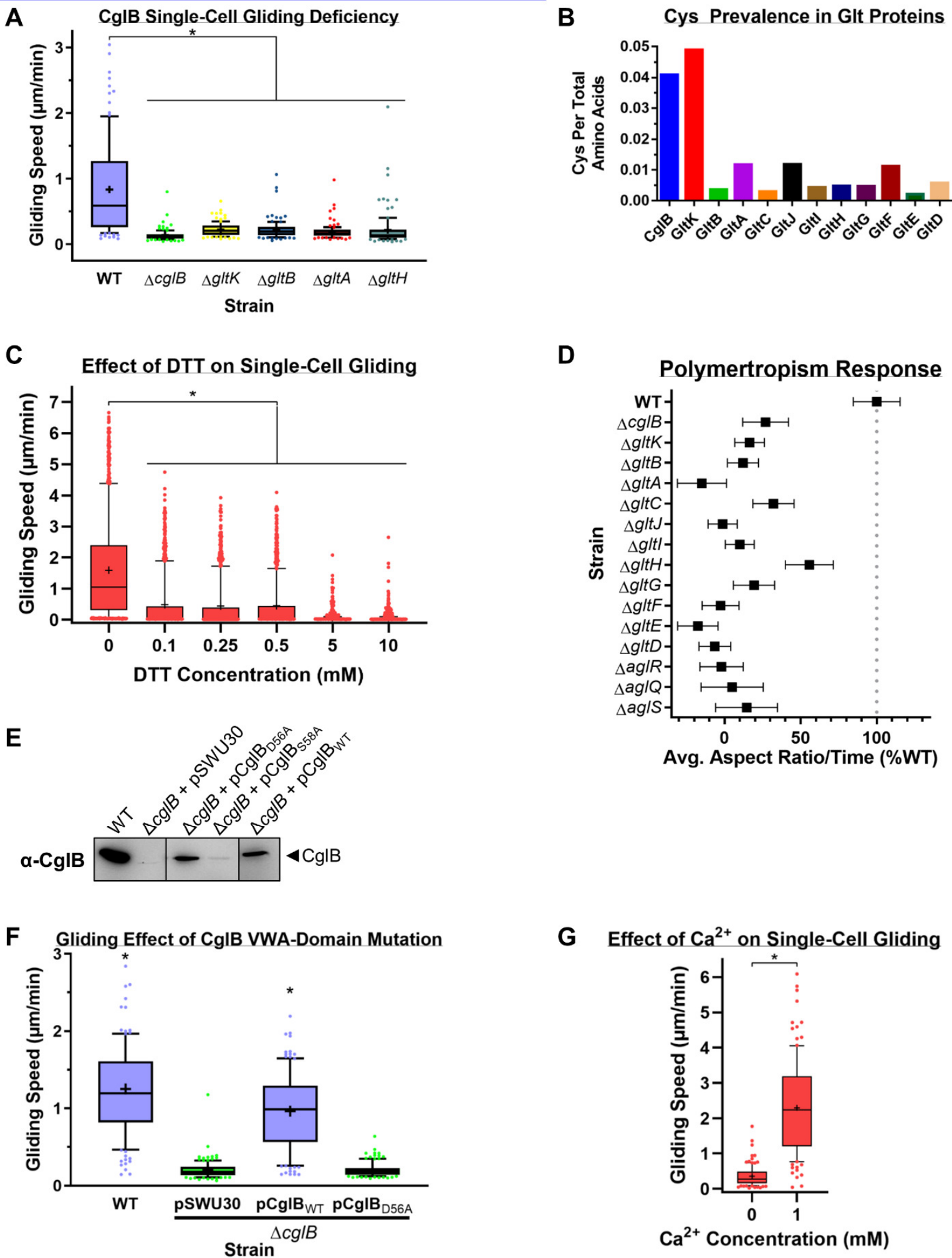
D) Polymertropism response for various *glt* and *agl* deletion mutant strains. Mean results are displayed +/− SEM, with the number of biological replicates (*n*) used to analyze each strain as follows: WT (20), Δ*cglB* (16), Δ*gltK* (19), Δ*gltB* (11), Δ*gltA* (15), Δ*gltC* (15), Δ*gltJ* (16), Δ*gltI* (15), Δ*gltH* (20) Δ*gltG* (18), Δ*gltF* (16), Δ*gltE* (14), Δ*gltD* (17), Δ*aglR* (11), Δ*aglQ* (6), Δ*aglS* (5).

E) α-CglB Western immunoblot of CglB MIDAS motif amino acid substitution mutants from whole-cell extracts. Non-adjacent lanes on the blot are separated by vertical black lines.

F) Single-cell gliding speeds on hard (1.5%) agar for *M. xanthus* DZ2 Δ*cglB* (n = 120 cells) complemented with CglB_{WT} or CglB_{D56A} ectopically expressed from the *attB* phage-attachment site in the chromosome. The lower and upper boundaries of the boxes correspond to the 25th and 75th percentiles, respectively. The median (line through centre of boxplot) and mean (+) of each dataset are indicated. Lower and upper whiskers represent the 10th and 90th percentiles,

1232   respectively; data points above and below the whiskers are drawn as individual points. Asterisks

1233   denote datasets displaying statistically significant dataset differences ($p < 0.0001$) compared to

1234   strains harbouring either the pSWU30 empty-vector control or the pCglB$_{D56A}$ VWA-domain

1235   mutant CglB complementation construct, as determined via 1-way ANOVA with Tukey's

1236   multiple comparisons test.

1237   **G)** Single-cell gliding speeds for *M. xanthus* DZ2 Ω*pilA* (n = 110 cells) on chitosan-coated glass

1238   in a PDMS microfluidic chamber in the presence and absence of Ca^{2+} ions. The lower and upper

1239   boundaries of the boxes correspond to the 25th and 75th percentiles, respectively. The median

1240   (line through centre of boxplot) and mean (+) of each dataset are indicated. Lower and upper

1241   whiskers represent the 10th and 90th percentiles, respectively; data points above and below the

1242   whiskers are drawn as individual points. Asterisk denotes datasets displaying statistically

1243   significant differences ($p < 0.0001$, as determined via two-tailed Mann-Whitney test.

SUPPLEMENTARY FIGURE S2

A CglB Single-Cell Gliding Deficiency

B Cys Prevalence in Glt Proteins

C Effect of DTT on Single-Cell Gliding

D Polymertropism Response

E

F Gliding Effect of CglB VWA-Domain Mutation

G Effect of Ca²⁺ on Single-Cell Gliding

1245 **Supplementary Figure S3: CglB Co-Occurrence and Gene Synteny in Bacteria**

1246 Taxonomic distribution and co-occurrence of *agl* and *glt* genes in bacteria. Bootstrap values at

1247 each node are indicated as follows: 100 (out of 100), ●; 90-99, ▲; 80-89, ■ ; 70-79, ⬟; 60-69,

1248 ⬟; 50-59, ★; < 49, ✗. Colour of gene hit indicates synteny with the G1 *gltDEFGHIJ* (*dark*

1249 *blue*) or G2 *gltKBAC* (*blue*) gene clusters or lack thereof (*light blue*), respectively; herein,

1250 synteny denotes a minimum of three genes in the vicinity of each other.

SUPPLEMENTARY FIGURE S2
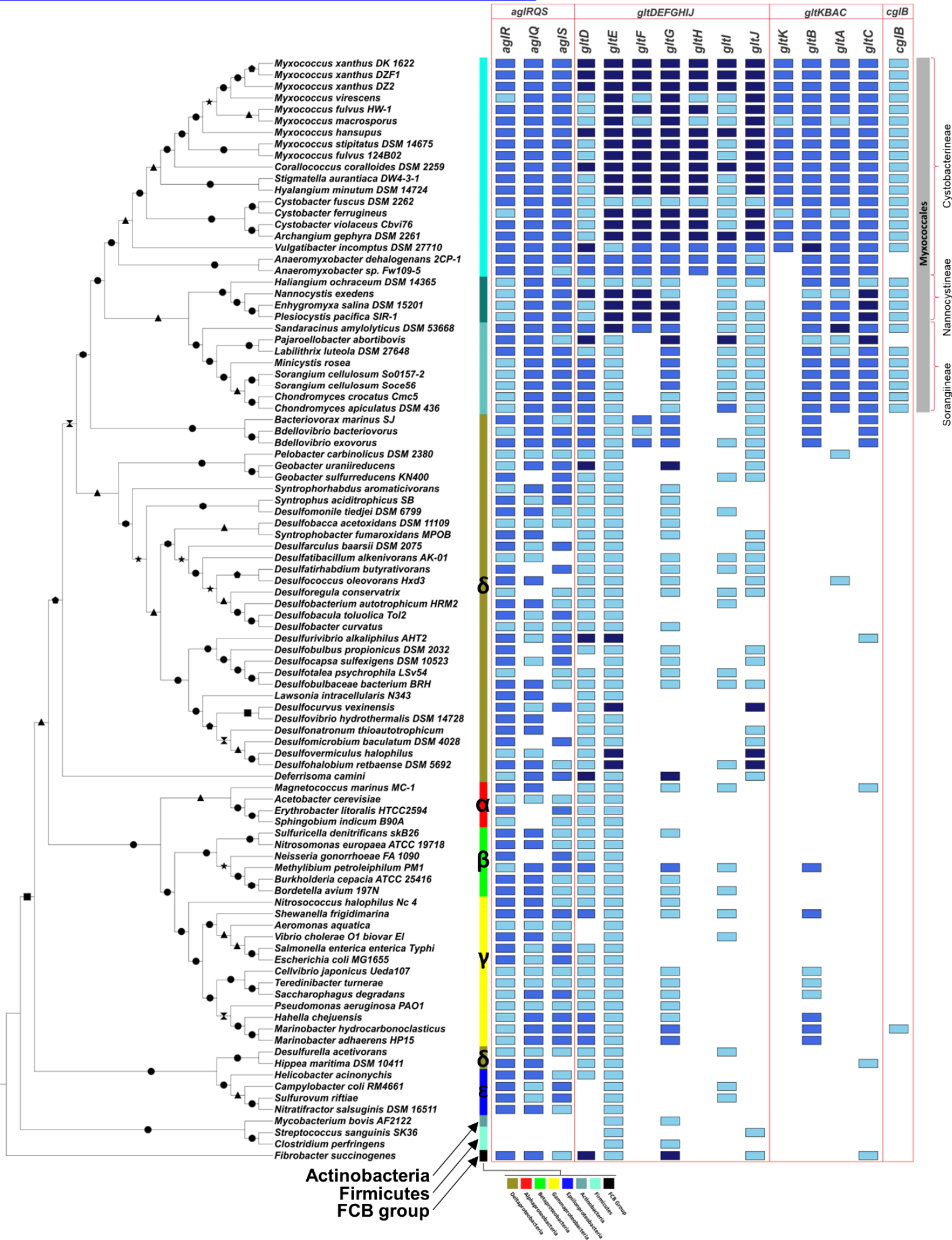
Supplementary Figure S4: Role of CglB in Agl–Glt Complex Localization and Directed Surface Transport

A) In-gel fluorescence (i) scan and (ii) densitometry analysis of AglZ-YFP levels in WT vs. Δ*cglB* crude-cell lysates resolved via SDS-PAGE. Fluorescence levels were analyzed across six biological replicates and are displayed +/− SEM. Despite a higher mean value for AglZ-YFP levels in Δ*cglB* cells than in WT cells, this difference was not statistically significant, as determined via Wilcoxon signed-rank test performed relative to "100" ($p = 0.0938$).
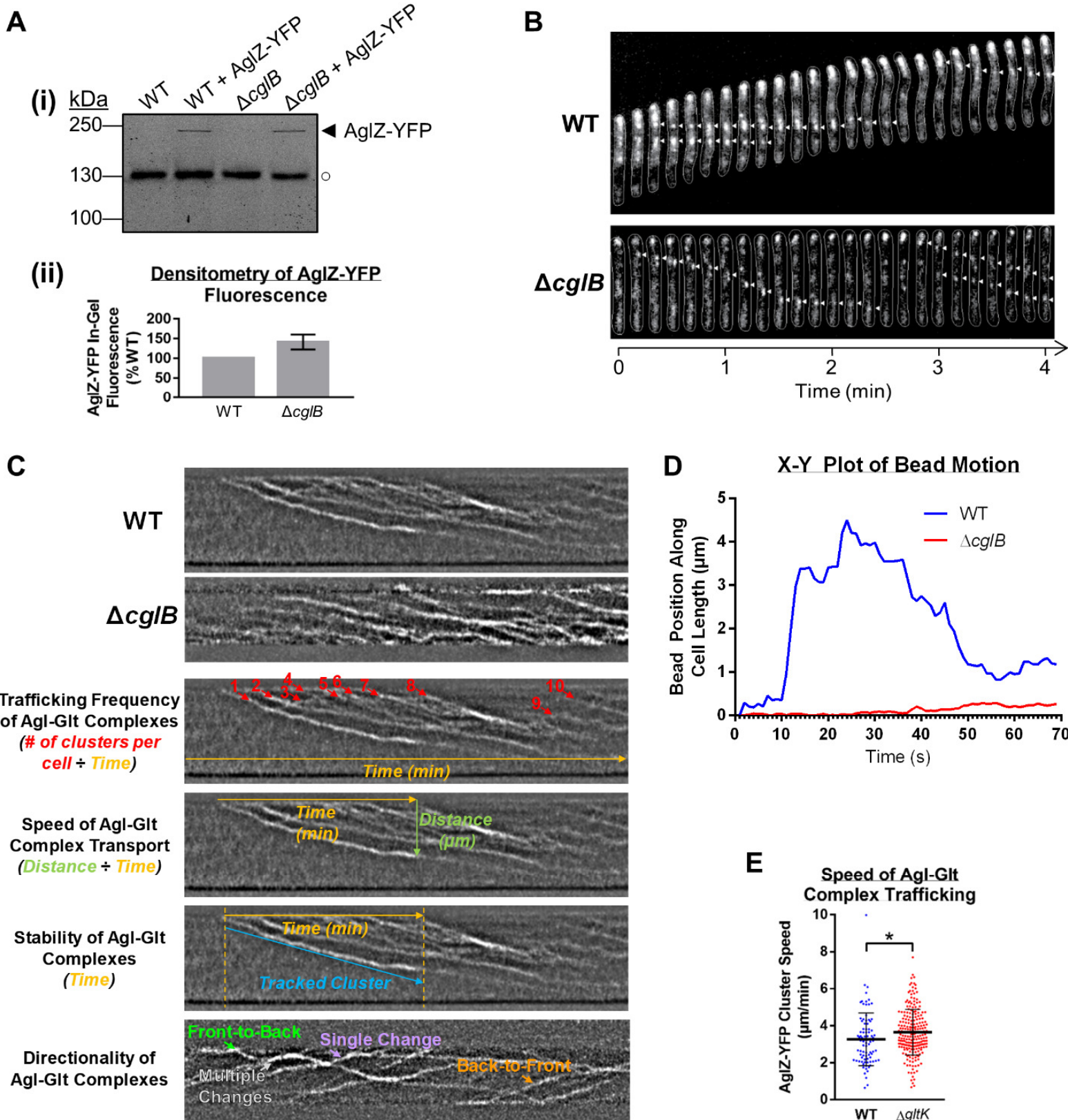
B) Montage of *M. xanthus* gliding time course displaying AglZ-YFP localization (white arrowheads) on hard agar via epifluorescence microscopy. Images were acquired at 10 s intervals. See also **Fig. 2C**.

C) Kymograph of AglZ-YFP localization in *M. xanthus* cells on chitosan-coated PDMS microfluidic chambers via TIRFM. Arrows in *orange* denote sequential kymograph slices over time. Arrows in *cyan* indicate positions of trafficked Agl–Glt clusters in the cell. The manner in which various fluorescent cluster tracking data were obtained have been indicated in the example images. See also **Fig. 2E-H**.

D) Representative time course of polystyrene bead position tracking along the length of a cell.

E) Speed of Agl–Glt complex trafficking via TIRFM (of AglZ-YFP) on chitosan-coated glass surfaces in PDMS microfluidic chambers for WT (n = 260 clusters) and Δ*gltK* (n = 371 clusters) strains. The distribution of the two datasets are is significantly different, as calculated via unpaired two-tailed Mann-Whitney U-test ($p < 0.05$). See also **Fig. 2F** and **Supplementary Fig. S4C**.

SUPPLEMENTARY FIGURE S4

A

(i) kDa — WT, WT + AglZ-YFP, ΔcglB, ΔcglB + AglZ-YFP — AglZ-YFP

250 — 130 — 100 —

(ii) Densitometry of AglZ-YFP Fluorescence — AglZ-YFP In-Gel Fluorescence (%WT) — WT, ΔcglB

B WT, ΔcglB — Time (min)

C WT, ΔcglB

Trafficking Frequency of Agl-Glt Complexes (# of clusters per cell ÷ Time)

Speed of Agl-Glt Complex Transport (Distance ÷ Time)

Stability of Agl-Glt Complexes (Time)

Directionality of Agl-Glt Complexes — Front-to-Back, Single Change, Multiple Changes, Back-to-Front

D X-Y Plot of Bead Motion — Bead Position Along Cell Length (µm) — Time (s) — WT, ΔcglB

E Speed of Agl-Glt Complex Trafficking — AglZ-YFP Cluster Speed (µm/min) — WT, ΔgltK

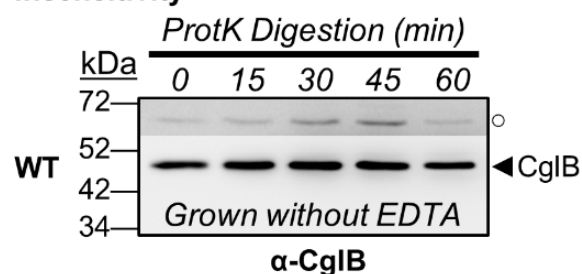**Supplementary Figure S5: Possibility of Post-Translational Processing of CglB**

A) Protein samples from WT cells resuspended in TPM buffer and digested with exogenous Proteinase K. Aliquots of the digestion mixture were removed at 15-min intervals and TCA-precipitated to stop digestion. The higher, darker zone on the blot corresponds to a section of the same blot image for which the contrast has been increased to highlight lower-intensity protein bands. Legend: ◄, full-length CglB; ○, loading control (non-specific protein band labelled by α-CglB pAb).

B) Concentrated supernatant samples from Δ*glt* mutants that exhibit depleted levels of cell-associated CglB. Equivalent supernatant volumes from different strains were concentrated in a centrifuge using 3 kDa-cutoff protein concentrator columns, followed by TCA treatment of precipitate proteins. Legend: ◄, full-length CglB. See also **Fig. 3B**.
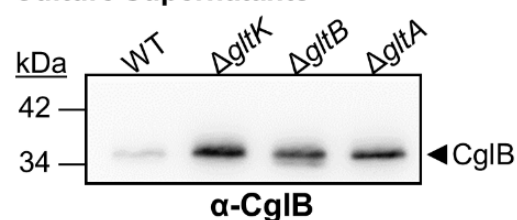
C) Peptides identified via trypsin/V8 digestion and mass spectrometric analysis of immunoprecipitated CglB from Δ*gltB* culture supernatant. Legend: *grey*, predicted signal peptide; *green*, trypsin-derived peptide; *cyan*, V8-derived peptide; *red box*, N-terminal tract of CglB unaccounted for by mass spectrometry.

D) α-CglB Western immunoblot demonstrating the resumption of CglB release from EDTA-grown cells upon transfer to an EDTA-free minimal buffer (TPM) or rich medium (CYE). Legend: ◄, full-length CglB; ←, CglB degradation band; P_{EDTA}, parent strain (WT or Δ*gltB*) grown in the presence of EDTA. See also **Fig. 3E**.

SUPPLEMENTARY FIGURE S5

A CgIB Intrinsic Proteinase K Insensitivity

ProtK Digestion (min)

kDa 0 15 30 45 60

72—
52—
42—
34—

WT ◄CgIB

Grown without EDTA

α-CgIB

B Spin Column-Concentrated Culture Supernatants

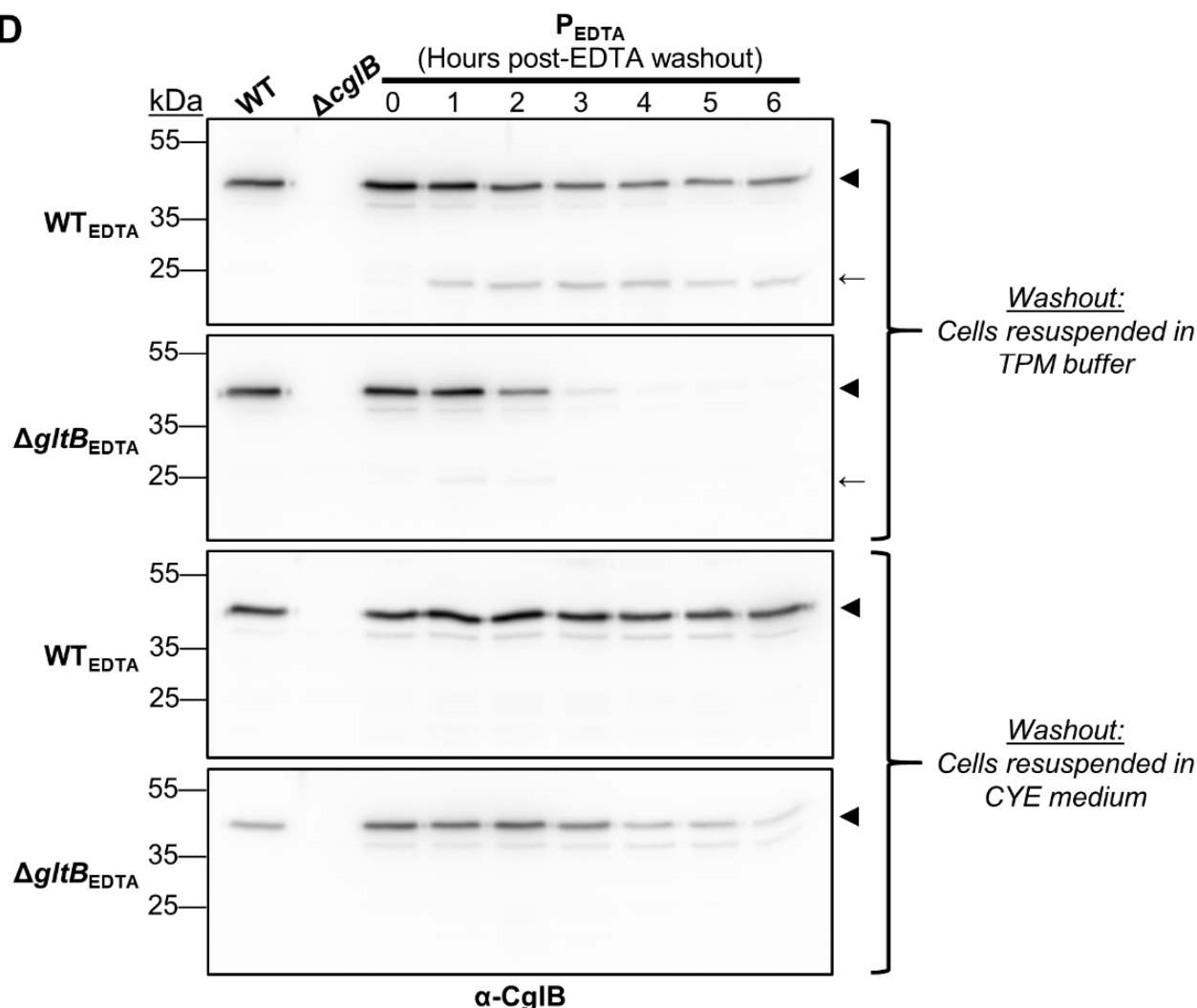kDa WT ΔgltK ΔgltB ΔgltA

42—
34— ◄CgIB

α-CgIB

C

>CglB
MRSKLPLLSALSVGAVVLACQTYDFEPVEPLAIAQTTVEEVIKARQSKPNIMLLVDTSGSMTYPVNPSPACDVEFKGSIVPCGGEAPCNVDVCPTRWTTLQSVVPQFLENSGRF

VRFALTTYPETRGGESIPDLCRASTAGALLKTLPEQEDDDSLLAHANEINTLLQGIPNGGEGRPLGGTPTSGSLNFVGGLEGLQDPDRENFVILLTDGLPNCNAANPNSGANPE

LCKCTIEGNQCQSGYLNRGCLDTDASVTAVRELHEKGIQTIVIGFGAETAVGDGPAVLEAMARAGGFKRTCSAERPCGEGDTCNPTTGLCNRSFFQAGNQAELASALEEISLAV

KIPEPCLIRLDGPQRPTDPKLLVVYVEGERTPSSDSTWTLNDDGVRFTGQICERILASTPEAPVKIEVRAIRQR

D

P_EDTA (Hours post-EDTA washout)

kDa WT ΔcglB 0 1 2 3 4 5 6

WT_EDTA
55—
35— ◄
25— ←

ΔgltB_EDTA
55—
35—
25— ← ◄

Washout: Cells resuspended in TPM buffer

WT_EDTA
55—
35— ◄
25—

ΔgltB_EDTA
55—
35— ◄
25—
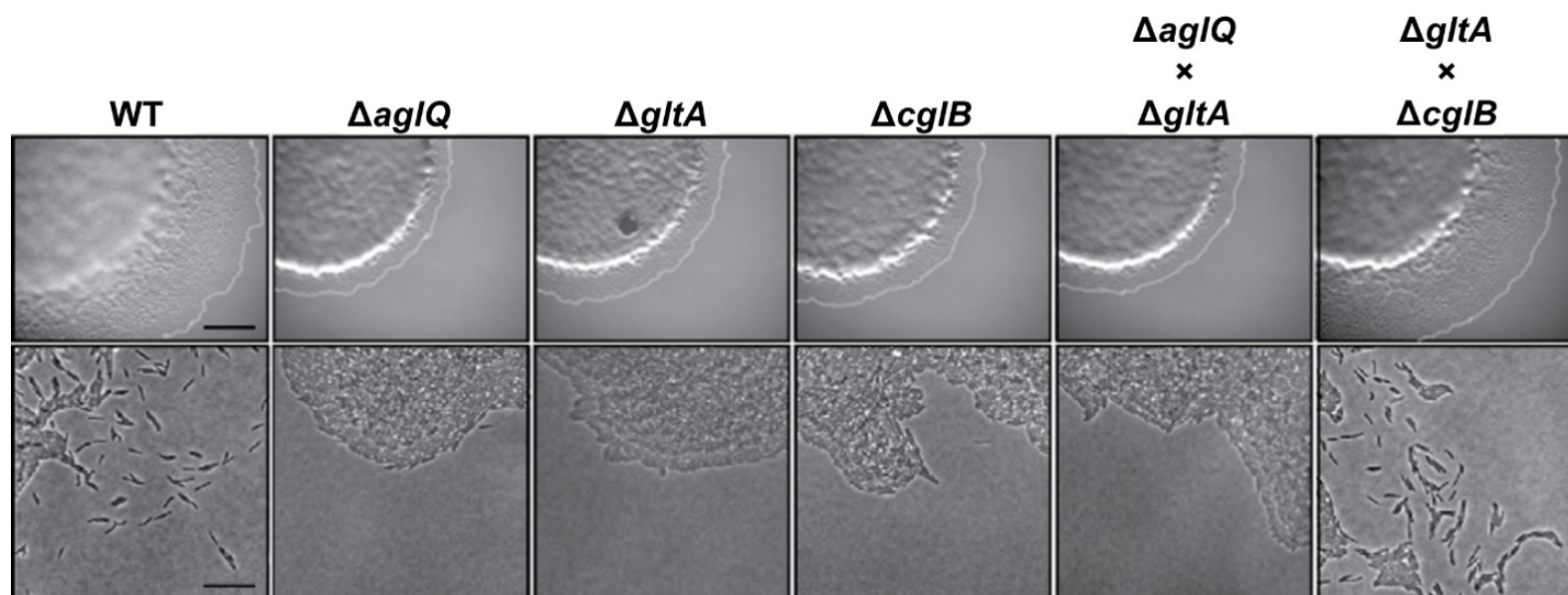
Washout: Cells resuspended in CYE medium

α-CgIB

1294   **Supplementary Figure S6: Exploration of GltK Function**

1295   Motility restoration in cells following *trans*-complementation via OME. Bottom panels (scale

1296   bar: 20 µm) are magnified views of the corresponding upper panels (scale bar: 1 mm).

SUPPLEMENTARY FIGURE S6

				Δ*aglQ* ×	Δ*gltA* ×
WT	**Δ*aglQ***	**Δ*gltA***	**Δ*cglB***	**Δ*gltA***	**Δ*cglB***

1298   **Supplementary Table S1: Strains of *M. xanthus* Used in this Study**

1299   *Myxococcus xanthus* strains used in this study.

Supplementary Table S1: *Myxococcus xanthus* strains used in this study

Strain	Genotype/Description	Construction	Source or Reference
DZ2	WT	Wild type	Laboratory collection
TM770	Ω*cglB*	DZ2 Ω*cglB*	This work
TM913	Δ*cglB*	DZ2 Δ*cglB*	This work
TM600	Δ*gltK*	DZ2 Δ*gltK* (pBJΔ*gltK*)	Luciano et al., 2011
TM603	Δ*gltB*	DZ2 Δ*gltB* (pBJΔ*gltB*)	Luciano et al., 2011
TM606	Δ*gltA*	DZ2 Δ*gltA* (pBJΔ*gltA*)	Luciano et al., 2011
TM570	Δ*gltC*	DZ2 Δ*gltC* (pBJΔ*gltC*)	Luciano et al., 2011
TM646	Δ*gltJ*	DZ2 Δ*gltJ*	This work
TM731	Δ*gltI*	DZ2 Δ*gltI*	This work
TM149	Δ*gltH*	DZ2 Δ*gltH* (pBJΔ*gltH*)	Luciano et al., 2011
TM135	Δ*gltG*	DZ2 Δ*gltG* (pBJΔ*gltG*)	Luciano et al., 2011
TM136	Δ*gltF*	DZ2 Δ*gltF* (pBJΔ*gltF*)	Luciano et al., 2011
TM148	Δ*gltE*	DZ2 Δ*gltE* (pBJΔ*gltE*)	Luciano et al., 2011
TM142	Δ*gltD*	DZ2 Δ*gltD* (pBJΔ*gltD*)	Luciano et al., 2011
TM1157	Δ*gltK* Δ*gltH*	TM149 Δ*gltK* (pBJΔ*gltK*)	This work
TM1156	Δ*gltB* Δ*gltH*	TM149 Δ*gltB* (pBJΔ*gltB*)	This work
TM1158	Δ*gltA* Δ*gltH*	TM149 Δ*gltA* (pBJΔ*gltA*)	This work
TM1154	Δ*gltB* Δ*gltA*	DZ2 pBJ114-Δ*gltBA*	This work
TM829	WT + *aglZ-YFP*	DZ2 pBJ*AglZ-YFP*	Mignot et al., 2007
TM1181	Δ*cglB* + *aglZ-YFP*	TM913 *aglZ-YFP* (pBJ*AglZ-YFP*)	This work
TM1159	Δ*cglB* + *cglB*$_{WT}$	TM913 *cglB*$_{WT}$ (pCglB$_{WT}$)	This work
TM1149	Δ*cglB* + *cglB*$_{D56A}$	TM913 *cglB*$_{D56A}$ (pCglB$_{D56A}$)	This work
TM1153	Δ*cglB* + *cglB*$_{S58A}$	TM913 *cglB*$_{S58A}$ (pCglB$_{S58A}$)	This work
TM1290	Δ*cglB* + IMss-mCherry	TM913 IMss-mCherry (pSWU10-PpilA-IMss-mCherry)	This work
TM1365	Δ*aglQ* Δ*gltK*	TM146 Δ*aglQ* (pBJΔ*gltK*)	This work
TM1367	Δ*cglB* Δ*gltK*	TM913 Δ*cglB* (pBJΔ*gltK*)	This work
TM1380	Δ*cglB* Δ*gltK* + IMss-mCherry	TM1367 Δ*cglB* Δ*gltK* (pSWU10-PpilA-IMss-mCherry)	This work

1301 **Supplementary Table S2: Fold-Recognition Hits of CglB to Various VWA-Containing**

1302 **Proteins**

1303 Select fold-recognition hits of structural homologues in the PDB to CglB, as identified via

1304 HHpred. The strength of the hits is anchored by the strong detection of a von Willebrand Factor

1305 type A domain.

Supplementary Table S2: HHpred fold-recognition hits in the PDB to CglB

PDB Hit	Name	Probability	E-value	Score	Aligned Coils	Identities	Similarity
1MF7_A	Integrin αM (*Homo sapiens*)	99.4	6.4E-14	123.84	187	19%	0.191
4FX5_A	von Willebrand factory type A (*Catenulispora acidiphila*)	99.36	3e-13	140.21	199	19%	0.161
4OKR_A	Micronemal protein MIC2 (*Toxoplasma gondii*)	99.34	3.8E-13	127.97	180	20%	0.242
4F1J_B	TRAP (*Plasmodium falciparum*)	99.18	1.1E-11	110.16	178	17%	0.257
3TXA_A	GBS104 tip pilin (*Streptococcus agalactiae* serogroup V)	99.13	7.2E-12	136.34	188	17%	0.239