Developmental and environmental regulatory pathways in alpha-proteobacteria

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1. ABSTRACT

Spatial and temporal control of cell differentiation and morphogenesis plays a key role in prokaryotes as well as eukaryotes. This is particularly important for bacteria that divide asymmetrically, as they generate two morphologically and functionally distinct daughter cells. Several alpha-proteobacteria, including the aquatic, free-living *Caulobacter crescentus*, the symbiotic rhizobia and the plant and animal pathogens *Agrobacterium* and *Brucella*, have been shown to undergo asymmetrical division. *C. crescentus* has become a model system for the study of the regulatory networks, in particular the control of the cell cycle, the cytokinetic machinery, the cytoskeleton and the functions required for duplication and differentiation in general. As the bulk of these regulatory networks and functions is conserved in most alpha-proteobacteria, we recapitulate the recent advances in understanding these spatially and temporally controlled processes, focusing on cell cycle progression, DNA replication and partitioning, cell division and regulation of specific phenotypes that vary during the cell cycle or in the case of different lifestyles (like extracellular polysaccharide production) in *C. crescentus* and other alpha-proteobacteria.

2. INTRODUCTION

Bacteria have not been viewed as spatially organized entities for a long time, but this view has been overhauled as recent research uncovered a myriad of temporally and spatially regulated processes, particularly those controlling the prokaryotic cell cycle, such as chromosome replication and partitioning and cell division. The Gram-negative aquatic alpha-proteobacterium *Caulobacter crescentus* has emerged as the preeminent model system for the study of cell cycle processes and asymmetric division. *C. crescentus* divides into a replication-competent stalked cell and a motile non-replicative swarmer cell at each cell division (Figure 1) (1, 2). Importantly, since recent evidence supports the notion that many (perhaps all) alpha-proteobacterial lineages divide asymmetrically, the principal regulatory mechanisms that determine an asymmetric cell division cycle, such as the tight coordination of DNA replication and segregation, polarity, morphogenesis and cytokinesis should also be conserved in this group (3, 4). The majority of these regulatory pathways were uncovered in *C. crescentus,* owing to its facile genetics and discernible differentiation cycle. Chromosome replication is initiated only once per cell cycle and confined to the stalked (replicative) cell. The

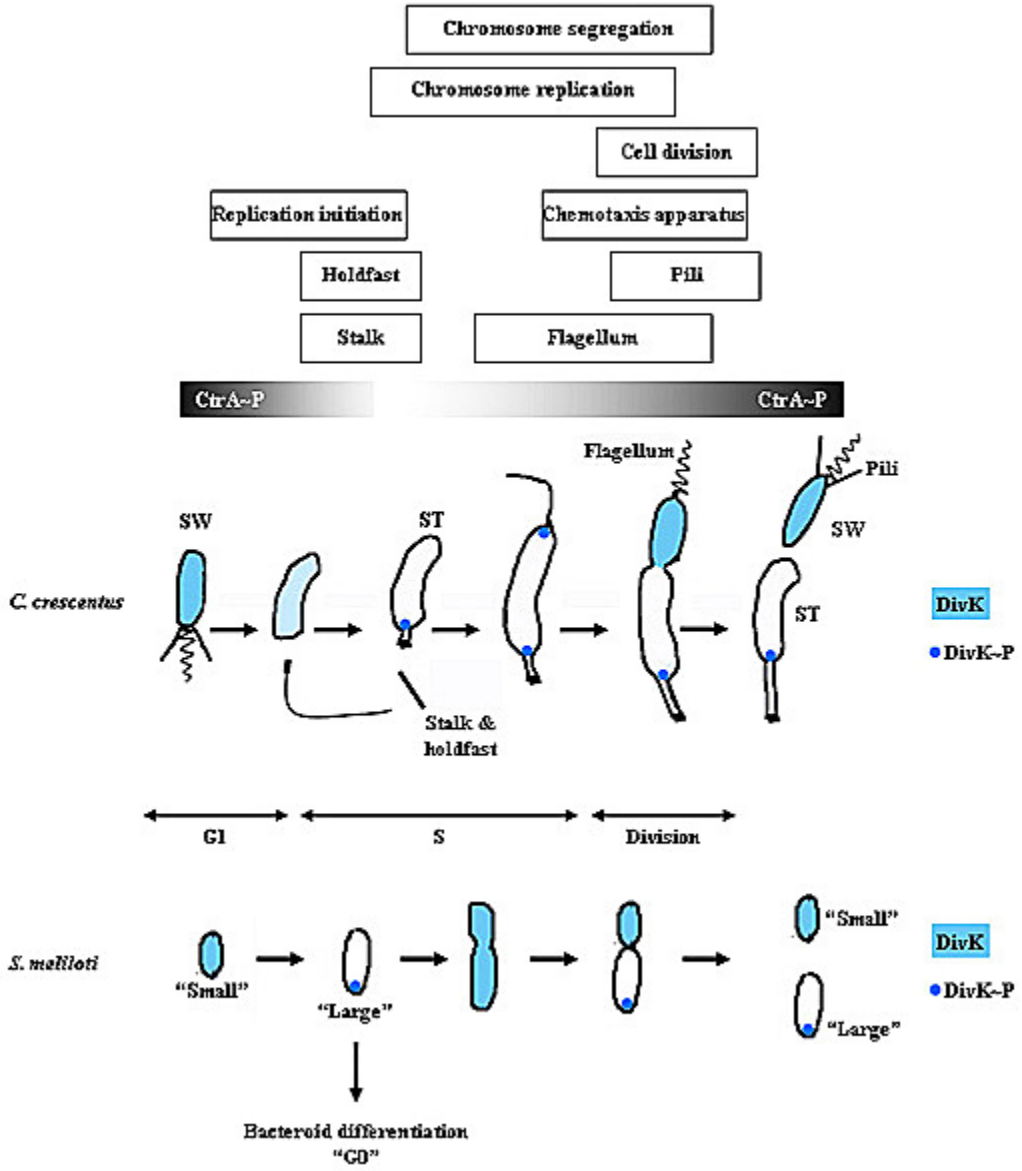

Figure 1. Schematic representation of the cell cycle in *C. crescentus* and *S. meliloti*. In *C. crescentus*, the motile swarmer cell (SW) harbors several pili and a rotating flagellum (thick wavy line). When entering the S phase, the swarmer cell differentiates into a sessile stalked cell (ST): the flagellum is shed, the pili lost and a stalk (capped with an adhesive polysaccharide, the holdfast) is synthesized at the pole previously occupied by the flagellum. The stalked cell then matures into a pre-divisional cell that assembles a flagellum at the pole opposite the stalk. The pre-divisional cell divides asymmetrically into a swarmer cell and stalked cell. CtrA~P is present in the swarmer cell and the incipient swarmer cell compartment of the pre-divisional cell. The localization of DivK during the progression of the cell cycle is determined by its phosphorylation state: unphosphorylated DivK is dispersed in the cytoplasm, whereas DivK~P is localized to the cell poles (55). Recently, *S. meliloti* has also been shown to divide asymmetrically, generating a "small" and a "large" cell. The localization of the *S. meliloti* DivK homolog indicates that the "small" and "large" cells correspond to the *C. crescentus* swarmer and stalked cells, respectively (55). During symbiosis, *S. meliloti* differentiate into nitrogen-fixing bacteroids. This process involves endoreduplication and subsequently a terminally differentiated state (G0), in which the cells are no longer capable of growth or DNA replication (Figure adapted from (90, 152)).

swarmer (dispersal) cell represents instead the non-replicative G1-like phase. To facilitate its dissemination and the subsequent colonization of a new surrounding, the swarmer cell is equipped with a single flagellum and multiple adhesive pili located at the same cell pole. The swarmer also goes through an obligate differentiation into a stalked cell, during which it ejects the flagellum, rids itself of pili and commences with the synthesis of an adhesive holdfast at the tip of a tubular cell envelope structure (the stalk) that is elaborated from the vacated pole (Figure 1).

Concomitantly, the nascent stalked cell also acquires DNA replication competence and assembles the initiation complex at the single origin of replication of its circular chromosome (*ori*). Linked to the progression of DNA replication is the synthesis of a flagellum, a chemosensory apparatus and the pilus secretion machinery at the pole opposite the stalk prior to cell separation (5). The availability of these diagnostic features for polarity and cell cycle stages has allowed the identification of global regulators involved in the control of cell cycle progression in *C. crescentus*. Most of the genes encoding orthologous regulatory pathways are not only conserved in the genomes of other alpha-proteobacteria but, more importantly, they are also functional, as detailed below.

Here, we review some of the recent advances in the understanding of spatially and temporally controlled processes – cell cycle progression, DNA replication and partitioning, cell division and other phenotypes that vary during the cell cycle or in the case of different lifestyles - in *C. crescentus* and other alpha-proteobacteria. Interestingly, the alpha subdivision defines a heterogeneous and functionally diversified group of bacteria, including plant pathogens (e.g *Agrobacterium*), animal pathogens (e.g. *Brucella* and *Rickettsia*) and plant symbionts (rhizobia). The fact that several members of this group, such as pathogens or symbionts, also divide asymmetrically suggests the intriguing possibility that asymmetric division is exploited by these bacteria to generate specialized daughter cells that play distinguished roles in host-microbe interactions.

3. CONTROL OF CELL CYCLE

3.1. CtrA and cell cycle regulators

In *C. crescentus*: CtrA, an essential response regulator with a DNA-binding output domain, synchronizes cell cycle progression with polar morphogenesis. CtrA is also encoded in the genomes of most other alpha-proteobacteria, but not in other proteobacterial subdivisions (3, 4). In *C. crescentus* CtrA directly controls the transcription of at least 95 genes and inhibits the initiation of DNA replication by binding to five sites at the origin of replication (*ori*) (6). As with other response regulators, DNA-binding of CtrA is stimulated by phosphorylation on a conserved aspartate (D51), but unlike most others, CtrA activity must be removed either by regulated proteolysis or by dephosphorylation to allow the initiation of DNA replication (7, 8). A failure to eliminate CtrA activity maintains the G1-state and the swarmer cell developmental program, resulting in cell death owing to a replication block (8). Phosphorylation is indispensable for CtrA activity and is governed by the hybrid histidine kinase/phosphatase CckA by way of the phosphotransfer protein ChpT (Figure 2) (9, 10). By contrast, the proteolytic regulatory pathway is dispensable, provided that the phosphorylation control for CtrA is intact (11, 12). In addition to the dispensable proteolytic pathway that clears CtrA during the swarmer-to-stalked transition, a complex regulatory circuit precisely determines the timing of transcription of the *ctrA* gene during the cell cycle (13). This circuit is composed of at least four essential and sequential nodes defined by the transcriptional regulators CtrA, DnaA, GcrA and CcrM. DnaA, a bifunctional replication initiator transcription factor, activates transcription of *gcrA*, whose gene product in turn activates transcription of the *ctrA* gene (14). CtrA then feeds back on this circuit by activating the transcription of *ccrM*, which encodes an adenine methyltransferase that regulates methylation-sensitive promoters (15-17). The activation of the *dnaA* promoter by CcrM through methylation closes the regulatory transcriptional loop. Despite the exquisite regulation acting on *ctrA* at the transcriptional level, which includes a direct auto-regulatory amplification loop on one of its two own promoters (13), constitutive expression of *ctrA* is not lethal in *C. crescentus*.

In other alpha-proteobacteria: Many alpha-proteobacterial genomes encode homologs of the four *C. crescentus* master transcriptional regulators; however, it remains to be determined whether they are also organized into a transcriptional circuit with comparable topology and whether constitutive expression of active CtrA compromises growth in these bacteria (4). The role of these regulators in cell cycle control remains largely unexplored in alpha-proteobacteria other than *C. crescentus*. Furthermore, whereas the CtrA protein is widely conserved and essential in many alpha-proteobacteria (including rhizobia, *Agrobacterium* and *Brucella* species), recent evidence indicates that there is some diversification in the targets and the functions it regulates. Some of the conserved functions controlled by CtrA are transcriptional auto-regulation, control of flagellar motility and chemotaxis, as well as the expression of a number of sensory proteins that interact with other proteins, small nucleotides or other small molecules (e.g. two-component systems, PAS, EAL and/or GGDEF domains) and transcriptional regulators (3, 4, 18). For example, the direct control of *ccrM* expression by CtrA has been shown in *C. crescentus*, *Sinorhizobium meliloti*, *Agrobacterium tumefaciens* and *Brucella abortus* (15, 19-21). In these species, CcrM is essential for viability, and its levels are cell cycle regulated; altering these levels results in aberrant cell division (19-23). The use of synchronized populations of *A. tumefaciens* has shown that methylation of the chromosome by CcrM occurs at a specific stage during the cell cycle, as is the case with *C. crescentus*. Moreover, several other processes appear to be cell cycle regulated in *A. tumefaciens*: DNA replication and cell division take place in a periodic manner as well, indicating that DNA is likely to be replicated just once per cell cycle, and motility varies during the cell cycle, in correlation with flagellin expression (19). On the other hand, it is also evident that in different species CtrA can regulate the same processes but through different target genes: for example, CtrA controls cell division through structural genes for cytokinesis (*ftsZ*, *ftsA*, *ftsQ* and *ftsW*) in *C. crescentus* and through the *minCD* regulatory operon and the *ftsE* structural gene in *B. abortus* (24-26). The variations in the regulation network of CtrA in different alpha-proteobacteria are almost certainly related to their different lifestyles; the increase in the sequenced genomes and the transcriptional data available for several members of this class – and especially for those belonging to orders other than *Caulobacterales* and

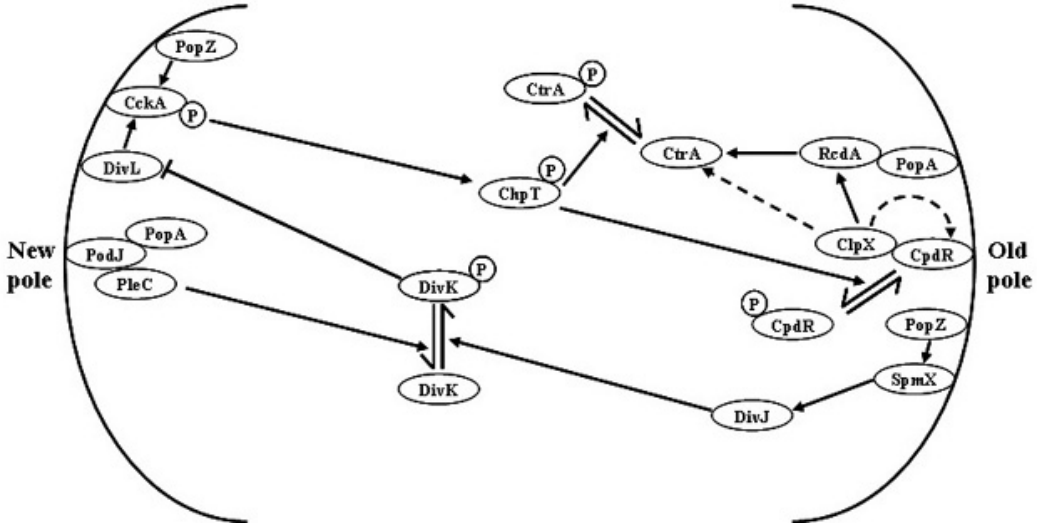

Figure 2. Control of CtrA phosphorylation, localization and proteolysis in *C. crescentus*. Direct interaction has been shown for CpdR/ClpX and PopA/RcdA. Dashed lines indicate proteolytic degradation of CtrA and CpdR by the ClpXP complex (see text for details). Note that it remains to be determined whether PopA and PleC physically associate with PodJ. By contrast, it is known that SpmX and DivJ reside in the same complex (52).

Rhizobiales - is helping to draw a more complete picture of the cell functions (and the specific target genes) controlled by the transcriptional regulators, and particularly by CtrA.

One example of diversification in CtrA functions is the recently characterized CtrA from *Rhodobacter capsulatus*, a purple nonsulfur photosynthetic bacterium (18). Despite its high degree of conservation (CtrA from *C. crescentus* and *R. capsulatus* share 71% sequence identity), CtrA from *R. capsulatus* is not essential and does not appear to regulate essential cell cycle genes as in other alpha-proteobacteria. Nevertheless, *R. capsulatus* CtrA positively regulates about 6% of the genes and *ctrA* mutant cells have a pleiotropic phenotype (18). Since the cell cycle control by CtrA has been lost in alpha-proteobacteria that do not divide asymmetrically (*Rhodobacterales* like *Rhodobacter* and *Ruegeria* species), it is possible that there is a causal relationship between the ability of CtrA to activate cell cycle genes (most notably the *fts* cell division genes) and the cellular capacity to divide asymmetrically (18).

3.2. Regulation of CtrA proteolysis by the ClpXP complex: RcdA, CpdR and PopA

In *C. crescentus*: The degradation of CtrA during the swarmer-to-stalked cell transition and in the stalked chamber of the compartmentalized pre-divisional cell is not only restricted in time, but also in space (11) (Figure 2 and 3). Fluorescently-labelled variants of CtrA that are degraded like wild type CtrA co-localize with its protease at the stalked pole, and at least three regulatory proteins – RcdA, CpdR and PopA (11, 12, 27) – are required for CtrA proteolysis and localization *in vivo*. However, it is not clear whether they also can influence the proteolysis *in vitro*. The AAA+ protease ClpXP, a complex formed by ClpP, the actual protease, and ClpX, responsible for substrate recognition and unfolding, is sufficient to degrade CtrA *in vitro* (28). RcdA, CpdR and PopA are all required for CtrA proteolysis and all co-localize with CtrA at the future stalked pole at the swarmer-to-stalked cell transition and in late pre-divisional cells, at the time when CtrA is proteolyzed (11, 12, 27). RcdA was first identified in a bioinformatic screen for uncharacterized proteins that are highly conserved in alpha-proteobacteria and under the direct transcriptional control of CtrA (11). Indeed, RcdA protein levels oscillate during the cell cycle in an anti-cyclical manner to CtrA levels, peaking at the swarmer-to-stalked cell transition. ClpX, PopA and and CpdR are all required for the polar localization of RcdA (11, 27). Moreover, RcdA interacts directly with PopA, a GGDEF domain protein that recruits RcdA to the pole to promote the degradation of CtrA at that site. This function of PopA depends on its ability to bind c-di-GMP (27), indicating that this nucleotide derivative plays a role in regulating cell cycle progression in *C. crescentus* via the degradation of CtrA. C-di-GMP binding of PopA is also required for its localization to the same cell pole as RcdA. Interestingly, PopA also localizes to the other cell pole, independently of c-diGMP but in a manner that depends on the presence of the PodJ polarity determinant (27).

Moreover, the function of RcdA is still not completely clear, as RcdA is not required for CtrA degradation by ClpXP *in vitro* (28), and the localization of RcdA can be decoupled from its effect on CtrA degradation (29). In fact, whereas the deletion of the last 19 residues of RcdA impairs both RcdA polar localization and CtrA proteolysis, other mutations (in two groups of highly conserved, charged surface residues) do not affect CtrA proteolysis but prevent RcdA polar localization (29). It was therefore suggested that the effect of RcdA on CtrA might be indirect and mediated by protein-protein interactions, as RcdA might inhibit a protein that blocks CtrA degradation or promote the activity of an adaptor (29). However, the

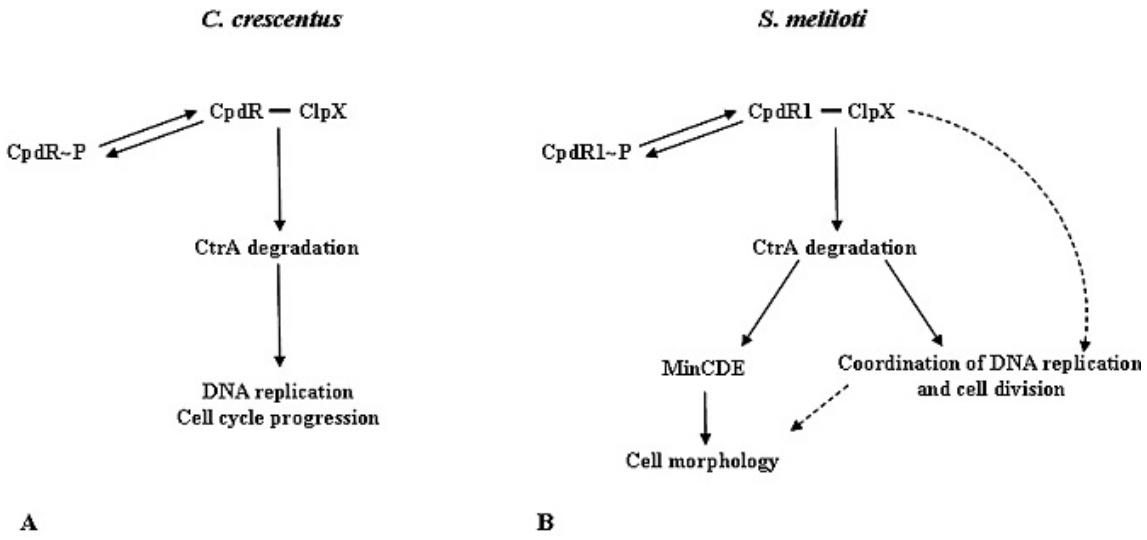

Figure 3. CpdR functions in *C. crescentus* (A) and *S. meliloti* (B). In *C. crescentus*, the absence of CpdR and the consequent stabilization of CtrA lead to a block in DNA replication, due to direct binding of CtrA next to *ori*. In *S. meliloti*, the absence of CpdR causes a loss of coordination between DNA replication and cell division. This results in polyploid cells and is presumed to be due to the lack of CtrA-binding sites near the origin of replication. In *S. meliloti* the loss of CpdR regulation by phosphorylation (in *cpdRD53A*) also results in aberrant cell morphology, at least partially because of unregulated expression of the *minCDE* operon that bears a CtrA-binding motif in the putative promoter (33).

interacting protein(s) have not been identified yet, as the only protein known to interact directly with RcdA is PopA, but none of the mutations that affect RcdA localization affects the interaction between RcdA and PopA (27, 29). Other scenarios are still possible: as some RcdA mutants are impaired in localization, but they promote all the same CtrA degradation (although CtrA localization itself is decreased or abolished), one hypothesis would be that CtrA localization is not actually essential for degradation, but as long as RcdA is present and able to form a complex with ClpXP, CtrA and any other required component, degradation occurs. This predicts that the proteolytic complexes can form in the cytoplasm as well, albeit at a lower rate than with all the components co-localized at the cell pole. Another possibility is that RcdA and CtrA localization in the *rcdA* site-specific mutants is only reduced but not completely abolished, and that partial localization is sufficient to cause a decrease in CtrA protein levels.

The single domain response regulator CpdR acts upstream of RcdA in the regulatory pathway for CtrA proteolysis, being required for the polar localization of ClpX (Figure 2) and directly interacting with ClpX in a bacterial two-hybrid assay (10, 12). In a *cdpR* mutant the polar localization of RcdA and CtrA is lost as well, probably as a consequence of the loss of ClpXP localization (12). The polar localization of CpdR is regulated by phosphorylation: unphosphorylated CpdR localizes to the pole, whereas phosphorylated CpdR is not sequestered at the pole. Furthermore, unphosphorylated CpdR localized at the stalked cell pole is itself a substrate for degradation by ClpXP, which releases ClpXP from the cell pole (12). Phosphorylation of CpdR depends on the same phosphorelay (CckA-ChpT) that phosphorylates CtrA, so the activation of CtrA and the block of its degradation occur at the same time (10). The single domain response regulator DivK is also involved in this regulation: by inhibiting the CckA-ChpT pathway, DivK promotes the accumulation of unphosphorylated CpdR and, as a consequence, its degradation by ClpXP (30). The same work has also shown that the essential function of DivK for *C. crescentus* viability is to allow CtrA degradation, as in the presence of an unphosphorylatable allele of CpdR (D51A), which does not require DivK for localization, DivK is not essential for viability (30). This indicates that cells depleted of DivK are arrested in G1 because CtrA cannot be degraded. Recent work showed that DivK regulates the activity of the essential CckA histidine kinase/phosphatase by directly interacting with the essential DivL protein, a histidine kinase homolog. When DivK is phosphorylated, it interacts with DivL and promotes CckA phosphatase activity, favouring the accumulation of dephosphorylated CpdR that then signals CtrA degradation (31, 32). Dephosphorylated DivK does not interact with DivL, so CckA functions as a kinase that phosphorylates CpdR to inhibit CtrA proteolysis.

In other alpha-proteobacteria: CpdR and RcdA are conserved in a cluster of alpha-proteobacteria (*Rhizobiales*, *Caulobacterales* and some *Rhodobacterales*), but not in all (4). In *S. meliloti* there are two homologs of *cpdR* (33) (Figure 3). While the function of CpdR2 is still unknown, as a *cpdR2* mutant had no phenotypes in the conditions tested, CpdR1 has a polar localization pattern and is involved in polar localization of ClpX, suggesting that CpdR1 is the CpdR ortholog of *S. meliloti* (33). Moreover, the *cpdR1* mutant is impaired in symbiosis with *Medicago sativa*, as *cpdR1* cells invade nodules but cannot differentiate into functional nitrogen-fixing bacteroids (33).

Mutant *cpdR1* cells also show an aberrant coccoid or swollen morphology, compared to the rod shaped wild type cells, and have a growth defect (33). Although CpdR functions are partially conserved in *C. crescentus* and *S. meliloti*, as in both cases the stabilization of CtrA in the absence of CpdR leads to a misregulation of genes involved in cell cycle control and as a consequence to the morphological defects observed in these mutants, there are also important differences. In fact, in *S. meliloti* the introduction of an unphosphorylatable variant ($CpdR_{D53A}$) in the *cpdR1* mutant does not rescue the morphological defects of this strain, and in the wild-type background $CpdR_{D53A}$ determines the appearance of highly branched cells with more than three poles (33). In *S. meliloti*, a branched phenotype is usually attributable to impaired division control that, in the case of the *cpdR1* mutant, partially stems from overexpression of the *minCDE*-like operon (which encode homologs of the *E. coli* division proteins that topologically regulate the assembly of the cytokinetic FtsZ-ring) (33, 34). Consistent with this model, no highly branched *S. meliloti* cells were observed when *minCDE* was disrupted in a *cpdRD53A* background (33). Also, consistent with a cell division defect, an *S. meliloti cpdR1* mutant shows an increase in DNA content to 2-5N (33). Not surprisingly such an effect is not observed in the case of *C. crescentus cpdR* mutant, as there are no *minC/E*-like genes in *C. crescentus* (27). As there are no CtrA binding sites at the replication origin of *S. meliloti*, a loss in CtrA degradation by the *cpdR1* mutant should not result in a block of DNA replication initiation. Instead, this mutation results in unregulated DNA replication that yields polyploid cells, indicating that *S. meliloti* CpdR1 co-ordinately regulates DNA replication and cell division (Figure 3) (33). This mechanism might be particularly relevant in the context of *S. meliloti* differentiation into nitrogen-fixing bacteroids, a developmental process that is accompanied by polyploidy, modification of cell shape and increased cell size, required to establish an efficient symbiotic interaction with plants. Several transcriptomic analyses have shown that during symbiosis many genes encoding proteins essential for cell cycle progression and cell division, like CtrA and FtsZ, are down-regulated (35-37).

3.3. CtrA phosphorylation

In C. crescentus: The phosphotransfer to CtrA described above is mediated by the membrane-integral hybrid histidine kinase CckA and the phosphotransfer protein ChpT, both highly conserved and found in most alpha-proteobacteria. CckA and ChpT levels do not oscillate during the cell cycle, but their activity is regulated through the (direct or indirect) interaction with the histidine kinase homolog DivL (38) (Figure 2). The CckA-DivL interaction is controlled by another two-component system comprising the histidine kinase/phosphatases PleC and DivJ, as well as the single domain response regulator DivK (10, 30, 32, 39). In *C. crescentus* swarmer cells there is a bias towards dephosphorylated DivK due to the presence of the PleC phosphatase, which is sequestered to the swarmer (flagellated) cell pole, and low levels of the DivJ kinase (Figure 1 and 2) (40). This imbalance ensures that the CckA-ChpT phosphorelay is active, leading to CtrA phosphorylation and activation, preventing *ori* from firing (10, 32). In the stalked cell, the imbalance is tilted towards phosphorylated DivK, owing to a low abundance of PleC and elevated levels of the DivJ kinase, localized to the stalked pole (41). DivK is phosphorylated by DivJ, interacts directly with DivL and this interaction reverses the phosphate flow through CckA-ChpT at or near the pole to inactivate and destabilize CtrA (via CpdR, see above) (42, 43). Several lines of evidence support this model. Inactivation of the *divJ* gene results in a decrease in the levels of phoshorylated DivK and an increase in CtrA phosphorylation and activity (44). Selecting for suppressors of the *divJ* mutation has yielded mutations in the *cckA* gene (44). Moreover, CckA is dispersed in swarmer cells, whereas it accumulates at the new pole in stalked and early pre-divisional cells (9, 38). This localization is then lost after cytoplasmic compartmentalization but before the two daughter cells separate (45). The CckA localization pattern is independent of CckA phosphorylation and kinase activity, as mutants in the two phosphorylatable residues of CckA (His322 in the His phosphotransfer domain and Asp623 in the receiver domain) can still localize at the new cell pole, although they cannot functionally substitute for wild type CckA to support viability (45). Instead, two other regions have been shown to be important for CckA localization, the putative PAS-(small molecule binding or protein-interaction) motif in the sensor domain and a linker region between beta-sheets in the ATP-binding domain (45). Mutations in these regions cause a striking increase in the cell-to-cell variability of CpdR levels and the disruption of the oscillation of CtrA levels, suggesting that CckA localization plays a role in its function, and therefore in cell cycle progression (45). As CckA and DivL co-immunoprecipitate and DivL also activates CckA auto-phosphorylation, it was proposed that DivL recruits CckA at the new pole, where it promotes its auto-phosphorylation and activity (31, 32). The fact that phosphorylated DivK directly binds to DivL and prevents CckA activation can also explain why *divL* mutants were identified as suppressors of the *divJ* mutation in the same screen that identified *cckA* mutations (44). Moreover, it is only possible to delete the *divL* or the *cckA* gene in a strain overproducing the phosphomimetic $CtrA_{D51E}$ (to overcome the impairment in CckA activity) (46).

It has recently been shown that also DNA replication initiation, but not chromosome segregation, is required for DivL and CckA localization to the new pole (47). Presumably a hitherto uncharacterized polar localization factor for DivL (and CckA) exists whose activity and/or accumulation is dependent on the assemblage or progression of the DNA replication machinery. In this context it is interesting to note that progression of the replication fork can influence gene expression from methylation-sensitive promoters (48). Moreover, the essential intiator protein DnaA, whose activity is stimulated at the onset of replication (49), is also known to activate transcription of genes in *C. crescentus* (50). It has also been reported that localization of DivL is dependent on the presence of the DivJ histidine kinase (51). As CtrA activity is high in *divJ* mutant cells, it is conceivable that the gene for the aforementioned

localization factor is repressed by CtrA or another transcriptional regulator that is DivJ-dependent, such as the TacA σ^{54}-dependent transcriptional activator (52). Furthermore, it is important to point out that, while the DivJ pathway controls the levels of phosphorylated DivK and thus CtrA activity, the GGDEF-domain containing response regulator PleD is also dependent on phosphorylation by (PleC and) DivJ for activation (53). Phosphorylation of PleD stimulates the di-guanylate cyclase activity in the GGDEF output domain, contributing to a burst in the cellular levels of c-di-GMP (53). Thus, the activity or accumulation of the localization factor could also be regulated by c-di-GMP-dependent receptor proteins acting at the level of gene expression or at the post-translational level (54).

In other alpha-proteobacteria: CckA and ChpT are conserved in most alpha-proteobacteria, and components of the DivK regulatory network – DivJ, PleC and DivK itself - are also well conserved (4). In *S. meliloti*, DivK features a dynamic cyclical localization, with two subsequent rounds of dispersed distribution and accumulation at the old pole per cell cycle (Figure 1) (55). However, there are also some differences in the localization and function of these proteins: for example, in *B. abortus* the localization pattern of PleC is different from the one observed in *C. crescentus*, and the main kinase responsible for DivK phosphorylation and positioning is PdhS, which is not present in *C. crescentus* (56). *Brucella* PdhS shares homology with DivJ and PleC, but in contrast to them it is essential for *Brucella* viability. PdhS is localized to the old pole of the large cell after *Brucella* asymmetric division and it co-localizes with DivK (56). *B. abortus* PdhS is also able to localize to the old pole of the large cell when expressed in *S. meliloti* or *C. crescentus*, suggesting that the polar features required for PdhS localization are conserved in several alpha-proteobacteria, including those that lack a PdhS homolog like *C. crescentus* (56). Some other alpha-proteobacteria like *S. meliloti*, *Mesorhizobium loti* and *A. tumefaciens* possess two homologs of PdhS, one long (PdhS1) and one short (PdhS2), which could have redundant functions, as in *S. meliloti* PdhS1 is not essential (56, 57).

3.4. Additional regulators of the CtrA pathway

In *C. crescentus*: PleC localization at the flagellated cell pole requires the polar localization factor PodJ, a coiled coil-rich bitopic membrane protein whose expression is repressed by CtrA (58). PodJ is synthesized and localized at the future flagellated pole during the swarmer-to-stalked cell transition and, upon cytokinesis, is processed to a shorter form ($PodJ_S$) by the periplasmic protease PerP, whose expression is activated by CtrA (59). $PodJ_L$ and $PodJ_S$ play at least partially different roles, with $PodJ_L$ being required for PleC recruitment and pili formation, presumably via the recruitment of the CpaE pilus assembly factor to the flagellated pole (60). By contrast, $PodJ_S$ is required for chemotaxis and holdfast formation (60, 61). $PodJ_S$ is then released from the membrane by the metalloprotease MmpA and degraded at the swarmer-to-stalked cell transition (59). In the absence of PleC, cells have an excess of phosphorylated DivK and as a result diminished levels of CtrA phosphorylation and activity. While the effect of a *podJ* deletion on the activity of the PleC kinase/phosphatase has not been measured directly, cells lacking PodJ also show reduced expression of many (but not all) CtrA-activated genes, such as the *pilA* gene (59, 62). These results suggest that there are at least two categories of CtrA-activated genes: those that require a high level of phosphorylated CtrA for activation and those that have a lower threshold.

Whereas PleC depends on PodJ for polar localization to the flagellated pole, the SpmX muramidase homolog recruits the DivJ kinase to the stalked pole (52). SpmX is a polytopic membrane protein whose production is triggered in swarmer cells by unphosphorylated DivK through a two-tiered transcriptional cascade involving CtrA > TacA > *spmX*. The SpmX translation product then accumulates and localizes to the developing stalked pole during the swarmer-to-stalked cell transition. SpmX then recruits and activates DivJ, therby promoting the phosphorylation of DivK and presumably aiding in the implementation of the stalked cell developmental program via CtrA (52). The muramidase domain of SpmX is required for its localization to the stalked pole, but does not require the presence of the stalk *per se*. SpmX can be co-immunoprecipitated with DivJ, indicating that both proteins reside in the same protein complex (52). Based on the finding that in a *pleC* mutant (that does not express *spmX*) the levels of phosphorylated DivK are elevated, a SpmX-independent pathway was predicted to activate DivJ as well (63). A screen for mutants with reduced DivJ activity in a *pleC* mutant background identified KidO, an oxidoreductase homolog that is required for optimal DivJ kinase activity and also regulates cell division through the cytokinetic tubulin homolog FtsZ (see below) (63). KidO and SpmX cooperate to control DivJ activity, and cells deprived of both KidO and SpmX are very filamentous and genetically unstable, indicating a severe defect in cell cycle progression and/or cell division (63).

In a recent brute force and unbiased approach, Christen *et al*. conducted a genome-wide and largely automated microscopic screen of transposon mutagenized cells to identify polar localization factors for DivJ, PleC and/or the polar pilus assembly regulator CpaE (64). In this screen transposon mutagenesis was carried out in cells expressing PleC-YFP, DivJ-mCherry and CFP-CpaE, and cells were imaged in 96- or 384-well format in order to identify, with a suitable computer algorithm, mutants with aberrant localization patterns (64). Satisfyingly, screening recovered PodJ as the main responsible for PleC and CpaE localization, and also confirmed that SpmX is required for DivJ localization. Moreover, this study implicated SpmX in the release of PleC and CpaE from the incipient stalked pole (64). However, this might also be attributable to an indirect effect, as the cells lacking SpmX are impaired in division control and seem to have a defect in the checkpoint that coordinates polar differentiation with division (52).

New regulatory insight on CtrA-activated genes in *C. crescentus* has recently emerged from two important studies uncovering the small and highly conserved

regulator SciP (65, 66). CtrA directly regulates the expression of at least 95 genes differentially during the cell cycle (67, 68). The expression of CtrA-repressed genes is usually restricted to a narrow window in cell cycle, corresponding to the initiation of DNA replication, whereas CtrA-activated genes are turned on later at the pre- and/or post-divisional stage (in the swarmer cell compartment) (65, 66). Expression of the *sciP* gene is activated by CtrA, so *sciP* mRNA and protein accumulate at the time of division (65). Upon cell division SciP is loaded into swarmer cells, where it interferes with CtrA-mediated transcriptional activation, while not affecting the repression of promoters by CtrA (65, 66). SciP then disappears during the swarmer-to-stalked cell transition (65, 66). Depletion of SciP results in the ectopic induction of CtrA-activated genes and, as a consequence, the disruption of the cell cycle. On the other hand, overexpression of SciP from a high copy plasmid is sufficient to inhibit CtrA-dependent transcription and also disrupts the cell cycle (66). Two models have been proposed to explain the mechanism by which SciP inhibits CtrA-activated genes: Gora *et al.* provided evidence that SciP does not bind DNA directly, and instead requires CtrA to bind DNA (66). SciP interacts directly with CtrA without affecting its stability or phosphorylation, suggesting that the interaction of SciP with CtrA competes with RNA polymerase. EMSA assays confirmed that the addition of SciP in the presence of CtrA disrupts the binding of RNA polymerase to the *fliF* promoter, indicating that CtrA is regulated by a protein-protein interaction critical for cell cycle progression (66). On the other hand Tan *et al.* proposed a model in which SciP can weakly bind to a DNA consensus motif [(A/C)-(N)3-GTCG(G/A)] on its own, presumably acting as a transcriptional repressor that competes with CtrA-mediated activation of promoters when SciP-binding sequences are near the TTAA-(N)7-TTAA consensus motif for CtrA (65, 67). Based on ChIP-chip experiments these authors proposed that at least 58 genes, including many flagellar and chemotaxis genes, are directly activated and repressed by CtrA and SciP, respectively. The presence of a putative SciP consensus motif within the promoter of the *ctrA* gene itself has led Tan *et al.* to propose that SciP overexpression causes a self-reinforcing shut-down of expression of the genes that are direct targets of CtrA and SciP, which ultimately causes cells to filament and die, akin to cells with a loss of function CtrA (7, 65).

In other alpha-proteobacteria: Whereas PodJ is conserved in alpha-proteobacteria, no obvious homologs of SpmX are discernible in rhizobia, *Agrobacterium* or *Brucella* species. In *B. abortus* PleC is localized to midcell, but experiments aimed at demonstrating that DivJ is localized have been unsuccessful (56). It would be very interesting to explore whether PodJ co-localizes with PleC at the division plane in *B. abortus*. Interestingly, when fluorescently labelled PodJ (YFP-PodJ) is constitutively expressed throughout the cell cycle in *C. crescentus*, midcell localization was observed at the time of constriction, indicating that *C. crescentus* PodJ also has the capacity to recognize the division septum (Viollier, unpublished). However, under normal conditions PodJ is only synthesized during a narrow window early in stalked cells and subsides as soon as CtrA accumulates. Thus, PodJ is synthesized in cells that have not yet begun constriction, perhaps explaining the absence of PodJ from the division plane and its preferred localization at the new (future flagellated) pole in *C. crescentus*. In light of the fact that the new poles are formed at the constriction site, the localization of PleC (and perhaps PodJ) in *C. crescentus* and *B. abortus* has much more in common than one might think. By contrast, the absence of a SpmX homolog in *B. abortus* might be related to the apparent absence of polar localization of DivJ. Intriguingly, however, another PleC-DivJ-like histidine kinase, PdhS, is encoded in the genome of *B. abortus* and many other alpha-proteobacteria (3). PdhS co-localizes with the DivK homolog at the old cell pole. Moreover, DivK interacts better with PdhS than with PleC and DivJ, suggesting that in *B. abortus* PdhS has taken over the function that DivJ performs in *C. crescentus*. If so, then PdhS might have a specialized localization that has co-evolved with it, rendering a putative SpmX homolog dispensable and resulting in the loss of its gene from the genome.

4. PHYR, AN ANTI-ANTI-SIGMA FACTOR

While the phospho-fluxes described above exhibit regular and predictive oscillations during each cell cycle, the regulatory fluxes of other systems are strongly modulated in response to environmental inputs and stresses that are generally stochastic. Recently, progress has been made in understanding how one such input feeds into a regulatory system comprising the EcfG-like sigma factor, the NepR anti-sigma factor and the PhyR response regulator that functions as anti-anti-sigma factor and can sequester NepR from EcfG (69-71). PhyR features a classical C-terminal receiver domain and an N-terminal output domain that resembles the N-terminal domain of EcfG (71). Phosphorylation of the N-terminal receiver domain stimulates the anti-anti-sigma factor activity of PhyR, ultimately causing the release of the sigma factor and allowing its association with RNA polymerase core enzyme to activate transcription of target genes (72). This model is supported by the work recently done on *Bradyrhizobium japonicum* USDA110, *S. meliloti* and *C. crescentus* systems (69-71) (Figure 4). The first alpha-proteobacterial PhyR was identified in *Methylobacterium extorquens*, and PhyR homologs were subsequently characterized also in rhizobial species and *C. crescentus* (70, 72-77). EcfG sigma factors, NepR and PhyR are conserved in all free-living alpha-proteobacteria, as the only species that do not possess this system are obligate symbionts (78, 79).

In C. crescentus: The model proposed for the PhyR/NepR/EcfG-like sigma factor pathway is supported by the work of Herrou *et al.*, who recently determined the X-ray crystal structure of PhyR from *C. crescentus*. *C. crescentus* PhyR is an anti-anti-sigma factor belonging to the signalling pathway of the ECF sigma factor homolog SigT, which is involved in acute osmotic and oxidative stress (73). In fact, in *C. crescentus* the effects of the *phyR* and *sigT* mutations are not additive, providing evidence that PhyR and SigT function in the same genetic pathway

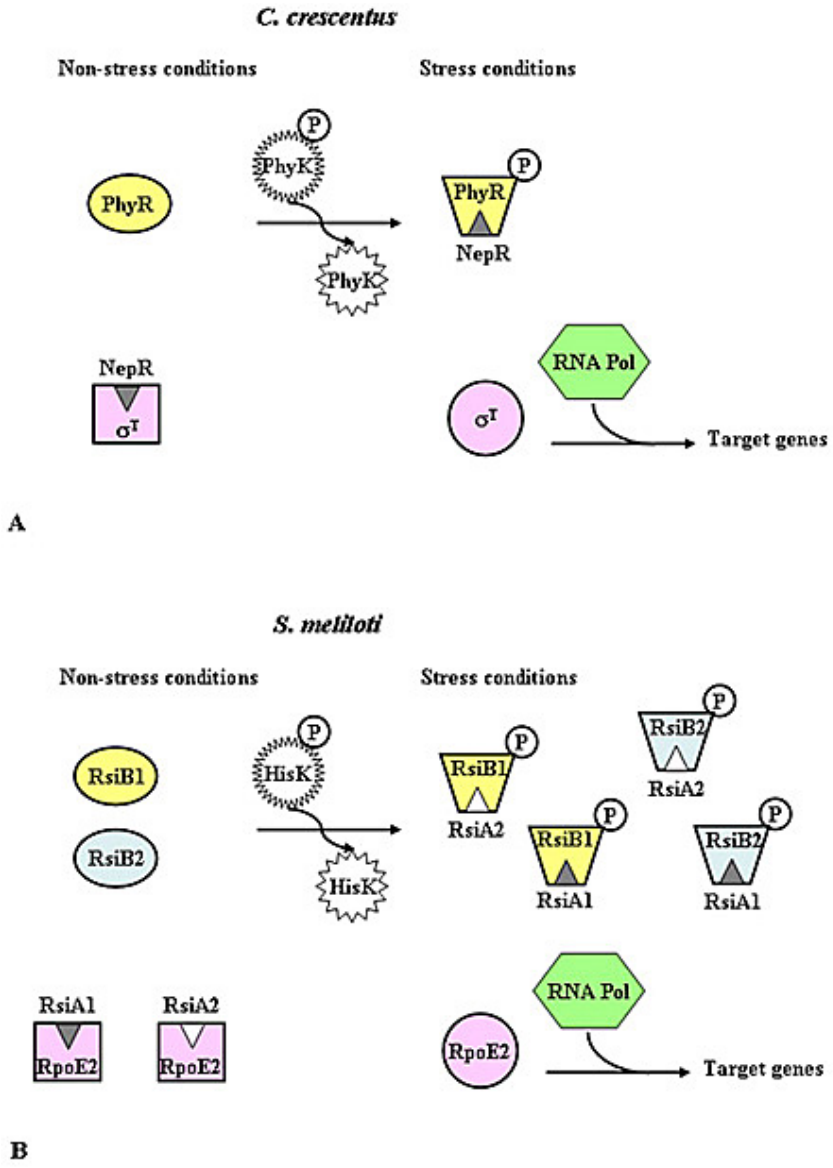

Figure 4. PhyR/NepR/EcfG-like sigma factor regulatory pathway in *C. crescentus* (A) and *S. meliloti* (B). In *C. crescentus*, in non-stress condition the anti-sigma factor NepR binds to sigma^T, preventing its association with RNA polymerase. In stress conditions the anti-anti-sigma factor PhyR, upon phosphorylation by the histidine kinase PhyK, binds to NepR, so that sigma^T is released and can recruit the RNA polymerase complex for the expression of target genes. In *S. meliloti* the activity of the EcfG-like sigma factor RpoE2 is regulated by two paralogous anti-sigma factors, RsiA1 and RsiA2, and two paralogous anti-anti-sigma factors, RsiB1 and RsiB2.

(71). The structure of PhyR in its unphosphorylated state revealed an N-terminal ECF sigma-like domain of seven alpha-helices and a C-terminal receiver domain with a central hydrophobic beta-sheet surrounded by five alpha-helices (71). The two domains are stacked against each other, but the interaction does not involve the area important for PhyR phosphorylation, suggesting that PhyR phosphorylation is required to open the inter-domain packing, making the N-terminal domain available to bind NepR (71). Based on homology to other regulators, the residues involved in propagating the conformational changes upon PhyR phosphorylation are highly conserved in *C. crescentus* PhyR, and the area that would undergo the largest modifications upon phosphorylation is located exactly at the surface that interacts with the N-terminal domain, supporting the hypothesis that phosphorylation would perturb this interaction (71). Also, a recent work by Lourenço *et al.* confirmed that PhyR and NepR act respectively as positive and negative regulators of SigT expression and function, and described the essential role of the His kinase PhyK in the regulation of the pathway (80).

In other alpha-proteobacteria: In *B. japonicum* USDA110, PhyR and EcfG (SigT homolog) are involved in

heat shock and desiccation resistance upon carbon starvation, but are also required for the establishment of an efficient nitrogen-fixing symbiosis (70). In fact, both *phyR* and *ecfG* mutants have symbiotic defects on *Glycine max* (soybean) and *Vigna radiata* (mungbean), two *B. japonicum* host plants. The nitrogenase activity measured in nodules of *G. max* or *V. radiata* two-three weeks after inoculation with *phyR* or *ecfG* mutants was significantly reduced compared to that found in nodules elicited by wild type *B. japonicum*. Four weeks after inoculation, however, soybean nodule development catches up to a large extent, showing a nitrogenase activity similar to that measured in nodules formed by the wild-type (70). This is not the case with *V. radiata*, where the nitrogenase activity remains low even five weeks after inoculation. Also, *B. japonicum phyR* and *ecfG* mutants give rise to the formation of aberrant nodules on both host plants, with ectopically emerging roots (70). PhyR and EcfG are part of the same signalling cascade, as phosphorylated PhyR and EcfG both interact with the anti-sigma factor NepR and transcriptome analysis using DNA microarray showed that the PhyR and EcfG regulons are congruent (70). Strikingly, a large fraction of genes of this regulon is of unknown function, indicating that still much remains to be uncovered about the mechanisms by which the PhyR/NepR/EcfG system promotes symbiosis (70).

In *S. meliloti*, the EcfG-like sigma factor RpoE2 is activated under different stress conditions, including heat shock, salt stress and entry into stationary phase upon carbon or nitrogen starvation (77). An *S. meliloti rpoE2* mutant is also more sensitive to desiccation and oxidative stress, which suggests that RpoE2 is involved in general stress response in *S. meliloti* (81, 82). However, this system presents an additional level of complexity, as RpoE2 activity is negatively controlled by two paralogous anti-sigma factors, RsiA1 (SMc01505, essential for *S. meliloti* viability) and RsiA2 (SMc04884, not essential for viability), and RpoE2 activation by stress is mediated by two redundant paralogous PhyR-type response regulators, RsiB1 (SMc01504) and RsiB2 (SMc00794) (69). RsiB1 and RsiB2 both interact with the anti-sigma factors RsiA1 and RsiA2, so it is likely that they relieve RpoE2 inhibition in response to stress by acting as anti-anti-sigma factors (69) (Figure 4). The function of RsiB1 as anti-anti-sigma factor is supported by the fact that the expression of the EcfG-like N-terminal domain of RsiB1 alone causes a strong increase in RpoE2 activity even in the absence of stress, whereas the mutation of the conserved phosphorylated Asp in the RsiB1 C-terminal phosphoreceiver domain determines the production of a non-functional protein that cannot complement an *rsiB1*/*rsiB2* double mutant (69). Therefore the model proposed for *S. meliloti* is similar to that suggested for *M. extorquens* and *B. japonicum*, but the presence of two pairs of anti- and anti-anti-sigma factors in this species provides an additional level of complexity, which probably allows the regulatory system to integrate multiple stimuli (69). At least two other sequenced alpha-proteobacterial genomes (*Sinorhizobium medicae* and *Sinorhizobium fredii* NGR234) seem to encode two pairs of anti- and anti-anti-RpoE2 sigma factors, suggesting that the complexity of this regulatory system is conserved and therefore important for symbiotic bacteria.

5. REGULATION OF DIVISION AND CHROMOSOME PARTITIONING

The localization and assembly of the tubulin homolog FtsZ into the cytokinetic FtsZ (Z-) ring lines the cytoplasmic membrane and determines the site where cell constriction occurs. The Z-ring plays an essential role in organizing the division machinery and in contributing force to drive the constriction process (34). While our knowledge of the spatial and temporally regulated mechanisms underlying Z-ring formation had been scarce in the past, tremendous progress has been made in the last five years in elucidating the fundamental processes of cell division. The identification of new players controlling cytokinesis in *C. crescentus* – particularly MipZ, DipM, KidO and FzlA – is unearthing the mechanisms that (alpha-proteo)bacteria use for the proper timing and positioning of the cytokinetic machinery (63, 83-85). Importantly, recent work has also illuminated how chromosome segregation occurs in *C. crescentus*, and how this process plays a central role in division control. A particularly exciting finding was the visualization of the ParA cytoskeletal filaments, a highly dynamic structure that is thought to drive the duplicated *ori* regions to the pole(s), in live cells. Moreover, Ingerson-Mahar *et al*. uncovered another filamentous cytoskeleton-like structure, composed of CTP synthase to regulate the curvature of the crescent-shaped *C. crescentus* cell (86, 87).

5.1. FtsZ targeting factors

In C. crescentus: MipZ, an essential protein of the ParA/MinD superfamily of ATPases, plays a major role in controlling the localization and activity of FtsZ (83). MipZ is cell cycle regulated at the level of transcription and it is conserved in all alpha-proteobacteria lacking MinC/E orthologs (68, 83, 88, 89). It allows the formation of medial Z-ring to be coordinated with the movement of the duplicated origin regions to opposite cell poles (83). MipZ forms a complex with ParB, the chromosome partitioning protein that binds the *parS* sequences clustered near *ori*. It is bound to the origin region via ParB and moves with *ori* during the segregation process. After the completion of segregation of origin regions to opposite poles, there is a bipolar disposition of MipZ. Interestingly, MipZ localization studies revealed an intracellular gradient of MipZ, peaking at the poles and decreasing towards midcell, that could be explained by MipZ shuttling between ParB-binding and unspecific DNA-binding states driven by the ATPase cycle (83). ATP binding by MipZ, but not ATP hydrolysis, also stimulates the GTPase activity of the FtsZ tubulin homolog. Since FtsZ polymerization requires a bound GTP, MipZ prevents FtsZ polymerisation *in vitro* and *in vivo* (83). Owing to the MipZ gradient, there is minimal interference of MipZ with FtsZ polymerization at midcell. Thus, the bipolar positioning of MipZ selects midcell as the future division site. The conservation of MipZ in alpha-proteobacteria suggests that the coordination of chromosome segregation and cell division is an essential function, especially important for bacteria like rhizobia, *Agrobacterium* and *Brucella* species that, like *C.*

crescentus, divide asymmetrically and replicate their chromosome only once per cell cycle (3, 19, 90).

Another recently identified protein that acts directly on FtsZ is KidO. KidO, a bifunctional regulator that controls both DivJ (see above) and FtsZ activity (63), is an oxidoreductase homolog that can bind NAD(H), but appears to have lost its enzymatic activity due to the mutation of a conserved and putative catalytic Tyr residue (63). CtrA activates the transcription of the *kidO* gene during the pre-divisional cell stage. Because KidO is degraded during the swarmer-to-stalked cell transition by the same ClpXP/CpdR/RcdA/PopA-dependent proteolytic pathway that degrades CtrA (63), KidO levels oscillate throughout the cell cycle with approximately the same phase as CtrA levels. Several experiments have shown that KidO directly interacts with and affects the polymerization of FtsZ: for example, whereas overexpression of KidO causes the disintegration of Z-rings, FtsZ mutants impaired in GTPase activity or potentially KidO-binding can divide despite high KidO levels (63). KidO localizes to cell constrictions in an FtsZ-dependent manner and NAD(H) binding is critical for KidO localization and regulation of FtsZ, but not for activation of DivJ (63). Therefore KidO is another key player that coordinates cytokinesis with the cell cycle, by influencing the timing of Z-ring formation and disassembly (63).

A microscopy-based localization screen to search for FtsZ-interacting proteins unearthed FzlA (and FzlC) as unknown regulator(s) of FtsZ (85). FzlA is a glutathione S-transferase (GST) family member, but binding to glutathione was not observed *in vitro*, suggesting that FzlA function does not involve glutathione. FzlC is a hypothetical protein with limited homology to known heparinases (85). Both FzlA and FzlC are widely conserved in alpha-proteobacteria. Also, both proteins co-localize with FtsZ at midcell in *C. crescentus*; this localization pattern is dependent on the presence of FtsZ, and both FzlA and FzlC interact with FtsZ *in vitro* (85). Whereas the mutation of *fzlC* does not cause any growth defect, FzlA is essential for *C. crescentus* viability, and depletion of FzlA results in long smooth filaments, in which Z-rings form but the inner membrane invagination fails to occur (85). *In vitro* FzlA inhibits FtsZ GTPase activity and promotes the formation of stable higher order structures of FtsZ, arcs and helical bundles that are not usually observed in the absence of FzlA; these structures are also more resistant to depolymerization by MipZ *in vitro* and less dynamic *in vivo* (85)). As the levels of FzlA vary during the cell cycle, the ratios between FtsZ, FzlA and MipZ (as well as other components of the division machinery) also significantly change during cell cycle progression; it is therefore likely that changes in the ratio or composition of FtsZ complexes dictate the dynamics of FtsZ polymerization and the function of the Z-ring. In fact, Z-rings first promote cell elongation through zonal growth of the peptidoglycan at midcell and only later drive cell envelope invagination as it switches to a mode of lateral (septal) peptidoglycan synthesis (85, 91). As FzlA levels actually decrease at the time when Z-rings switch function, it is possible that this switch in FtsZ activity occurs as a result of the fluctuations in FzlA abundance (85). The work on FzlA therefore suggests that modifications in FtsZ structure and function during cell cycle progression can be determined by changes in the ratio between the different components of the cell division machinery (85).

In other alpha-proteobacteria: The elucidation of the mechanisms by which FtsZ is regulated is particularly important because the functions of FtsZ, as well as those of other components of the cell division apparatus like FtsA, are generally conserved. For example, *S. meliloti* or *A. tumefaciens* FtsZ localize at the Z-ring even when expressed in *E. coli*, indicating that they can probably co-assemble with *E. coli* FtsZ (92). On the other hand, FtsA from *S. meliloti* or *A. tumefaciens* does not localize at Z-rings in *E. coli*, unless *ftsZ* from *S. meliloti* is co-expressed; the fact that FtsA from *S. meliloti* or *A. tumefaciens* can interact with FtsZ from either species, but not with FtsZ from *E. coli*, indicates that FtsZ-FtsA interactions co-evolve (92). It has also to be noted that in rhizobia and *Agrobacterium* species, in contrast to *C. crescentus* and *Brucella* species, there are two copies of FtsZ: a longer one (FtsZ1), that contains a C-terminal extension compared to FtsZ from *E. coli* and *Bacillus subtilis*, and a shorter one (FtsZ2), which lacks this C-terminal domain. *C. crescentus* possesses a single FtsZ, similar in length to FtsZ1 from rhizobia. FtsZ1 and FtsZ2 could have functions at least partially redundant, although this has not been deeply investigated. Depletion and overexpression of *ftsZ2* in *S. meliloti* seem to give phenotypes less severe than those due to *ftsZ1*, but the results are dependent on the conditions tested (93).

5.2. Fission of the cell envelope

In Gram-negative bacteria cell division requires the coordination of Z-ring constriction with the invagination and splitting of the three cell envelope layers (the inner membrane, the peptidoglycan wall and the outer membrane). The Z-ring not only generates constrictive force to drive inner membrane invagination, but it is also the scaffold structure that recruits downstream components of the division machinery (94-97). These factors are involved in the stabilization of the Z-ring, the synthesis and remodelling of peptidoglycan, the coordination of cytokinesis with chromosome segregation and the invagination of the outer membrane, but the precise role(s) of each have not been completely elucidated. For example, in *C. crescentus* FtsZ is required for the localization of DipM and the Tol-Pal complex (84, 98-101). DipM (for Division Involved Peptidase with LysM domains) is a recently characterized protein, essential for *C. crescentus* cell division as it seems to be involved in peptidoglycan remodelling (84, 99, 100). Tol-Pal is a protein complex highly conserved in Gram-negative bacteria, where it plays multiple roles such as keeping the three layers of the cell envelope in contact (98, 101). However, whereas the *C. crescentus* Tol-Pal complex is essential for viability, in *E. coli* this is not the case (102-107).

In *C. crescentus* DipM and the Tol-Pal complex are positioned mainly at the division plane and then remain at the new pole of the two daughter cells upon cytokinesis

(84, 98-101). Whereas DipM seems to be required for splitting the septal peptidoglycan, the envelope-spanning Tol-Pal complex is required to maintain the integrity and the coordinated invagination of the three envelope layers during cytokinesis. *C. crescentus* cells depleted of Pal or TolA form chains, because of incomplete cell separation, and show blebs at the division site and cell poles. Cryo-EM images have shown that the outer membrane looks separated from the inner membrane in Pal depleted cells, and the peptidoglycan layer seems to adhere to the inner membrane (98, 101). In TolA-depleted cells, the peptidoglycan seems to adhere to the outer rather than the inner membrane, and the outer membrane blebs are more extended, even along the lateral surface of the cells (101). Cell envelope defects also arise from overexpression or depletion of DipM. DipM overexpression causes cells to first elongate, then become round and finally lyse, probably because of an excess of peptidoglycan lysis (84, 99, 100). By contrast depletion of DipM results in an increase in the average distance between the inner and outer membrane, probably due to a loss of coordination in peptidoglycan remodelling. In fact, the peptidoglycan layer in sacculi isolated from DipM depleted cells is thicker than in sacculi isolated from wild type cells, especially at the division site, where the peptidoglycan appears disordered and multilayered (84, 100). Because of this increased distance between the inner and outer membrane the interactions between the members of the Tol-Pal complex, which are required for the trans-envelope connections, might be lost. As the Tol-Pal complex is also required for the localization of polarity factors such as TipN and the histidine kinase/phosphatase PleC the role of DipM and the Tol-Pal complex goes beyond their functions in cytokinesis in the current cell cycle, influencing polarity-dependent processes in the ensuing one (101).

5.3. Segregation and anchoring of the *ori*-region at the cell pole

In *C. crescentus*: Chromosome segregation in *C. crescentus* is a multistep process that occurs concurrently with replication (108-110). An essential function is provided by the ParA-ParB proteins and the *cis* acting *parS* sequence, the target of ParB, located near *ori*. The first step is the initial release of the *ori*-region from the old cell pole, followed by its duplication, including the *parS* site. The two duplicated *parS* sites are bound by ParB and have different fates: while one retracts to the nearby pole again, the other is ultimately driven to the new pole. This latter event involves a ParA-mediated translocation mechanism of the *ori*-proximal ParB/*parS* complex (109). Shebelut *et al.* observed that ParA forms a polar gradient with its maximum at the distal pole; when ParB arrives at the edge of the ParA gradient, the association between ParA and ParB induces ParA retraction and pulls ParB - with the associated chromosome - to the distal pole (109). This conclusion is further supported by the fact that ParA can form linear polymers *in vitro* and narrow linear structures *in vivo*, as visualized by single-molecule fluorescence imaging (111). *In vitro* and *in vivo* experiments with ParA site-specific mutants suggest a model in which Apo-ParA binds ATP and dimerizes, then ParA homodimers can polymerize, bind to DNA or interact with ParB; finally the interaction with ParB stimulates ParA ATPase activity or nucleotide exchange, which release ParA monomers (111-114).

The movement and anchoring of the *ori*-region also involve two polarity factors: the proline-rich polymeric PopZ anchoring protein and the coiled-coil rich bitopic membrane protein TipN (112, 115, 116). Whereas TipN is localized to the new pole, PopZ is bipolar throughout most of the cell cycle. Both localization patterns reflect their respective effects on the *ori*-region. Polar PopZ captures and fastens the *ori*-region at the poles by directly associating with ParB, while TipN influences the localization of ParA and the uninterrupted directional movement of ParB/*parS* translocation complex to the new pole. Based on the observation that TipN and ParA interact directly *in vitro* and *in vivo*, the current model posits that in the absence of TipN ParA structures reform behind the ParB/*parS* complex, which therefore stops moving and even reverses direction, resulting in erratic and incomplete *ori* translocation (111, 112). Like TipN, PopZ also has affinity for forms of ParA that do not bind DNA and thus may contribute to regulation of the ParA-mediated directionality (112). In support of this idea, PopZ and TipN act synergistically, and deletion of both genes is lethal due to a severe defect in chromosome segregation (111, 112). In addition to its role in anchoring the *ori*-region, a function of PopZ in stalked pole maturation has been proposed, based on the observation that *popZ* mutant cells do not form stalks, and often do not release TipN from the new pole (116). Moreover, at least seven proteins show localization defects in a *popZ* mutant strain: SpmX, DivJ, DivK, CckA, RcdA, CpdR and ClpX (108, 116). Since DivJ and CckA also co-immunoprecipitate with PopZ, it seems that PopZ is at the top of a hierarchical recruitment process, or that PopZ forms a matrix that captures other proteins once they have been attracted to the pole by other mechanisms (108, 116). Furthermore, although PopZ is a cytoplasmic protein, its influence on polar assembly seems to extend to the periplasm, as a truncated form of SpmX, possessing only the periplasmic muramidase domain, is also delocalized in a *popZ* mutant, suggesting that PopZ could affect the synthesis or modification of peptidoglycan as well (108).

In other alpha-proteobacteria: While the distribution of PopZ and TipN homologs in other alpha-proteobacteria is relatively narrow, the *parS*/ParB/ParA system is broadly conserved throughout most proteobacterial subdivisions (117, 118). Not surprisingly, ParA/MinD ATPases are known to be widely used for plasmid and chromosome partitioning, but are also implicated in other processes including virulence regulation in *A. tumefaciens*. VirC1, a factor required for efficient DNA transfer in *A. tumefaciens*, has sequence motifs characteristic of ParA/MinD-like ATPases (119). VirC1 stimulates the generation of multiple copies per cell of the T-complex (VirD2-T-strand particles), probably through its interaction with other Vir proteins (119). VirC1, like MinD and in contrast to ParA, possesses a C-terminal amphipatic helix and is associated with the polar membrane. It recruits T-complexes to the cell poles, where the VirB/D4 type IV

secretion system (T4SS) assembles (119, 120). VirC1 can also bind to the Ti plasmid, and this binding seems to be stimulated by VirC2; this suggests that the VirC1/VirC2/Ti-DNA complex could function in an analogous way to the ParA/ParB/*parS* complex (119). The mechanism by which this complex would be recruited to the cell pole is not known, as pole-to-pole oscillations or formation of filaments have not been detected for VirC1 in the experimental conditions tested, but they remain an interesting possibility (119).

In contrast to *C. crescentus*, which only possesses a single circular chromosome, multiple replicons are present in rhizobia and agrobacterial species, making it much more challenging to dissect the underlying positioning and segregation mechanisms. *A. tumefaciens* has a circular chromosome, a linear chromosome, a cryptic plasmid (pAtC58) and the tumor-producing Ti plasmid (121). *S. meliloti* possesses a circular chromosome and two megaplasmids (122). In both cases, the circular chromosome has an origin of replication resembling that of *C. crescentus*, whereas the linear chromosome of *A. tumefaciens* and the (mega)plasmids carry a plasmid-like replication system of the *repABC* family – in which RepA and RepB are responsible for partitioning and RepC is required for the initiation of replication (121-123). In *A. tumefaciens*, all replicons are duplicated at the same time during the cell cycle and preferentially localize at, or near, the cell pole, although their positions do not overlap (19, 121, 124, 125). Similarly, in *S. meliloti* the replication origins of all three replicons preferentially localize at the cell pole, with a higher polar bias for the chromosome origin (124). The polar localization of the replicons origins seems to be conserved in alpha-proteobacteria and might contribute to their stability and inheritance. However, the pole is not just a general site for origin localization, as an RK2-based broad host range multicopy plasmid is positioned predominantly at mid- and quarter-cell sites in *C. crescentus*, *A. tumefaciens* and *S. meliloti* cells, as is the case for *E. coli* cells (124, 126).

6. REGULATION OF MUCOIDY AND CELL DENSITY

In *C. crescentus*: Determining the complete genome sequence of a *C. crescentus* wild isolate (CB15) and a derived laboratory strain (NA1000) unearthed a 26-kb prophage-like insertion, probably a mobile element, that confers several distinct properties to NA1000 compared to CB15. These NA1000 distinctive phenotypes include the mucoidy on high-sugar medium, a reduced sensitivity to phage ΦCr30 and the sedimentation properties which are exploited to physically separate swarmer and stalked cells by cell density centrifugation (127). The 26-kb prophage region encodes a number of putative ORFs predicted to be involved in polysaccharide synthesis and export (127). The presence of these genes could alter the capsular properties of *C. crescentus*, and as a consequence interfere with phage ΦCr30 attachment to the S-layer. Moreover, the presence of a modified capsule on the cell surface might affect the cell density and hydrodynamic properties, which in turn would be responsible for the change in the sedimentation properties (127).

In other alpha-proteobacteria: While little is known about the regulation of capsular polysaccharides production in *C. crescentus*, this is a particularly important topic in the realm of understanding the symbiotic relationship of rhizobia with their hosts. In rhizobia, the mucoid phenotype is often related to the production of exopolysaccharides (EPS). In the case of *S. meliloti*, two types of EPS are produced: succinoglycan (EPS I) and galactoglucan (EPS II) (128, 129). Their production is under control of a number of environmental signals, including phosphate concentrations and quorum sensing, and EPS play an important role in the establishment of an efficient symbiotic interaction with host plants as well (90, 130-132). A small transcriptional regulator with a C_2H_2 zinc finger domain, MucR, has also been implicated in the regulation of both types of EPS in *S. meliloti*: MucR has a positive effect on the production of succinoglycan, by binding to a short DNA region upstream of *exoH* and *exoY* (involved in EPS I biosynthesis), whereas it represses EPS II production (133-135). The EPS II gene cluster contains four operons, *wge*, *wga*, *wgd* and *wggR*-*wgcA* (135, 136). The proteins encoded by the *wge*, *wga* and *wgd* transcriptional units have biosynthetic functions, whereas WggR is a transcriptional regulator that promotes EPS II synthesis and WgcA is a glycosyl transferase involved in the production of low molecular weight EPS II (135-137). Upstream of each of these operons there are two promoters, controlled by PhoB, WggR and MucR: the proximal promoters are cooperatively induced by PhoB and WggR, driving EPS II production under phosphate-limiting conditions (136). On the other hand MucR can strongly inhibit the distal promoters and binds to a DNA sequence next to the distal transcription start sites, inhibiting galactoglucan synthesis in phosphate-sufficient conditions (136).

In *S. meliloti* MucR is also involved in the control of motility, which is required for the interaction with host plants (138). MucR represses the expression of *rem*, which encodes an activator of motility gene expression with targets such as *flaF* (a flagellar biosynthesis regulatory protein) and *flgG* (a component of the basal body rod) (138-140). Bahlawane *et al*. have shown the direct binding of MucR to the *rem* promoter, and that the MucR and Rem consensus sequences overlap, so that the competition between MucR and Rem for binding would lower Rem auto-induction and thus motility (138). Coordinated regulation of EPS production and motility has been demonstrated in other bacteria such as *Ralstonia solanacearum*, *Vibrio cholerae* and *Salmonella enterica*, and is considered to play an important role in promoting bacteria-host interactions (141-144). It has also to be noted that *S. meliloti* is motile mainly in exponential phase, and its motility progressively decreases in stationary phase, in response to cell density (145). Actually, the ExpR/Sin and ExoR/ExoS/ChvI quorum sensing systems regulate both motility and EPS production as a well as a number of other functions according to the growth phase (146-151). However, it is important to highlight that ExpR/Sin

abolishes the repression of MucR on galactoglucan biosynthesis but does not affect the role of MucR on a number of other functions that promote symbiosis, such as repression of motility and increase in EPS I and Nod factors production (137). Therefore, *S. meliloti* MucR is a key player in the coordination of bacterial functions, as it represses genes specifically intended for expression during symbiosis and promotes root nodule formation. While there are no typical quorum-sensing systems encoded in the *C. crescentus* genome, there are three *mucR*-like genes (CC_0933, CC_0949 and CC_1356), raising the possibility that an intricate MucR-regulated relationship also exists for polysaccharides production and motility in *C. crescentus*.

7. CONCLUSION

Spatial and temporal regulation of developmental and differentiation processes has acquired an outstanding importance in bacteria. *C. crescentus* is a model system for cell cycle control and cell division, and its study is helping to elucidate the same processes in other alpha-proteobacterial species, as the fundamental components of regulatory networks and cell division machinery are highly conserved. Conversely, identifying the genetic basis for specialized properties such as virulence, symbiosis, DNA transfer by a type IV secretion system and EPS production in other alpha-proteobacteria will also be very helpful in illuminating the regulation of mucoidy, cell density and type IV secretion in *C. crescentus* for which little is known currently.

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