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A multi-protein complex from *Myxococcus xanthus* required for bacterial gliding motility

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Summary

Myxococcus xanthus **moves by gliding motility powered by Type IV pili (S-motility) and a second motility system, A-motility, whose mechanism remains elusive despite the identification of ~40 A-motility genes. In this study, we used biochemistry and cell biology analyses to identify multi-protein complexes associated with A-motility. Previously, we showed that the N-terminal domain of FrzCD, the receptor for the *frizzy* chemosensory pathway, interacts with two A-motility proteins, AglZ and AgmU. Here we characterized AgmU, a protein that localized to both the periplasm and cytoplasm. On firm surfaces, AgmU-mCherry colocalized with AglZ as distributed clusters that remained fixed with respect to the substratum as cells moved forward. Cluster formation was favoured by hard surfaces where A-motility is favoured. In contrast, AgmU-mCherry clusters were not observed on soft agar surfaces or when cells were in large groups, conditions that favour S-motility. Using glutathione-S-transferase affinity chromatography, AgmU was found to interact either directly or indirectly with multiple A-motility proteins including AglZ, AglT, AgmK, AgmX, AglW and CglB. These proteins, important for the correct localization of AgmU and AglZ, appear to be organized as a motility complex, spanning the cytoplasm, inner membrane and the periplasm. Identification of this complex may be important for uncovering the mechanism of A-motility.**

Introduction

Myxococcus xanthus is a rod-shaped Gram-negative soil bacterium with a complex life cycle that includes predation, vegetative growth and development (fruiting body formation). During vegetative growth, *M. xanthus* cells move in organized groups known as swarms, and feed on lysed microorganisms or organic matter by secreting hydrolytic enzymes and antimicrobials. When nutrients or prey are scarce, *M. xanthus* cells enter a developmental pathway that results in cellular aggregation in which 10^5–10^6 cells form fruiting bodies that contain spores (Shimkets, 1999; Kaiser, 2006; Mauriello and Zusman, 2007; Zusman *et al.*, 2007; Berleman and Kirby, 2009). Directed motility is essential for vegetative swarming, predation and development.

Myxococcus xanthus cells move by gliding motility and do not have flagella (Henrichsen, 1972). Hodgkin and Kaiser, over 30 years ago, showed that *M. xanthus* cells utilize two genetically distinct motility systems (Hodgkin and Kaiser, 1979). One system, called social (S)-motility, is required for the movement of cells in groups and is now known to be powered by the retraction of polar Type IV pili, similar to twitching motility in *Pseudomonas aeruginosa* (Henrichsen, 1983; Sun *et al.*, 2000; Li *et al.*, 2003). The other system, called adventurous (A)-motility, is required for the movement of isolated cells (Hodgkin and Kaiser, 1979; Zusman *et al.*, 2007). However, the mechanism of A-motility is still unknown, although several hypotheses have been proposed (Wolgemuth *et al.*, 2002; Mignot *et al.*, 2007). These two motility systems enable *M. xanthus* cells to move selectively on different agar surfaces: A-motility works best on relatively hard, dry surfaces, whereas S-motility is favoured on soft moist agar surfaces (Shi and Zusman, 1993) or when cells are submerged in methylcellulose (Sun *et al.*, 2000). With both motility systems, cells periodically reverse their gliding direction. During reversals, the polarity of the cells is inverted so that the leading cell pole becomes the lagging cell pole and the old lagging cell pole becomes the new leading cell pole. The co-ordination of the two motility systems is essential for directed motility. The reversal frequencies of both the S- and A-motilities are regulated by the *frizzy* (Frz) chemosensory pathway (Zusman *et al.*, 2007). Little is known about how these two motility systems are co-ordinated or how cells switch between these systems as they encounter different surfaces.

Several models of A-motility have been proposed based on experimental observations: (i) In the 'slime gun' model, cells actively secrete a polyelectrolyte gel (slime)

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through 'slime nozzles' located at the lagging cell pole; according to this model, the hydration of the slime propels cells forward (Wolgemuth *et al*., 2002). (ii) Another model, proposed by our laboratory, is the 'focal adhesion' model, based on the cytological observations of the A-motility protein AglZ (Yang *et al*., 2004; Mignot *et al*., 2007). Mignot *et al*. reported that when cells move forward on 1.5% agar, AglZ clusters remain stationary with respect to the substratum (Mignot *et al*., 2007). This observation suggested a model in which the A-motility engines push against 'focal adhesion' complexes that span the cell envelope and connect to an internal cytoskeleton (Mignot, 2007; Mauriello *et al*., 2010). Although these two models are very different, both require a protein complex spanning the cell envelope to power A-motility. Characterization of this complex will be a crucial step towards uncovering the mechanism of A-motility. About 40 genes have been identified as playing a role in A-motility (Youderian *et al*., 2003; Yu and Kaiser, 2007; Hartzell *et al*., 2008). However, it is still unknown how the proteins encoded by these genes are organized into functional A-motility complexes. In this study, we report that AgmU, a protein that interacts with FrzCD, is part of an envelope-spanning complex with AglZ, AglT, AgmK, AgmX, AglW and CglB. This work provides the basis for assigning function to several important A-motility proteins.

Results

AgmU interacts with the N-terminal region of FrzCD

In a previous study, we used affinity chromatography with glutathione-S-transferase (GST)-tagged FrzCD as bait to identify proteins that interact with FrzCD, the receptor for the Frz pathway. In that study, we identified two A-motility proteins, AglZ and AgmU as interacting partners with the N-terminal domain of FrzCD (Mauriello *et al*., 2009a). In the present study, the interaction between AgmU and FrzCD was characterized.

Figure 1A shows that AgmU, a large protein of 1218 amino acids, contains two clusters of tetratricopeptide repeats (TPR) in its N-terminal domain. The first cluster contains five TPR motifs (residues 163–400) and the second, four repeats (residues 620–810) (Fig. 1A). TPR domains have been previously shown to be important in protein–protein interactions (D'Andrea and Regan, 2003), suggesting that these TPR clusters in AgmU might play a similar role together with other motility proteins. The AgmU C-terminal domain lacks a predicted function.

In order to investigate the interaction between AgmU and FrzCD, the two TPR clusters and the C-terminal domain of AgmU, and full-length AgmU were expressed and purified in *Escherichia coli* (TPR I, TPR II, C-ter and AgmU in Fig. 1A). We then examined their interactions with purified FrzCD by *in vitro* formaldehyde cross-linking. Figure 1B shows a Western blot in which anti-FrzCD antibodies were used to show that full-length AgmU and both TPR clusters of AgmU interacted with the N-terminal domain of FrzCD. In contrast, no evidence for an interaction between AgmU and the C-terminal domain of FrzCD was observed (Fig. 1C).

AgmU is an essential component of the A-motility machinery in M. xanthus

The *agmU* gene was first identified by Youderian *et al*. (2003) as an A-motility-related gene through a genome wide screen using the transposon, *magellan-4*. In this study, we constructed an *agmU* in-frame deletion mutant that lacks the coding region from amino acids 72 to 1206. The *agmU* deletion mutant, constructed in a strain lacking S-motility because of a *pilA::tet* insertion, showed very few single cells at the edge of colonies on 1.5% agar. This indicates that it has a defect in A-motility (Hodgkin and Kaiser, 1979) (Fig. 2). However, *agmU pilA*$^+$ cells showed wild-type S-motility swarming on soft (0.5%) agar, indicating that S-motility was not defective in the mutant (data not shown). To study the biological function of the AgmU-FrzCD interaction, an *agmU frzCD pilA::tet* triple mutant was constructed. Figure 2 shows that in this strain, A-motility was restored, suggesting that AgmU, like AglZ, negatively regulates A-motility through its interaction with FrzCD (Mauriello *et al*., 2009a).

Because *agmU frzCD pilA::tet* and *aglZ frzCD pilA::tet* triple mutants both showed restored A-motility, we constructed an *agmU aglZ frzCD pilA::tet* quadruple mutant. To our surprise, this quadruple mutant showed no A-motility (Fig. 2). The phenotype of the *agmU aglZ frzCD pilA* quadruple mutant indicates that either AgmU or AglZ is absolutely required for A-motility. This result suggests that AgmU and AglZ belong to the same A-motility machinery. We note that both AgmU and AglZ are proteins of more than 1000 amino acids and have multiple domains, suggesting that they could have both regulatory and structural functions.

AgmU has two distinct localization patterns in vivo

In order to investigate the localization of AgmU *in vivo*, an *agmU::mCherry* strain was constructed that encoded a mCherry tag fused to the C-terminus of AgmU. The gene fusion was inserted at the endogenous locus of *agmU*; this strain showed no defect in A-motility (Fig. 2), S-motility or fruiting body formation (data not shown). The localization of AgmU-mCherry on 1.5% (w/v) agar (or agarose) was then monitored by fluorescence microscopy. Interestingly, we observed that AgmU-mCherry localized in two distinct patterns depending on

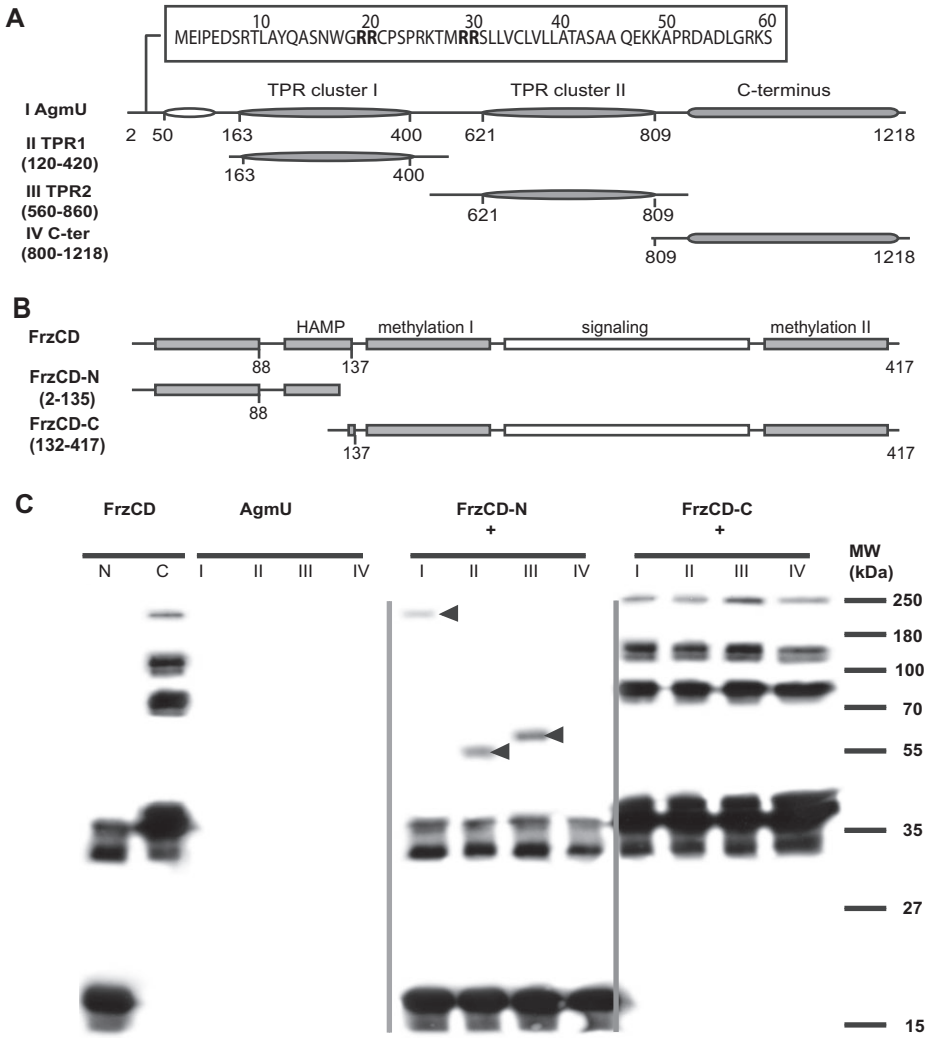

Fig. 1. The two TPR clusters of AgmU interact with the N-terminus of FrzCD.
A. Schematic representation of the different AgmU fragments used in the *in vitro* cross-linking shown in (C). Each protein is fused to an N-terminal His_6 tag. The first 60 amino acids of AgmU are shown in the inset. The possible recognition sites for the Tat secretion pathway (twin arginines) are shown in bold characters.
B. Schematic representation of the N-terminal and C-terminal regions of FrzCD used in the *in vitro* cross-linking shown in (C). Each protein is fused to an N-terminal His_6 tag.
C. Anti-FrzCD immunoblotting of the *in vitro* formaldehyde cross-linking experiments with AgmU and FrzCD fragments. Purified FrzCD N-terminal (FrzCD-N) or C-terminal (FrzCD-C) regions and/or purified AgmU fragments [roman numerals indicate AgmU domains shown in (A)] incubated in the presence of the cross-linker (10 mM formaldehyde). Lanes 1–6, FrzCD or AgmU proteins incubated alone; lanes 7–10, FrzCD-N co-incubated with different AgmU fragments; lanes 11–14, FrzCD-C co-incubated with different AgmU fragments. The arrowheads indicate bands only seen after co-incubating FrzCD-N with full-length AgmU or the TPR clusters of AgmU and cross-linker.

the environment of the cells: (i) In large groups of cells (usually more than 100 cells per group), AgmU-mCherry was localized primarily near the cell envelope (Fig. 3A–C, movie S1). This observation was confirmed by *trans*-section scans of 20 individual cells, which showed two fluorescence peaks that correspond to the location of the cell envelope (Fig. 3B). In these cells, AgmU appeared to be slightly more concentrated towards the lagging cell pole (Fig. 3C). (ii) In small cell groups (usually less than 20 cells per group) or isolated cells, AgmU-mCherry was again observed near the envelope, but was also present as distributed clusters (Fig. 3D–F, movie S2). These AgmU-mCherry clusters appeared to be very dynamic, frequently appearing and disappearing (data not shown). Trans-section scans taken between these clusters showed two peaks, corresponding to the location of the cell envelope (Fig. 3E), while scans across clusters gave an additional peak in the centre (Fig. 3F).

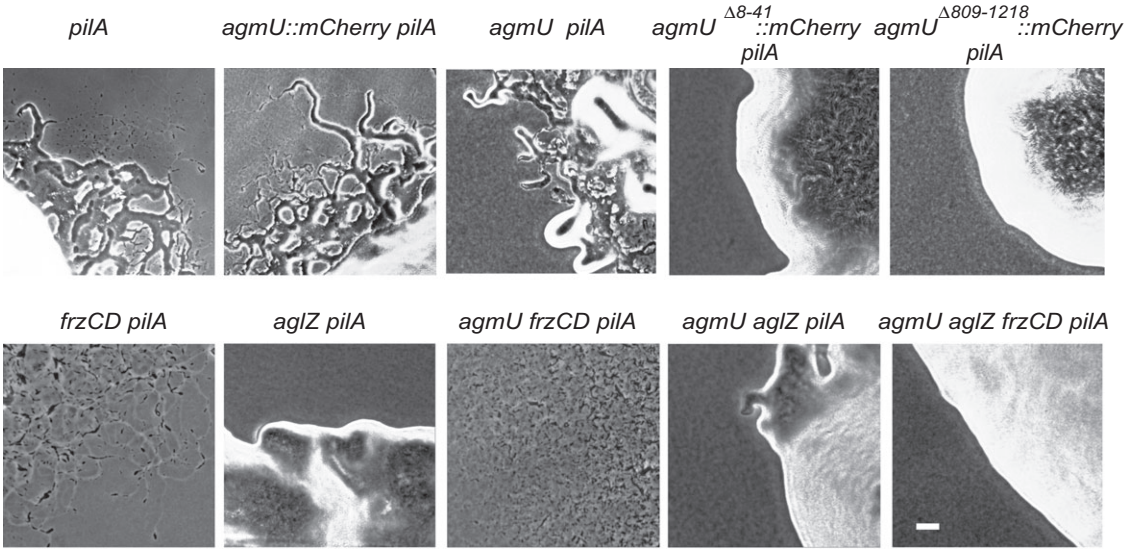

Fig. 2. A-motility analysis of *M. xanthus* strains DZ2 (wt), *frzCD*, *aglZ* and *agmU* mutants. In the S^- background (*pilA*), single cells swarming out from the edge of colonies on 1.5% agar (or agarose) were monitored as an indicator of A-motility (Hodgkin and Kaiser, 1979). Cells (10 μl), at a concentration of 4×10^9 cfu ml^{-1}, were spotted on CYE plates containing 1.5% (w/v) agar (or agarose), incubated at 32°C and photographed after 48 h with a WTI CCD-72 camera, using a Nikon Labphot-2 microscope. Scale bar, 40 μm.

AgmU localizes in both the periplasmic and cytoplasmic fractions

In order to study the unexpected localization pattern of AgmU, we subjected *M. xanthus* cells to osmotic shock and investigated the localization of AgmU in the various cell fractions. We used the shock procedure, described by Nelson *et al*. (Nelson *et al*., 1981), which involves treating *M. xanthus* cells with buffer containing 25% (w/v) sucrose, and then rapidly resuspending the cells in buffer lacking sucrose. The whole cell, periplasmic, cytoplasmic and membrane fractions were then analysed by SDS polyacrylamide gel electrophoresis and Western immunoblotting using anti-AgmU, anti-FrzE and anti-MbhA (Nelson *et al*., 1981) antibodies. Figure 4 shows the detected bands of AgmU, FrzE and MbhA cut from each Western blot. AgmU from wild-type cells was found in the periplasmic, cytoplasmic and membrane fractions. Because AgmU lacks a transmembrane domain, the observed localization pattern suggests that some of the AgmU molecules might be secreted and anchored to the cytoplasmic or outer membrane, and other molecules remain in the cytoplasm. In contrast, FrzE, a cytoplasmic histidine kinase, was only found in the cytoplasmic fraction, indicating that there was very little lysis of protoplasts during the shock procedure (Fig. 4). Additionally, the periplasmic hemagglutinin, MbhA (Nelson *et al*., 1981), was observed in the periplasmic and membrane fractions, but not in the cytoplasmic fraction, indicating that the osmotic shock separated cytoplasmic and periplasmic proteins effectively (Fig. 4). The observation that AgmU-mCherry shows a dual localization pattern is consistent with our fractionation experiments in which cells cultured in liquid showed AgmU to be present in both the periplasmic and cytoplasmic fractions.

Analysis of the AgmU clusters

The N-terminal sequence of AgmU shows some similarity with the signal sequences of lipoproteins (Fig. 1A). To investigate the dual periplasmic and cytoplasmic localization pattern of AgmU, we constructed strains in which the amino acids 8–41 of AgmU or the AgmU C-terminal domain (amino acids 809–1218) were deleted from the *agmU::mCherry* strain. The *agmU*$^{\Delta 8-41}$*::mCherry pilA*$^-$ and *agmU*$^{\Delta 809-1218}$*::mCherry pilA*$^-$ mutants showed dramatic defects in A-motility, defects that were more severe than deletions that spanned the entire *agmU* gene (Fig. 2). We speculate that the truncated AgmU$^{\Delta 8-41}$ and AgmU$^{\Delta 809-1218}$ proteins interfere with overlapping functions of other A-motility proteins, such as AglZ, thereby causing a more severe defect in A-motility. Cell fractionation experiments showed that AgmU$^{\Delta 8-41}$-mCherry is found only in the cytoplasm, while AgmU$^{\Delta 809-1218}$-mCherry is found in the periplasm, cytoplasm and membrane, indicating that the deletion of the C-terminal domain does not change the localization patterns of these proteins (Fig. 4). We also followed the localization of AgmU$^{\Delta 8-41}$-mCherry in living cells on 1.5% (w/v) agar (or agarose) by fluorescence microscopy. In these cells, AgmU$^{\Delta 8-41}$-mCherry no longer localized near the cell envelope but instead formed larger centrally located clusters within the cell (Fig. 3G–I). We analysed the clusters by *trans*-section scanning between

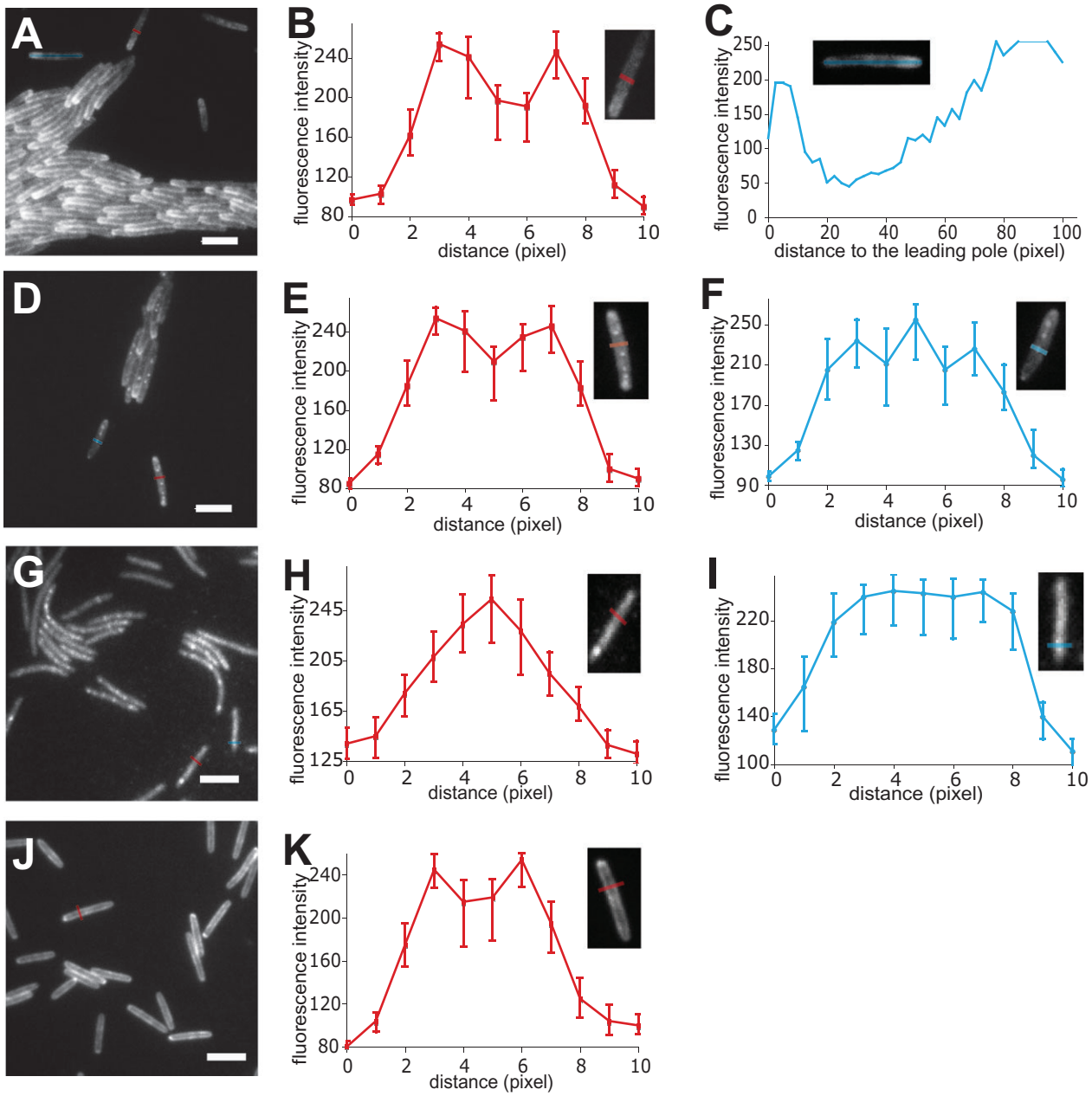

Fig. 3. AgmU-mCherry shows two distinct localization patterns.
A. In large cell groups on 1.5% (w/v) agar (or agarose), AgmU-mCherry concentrates near the cell envelope. Images were taken with an Olympus IX70 DeltaVision microscope.
B. Statistical analysis of the *trans*-section fluorescence scans in the same condition as (A) gives two fluorescence peaks at the location of the membrane. Twenty individual cells were scanned with ImageQuant software (GE healthcare) and the highest fluorescence density of each scan was normalized to 255 (same below). The inset shows a typical scan position in (A).
C. Fluorescence scan along the long axis of one typical cell in (A) shows the relative high concentration of AgmU-mCherry in the posterior half of the cell.
D. In small groups or isolated cells on 1.5% (w/v) agar (or agarose), AgmU-mCherry shows two localization patterns. Besides the envelope-associated localization, protein clusters distributed along the cells were also seen.
E. Statistical analysis of the scans between clusters in the condition of (D).
F. Statistical analysis of the scans across clusters in the condition of (D).
G. When the N-terminal sequence of AgmU is deleted, AgmU$^{\Delta 8-41}$-mCherry only forms cytoplasmic clusters.
H. Statistical analysis of the scans between clusters in the condition of (G).
I. Statistical analysis of the scans across the clusters in the condition of (G).
J. On 1.5% agar (or agarose), no clusters formed by AgmU$^{\Delta 809-1218}$-mCherry are evident in either grouped or isolated cells, while the periplasmic localization is retained in this mutant.
K. Statistical analysis of the scans in the condition of (J). Scale bars, 5 μm.

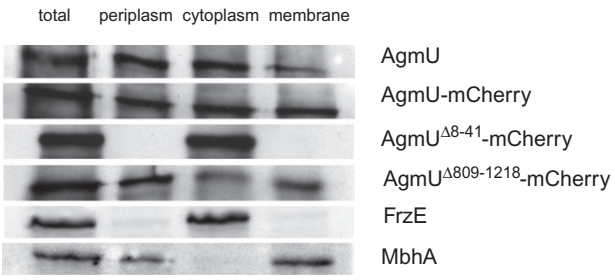

Fig. 4. AgmU localizes to both the periplasm and cytoplasm. Western immunoblots of cell fractions following osmotic shock show that wild-type AgmU, AgmU-mCherry and AgmU$^{\Delta 809–1218}$-mCherry (AgmU C-terminus deletion) localize in both cytoplasmic and periplasmic fractions. In contrast, the protein with the hypothetical signal sequence deleted (AgmU$^{\Delta 8–41}$-mCherry), only localizes in the cytoplasm. The cytoplasmic protein FrzE and lipoprotein MbhA are also shown as controls.

clusters (Fig. 3H) or across clusters (Fig. 3I). In both cases, the fluorescence peaks corresponding to the cell envelope disappeared (Fig. 3H and I). These observations confirm that the N-terminal sequence is required for the proper localization of AgmU in the periplasmic space. In contrast, AgmU$^{\Delta 809–1218}$-mCherry, which contains the N-terminal sequence, was found concentrated near the cell envelope (Fig. 3J and K). However, AgmU$^{\Delta 809–1218}$-mCherry did not form fluorescence clusters in the centre of cells (Fig. 3K), although many cells showed high fluorescence intensity near the cell poles (Fig. 3J). Since AgmU$^{\Delta 809–1218}$-mCherry is found in the periplasm, cytoplasm and membrane (Fig. 4), it is possible that the cytoplasmic fraction of AgmU$^{\Delta 809–1218}$-mCherry concentrates near the cell poles. These results suggest that the C-terminal domain of AgmU is required for the formation of distributed cytoplasmic clusters.

AgmU-mCherry clusters colocalize with AglZ-GFP but not with FrzCD-GFP

Because *agmU pilA* and *aglZ pilA* strains showed similar A-motility phenotypes and both AgmU and AglZ proteins form clusters, we were interested in determining whether these two proteins interact with the same A-motility machinery. To explore this possibility, a double-labelled *agmU::mCherry aglZ::gfp* strain was constructed that showed wild-type motility (Fig. 7B). As shown in Fig. 5A and movie S3, the clusters of AgmU and AglZ overlapped with each other. Scans of fluorescence density along the long axis of cells indicated that the fluorescence peaks of AgmU and AglZ were located at the same positions, except for the lagging cell pole, where the fluorescence intensity of AglZ-GFP was usually low (Mignot *et al*., 2007) while the fluorescence intensity of AgmU-mCherry was higher (Fig. 3A and C). In contrast, AgmU and FrzCD did not appear to colocalize. A doubly labelled *agmU::mCherry frzCD::gfp* strain showed the AgmU-mCherry clusters localized in distributed positions along the cell length and at the cell poles, while the FrzCD-GFP clusters localized in different positions and were always non-polar, as previously described (Mauriello *et al*., 2009b). Figure 5C and D show that in merged images, the two proteins occupied mutually exclusive positions similar to AglZ and FrzCD (Mauriello *et al*., 2009a).

To determine whether AgmU shows a localization pattern that is consistent with focal adhesion sites as described previously for AglZ (Mignot *et al*., 2007), we imaged *agmU::mCherry* cells every 30 s for 10 min by fluorescence microscopy. By analysing the images, we found that when cells were moving, the AgmU clusters appeared to remain relatively fixed with respect to the substratum, rather than moving forward with the cell. Figure 5E and movie S4 show typical time-lapse image sequence of AgmU-mCherry in a moving cell. These results suggest that AgmU is localized at the same focal adhesion sites as AglZ (Mignot *et al*., 2007).

Surface hardness influences AgmU localization

As described above and in Fig. 3A, clusters of AgmU-mCherry on 1.5% agar (or agarose) were only observed in isolated cells or in small cell groups, but never observed in large groups. These observations suggest that AgmU cluster formation is regulated by signals from the environment or by cell–cell contact. To test the effect of the environment, we floated isolated cells in 1% methylcellulose (Fig. 6A), CYE liquid medium or water (data not shown): none of these cells formed AgmU clusters. The distinguishing difference between these media and 1.5% agar or agarose is the physical hardness of the environment around the cells. To test the effect of substratum hardness on the formation of AgmU clusters, we placed cells on 5% agar or agarose. On these surfaces, almost every isolated cell showed AgmU-mCherry clusters (data not shown). Surprisingly, under these conditions, almost every cell in large groups also showed AgmU-mCherry clusters (Fig. 6B; note that only grouped cells in monolayers were imaged); in contrast, these clusters were never seen when large cell groups were examined on 1.5% agar or agarose (Fig. 3A, movie S1). These results indicate that the hardness of the substratum serves as a physical signal that regulates the localization of AgmU. To further verify the effect of substratum hardness on AgmU localization, we followed about 50 cells spotted directly onto glass microscope slides. On this surface, cells did not move. However, very large AgmU-mCherry clusters were observed in almost every cell. Figure 6C shows four typical cells. AgmU$^{\Delta 8–41}$-mCherry did not form clusters in 1% methylcellulose

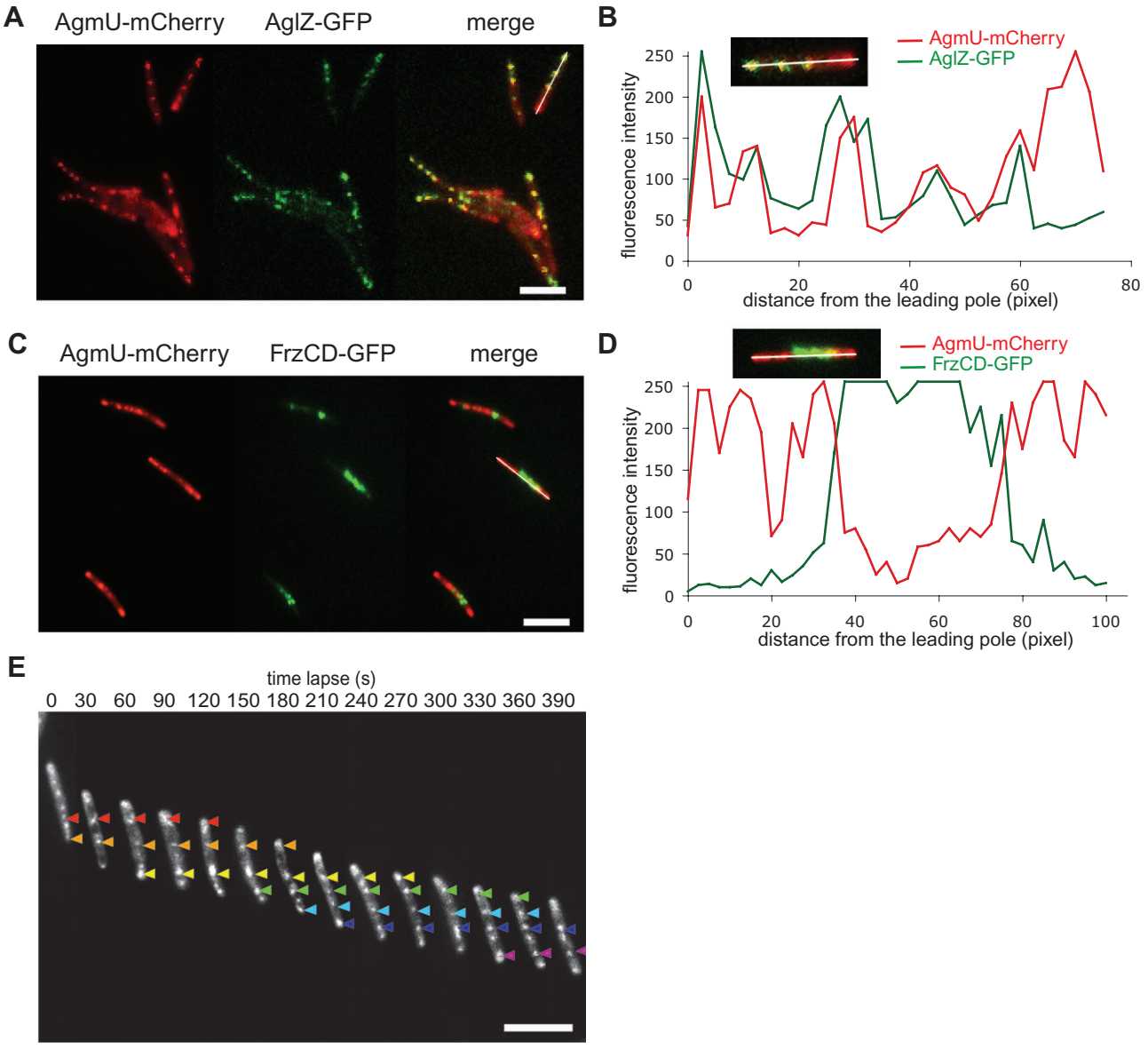

Fig. 5. The clusters of AgmU identify the same 'focal adhesion' sites as AglZ.
A. The localization of AgmU-mCherry clusters overlaps with those of AglZ-GFP *in vivo*.
B. Fluorescence scans along the cell length in (A). mCherry and GFP signals are scanned separately with ImageQuant (GE Healthcare). The highest fluorescence density of each scan was normalized to 255. The inset shows the twofold magnification of the cell scanned in (A).
C. AgmU-mCherry clusters localize differently from that of FrzCD-GFP *in vivo*.
D. A fluorescence scan along the cell length in (C). The inset shows the twofold magnification of the cell scanned in (C).
E. When cells move forward, AgmU-mCherry clusters remain fixed relative to the substratum. The fluorescent signal of mCherry in a single cell on 1.5% (w/v) agar (or agarose) is monitored with an Olympus DeltaVision IX70 microscope and recorded every 30 s. The positions of each cluster during a 390 s time-course are marked with arrows in different colours. Scale bars, 5 μm.

(Fig. 6D), suggesting that AgmU does not sense the surface hardness directly.

Previously, it was shown that AglZ forms distributed clusters in isolated cells, while in large cell groups or methylcellulose, AglZ was diffuse along the cell length, only forming dominant clusters at the leading cell pole (Mauriello *et al.*, 2009a). To test whether the localization of AglZ is also regulated by the hardness of the substratum, we spotted the *aglZ::gfp* cells on hard (1.5%) or very hard (5%) agar or agarose. We observed no significant difference in cluster formation between 1.5% and 5% agar or agarose, suggesting that the localization of AglZ was not directly regulated by substratum hardness (data not shown).

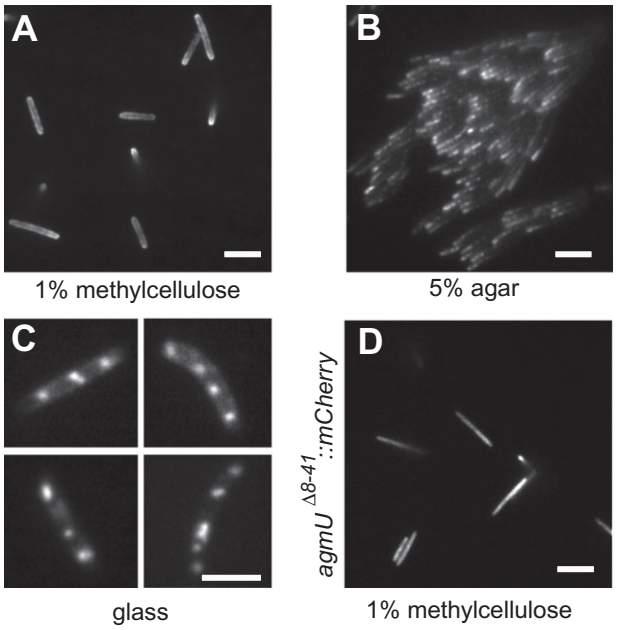

Fig. 6. The formation of AgmU clusters is regulated by the hardness of the substratum.
A. AgmU never formed clusters in 1% (w/v) methylcellulose.
B. On 5% (w/v) agar (or agarose), AgmU-mCherry formed clusters even in large cell groups.
C. Super-sized AgmU-mCherry clusters are found when cells are placed on a glass surface. Four individual cells are shown, but more than 50 cells were observed.
D. AgmU$^{\Delta 8-41}$-mCherry did not form clusters in 1% (w/v) methylcellulose. Scale bars, 5 μm.

Genes downstream of agmU *are required for functional localization of AgmU and AglZ*

As shown in Fig. 7A, *agmU* is the first gene of an eight-gene cluster (http://www.wikimods.org/organism/myxococcus-xanthus/gene-page?zoom=5&offset=0&locus=MXAN_4870). The downstream genes *aglT* (Youderian *et al*., 2003), *agmK* (Youderian *et al*., 2003), *agmX* (Youderian *et al*., 2003), *agmV* (Hartzell *et al*., 2008) and *pglI* (Yu and Kaiser, 2007) were previously reported to be required for A-motility during the characterization of a collection of mutants with transposon insertions; the function of the genes *mxan_4868* and *mxan_4864* was not reported. For some genes encoding multiple domains (i.e. *agmX* and *agmK*), different insertions caused different phenotypes (Youderian *et al*., 2003). In this study, to more carefully analyse the function of each gene, we constructed in-frame deletions in all of the seven genes downstream of *agmU* in strains carrying the *agmU::mCherry aglZ::gfp* and *pilA::tet* fusions. These constructs allowed us to determine the motility phenotypes of the various mutants as well as the effect of each mutation on the localization of AgmU and AglZ.

Figure 7B shows that all seven of the deletion mutants showed A-motility defects to some degree. Figure 7C shows the effect of these mutations on the localization of AgmU and AglZ. AgmU::mCherry localization was clearly defective in the *aglT*, *pglI* and *agmK* mutants, but relatively unchanged in the other mutants and in the *aglZ* mutant. Western immunoblot analysis using anti-AgmU antibodies showed that the *aglT* mutant produced very little AgmU (Fig. S2). Because AglT may be a lipoprotein, we speculate that AglT regulates the folding of AgmU and/or protects mature AgmU from digestion by periplasmic proteases. The *pglI* and *agmK* mutants showed AgmU localized near the membrane and in aberrant clusters at the leading pole or at both the leading and lagging poles (Fig. 7C). However, these mutants showed no significant change in the amount of AgmU found in the periplasm or cytoplasm (data not shown), suggesting that the cytoplasmic AgmU was concentrated at the cell poles. Interestingly, the *pglI* and *agmK* mutants failed to form distributed clusters along the cell length. Additionally, they did not form clusters on 5% agar (Fig. S3). The localization of AgmU in these mutants suggests that PglI and AgmK serve to sense the hardness of the substratum or function in the positioning of the 'focal adhesion' sites. PglI is a TonB homologue, while AgmK is a large TPR protein with unknown function.

Figure 7C (second row) shows that all of the seven genes downstream of *agmU* were required for AglZ to localize normally: (i) deletions in *aglT*, *pglI*, *mxan_4864* and *agmK* caused AglZ to form a single cluster at the leading cell pole, (ii) deletions in *mxan_4868* and *agmV* showed diffuse AglZ-GFP that did not form clusters and (iii) deletions in *agmU* and *agmX* caused AglZ-GFP clusters to be more diffused than in the wild type (Fig. 7C).

AgmU and AglZ are components of the same A-motility complex

The altered localization of AgmU and AglZ in the various A-motility mutants described above suggests that many proteins interact to control the localization of the adhesion complexes associated with A-motility. In order to identify these interacting proteins, a series of GST affinity chromatography experiments were performed using GST-tagged fragments of AgmU, AglT, AgmX and AgmK as baits, which were heterologously overexpressed and purified from *E. coli*. Because the GST-tagged full-length AgmU was difficult to express and purify, we used the N- and C-terminus of AgmU (AgmU-N, amino acids 52–860, including both the TPR clusters but not the sequence; AgmU-C, amino acids 800–1218) mixed in the ratio of 1:1 as bait. We also used the GST-tagged full-length AglT only lacking the putative signal sequence (amino acids 33–478); the N-terminus of AglX (amino acids 2–373); and an AgmK fragment (amino acids 2681–3364) containing five TPR motifs.

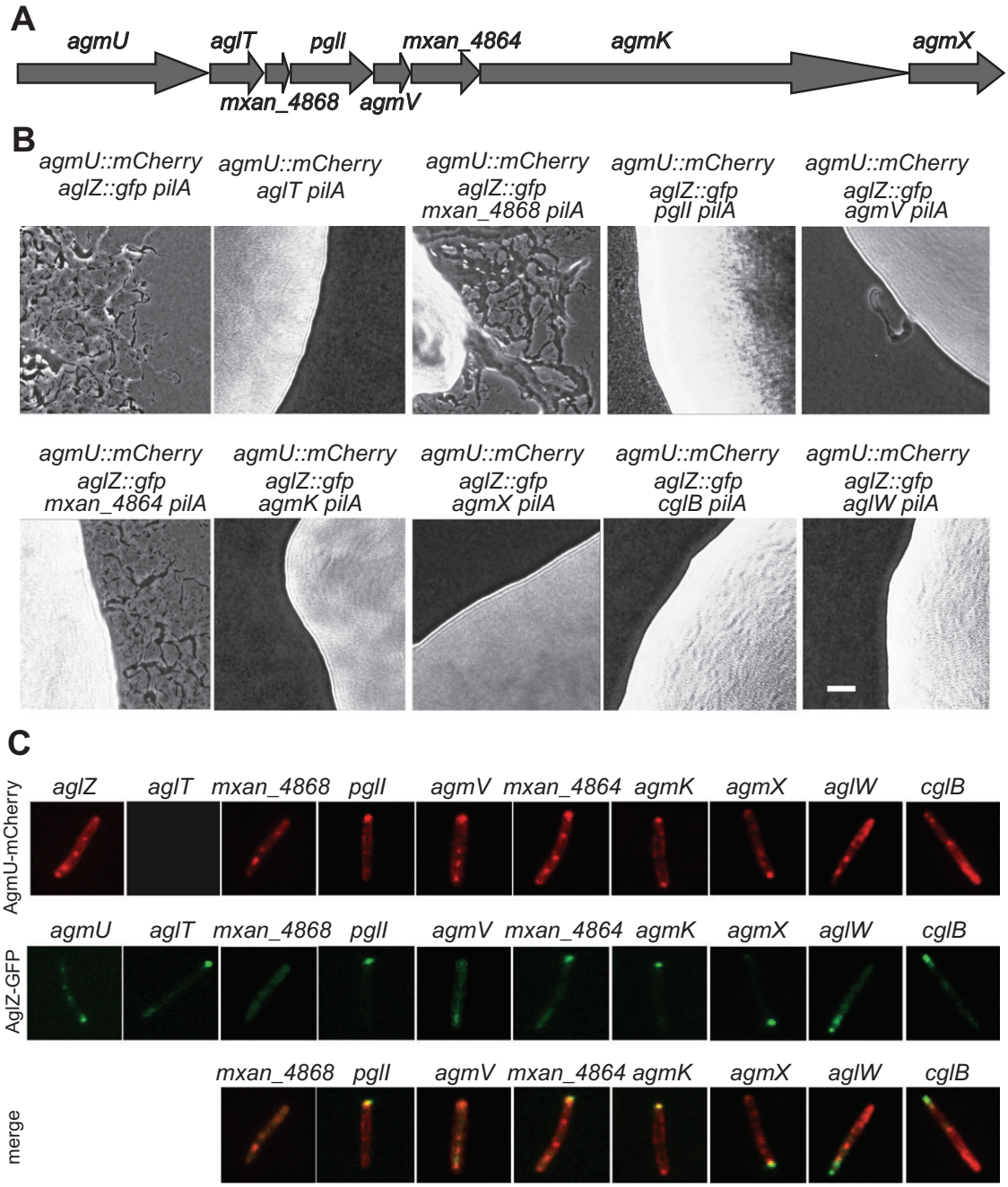

Fig. 7. The genes that encode proteins interacting with AgmU are all required for A-motility.
A. *agmU* is the first gene of a large A-motility-related gene cluster.
B. A-motility analysis of the parental strain *agmU::mCherry aglZ-gfp::kan*, the additional deletion mutants of the genes downstream of *agmU*, and the additional deletion mutants of *cglB* and *aglW*. The movement of (*pilA*) cells that lack S-motility were monitored as an indicator of A-motility on 1.5% agar (or agarose) (Hodgkin and Kaiser, 1979). Samples were cultured and photographed as described in Fig. 2. Note that the strains *aglT agmU-mCherry::kan* and *aglT aglZ-gfp::kan* have similar phenotypes and only the phenotype of *aglT agmU-mCherry::kan* is shown. Scale bar, 40 μm.
C. The products of a series of genes effect the localization of AgmU and AglZ. All the gene products regulate the localization of AglZ while only AglT, PglI and AgmK affect the localization of AgmU. In the *aglT* background, almost no AgmU-mCherry is detectable (see Fig. S2).

Table 1. Summary of the GST affinity chromatography.

Prey \ Bait	AgmU	AglT	AgmX	AgmK (AA 2681–3364)
FrzCD	✓	×	×	×
AglT	✓	–	×	×
AglW	✓	✓	✓	✓
AglZ	✓	✓	✓	✓
AgmK	✓	✓	✓	–
AgmU	–	✓	✓	✓
AgmX	✓	×	–	×
CglB	✓	✓	×	✓

'✓', co-purified with the bait; '–', not determined; '×', not co-purified with the bait.

The proteins that interacted with the baits were purified by affinity chromatography from wild-type (strain DZ2) lysates and identified with MS/MS mass spectrometry (MS/MS, Proteomics/Mass Spectrometry Laboratory, UC Berkeley) as described (Mauriello *et al.*, 2009a). The chromatography experiment with each GST-tagged bait was performed twice in parallel. Mass spectrometry identified as many as 100 proteins that co-purified with each bait (data not shown). Only the annotated A-motility proteins which were identified in both the two parallel chromatographic experiments were listed in Table 1. AgmU interacted with FrzCD as expected, but also with AglT, AgmK, AgmX and AglZ. Among these proteins, AgmK and AgmX were predicted to contain transmembrane fragments. We were concerned that some membrane vesicles containing AgmK or AgmX might be co-purified with AgmU, yielding false positive results. This possibility was excluded by using soluble fragments of AgmK and AgmX as baits, confirming the fidelity of the interacting proteins (Table 1). Additionally, two other A-motility-related lipoproteins were identified with AgmU and other baits: (i) AglW (Youderian *et al.*, 2003), a TolB homologue that co-purified with GST-tagged AgmU, AglT, AgmK and AgmX and (ii) CglB (Rodriguez and Spormann, 1999), a contact stimulatable motility protein that co-purified with GST-tagged AgmU, AglT and AgmK.

To study the function of AglW and CglB in A-motility, we constructed in-frame deletions in *aglW* and *cglB* in strains carrying the *agmU::mCherry*, *aglZ::gfp* and *pilA::tet* fusions. Both the *aglW* and *cglB* deletion mutants showed defective A-motility (Fig. 7B). In these strains, AglZ formed single clusters at the cell poles rather than distributed clusters (Fig. 7C). AgmU localization was unaffected in both mutants (Fig. 7C). Interestingly, the *aglW* strain showed the same growth rate as wild type (data not shown), but the cells formed accordion waves, also known as ripples (Shimkets and Kaiser, 1982; Welch and Kaiser, 2001; Berleman *et al.*, 2006) on rich media (Fig. S4). Since these waves have been shown to be associated with feeding on lysed prey cells or macromolecules, their presence suggests that *aglW* cells release macromolecules into the environment (Berleman *et al.*, 2006).

Discussion

Myxococcus xanthus glides on surfaces by two distinct motility mechanisms, A-motility and S-motility. To achieve efficient locomotion, these motility systems must be co-ordinated. The Frz chemosensory pathway controls reversals for both motility systems, but it is not known how this pathway interacts with the motility engines. FrzCD, the methyl-accepting chemotaxis receptor for the Frz pathway, was previously shown to interact with two A-motility proteins, AglZ and AgmU (Youderian *et al.*, 2003; Yang *et al.*, 2004; Mauriello *et al.*, 2009a). In this study, we examined the role of AgmU in controlling A-motility and showed that, like AglZ, it acts as a negative regulator of FrzCD activity, coupling A-motility to the Frz chemosensory pathway. For example, although *agmU* and *aglZ* mutants are defective in A-motility, *agmU frzCD* and *aglZ frzCD* double mutants show restored A-motility. However, *agmU aglZ frzCD* triple mutants show no A-motility, indicating that while the regulatory activities of AgmU and AglZ appear to be redundant with respect to the Frz pathway, together they are essential for A-motility. We speculate that both AgmU and AglZ have other functions in addition to the regulation of the Frz pathway, because *in vivo* fluorescence microscopy showed that both AgmU and AglZ did not colocalize with FrzCD; this suggests that only a small portion of AgmU and AglZ function as regulators through the interactions with FrzCD (Fig. 5) (Mauriello *et al.*, 2009a). This hypothesis is consistent with our *in vitro* cross-linking experiments, which show that only a small proportion of AgmU and AglZ could be directly cross-linked with FrzCD (Fig. 1 and Mauriello *et al.*, 2009a).

The data presented suggest that AgmU and AglZ work together as partners. Indeed, AgmU and AglZ both interact directly with the N-terminus of FrzCD coupling the activity of the Frz pathway in the regulation of A-motility (Mauriello *et al.*, 2009a) (Fig. 1C). Moreover, these two proteins colocalize in the previously described 'focal adhesion' sites associated with A-motility; these sites remain fixed with respect to the substratum rather than with their cellular positions as cells move forward (Fig. 5). To identify additional interaction partners in these 'focal adhesion' sites, we used GST affinity chromatography with GST-AgmU as bait. These pull-down experiments, summarized in Table 1, show that AgmU interacted with six proteins: AglT, AglW, AglZ, AgmK, AgmX, CglB, in an A-motility complex that spans the cytoplasm, inner membrane and periplasm. These interactions were further confirmed by additional pull-down experiments with GST-tagged AglT, AgmK and AgmX fragments as baits. A schematic representation of the A-motility complex

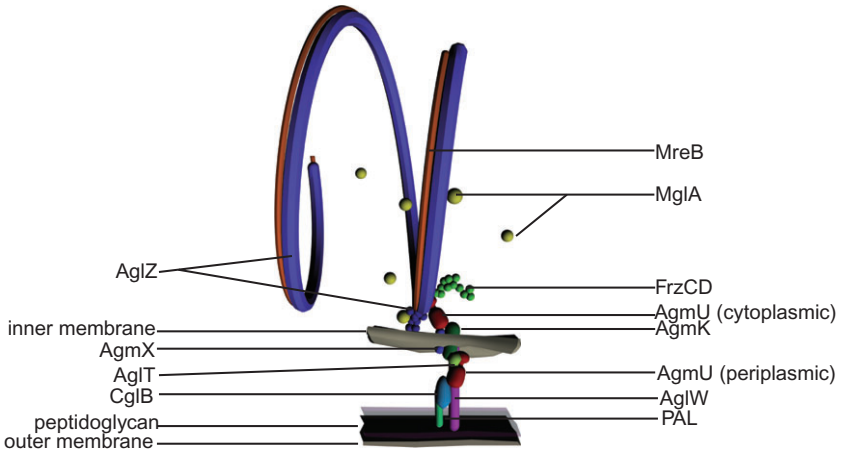

Fig. 8. A working model for the A-motility complex. The proteins identified in this report and previous studies are summarized in this model. The outer membrane, pepidoglycan layer and the inner membrane near the 'focal adhesion' site are shown in fragments. Previously, FrzCD was shown to interact with both AgmU and AglZ (Mauriello *et al*., 2009a); AglZ was shown to interact with the cytoskeleton protein MreB and the GTPase MglA (Hartzell and Kaiser, 1991; Mauriello *et al*., 2010). Data from *E. coli* TolB protein (AglW in this study) suggest an interaction of this protein with PAL (Bonsor *et al*., 2009), whose homologue in *M. xanthus* is MXAN_4581. Note that the proteins and structures in this model are not represented proportionally to their actual sizes.

formed by AgmU and the other six A-motility proteins is presented in Fig. 8. MreB, an actin-like protein (Carballido-Lopez, 2006), and MglA, a Ras family GTPase, which also interact with AglZ (Yang *et al*., 2004; Mauriello *et al*., 2010), are also involved in this complex. As another 30 proteins have been shown to be associated with A-motility (Hodgkin and Kaiser, 1979; Macneil *et al*., 1994; Youderian *et al*., 2003), the complex that we propose may represent just a small piece of the A-motility complex. Figure 8 also suggests putative functions for the different components of the A-motility complex:

i. AglZ is a cytoplasmic protein which contains an N-terminal pseudo-receiver domain and a long C-terminal coiled-coil domain, showing similarity with FrzS, a S-motility protein (Ward *et al*., 2000; Mignot *et al*., 2007). AglZ-YFP forms distributed clusters that remain stationary with respect to the substratum as cells move forward. The localization of AglZ clusters requires direct interactions with the cytoskeleton protein, MreB, and the Ras-like GTPase, MglA (Yang *et al*., 2004; Mauriello *et al*., 2010).
ii. AglT is a putative lipoprotein with six tandem TPR motifs. The amount of AgmU in *aglT* cell lysates was very low compared with the wild type. This result suggests that AglT serves to maintain AgmU in the correct conformation or to protect it from periplasmic proteolytic activities. AglT and AgmU might directly interact through their TPR motifs.
iii. PglI is a TonB-like transporter with a forkhead-associated (FHA) domain at its N-terminus and a collagen domain near its C-terminus. The presence of the FHA and collagen domains suggests additional functions in mediating protein–protein interactions in the A-motility complex (Durocher and Jackson, 2002; Heino, 2007).
iv. AgmK is a protein of extraordinary size (3812 amino acids) with two potential transmembrane fragments near its C-terminus (amino acids 3491–3506, 3550–3570) and at least 17 TPR motifs. The structural complexity and the putative transmembrane topology of AgmK make it an ideal candidate for a structural scaffold that anchors AgmU at 'focal adhesion' sites and/or as a sensor for the hardness of the substratum.
v. AgmX shows limited similarity with plant extensins, which are usually found in the extracellular matrix (Kieliszewski and Lamport, 1994).
vi. CglB is a lipoprotein required for A-motility. A-motility of a *cglB* mutant could be transiently restored by extracellular complementation upon mixing with *cglB*$^+$ cells (Rodriguez and Spormann, 1999; Spormann and Kaiser, 1999).
vii. AglW is a TolB homologue containing the Trp-Asp (WD) repeats, which form a β-propeller structure that is a common binding site for TPR motifs (Neer *et al*., 1994; Smith, 2008). In *E. coli*, TolB proteins participate in maintaining the integrity of the outer membrane through interactions with the peptidoglycan-associated lipoprotein (PAL) (Lazzaroni *et al*., 1999; Bonsor *et al*., 2009). The function of AglW might be supplying an assembly anchor for the A-motility complex at the peptidoglycan layer. We note that *aglW* is the last gene of an A-motility-related operon. The genes upstream of *aglW*, *aglX* and *aglV* encode a TolQ/TolR pair (Youderian *et al*., 2003), homologous to the flagella motor MotA/MotB (Cascales *et al*., 2001).
viii. MglA is a Ras-like GTPase which is required for both A- and S-motility (Hodgkin and Kaiser, 1979). MglA was reported to regulate the localization of AglZ and FrzS through direct interactions. The localization of MglA is dependent on the cytoskeleton protein MreB (Mauriello *et al*., 2010).
ix. MreB is an actin-like cytoskeleton protein which forms helical filaments in rod shaped bacteria and is

involved in several important cellular processes such as cell shape determination, cell polarity and DNA segregation (Jones *et al*., 2001; Kruse *et al*., 2003; Gitai *et al*., 2004; Gitai *et al*., 2005; Carballido-Lopez, 2006; Shih and Rothfield, 2006; Madabhushi and Marians, 2009). In *M. xanthus*, perturbation of the MreB cytoskeleton blocks both A- and S-motility (Mauriello *et al*., 2010). MreB is required for the functional localization of AglZ, FrzS and MglA; it forms a double helix with the same periodicity as the AglZ clusters and directly interacts with AglZ (Mauriello *et al*., 2010).

Among the identified A-motility proteins, AglT, AgmK, AgmX, AglZ, AglW and CglB were all found to interact with AgmU (Table 1). They are predicted to form a large complex that spans the cytoplasm, membrane and periplasm. Although not identified by mass spectrometry, the other four proteins, MXAN_4868, PglI, AgmV and MXAN_4864 may play roles in conjunction with this complex, because they are required to form functional AglZ 'focal adhesion' clusters; PglI is also required for AgmU cluster localization (Fig. 7C). All the genes in the *agmU* gene cluster are well conserved among myxobacteria species (e.g. *Stigmatella aurantiaca, Anaeromyxobacter dehalogenans* and *Sorangium cellulosum*), suggesting an essential function of this A-motility complex. We note that it is difficult to judge from our pull-down experiments whether the interactions between proteins are direct or indirect. Additionally, some proteins may interact with this A-motility complex but fail to be pulled down, as detergent was not used in our experiments and some membrane proteins might therefore have been excluded due to their insolubility.

AgmU, unlike AglZ, is found in the periplasmic, cytoplasmic and membrane fractions of cells. *In vivo* experiments with AgmU-mCherry confirmed this dual localization. Because *agmU* mutants that lacked the N-terminal sequence (*agmU*$^{\Delta 8-41}$*;;mCherry*) or the C-terminal domain (*agmU*$^{\Delta 809-1218}$*;;mCherry*) showed severe A-motility defects, both the periplasmic and cytoplasmic localizations of AgmU are required for functional A-motility. AgmU$^{\Delta 8-41}$-mCherry was not present in the periplasmic fraction (Fig. 4), but still formed clusters observable by fluorescence microscopy (Fig. 3G–I), indicating that transport of the protein to the periplasm is not essential for cluster formation. In contrast, AgmU$^{\Delta 809-1218}$-mCherry maintained periplasmic, cytoplasmic and membrane localization in fractionation assays, but lost the ability to form distributed clusters (Figs 3J and K and 4, and Fig. S1), suggesting that the C-terminal domain is required for cluster formation.

It is still not clear if AgmU is a lipoprotein or how AgmU is secreted. The N-terminal sequence of AgmU contains the positively charged N-terminus (n-region) and the hydrophobic core (h-region) of a typical Sec pathway signal sequence, but lacks the polar C-terminus (c-region) and the conserved cystine residue (Natale *et al*., 2008). This N-terminal sequence also contains two pairs of arginine residues (Fig. 1A), which are potential recognition sites for the twin-arginine translocation (Tat) system. However, both of the arginine pairs lack the hydrophobic residues that normally follow and the Sec 'avoidance signal' (usually a positively charged residue in the c-region) (Natale *et al*., 2008). Taken together, the poorly conserved 'signal sequence' may delay the secretion of AgmU and generate the formation of cytoplasmic clusters. The mechanism for dual periplasmic and cytoplasmic localization of AgmU is unknown. Similar dual localization patterns were reported for the *Helicobacter pylori* KatA protein (Harris and Hazell, 2003) and the *E. coil* GroESx protein (Lee and Ahn, 2000).

An unexpected finding in this study was that the formation of AgmU clusters is regulated by the physical hardness of the substratum. On 1.5% agar (or agarose), a substrate that facilitates both A- and S-motility, AgmU clusters were only observed in isolated cells or in small cell groups (Fig. 4D–F). In contrast, in large cell groups, where S-motility is dominant, clusters were never observed (Fig. 4A–C), except for some cells at the very 'edge' of the group (data not shown). But when cells were spotted on 5% agar (or agarose), AgmU clusters were present in almost every cell, even in large cell groups (Fig. 6B). This observation suggests that the formation of AgmU clusters is regulated by the hardness of the surface on which cells are gliding and it may play an important role in the switch favouring A-motility. This hypothesis is consistent with three additional experiments: First, in 1% methylcellulose, a condition that favours S-motility, distributed AgmU clusters were never observed (Fig. 6A). This experiment ruled out the possibility that cell–cell contact may inhibit AgmU cluster formation. Second, in CYE liquid culture or water, where no gliding motility is present, AgmU clusters were never observed, indicating that the formation of AgmU clusters was not inhibited by any chemical content of the substratum (data not shown). Third, on a glass surface, AgmU formed extraordinary large clusters (Fig. 6C).

In large groups, cells are enveloped in a soft extracellular matrix, which inhibits the formation of AgmU clusters. In contrast, there is very little extracellular matrix around single cells and small cell groups, where the AgmU clusters appear. We note that AgmU clusters were not always observed in isolated cells on 1.5% agar (or agarose), possibly due to the following factors: First, under the experimental conditions used for imaging cells (liquid cell culture dropped on an agar pad), it is difficult to make the environment of each cell (especially the hardness of the surface) absolutely identical. However, with 5% agar (or agarose) or glass, the effect of surface hardness stands

out and AgmU clusters were observed in every single cell. Second, AgmU clusters appear to form in a thin layer right above the surface of the substrate (data not shown), which makes it impossible to focus on the clusters of every cell in the same focal plane.

The experiments reported in this paper support the hypothesis that A-motility is regulated and powered, at least in part, by distributed motility proteins that act together as part of a complex. This complex could also work together with a proposed 'slime secretion' motility system (Kaiser, 2009) or act as a 'motility sensor' for the Frz pathway. We have identified many of these proteins but clearly many additional proteins remain to be characterized. Of particular interest are the elusive motor proteins that are presumed to power cell movement. We note that the *M. xanthus* genome encodes eight MotAB/TolQR homologues. These homologues are excellent candidates for motor proteins as MotAB from *E. coli* powers flagellar rotation (Minamino *et al*., 2008).

Experimental procedures

Strains and growth conditions

Bacterial strains and plasmids are listed in Table 2. *M. xanthus* strains were cultured in CYE medium, which contains 10 mM MOPS pH 7.6, 1% (w/v) Bacto Casitone (BD Biosciences), 0.5% Bacto yeast extract and 4 mM $MgSO_4$ (Campos *et al*., 1978). For A-motility assays, 10 μl of cells of each strain in the concentration of 4×10^9 colony forming units (cfu) ml^{-1} were spotted on CYE plates containing 1.5% (w/v) agar (or agarose), incubated at 32°C for 48 h and photographed with a WTI charge-coupled device (CCD)-72 camera, on a Nikon Labphot-2 microscope.

Double and triple mutants were constructed by electroporating *M. xanthus* cells with 4 μg of plasmid DNA or 1 μg of chromosomal DNA. Transformed cells were plated on CYE plates supplemented with 100 mg ml^{-1} sodium kanamycin sulphate. To construct the in-frame deletion or insertion strains, in-frame deletion or insertion cassettes were amplified with polymerase chain reaction (PCR) using chromosomal DNA as template, digested and inserted into plasmid pBJ113. All constructs were confirmed by DNA sequencing. Transformants were obtained by homologous recombination as previously described (Bustamante *et al*., 2004). To construct *pilA::tet* and *aglZ-gfp::kan* insertions, chromosomal DNA of strain TM7 (Mignot *et al*., 2007) and DZ4760 (which carries an *aglZ-gfp::kan* insertion, T. Mignot *et al*., unpublished; Table 2) were electroporated into the parental strains. The primers used in the constructions of the in-frame deletions and insertions are summarized in Table S1.

Protein expression and purification

The coding sequence of each protein or protein fragment was amplified by PCR from genomic DNA of DZ2 strain, digested and inserted into pET28a (Navagen), pGEX-KG (Guan and Dixon, 1991) or pGEX-2TK (GE Healthcare) vectors. The primers used in the cloning for protein expression are listed in Table S2. All constructs were confirmed by DNA sequencing. Expression and purification of the recombinant proteins were performed as described (Mauriello *et al*., 2009a), except that CHAPS and glycerol were only used for purification of FrzCD fragments.

In vitro *protein cross-linking*

In vitro protein cross-linking reactions were performed as described (Mauriello *et al*., 2009a). Proteins were diluted to the following concentrations to keep them at a 1:1 molar ratio: FrzCDN-ter, 2.5 μg ml^{-1}; FrzCDC-ter, 2.5 μg ml^{-1}; AgmU, 25 μg ml^{-1}; AgmU-TPR1, 5 μg ml^{-1}; AgmU-TPR2, 5 μg ml^{-1}; AgmU-C-ter, 10 μg ml^{-1}.

Osmotic shock fractionation

Osmotic shock fractionation was performed as described (Nelson *et al*., 1981). For AgmU and FrzE, cells were harvest from CYE liquid culture at the concentration of 4×10^8 cfu ml^{-1} by centrifugation at 8000 r.p.m. at 4°C for 10 min. For MbhA, cells were harvest from CF plates after 48 h of development (Nelson *et al*., 1981). The pellet was weighted and re-suspended into ice-cold 10 mM Tris-HCl pH 7.5 to a concentration of 10% (w/v). After 10 min incubation on ice, the buffer was changed to 10 mM Tris-HCl pH 7.5 25% (w/v) sucrose after 10 min of centrifugation at 8000 r.p.m., 4°C. The cells were incubated on ice for 15 min with gentle shaking. The cells were collected with centrifugation (8000 r.p.m., 4°C, 10 min) and shocked on ice with 10 mM Tris-HCl pH 7.5 for 10 min with gentle shaking. The cells were again subjected to centrifugation (8000 r.p.m., 4°C, 10 min) and the supernatant was kept as the periplasmic fraction. The pellet was later sonicated and the supernatant of high-speed centrifugation (100 000 *g*, 4°C, 30 min) was collected as the cytoplasmic fraction. 10 μl of each fraction was loaded into 10% SDS PAGE and Western blot was performed using polyclonal anti-AgmU antibodies.

Immunoblot analysis

Immunoblotting were performed as described (Mauriello *et al*., 2009a). AgmU polyclonal antibodies were produced from Covance Co. using 10 mg of protein mixture containing AgmU-TPR1, AgmU-TPR2 and AgmU-C-ter in the molar ration of ~1:1:1.

GST affinity chromatography and mass spectrometry

Glutathione-S-transferase affinity chromatography was performed as described (Mauriello *et al*., 2009a), except that CHAPS and glycerol were not added to the PBS buffer. 0.1 mg of each purified GST-tagged protein was injected into a 1 ml GSTrap™ HP column (GE Heathcare); in the case of AgmU, 50 μg GST-tagged AgmU N- and C-terminal domains were mixed and injected. MS/MS was performed in the Proteomics/Mass Spectrometry Laboratory of UC Berkeley as described (Scott *et al*., 2008; Mauriello *et al*., 2009a).

Table 2. Strains and plasmids used in this study.

Strains/plasmids	Genotype	Reference/source
M. xanthus strains		
DZ2	Wild type	Campos *et al.*, 1978
DZ4469	*pilA::tet*	Vlamakis *et al.*, 2004
TM7	*aglZ::kan*	Mignot *et al.*, 2007
DZ4725	*aglZ::kan pilA::tet*	Mauriello *et al.*, 2009a
DZ4760	*aglZ-gfp::kan*	T. Mignot *et al.*, unpublished
DZ4769	*frzCD pilA::tet*	This study
DZ4771	*ΔagmU pilA::tet*	This study
DZ4772	*agmU::mCherry pilA::tet*	This study
DZ4773	*agmU$^{\Delta 8-41}$::mCherry pilA::tet*	This study
DZ4774	*agmU$^{\Delta 809-1218}$::mCherry pilA::tet*	This study
DZ4775	*ΔagmU ΔfrzCD pilA::tet*	This study
DZ4776	*ΔagmU aglZ::kan pilA::tet*	This study
DZ4777	*ΔagmU ΔfrzCD aglZ::kan pilA::tet*	This study
DZ4778	*agmU::mCherry aglZ-gfp::kan*	This study
DZ4779	*agmU::mCherry frzCD::gfp*	This study
DZ4780	*agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
DZ4781	*ΔaglT agmU-mCherry::kan pilA::tet*	This study
DZ4782	*ΔaglT aglZ-gfp::kan pilA::tet*	This study
DZ4783	*Δmxan_4868 agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
DZ4784	*ΔpglI agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
DZ4785	*ΔagmV agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
DZ4786	*Δmxan_4864 agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
DZ4787	*ΔagmK agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
DZ4788	*ΔagmX agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
DZ4789	*ΔaglW agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
DZ4790	*ΔcglB agmU::mCherry aglZ-gfp::kan pilA::tet*	This study
E. coli strains		
DH5α	Host strain for molecular cloning	Invitrogen
BL21 (DE3) Tuner	Host strain for protein expression	Novagen
Plasmids		
pET28a	His-tagged protein expression vector	Novagen
pGEX-KG	GST-tagged protein expression vector	Guan and Dixon, 1991
pGEX-2TK	GST-tagged protein expression vector	GE Healthcare
pBJ113	Plasmid for gene deletions/insertions, galK^{S}, kan^{R}	Julien *et al.*, 2000
pBN3	pET28a with *his$_6$::frzCD* (AA2-135)	Mauriello *et al.*, 2009a
pBN4	pET28a with *his$_6$::frzCD* (AA132-417)	Mauriello *et al.*, 2009a
pBN9	pBJ113 with *agmU* deletion cassette	This study
pBN10	pBJ113 with *agmU::mCherry* insertion cassette	This study
pBN11	pBJ113 with *agmU$^{\Delta 8-41}$* deletion cassette	This study
pBN12	pBJ113 with *agmU$^{\Delta 809-1218}$* deletion cassette	This study
pBN13	pBJ113 with *aglT* deletion cassette	This study
pBN14	pBJ113 with *mxan_4868* deletion cassette	This study
pBN15	pBJ113 with *pglI* deletion cassette	This study
pBN16	pBJ113 with *agmV* deletion cassette	This study
pBN17	pBJ113 with *mxan_4864* deletion cassette	This study
pBN18	pBJ113 with *agmK* deletion cassette	This study
pBN19	pBJ113 with *agmX* deletion cassette	This study
pBN20	pBJ113 with *aglW* deletion cassette	This study
pBN21	pBJ113 with *cglB* deletion cassette	This study
pBN22	pET28a with *his$_6$::agmU*	This study
pBN23	pET28a with *his$_6$::agmU* (TPRI, AA120–420)	This study
pBN24	pET28a with *his$_6$::agmU* (TPRII, AA560–860)	This study
pBN25	pET28a with *his$_6$::agmU* (C-terminal, AA800–1218)	This study
pBN26	pGEX-KG with *gst::agmU* (N-terminal, AA52–860)	This study
pBN27	pGEX-KG with *gst::agmU* (C-terminal, AA800–1218)	This study
pBN28	pGEX-KG with *gst::aglT* (AA33–478)	This study
pBN29	pGEX-2TK with *gst::agmX* (AA2–373)	This study
pBN30	pGEX-KG with *gst::agmK* (AA2681–3364)	This study

Time-lapse fluorescence microscopy

Time-lapse fluorescence microscopy was performed as described (Mauriello *et al.*, 2009a). For the fluorescence microscopy on a glass surface, 3 μl of cells from liquid CYE culture in the concentration of 4×10^8 cfu ml^{-1} were spotted on glass slide and covered with cover slide immediately. In order to avoid the evaporation

of water, nail polish was used to seal the cover slide.

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Competing interest

The authors declare no competing interest.

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