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REVIEW

Unorthodox regulation of the MglA Ras-like GTPase controlling polarity in *Myxococcus xanthus*

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Motile cells have developed a large array of molecular machineries to actively change their direction of movement in response to spatial cues from their environment. In this process, small GTPases act as molecular switches and work in tandem with regulators and sensors of their guanine nucleotide status (GAP, GEF, GDI and effectors) to dynamically polarize the cell and regulate its motility. In this review, we focus on *Myxococcus xanthus* as a model organism to elucidate the function of an atypical small Ras GTPase system in the control of directed cell motility. *M. xanthus* cells direct their motility by reversing their direction of movement through a mechanism involving the redirection of the motility apparatus to the opposite cell pole. The reversal frequency of moving *M. xanthus* cells is controlled by modular and interconnected protein networks linking the chemosensory-like frizzy (Frz) pathway – that transmits environmental signals – to the downstream Ras-like Mgl polarity control system – that comprises the Ras-like MglA GTPase protein and its regulators. Here, we discuss how variations in the GTPase interactome landscape underlie single-cell decisions and consequently, multicellular patterns.

Keywords: cell motility; cell polarity; GAP; GEF; MglA; *Myxococcus xanthus*; Ras-like GTPase; Roadblock/LC7 domain

In all kingdoms of life, guanine nucleotide-binding proteins (G proteins) known as phosphate-binding P-loop GTPases regulate multiple aspects of signalling pathways and cellular processes. Based on phylogenetic and structure analyses, they were mainly classified into two large superclasses: the SIMIBI (signal recognition GTPase and the MinD and BioD) superclass and the TRAFAC (translation factor) superclass [1]. Small GTPases of the Ras (Rat sarcoma) superfamily belong to the TRAFAC superclass and are now widely known as nucleotide-dependent molecular switches regulating complex signal transduction pathways [2–4]. They were originally studied in eukaryotes for their fundamental role in regulating cellular processes including motility, polarity, nuclear and vesicular transport as well as signal transduction [5–7]. Later on, single-domain GTPases were identified in prokaryotes and were demonstrated to be crucial for various processes ranging from cell motility and polarity to predation, development and antibiotic resistance [1,8–10].

The first three-dimensional structure of Ras was presented in 1988 and soon later was proven to share the same fold as one of the domains of the bacterial elongation factor EF-Tu (that regulates ribosome

Abbreviations
A-motility, adventurous motility; Ct helix, C-terminal helix; G domain, GTP-binding domain; G protein, guanine nucleotide-binding protein; GAP, GTPase-activating protein; GDI, guanine dissociation inhibitor; GDP, guanosine diphosphate; GEF, guanine exchange factor; GTP, guanosine triphosphate; *M. xanthus*, *Myxococcus xanthus*; MCP, methyl-accepting chemotaxis protein; MglA, mutual gliding motility A; MglB, mutual gliding motility B; Ras, Rat sarcoma; RomRX, *required for motility* protein R and X complexes; S-motility, social motility; TFP, type-IV pili; TPR, tetratricopeptide repeat domain.

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biosynthesis) [11,12]. Since then, the number of solved structures of Ras proteins have remarkably increased and currently represents a considerable source of structural and mechanistic information to understand the regulation of these proteins [13].

The fundament of all G-proteins resides in their canonical GTP-binding domain (G domain), an approximately 20 kD domain that carries out nucleotide binding and the associated conformational changes. It consists of a six-stranded β-sheet and five α-helices which contain four or five highly conserved sequence motifs (G_1–G_5) [14,15] (Fig. 1A). G_1 corresponds to the Walker A/phosphate-binding loop motif more commonly called P-loop (GxxxxGKS/T). It constitutes

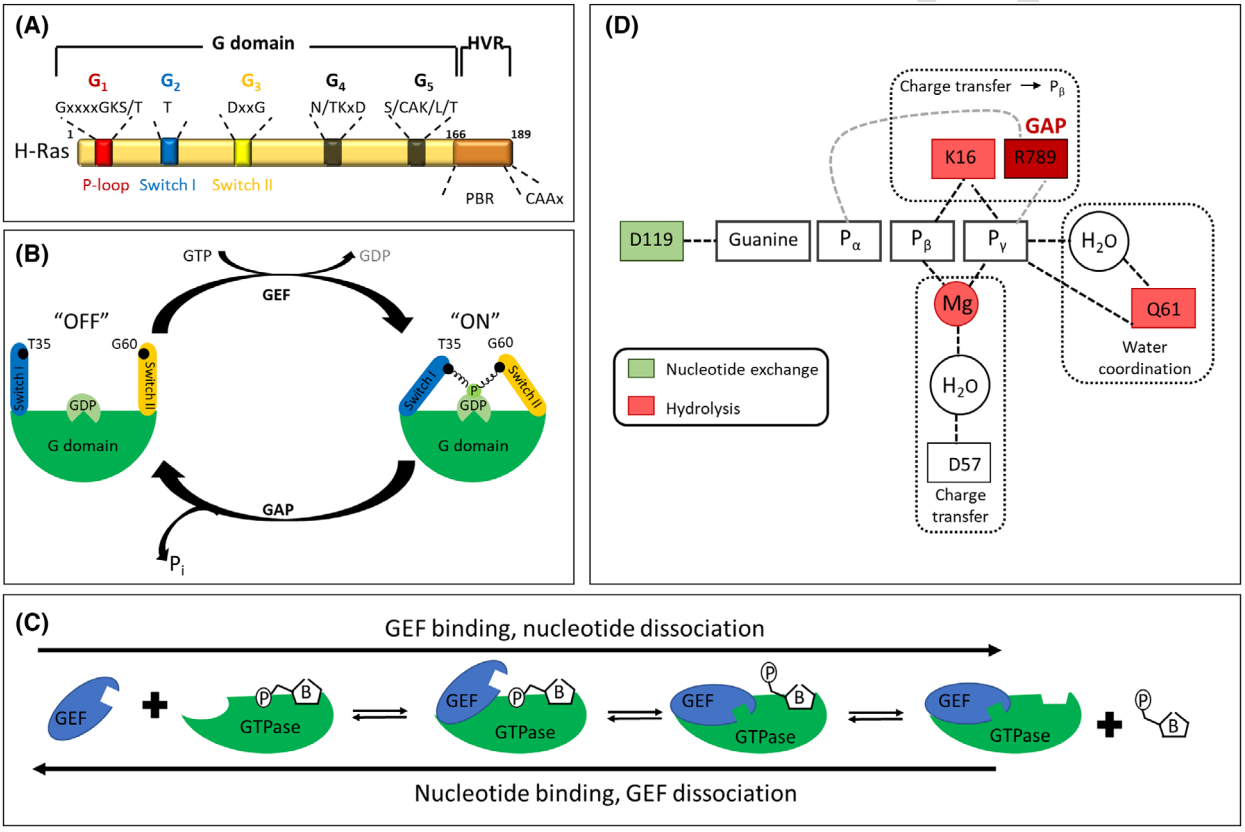

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Fig. 1. General features of small GTPase proteins. (A) Schematic of the prototypic G domain of H-Ras. The five conserved sequence motifs are highlighted from G1 to G5. G1 is also known as P-loop (red bar) while G2 and G3 as switch I and switch II respectively (blue and yellow bars). G4 and G5 make specific contact with the guanine base to differentiate guanine from other types of nucleotides (black bars). The hypervariable region (HVR) including a polybasic region and a CAAx motif is highlighted in orange. Corresponding conserved amino acids for each motif are indicated above with x representing any amino acid residue. (B) Schematic of the canonical cycle of small Ras GTPase. GEF binding activates Ras ('ON') by catalysing the exchange from GDP to GTP. GAP promotes GTP hydrolysis and thus deactivates RAS ('OFF'). The switch I and switch II involved in the nucleotide-dependent structural changes are shown in blue and yellow respectively. The conserved threonine 35 and glycine 60 residues in switch I and switch II contact the γ-phosphate *via* two main chain hydrogen bonds shown as springs. (C) The current model of GEF-induced guanine nucleotide exchange: the high affinity of the GTPase (green) to guanine nucleotide makes the nucleotide dissociation very slow, and the GEF (blue) accelerates the dissociation by reducing the nucleotide affinity. The nucleotide is strongly bound to the GTPase *via* its base (B) and its phosphate moieties (P). The GEF first associates with a low affinity to the Ras–nucleotide complex, therefore, inducing conformational changes. These structural changes induce the release of the nucleotide and leave the GEF tightly bound to the nucleotide-free GTPase. The GTPase will then bind GTP thus promoting the dissociation of the GEF and keeping the GTPase in the active GTP state (the GEF does not favour the rebinding of GDP or GTP but since the concentration of GTP *in vivo* is higher than GDP concentration, the binding of GTP to the nucleotide-free GTPase is favoured). (D) Mechanism of action of GAP-induced GTP hydrolysis: crucial residues for nucleotide exchange and hydrolysis are highlighted in green and red respectively. The conserved glutamine (Q) residue (in switch II of the GTPase) orients the nucleophilic water molecule for the in-line attack of the γ-phosphate of the GTP contributing to catalysis. The conserved positively charged lysine and the arginine residue (termed arginine finger) provided in trans by the GAP neutralize the negative charges that develop at the transition state. Further charge transfer is caused by the magnesium ion that is positioned by a conserved aspartic acid.

one of the most frequent sequence motifs in the database because it is not restricted to GTP-binding proteins but is also present in ATP-binding proteins [16]. The P-loop includes residues that contact the α and β phosphates of the guanine nucleotides. G_2 comprises the switch I region (xTx) and G_3 constitutes the aspartate-containing motif (DxxG) which is the motif comprising the switch II region. Switch I and switch II regions commonly called 'the switch regions' are generally involved in the interaction of the GTPase with its regulators and they adopt different conformations when cycling between the GTP-bound active state and the GDP-bound inactive state [15,17,18] (Fig. 1B). The P-loop together with the switch regions interact with the β- and γ- phosphate group of the guanine nucleotide and with the magnesium ion Mg^{2+}. The G_4 (N/T)(K/Q)xD motif forms hydrogen bonds only with the guanine rings. G_5 (SAx) is the least conserved motif, it interacts with the guanine *via* water-mediated hydrogen bonds. Thus, G_4 and G_5 are essential for the high-affinity binding of the GTPase to guanine nucleotides.

Therefore, small Ras-like GTPases are single-domain molecular switches that can alternate between two conformational states, a GTP-bound active conformation and a GDP-bound inactive conformation [19]. Their activation allows them to interact with a myriad of downstream effectors to generate a specific response. The activation level of Ras and therefore the nucleotide-binding status have been linked to the ratio between nucleotide exchange and hydrolysis [20]. However, Ras is itself a weak GTPase because the intrinsic reaction rates for hydrolysis and nucleotide dissociation are very slow compared to the observed kinetics of Ras activation in cells (reaction half-life of about 30 min compared to seconds to minutes in cells) [21]. This is because the activation of Ras is highly regulated by molecules that catalyse the GDP-GTP cycle. These molecules include guanine exchange nucleotide factors (GEFs) that accelerate the exchange from GDP to GTP-bound G protein (Fig. 1C) and GTPase-activating proteins (GAPs) that stimulate the low intrinsic GTP hydrolysis (Fig. 1D) [18]. The third class of molecules known as guanine dissociation inhibitors (GDIs) can also regulate some GTPases by inhibiting the dissociation of the GDP bound to the GTPase, therefore maintaining the GTPase in its inactive state [22,23]

The first bacterial small Ras-like GTPase to be identified is the mutual gliding motility A (MglA), discovered over 30 years ago by Hartzell and Kaiser in the bacterium *Myxococcus xanthus* [24]. Since then, MglA has been extensively studied due to its role in regulating polarity reversals during *M. xanthus* motility. *M. xanthus* cells move on surfaces directionally along their long axis, changing their direction by a process called reversal during which cell polarity is rapidly inverted [25–27]. At the molecular level, cellular reversals depend on the pole-to-pole exchange of dynamically and polarly localized motility complexes (the reader is referred to [27–29] for more details on the molecular aspects of these complexes). The localization of these complexes is under the control of MglA and proposed regulators of its nucleotide state, which together are suggested to determine a polarity axis. MglA-GTP localizes at the leading pole, recruited by polar proteins RomR and RomX (RomRX hereafter), which have been proposed to function as an MglA GEF. At the lagging pole, MglA is excluded by MglB, a proposed GTPase-activating protein, that spatially activates GTP hydrolysis and thus the inactivation of MglA [8,30,31]. As we will discuss, the exact function of the MglA regulators remains to be established. When cells reverse, MglA and its regulators are switched from one pole to the other. This process is only partially resolved and depends on the activity of the Frz chemosensory-like pathway.

In this review, we describe the current understanding of the molecular mechanisms underpinning the dynamic regulation of polarity in *Myxococcus* cells with a particular focus on the role of the key GTPase MglA. Based on available biochemical and structural data, we highlight our current understanding of the mechanisms controlling the nucleotide state of MglA. In light of this analysis, we question the exact function of the RomRX complex and MglB and discuss research perspectives to revise the current model.

M. xanthus motility is powered by two distinct molecular machineries

Myxococcus xanthus adopts a sophisticated multicellular lifecycle showing cooperative predation and multicellular development based on nutrient availability [32]. *M. xanthus* cells spread coordinately across surfaces and acquire nutrients by preying on other bacteria by contact-dependent killing [33] and saprophytic use of the degradation products [34]. In depleted conditions, the bacteria initiate a multicellular developmental cycle wherein cells aggregate to form spore-filled fruiting bodies [32,35]. Once the nutrient condition becomes favourable, myxospores can germinate to form vegetative cells leading to a new community [36]. These processes depend on the coordinated movement of cells that is powered by two genetically distinct motility machineries with well-defined front-rear polarity. S(ocial)-motility driven by the so-called Type-IV

pili (TFP) that assemble at the leading pole and, promote the coordinated movement of large cell groups [37,38]. In this process, the pili are polymerized by a multiprotein apparatus and adhere *via* their tips to a self-secreted exopolysaccharide [39–41]. After adhesion, pilus retraction by depolymerization pulls the cells forward [42–47] (Fig. 2A). A(dventurous)-motility promotes the movement of single cells at the colony edges. The A-motility machinery, the so-called Agl-Glt motor, also assembles at the leading pole and moves along the cell axis along helical trajectories [48]. When the complex adheres to the substrate, it forms the so-called bacterial focal adhesions, which propel the cell forward *via* a screw-like rotational movement. When it reaches the lagging pole, the protein complex disassembles [25,26,49–51] (Fig. 2B). For more details about the structure and function of these molecular machineries, the reader is referred to [27,29,47,49]; here, we will mainly discuss their activation mechanism *via* MglA-GTP.

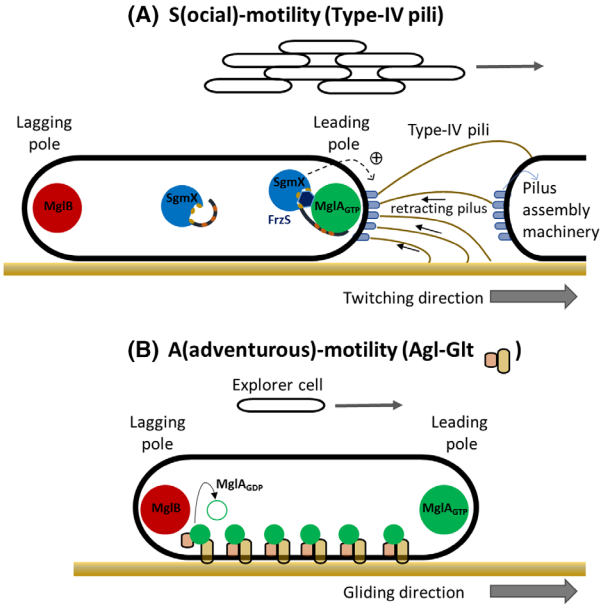

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Fig. 2. Schematic representation of motility machineries in *Myxococcus xanthus*. MglA activates two motility machineries at the bacterial cell pole. (A) Collective motility (referred to as Social-motility) involves Type-IV pili that undertake cycles of extension and retraction at the leading pole to pull the cell forward. MglA complexed to GTP interacts and recruits the newly identified TFP regulator protein SgmX, therefore unmasking the FrzS-binding domain of SgmX to activate S-motility machinery at the bacterial cell pole [52]. The different domains of SgmX are represented as follows: blue is the Tfpa-activating domain, black tail represents the C-terminal domain and the dark-orange and yellow circles represent the TPR domain. (B) Single-cell gliding motility (referred to as Adventurous motility) is driven by the Agl-Glt complexes that assemble at the leading cell pole and move directionally towards the lagging cell pole. The moving complexes adhere to the underlying substrate to propel the cell forward. When the active Aglt-Glt complexes reach the lagging pole, they are disassembled by the action of MglB. During a reversal, *M. xanthus* cells invert their direction of movement by changing the polarity of the motility machineries that simultaneously disassemble from the old leading pole and re-assemble at the old lagging pole (which becomes the new leading pole).

Both motility machineries are polarized and only assemble at the leading pole under the control of the small Ras-like GTPase MglA [8,31], allowing a persistent unidirectional movement of *M. xanthus* with a well-defined polarity axis. Deletion of *mglA* induces defective localization of both A- and S- motility proteins and during reversals the function of MglA is to redirect proteins of each motility machinery to the opposite pole [26]. We summarize below the current knowledge of the mechanisms of S- and A-motility activation.

S-motility

Recent studies elucidated the mechanism by which MglA-GTP activates Tfps at the leading cell pole. MglA recruits an essential Tfp activator, SgmX, at the leading cell pole (Fig. 2A). The localization mechanism is now well understood: The SgmX C-terminal domain, consisting of three tetratricopeptide repeat (TPR) domain – a well-known protein–protein interaction, structural motif –, forms an MglA-GTP-binding domain, which when binding unfolds an otherwise hidden secondary binding site to the polar landmark protein FrzS [52]. This allows recruitment of SgmX to the cell pole where it activates the TFP machinery *via* its N-terminal activation domain [52]. The exact activation mechanism is still under study and could be linked to dynamic interactions between SgmX and the PilB pilus extension motor [37,38].

A-motility

MglA plays a central role as it is directly incorporated into the machinery in its GTP-bound form and it is necessary for its stability [53]. Assembly occurs at the leading pole by a yet undefined mechanism where MglA-GTP forms a so-called cytoplasmic platform for Agl-Glt machinery assembly. The exact interactions within the platform remain to be discovered but polar assembly involves a sequence of interactions among MglA-GTP, the AglZ coiled–coil protein and the actin-like-protein MreB [50,53–56]. Studies are underway to understand how the cytoplasmic subcomplexes MglA, MreB and AglZ connect to the rest of the

gliding motility machines [53,57]. Disassembly at the lagging cell pole is driven by the collapse of the cytoplasmic platform *via* the spatial inactivation of MglA-GTP by MglB (see below).

The polarity axis proteins MglB and RomRX

As discussed above, each of the motility machineries is activated independently by MglA-GTP at the cell pole, which can be switched during reversal. The unipolarity of MglA is centrally controlled by its regulators MglB and the RomRX complex.

At the cellular level, experimental studies have shown that all four proteins localize asymmetrically to the cell poles. While MglA is unipolar in its GTP-bound state and delocalized in the cytoplasm when bound to GDP [8,31], RomRX and MglB localize in a bipolar asymmetric pattern with a large cluster at the lagging pole and a small cluster at the leading pole [8,30,58,59]. These four proteins constitute the core regulatory components of the polarity control network. This is especially evident in mutants lacking one or two components of the system: MglA-GTP becomes diffuse in the absence of RomRX and bipolar in the absence of MglB [59,60]. In a double *romR mglB* mutant, MglA-GTP is diffuse, which led to suggest that RomRX acts as the main polar determinant of MglA-GTP. But, how do these proteins establish a polarity axis?

Current models suggest that despite sharing similar localization patterns (except immediately after reversals, see below), the functions of MglB and RomRX are spatially separated [25,30,58]. MglB was proposed to inactivate MglA by acting as a GAP with a dominating activity at the lagging pole [8,31]. RomRX function instead was suggested to dominate at the leading pole, functioning as a GEF and thereby enhancing the pool of MglA-GTP at this location [30].

Yet, this picture is likely over-simplistic [28] and complex regulations must take place to restrict the localization of MglA at a single cell pole. While the choreography of each of these proteins following cell reversals has been precisely established by several studies, there are still several critical aspects of the reversal cycle that should be considered [30,58,61]:

1 The localization of RomRX is highly dynamic. RomRX is localized at the leading cell pole immediately after cells reverse, but the complex rapidly moves to the lagging cell pole, presumably due to direct interaction with MglB. Thus, after a relaxation period (a fixed time delay during which the majority of the RomRX pool shifts to the lagging pole), the cell is polarized such that most of the MglA pool is at the leading pole and most of the RomRX and MglB pools are at the lagging pole (Fig. 3A). This situation remains stable until reversal signals from the Frz complex induce a new polarity switch.
2 When Frz signals a reversal, MglA detaches from the leading cell pole and relocalizes to the lagging pole where both MglB and RomRX are present. This step is rapidly followed by the switch of MglB to the opposite pole, which then provokes the reversal. As in the previous cycle, RomRX slowly delocalizes, completing the polarity switch (Fig. 3A).

This sequence of events shows that a simple model such as that presented above cannot explain the polarity mechanism and its switch. Several questions arise which we will discuss in subsequent sections of this review:

1 How is MglA localized to the pole and how does RomRX promote this localization?
2 How are interactions regulated at the lagging pole and, what is the exact function of MglB?
3 How can Frz signalling promote a concerted switch in the localization of these proteins?

Interactions at the leading cell pole: the MglA-RomRX complex

MglA is recruited at the pole by the RomRX complex. RomR interacts with RomX, which interacts with MglA-GTP, thus forming a heteromeric complex where ROMX is sandwiched between MglA-GTP and RomR. The complex was suggested to carry out a GEF activity which is mediated by RomX and enhanced by RomR in the RomRX complex [30]. However, this function can be disputed because conventional GEFs act as catalysts in a two-step process:

1 First, by a reaction where a complex is formed between the GDP-bound GTPase and the GEF. This interaction leads to the loss of the bound nucleotide, leaving the GEF in a high-affinity complex with the nucleotide-free GTPase.
2 Second, by insertion of GTP, which displaces the GEF [18,21,23] (Fig. 1C).

Yet, while RomX and the RomRX bind to MglA-GTP [30], neither of them shows an interaction with GDP-bound MglA, which constitutes the fundamental of a GEF-based reaction. Thus, it is possible that rather than acting as a *bona fide* MglA GEF, RomRX

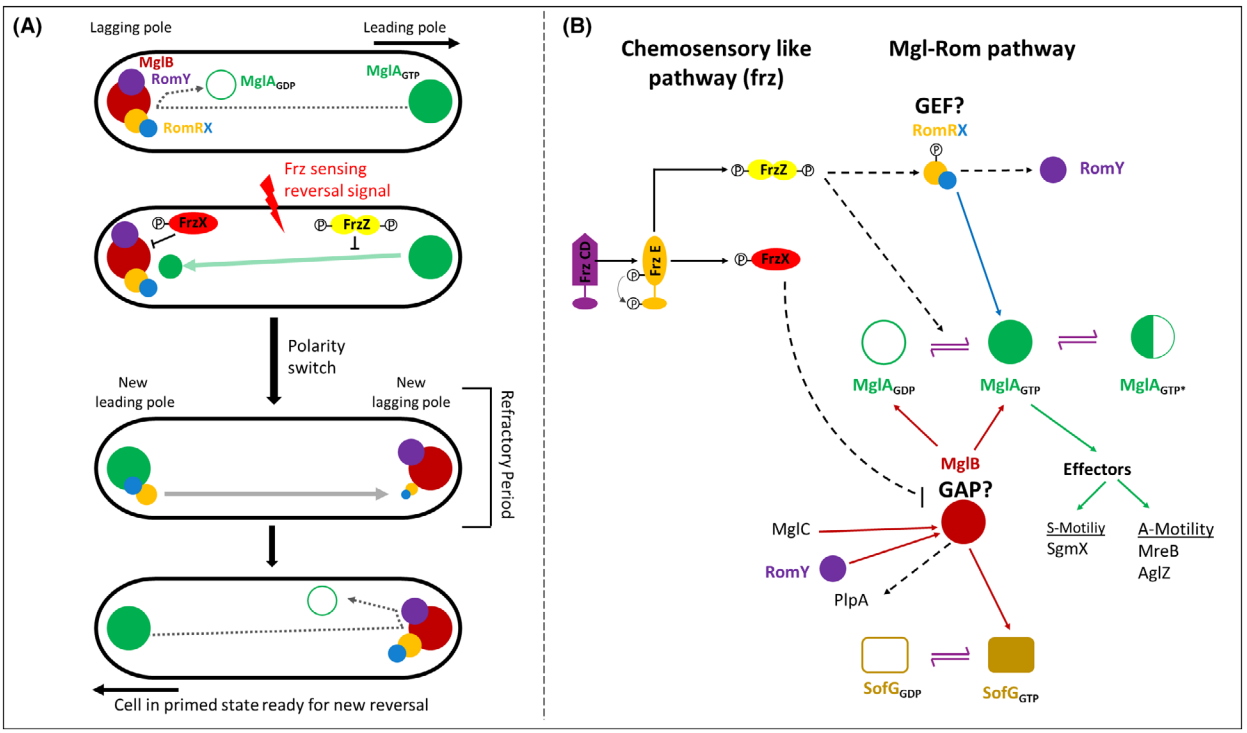

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Fig. 3. Molecular mechanism underpinning cellular reversals in *Myxococcus xanthus*. (A) *M. xanthus* cell polarity switch is controlled by a gated-relaxation oscillator. Before a reversal, MglA-GTP (in green) assembles at the leading cell pole, and MglB (in dark red) and RomRX (in orange and blue, respectively) are at the lagging cell pole. The cell cannot reverse because the GATE is closed. MglB by functioning as a GAP together with RomY (in purple) deactivates MglA by converting it to the GDP-bound inactive state (open green circle). When a reversal signal is perceived by the Frz system, phosphorylated FrzX (in light red) localizes at the lagging pole and opens the GATE. MglA is then recruited by the RomRX complex to the new leading pole, leading to a reversal. After the reversal, RomRX slowly detaches from the pole to accumulate at the opposite pole and interact with MglB. This process defines the relaxation step for the system and introduces a refractory period during which no new reversal can happen. Phosphorylated FrzZ (in yellow) localizes at the leading pole and acts to limit the duration of the refractory period set by RomRX (adapted from Ref. [61]). (B) Molecular interaction networks underlying the polarity switch. Upon activation, nucleoid-bound Frz receptor-kinase complexes phosphorylate the two diffusible response regulators FrzX and FrzZ. FrzX-P localizes at the lagging pole and favours the accumulation of MglA-GTP, while FrzZ-P localizes at the leading pole and possibly dissociates MglA from the pole and limits the length of the refractory period set by RomR. The RomRX complex has been proposed to recruit MglA at the leading pole and function as a MglA GEF, while MglB is suggested to be a GAP that spatially activates GTP hydrolysis and thus inactivation of MglA. The function of the RomRX complex and MglB is still not clear and requires other regulators such as MglC, PlpA and RomY.

functions as an MglA effector, interacting with MglA-GTP and thus stabilizing the GTP-bound conformation, an effect that has been observed upon the interaction between an effector and its cognate GTP-bound G protein [22]. Neither RomR nor RomX show homology with canonical GEF proteins. According to predictions made using AlphaFold2 [62], the structure of RomX resembles that of an alpha-helical protein with no match to any currently characterized protein domains [28]. The structure of RomR could not be predicted with AlphaFold due to the presence of an extended proline-rich region (natively unfolded) that separates the N-terminal receiver domain and the highly conserved α-helical C-terminal region [60,63]. The proline-rich region and the C-terminal region were demonstrated to be individually sufficient for polar targeting of RomR and required for motility [60]. However, how RomR interacts with RomX and how in turn RomX interacts with MglA-GTP remains to be elucidated. In addition, RomR interacts with MglB [58,60], possibly explaining the relocalization of RomRX at the lagging cell pole. Again, structural knowledge of this interaction is not available.

The proposed function of RomRX as an MglA localization factor also suffers from a major unresolved conundrum. While it is clear that the presence of RomRX is required for MglA polar localization, RomRX and MglA only co-localize at the leading cell pole during a short window of time. It is therefore likely that RomRX is not *per se* an MglA anchor at

the pole, but rather supports the loading of MglA to the cell pole, for example, allowing it to interact with polar motility-specific effectors present in the A- and S-motility complex. Such polar anchors have not been identified, but consistent with their possible existence, MglA is not polarly localized in strains that lack Tfps [64].

Altogether, these findings reveal that MglA polarity cannot be solely a result of the presence of RomRX at the pole because these proteins are only transiently co-localized. In addition, the function of RomRX as a GEF is questionable. Even more questions arise when the properties of the MglA-MglB complex are scrutinized.

Interactions at the lagging cell pole: insights from the MglA-MglB complex

The structure of MglA and MglB alone or in the complex has been reported with both *Thermus thermophilus* and *M. xanthus* proteins. In *T. thermophilus*, 9 the function of MglA is not well characterized and it appears to also regulate the activity of TFPs [65,66]. As we will see, while the reported structures are very similar, their comparison reveals intriguing features of the *Myxococcus* MglA-MglB complex.

MglB is a 17 kDa small protein with no homology to known GAPs [31]. Structural analysis has shown that MglB is part of the Roadblock/LC7 protein family involved in the regulation of NTPase activity and with members in all three domains of life [67]. Contrary to other G protein/GAP complex binding in a 1 : 1 stoichiometry, a dimer of MglB interacts with an MglA monomer in 2 : 1 stoichiometry, forming an asymmetrical interface where MglA mostly occupies one side of the MglB dimer (this possibly allows the binding of additional regulators, as discussed below) [65,68,69]. Some GAPs also form dimers, for example, RapGAP1 and RopGAP2 are constitutive dimers but they form a 2 : 2 complex with their simultaneous Rap1 and Rop GTPases [70–72]. In addition to forming a dimer, MglB was also observed to form high-order oligomers but their biological relevance is unclear [61,68].

So how does MglB activate GTP hydrolysis in MglA? MglA-GDP is inactive because its switch 1 region is retracted and unavailable for interaction with the effectors. The switch 2 region is also in a conformation that prevents binding of both GTP and Mg^{2+} [65] (Fig. 4A). In contrast, the binding of GTPγS induces a shift in the switch 1 region as well as a displacement in the switch 2 region, which now creates space for the γ-phosphate and Mg^{2+} [69] (Fig. 4B). In this conformation MglA can interact with its effectors, for example, SgmX [37,38].

The structure of the MglAGTPγS-MglB complex reveals that MglA interacts with an MglB dimer such that switch 1 and switch 2 establish contact with each of the MglB monomers [69]. However, one MglB monomer also makes additional contact with another region of the MglA protein which leads to several changes in the conformation of MglA:

1 A new orientation of the Thr54 and Asp58 residues, which promotes the coordination of Mg^{2+} ion in the presence of GTPγS (Fig. 4B inset i). Interestingly, it was recently proven that Asp58 functions as the Walker B acidic residue of MglA and is crucial for nucleotide binding [73].
2 A repositioning of the critical MglA arginine (Arg53) into the nucleotide-binding pocket. This latter event explains the GAP function of MglB. In contrast to conventional GAPs, which typically insert an arginine finger (Rho-, Ras-, Arf-, Rab-, Arl- and Rab-specific GAP's) or a so-called asparagine thumb (Rap- and Rheb-specific GAP's) into the nucleotide-binding pocket of the corresponding GTPase to reconstitute a full catalytic site [18,68,74], MglB functions by reconstituting a full MglA catalytic site by changing the conformation of the MglA Arg53. In the absence of MglB, Arg53 is oriented away from this pocket in the MglA-GDP and MglA-GTPγS structures which explains why it is a weak GTPase (Fig. 4A,B).

While these structural insights solve the mechanism by which MglB promotes GTP hydrolysis, additional oddities suggest the existence of complex regulation at the level of MglB. Baranwal et al. [65] also solved the structure of the MglAGTPγS-MglB complex (Fig. 4C) and observed that one of the two MglB monomers (MglB1) interacts with MglA *via* a short C-terminal helix (Ct helix). This Ct helix wrapping around MglA was not observed in the *T. thermophilus* MglA-MglB structure, suggesting that it is a specific feature of the *Myxococcus* MglA-MglB complex [31,68]. *In vitro*, the helix confers remarkable properties: it allows MglB to bind both MglA-GDP and MglA-GTP, and it not only enhances the GAP activity of MglB but also facilitates nucleotide exchange, GDP as well as GTP, allosterically (although this particular effect was not observed in another study, [69]). *In vivo*, expression of a stable MglB lacking the helix abolishes cell polarity, and MglA becomes trapped at both cell poles [65]. It is currently difficult to reconcile the *in vitro* and *in vivo* data into a coherent model but the discovery that MglB binds to both, the active and inactive, states of MglA challenges

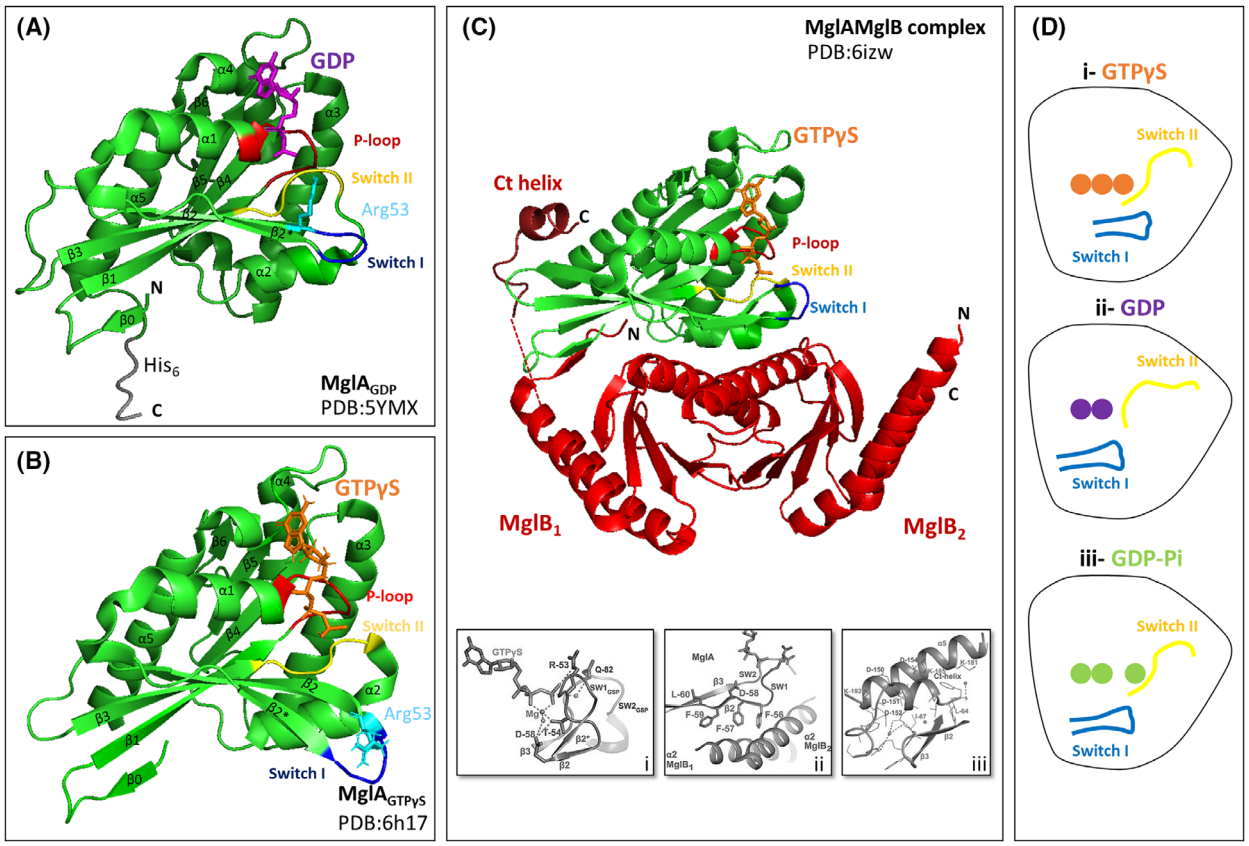

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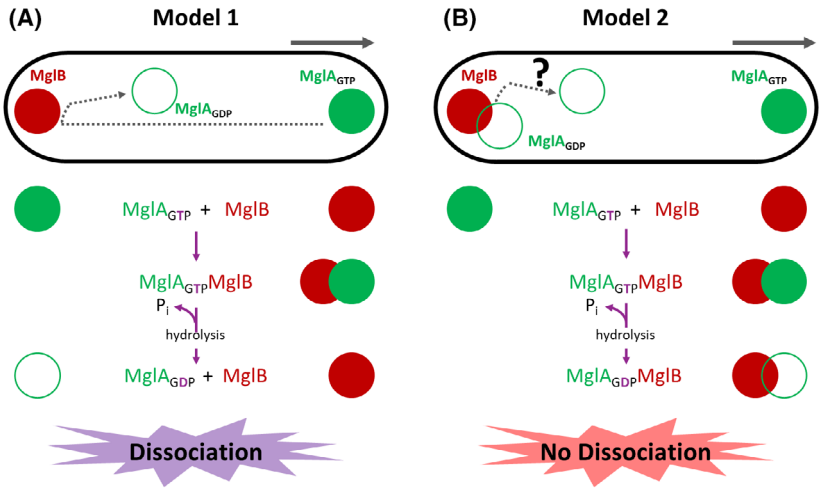
Fig. 4. Structural characterization of MglA and MglA-MglB complex. (A) Ribbon diagram of MglA loaded with GDP (adapted from Ref. [65]). The six-stranded β-sheet and the five α-helices are labelled. The catalytic loops are labelled and colour coded as follows: P-loop (red), switch I (dark blue) and switch II (yellow). GDP is labelled and represented as magenta sticks. The C-terminal hexahistidine (His_6), which is ordered in the crystal structure, is highlighted in grey. The catalytic arginine finger is labelled (Arg53) and highlighted in light blue. (B) Ribbon diagram of MglA loaded with GTPγS (adapted from [69]). The six-stranded β-sheet and the five α-helices are labelled. The catalytic loops are labelled and colour coded as follows: P-loop (red), switch I (dark blue) and switch II (yellow). GTPγS is labelled and represented as orange sticks. (C) Ribbon diagram of MglA (green) bound to GTPγS (orange) and MglB dimer (red) (adapted from Ref. [65]). The two protomers of MglB are labelled as MglB1 and MglB2. The C-terminal helix of MglB is labelled (Ct helix) and highlighted in dark red. The corresponding N and C terminals of MglA and the two MglB protomers are labelled. GTPγS is labelled and represented as orange sticks. The dotted red line connects the ends on either side of the stretch of disordered residues in MglB1. Inset (i) GAP activity of MglB induces the repositioning of the active site residues Arg53 and Gln82 of MglA. Thr54 and Asp58 reorient allowing the coordination of Mg^{2+}-ion bound to GTPγS. Inset (ii) The central β2 strand repositions exposing the three hydrophobic phenyl residues (Phe 56, Phe 57 and Phe59) of MglA to form an interacting surface with MglB. Inset (iii) Residues involved in the interaction between the Ct helix of MglB and the β2-β3 loop of MglA. (D) MglA can adopt three conformational states: (i) active GTPγS-bound state, (ii) inactive GDP-bound state and (iii) a possible GDP-Pi-bound conformation combining both active and inactive features. The black trace around each schematic represents the nucleotide-binding site.

the current polarity model. GAPs typically bind GTP-bound small proteins and dissociate once catalysis has occurred (Fig. 5A). MglB also has other targets in the cell, for example, it can also function as a GAP for SofG, another small GTPase in *M. xanthus*. But in this case, MglB acts canonically because it only binds to SofG-GTP and the MglB Ct helix does not play a major role [75]. The significance of this regulation *in vivo* is unclear; SofG has been proposed to regulate the localization of the TFP motor proteins PilB and PilT in a Bactofilin-dependent manner *via* a mechanism that remains to be clarified [75]. Thus, it is a particularity of the MglA-MglB complex not to dissociate following hydrolysis. This raises the possibility of additional regulatory mechanisms (Fig. 5B).

The binding of MglB to MglA-GDP has several important consequences. Because this binding favours GTP insertion, it can be imagined that MglB could function as an MglA GEF in specific situations. For example, if its effect on the positioning of the MglA Arg53 in the nucleotide-binding pocket is blocked. There is currently no evidence for such regulation.



Color

Fig. 5. Binding of MglB to MglA-GDP affects the polarity model. MglA localizes at the leading pole in its GTP active state while MglB resides at the lagging pole. At the lagging pole, MglA GTPase activity is promoted by the interaction with MglB. (A) The previous model. It was proposed that MglA cannot accumulate at the lagging pole because MglA-GDP dissociates following the GAP reaction. This could explain MglA polarity. (B) The proposed model. The binding of MglB to MglA-GDP requires an additional dissociation step. Since the GAP reaction is not sufficient to dissociate the MglA-MglB complex, an additional step must be added to explain polarity.

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Nevertheless, recent work from Szadkowski et al. [76] showed that although the MglB GAP activity is observed *in vitro* in absence of other factors, it might require other proteins *in vivo*. One of them, RomY both activates GAP activity *in vitro* and critically, it is essential for GAP activity *in vivo*. The exact mechanism is not known, but an AlphaFold model of RomY in complex with MglA-MglB predicts that an N-terminal domain of RomY binds to the second MglB monomer, in the MglA-free interface. Such binding will have to be demonstrated but it nicely suggests that the MglA-MglB complex can interact with additional regulators to enhance the GAP activity. In theory, it could also be conceived that such kind of additional regulation, *via* other binding factors, could also convert MglB into a GEF.

Another intriguing observation further suggests complex regulations of the MglA GAP. *In vitro*, it was observed that MglB readily promotes GTP hydrolysis, but not when MglA-GTP has been previously incubated at room temperature. This suggests that MglA can mature into a form that is resistant to MglB action (named MglA-GTP*, [69]). Remarkably, MglA-GTP* is not a denatured or aggregated form of MglA because its GTP hydrolysis can be rescued by the addition of fresh MglA-GDP to the reaction. The molecular basis of this effect is not known but a third MglA structure, revealed that it can adopt a third possible mixed conformation where the switch 1 is retracted and the switch 2 is in an active conformation [69] (Fig. 4D). It remains to be shown whether this third conformation is the conformation of MglA-GTP*, but this new structure does reveal that MglA can adopt two distinct GTP-bound states [69]. *In vivo*, there is evidence that MglA-GTP* participates in the regulation [69].

In summary, MglB shows four unique characteristics which separate it from other known GAP proteins:

1 MglB binds MglA in a 2 : 1 stoichiometry which was not seen before for GTPase/GAP complexes.
2 MglB lacks the active site residues (glutamine and arginine) that were found in MglA itself which indicates that MglB does not engage directly in hydrolysis but properly orients and/or stabilizes the catalytic machinery.
3 MglB is not a *bona fide* GAP. It forms a stable complex with both GTP and GDP. In addition, GTP hydrolysis of MglA in presence of MglB was stimulated only about 50-fold [65,68] compared to other Ras GTPase GAPs that increase the rate up to 10^5-fold [77,78]. Accessory factors such as RomY are necessary *in vivo*.
4 MglB is sensitive to the state of MglA, which can be resensitized by a feedback mechanism involving MglA-GDP.

The Frz system regulates spatial switching of the MglA module

Cells moving by A- or S-motility are capable of periodically reversing their direction of movement and thus, reorienting the direction of their track. Periodic reversals are genetically controlled by the so-called

'frizzy' (*frz*) genes first identified by Zusman in 1982 [79]. The *frz* genes encode proteins of a chemosensory-like pathway [80,81] and consist of a methyl-accepting chemotaxis protein (MCP or chemoreceptor, FrzCD) associated *via* a coupling protein (FrzA) with a CheA-type histidine kinase (FrzE).

Intriguingly, FrzCD does not localize to the membrane as most bacterial MCPs, but forms signalling complexes at the surface of the *Myxococcus* chromosome. How Frz becomes activated is still a mystery, but the localization of FrzCD suggests a function as an intracellular signalling hub rather than a direct sensor of extracellular signals [82,83]. As an MCP, FrzCD activity is regulated by methylation, promoted by the methyltransferase, FrzF (a CheR homologue), and demethylation, promoted by the methylesterase FrzG (a CheB homologue) [84]. The exact function of the methylation/demethylation systems remains to be understood: methylation of FrzCD appears to be essential for signalling, which is unexpected for an MCP [85]. This is particularly intriguing given that FrzG is only important in specific circumstances such as during rippling [85]. Nevertheless, as in other chemotaxis systems, activation of the MCP leads to the autophosphorylation of the FrzE kinase from ATP, which in turn phosphorylates two downstream receiver domain proteins (CheY), FrzX and FrzZ.

FrzX and FrzZ are believed to act as diffusible messengers that promote reversals *via* interactions with the Mgl polarity complex. Both regulators act complementarily, FrzX-P is absolutely required to activate reversals, while FrzZ-P is required to obtain a high reversal frequency at high levels of Frz activation [86]. Remarkably, each regulator binds at opposite poles in its phosphorylated form [61,87]. FrzX-P binds at the lagging cell pole in an MglB-dependent manner, which provokes the relocalization of MglA and thus, FrzX is a reversal trigger. In contrast, FrzZ-P binds to the leading cell pole, which could facilitate the liberation of MglA (Fig. 3A). The exact molecular targets of these regulators are still unknown. Below we discuss their potential mode of action with regard to the polarity complex.

Towards an integrated polarity model

Above we have described evidence that *Myxococcus* motility is regulated by an intricate molecular network consisting of chemosensory-like signal transduction proteins acting upstream from an invertible polarity complex formed at its core by a small GTPase switch (Fig. 3B). While it is clear that the main molecular players have been identified, the exact regulatory network connecting these proteins is at best partially identified. Indirect evidence suggests that the Frz pathway is activated by cell–cell contacts [88–90], but the molecular players involved are not characterized. Thus, the 'natural' Frz-inducing signals (FrzCD can be artificially activated by short-chain alcohols, such as isoamyl alcohol, see below) remain unknown. Below we try to integrate the current data into a possible regulatory model, proposing new research perspectives for the future.

Several critical properties of the regulatory network need to be considered to obtain a holistic view of its operation. Guzzo et al. [61] observed that the Frz-Mgl system behaves as a so-called 'gated relaxation oscillator'. To explain this concept, we must first define two types of systems that function very differently, relaxation oscillators and toggle switches. A relaxation oscillator is a system that spontaneously returns to equilibrium after being disturbed [91]. A short impulse (i.e. an activating signal) will perturb the system, which will slowly return to equilibrium to do an embedded periodic (relaxation) component. In a toggle switch, a signal impulse separates two stable-steady states, ON and OFF, without intermediate transitions [92]. A gated-relaxation oscillator combines both the properties of a toggle switch and a relaxation oscillator. The Frz Mgl system functions as a toggle switch at low signal concentrations and turns into a relaxation oscillator at high signal concentrations. These properties depend on the RomRX complex and the response regulators FrzX and FrzZ. The relocalization of RomRX to the lagging cell pole occurs at a constant speed and this creates a limiting step in the reversal cycle: new reversals cannot be provoked until RomRX has attained a sufficient concentration at the lagging cell pole [61]. This creates a so-called refractory period during which new reversals cannot be provoked. However, the accumulation of RomRX at the lagging pole is not itself sufficient to provoke another reversal. This requires the actions of both FrzX and FrzZ (Fig. 3A).

Studies *in vitro* where Frz signalling could be artificially stimulated in a dose-dependent manner by the addition of iso-amyl alcohol (IAA, an artificial signal known to activate FrzCD, [86]) led to suggest the following properties for the system:

1 At low signal levels (i.e. when environmental signals are in low abundance, *in vitro* at low IAA concentrations), the system functions as a toggle switch: the levels of FrzX-P and FrzZ-P are low and limiting; the cells are ready to reverse with the RomRX complex at the lagging cell pole. If the Frz complex

becomes activated, a sharp rise in [FrzX-P] and [FrzZ-P] opens 'the gate' and provokes the reversal.
2 At high signal levels (i.e. when environmental signals are abundant, *in vitro* at high IAA concentrations), the system functions as an oscillator, the activity of the FrzE kinase is high and thus [FrzX-P] and [FrzZ-P] are high; therefore, the gate is open. However, the slow dynamics of RomRX create a limiting step because the cell only reverses when the [RomRX] has reached a sufficient level at the lagging cell pole. As soon as the cell reverses, RomRX detaches again and this creates an oscillation. Remarkably, the slow dynamics of RomRX are bypassed by the activity of FrzZ-P, the action of which limits the minimum RomRX threshold, allowing cells to attain high reversal frequencies.

Several outstanding questions will have to be resolved to understand the molecular basis of these regulations:

1 The exact interaction network between MglA and its regulators underlying the formation of the polarity axis needs to be resolved. Recently, Carreira et al. [59] proposed that cell polarity could be established by a mechanism where MglA-GTP inhibits the formation of a RomR-MglB complex, and *vice versa*, RomR-MglB blocks MglA-GTP accumulation at the lagging cell pole. While attractive, the molecular basis for such effects is missing and arguably, the existence of a RomR-MglB complex remains to be characterized. This problem will not be solved until the exact functions and regulations of MglB, the role of its C-terminal helix and the regulation by RomY are understood. The exact function of RomRX must also be determined as well as that of the RomRX-MglA and possible RomRX-MglB complexes. Other possible regulators are not discussed here, mainly because their function remains unclear, such as MglC or PlpA which also appear to be part of the polarity mechanism and localize at the lagging cell pole [64,93,94]. In summary, although the major players have been identified, how they promote polarity is arguably still very poorly understood.
2 How is the polarity axis inverted by the Frz signalling? What is the mode of action of FrzX-P and FrzZ-P, and what are their targets? Although it is clear that these proteins act upstream from the polarity proteins, their mode of action remains elusive. FrzX-P, in particular, is essential to trigger reversals and acts at the lagging cell pole likely by antagonizing a molecular complex that blocks MglA accumulation. At the leading pole, FrzZ-P exerts a complementary action, possibly favouring the detachment of MglA. How that occurs will require identification of the mechanism by which MglA is retained at the cell pole.

Conclusions and perspectives

Here, we discussed the molecular architecture of a cellular compass allowing single *Myxococcus* cells to navigate in their environment. However, there is an additional layer of complexity when it comes to understanding how this system promotes the concerted movements of thousands of cells, promoting all the major developmental transitions during the *Myxococcus* lifecycle, from predation and the formation of rippling waves to the formation of fruiting bodies. In the future, it will be important to elucidate how the signalling properties of the Frz-Mgl complex (the above-mentioned gated-relaxation oscillator) can promote such transitions. For this, it will be necessary to identify the reversal-inducing signals.

Beyond, studies of the MglA-MglB regulations at molecular scales can open perspectives to understand the mechanisms by which the LC7/roadblock superfamily of proteins frequently regulate small GTPases. Proteins with a roadblock domain are found to be conserved in the three domains of life and present in the last universal common ancestor [5,67,95–97]. Together with their structurally related longin domain-containing proteins, roadblock domain-containing proteins form heteromeric complexes with small Ras-like GTPases. Intriguingly, they appear to function as GEFs more frequently than they function as GAPs [95]. A striking example is a pentameric complex known as Ragulator that promotes untypical Rag GEF activity. Understanding the mechanism of Ragulator function is difficult because it contains up to four subunits with roadblock fold and assembles in heterodimers. Interestingly, and perhaps similar to MglB, the Ragulator complex also communicates allosterically with the G domain of the RAG GTPase [98]. Moreover, it was reported that the Rag family proteins could have evolved from the MglA protein family [73,96]. Other roadblock domain-containing complexes include GATOR1 (Gap activity towards Rags) and FLCN (folliculin)/FNIP2 (folliculin-interacting protein 2) complex. Both are constituted of several subunits and exert a GAP activity simultaneously on RagA and RagC GTPases. Interestingly, an arginine residue required for the GAP activity was identified in both

GATOR1 and the folliculin complex, however, it was seen to be located far from the nucleotide-binding pocket [99–101]. Thus, the GAP activity of these proteins could also be performed through an allosteric mechanism like in the MglA-MglB complex [102].

Interestingly, the MglA-MglB interaction shows two typical characteristics of a roadblock domain protein/GTPase interaction: first, MglB regulates the MglA nucleotide state indirectly, and second, its binding site for MglA is situated on its top surface (the surface containing the α_2 helix from each roadblock domain of the two MglB monomers). This is also true for longin and roadblock domains throughout evolution with some exceptions [95]. For example, the crystal structure of the roadblock domains mediating the dimerization of Rag GTPases also shows an interaction of the GTPase with the helices on the top surface of the roadblock [103,104]. However, we are still lacking a lot of knowledge on the function of roadblocks containing proteins, their interaction and their evolution, especially due to the absence of simple biochemical assays. Thus, understanding how MglB regulates MglA at the molecular level could be a building block for understanding the function of this universally conserved protein domain. As discussed above, MglB also functions in a complex with RomY [76] and further interacts with another roadblock domain-containing protein MglC [93,94], both of which have been implicated in regulating *M. xanthus* reversals. Therefore, MglB engages in multiple interactions, which suggests that rather than being a conventional GAP, MglB could form a signalling hub in complex with MglA. It is thus tempting to imagine further parallels with the Ragulator complex where several roadblock domains and other regulators modulate GTPase function. Further studies still need to be performed to understand how these proteins organize inside the complex and how they act together to exert a specific function in time and space within the cell.

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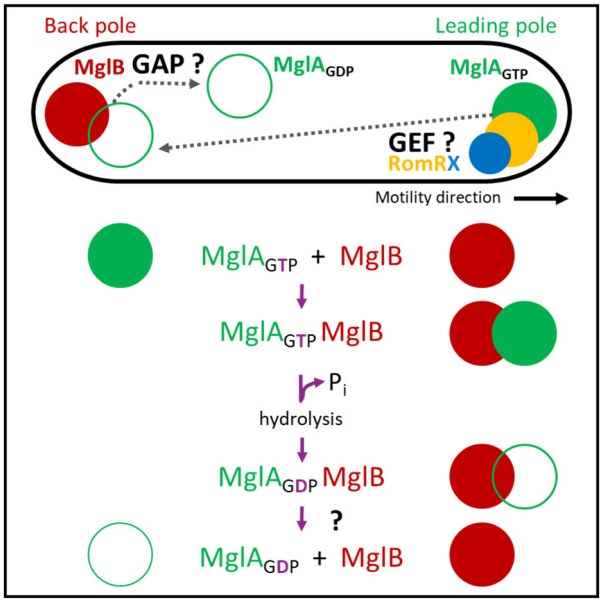

In eukaryotic cells, small GTPases act as molecular switches alternating between a GTP-bound 'ON' and a GDP-bound 'OFF' state to regulate cell motility. This is also the case with the social predatory bacterium *Myxococcus xanthus* which can change its direction of movement by a polarity switch. This phenomenon is centrally controlled by an atypical small Ras GTPase MglA and regulators of its nucleotide state (MglB, GAP and ROMRX GEF). In this review, we summarize the molecular mechanism of this GTPase and highlight current gaps and conundrums in the understanding of the system.