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More than a Tad: spatiotemporal control of *Caulobacter* pili

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The Type IV pilus (T4P) is a powerful and sophisticated bacterial nanomachine involved in numerous cellular processes, including adhesion, DNA uptake and motility. Aside from the well-described subtype T4aP of the Gram-negative genera, including *Myxococcus*, *Pseudomonas* and *Neisseria*, the Tad (tight adherence) pilus secretion system reshuffles homologous parts from other secretion systems along with uncharacterized components into a new type of protein translocation apparatus. A representative of the Tad apparatus, the *Caulobacter crescentus* pilus assembly (Cpa) machine is built exclusively at the newborn cell pole once per cell cycle. Recent comprehensive genetic analyses unearthed a myriad of spatiotemporal determinants acting on the Tad/Cpa system, many of which are conserved in other α-proteobacteria, including obligate intracellular pathogens and symbionts.

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Introduction

Biological nanomachines are assembled from dissimilar subunits in tight temporal order and often at precise subcellular locations [1,2]. Bacterial pili are ubiquitous fibrillary organelles of major clinical importance as they are involved in a myriad of cellular processes such as adhesion, non-flagellar (gliding or twitching) motility, DNA uptake and biofilm formation [3–6]. The pilus secretion apparatus is an envelope-spanning structure. An assembly platform embedded in the cytoplasmic membrane polymerizes a filament from pilin subunits that traverses the cell wall (peptidoglycan, PG) layer and that protrudes the outer membrane of diderm (Gram-negative) bacteria via a (gated) pore [7••]. As the spatiotemporal regulation of most pilus systems is still poorly understood, we review here recent insight revealing how the Tad/Cpa piliation system of the free-living Gram-negative α-proteobacterium *Caulobacter crescentus* is regulated in time and space.

The *C. crescentus* paradigm

C. crescentus is the preeminent model system for bacterial cell cycle and cell polarity studies. The crescent-shaped predivisional cell is polarized, featuring a flagellum and Tad/Cpa secretion machine at the newborn pole and a cylindrical extension of the cell envelope (the stalk) at the old cell pole [8–10]. Upon cytokinesis two distinct daughter cells emerge, a motile swarmer (SW) cell and a sessile stalked (ST) cell with an adhesive holdfast at the tip of the stalk. The SW cell harbours a flagellar filament and several pili secreted by the flagellar type III and Tad/Cpa system, respectively, at the same pole (Figure 1a). While the SW cell lacks replication competence [11], the ST cell initiates replication of the circular chromosome and proceeds to cell division (Figure 1a). Coincident with the assembly of the cytokinetic apparatus, the flagellar and Tad secretion machines are built opposite the stalk. The non-replicative (G1-like) state is transient and its duration is dictated by the regulated synthesis and degradation of key cell cycle factors that reprogram transcription. The G1-phase transcriptional state is induced by the transcription factor CtrA in SW cells, while the S-phase program is implemented by the transcriptional regulator GcrA in ST cells (see below) [12]. The G1 → S (CtrA → GcrA) transcriptional switch also accompanies the remodelling of the cell poles: the flagellum is shed, pili are lost and a stalk is subsequently elaborated from the denuded cell pole. New flagellar and Tad/Cpa secretion components [9,13] are synthesized and the corresponding export machineries are assembled at the newborn pole opposite the stalk later in S-phase when CtrA reaccumulates. However, pili are only elaborated coincident with cell separation in the SW cell progeny, but rarely during the predivisional cell phase [8,14,15].

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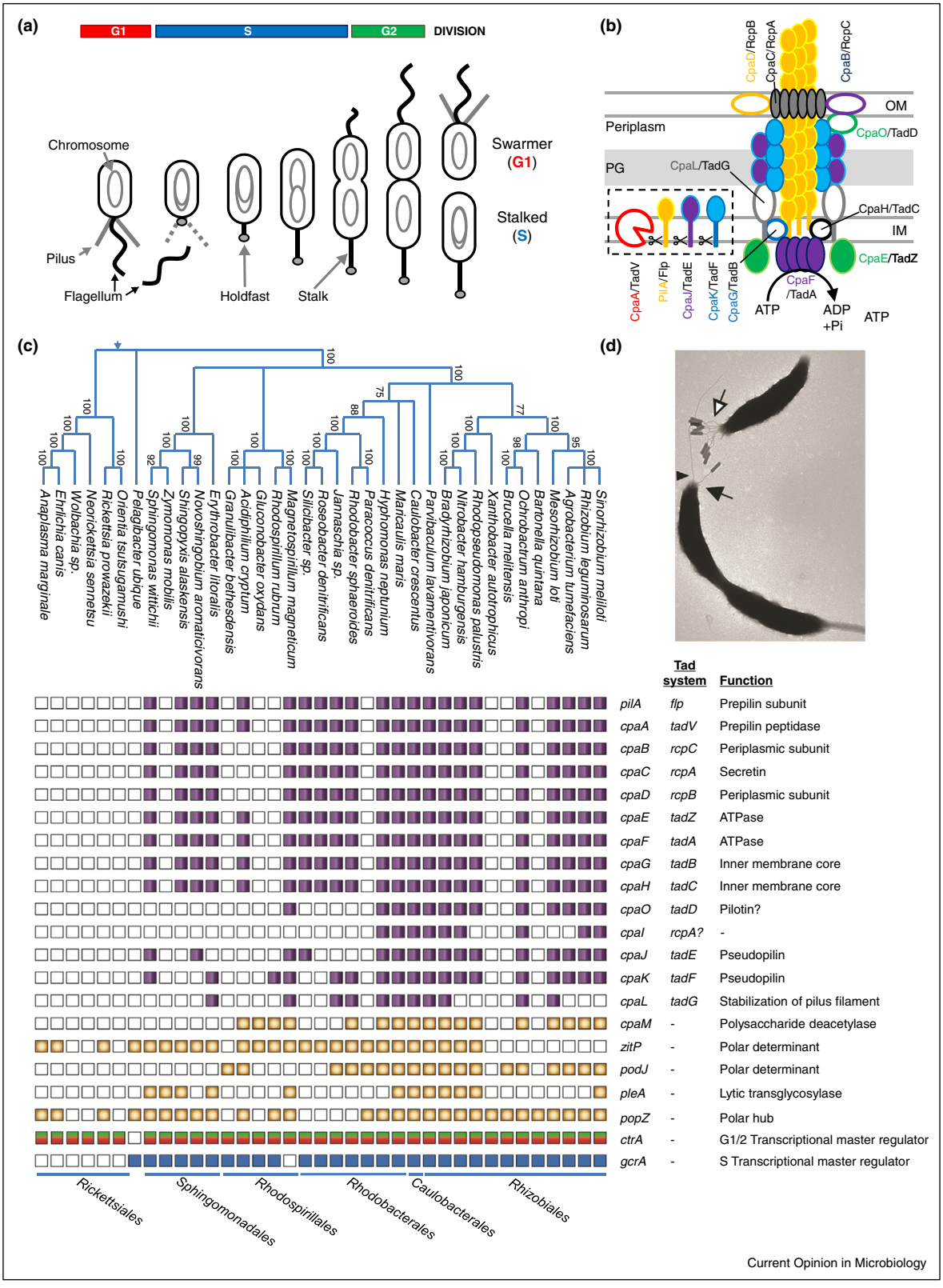
Figure 1



Tad pilus structure and conservation in α-proteobacteria. **(a)** Schematic of the *C. crescentus* cell cycle. The bacterium exhibits a dimorphic life cycle giving rise to two progeny cells with distinct sizes and fates. The smaller swarmer (SW) cell harbours pili and a flagellum at the same pole. This motile SW cell is temporarily arrested in a quiescent G1-like phase, unable to initiate replication or to divide, and must differentiate into the

The components of the Tad/Cpa system

The Tad/Cpa secretion system plays a role in biofilm formation, pathogenesis, adhesion or natural transformation in different bacterial lineages [16,17,18•,19] (Figure 1b,c). Despite its abundance (425 Tad/Cpa systems encoded in 323 genomes [6]), it is less well-studied than other secretion systems. Phylogenetically, it shares similarities with the Type II secretion system, Type IV secretion and T4P subunits [6]. However, several of its components do not have counterparts in other secretion systems, pointing to a distinct molecular ancestry and likely differences in the assembly process. The core components of the Tad/Cpa-dependent piliation system (Figure 1b) are usually encoded within one genetic cluster [6], but trans-encoded accessory factors serve to regulate the system in time and space (Figure 2a).

The TadA/CpaF ATPase is predicted to couple ATP hydrolysis to the secretion of pilus components, while TadB/CpaG and TadC/CpaH are two putative scaffolding factors in the inner (cytoplasmic) membrane [20,21] (Figure 1b). By contrast, TadZ/CpaE is a cytoplasmic protein that associates peripherally with the membrane-anchored Tad/Cpa machine (Figure 1b). TadZ/CpaE features a proline-rich N-terminal domain followed by an atypical domain resembling response regulator receivers, an ATPase domain with a deviant Walker A motif [2,22] and finally a C-terminal amphipathic helix that promotes peripheral interactions with the cytoplasmic membrane [23•]. The ATP-bound state of TadZ/CpaE is thought to mediate its dimerization [24•] and functional fluorescent protein fusions to TadZ/CpaE revealed that it is polarly localized. This localization occurs independently of other known Tad/Cpa factors [9,23•,25], consistent with the notion that other deviant Walker A-type ATPases direct the spatial organization of protein and protein-DNA complexes in other bacteria [2,22]. Indeed, TadZ/CpaE is required for the assembly of the polar RcpA/CpaC secretion channel in the *C. crescentus* outer membrane [9,25] and for efficient polar localization of TadA/CpaF in *Aggregatibacter actinomycetemcomitans* [23•]. Thus, this cytoplasmic factor represents the first milestone of pilus biogenesis within the Tad/Cpa system which, akin to the construction of other trans-envelope complexes [1], seems to follow an inside-out assembly path.

A second striking feature of the Tad/Cpa pilus is the fact that Flp/PilA (pre)pilins form a unique monophyletic clade in the Type IVb prepilin family [8,26]. Flp/PilA prepilins are inserted into the cytoplasmic membrane, matured by the TadV/CpaA peptidase [27] and elaborated as (often bundled) polymeric pilin fibers through the RcpA/CpaC secretion channel (Figure 1b) [28]. Cells lacking CpaC are unable to display pili on the surface [8] even though they accumulate PilA [9]. The TadE/CpaJ and TadF/CpaK pseudopilins, that are likely also processed by TadV/CpaAs, are required for pilin extrusion from the cell surface [29•], but they are not major constituents of the pilus fiber [8,27]. The cytoplasmic membrane protein TadG/CpaL likely mediates protein–protein interactions between the polymerized pilin fiber and a component of the cell envelope, possibly connecting the pilus to the PG [30]. While no function can be predicted for the other Tad/Cpa proteins, it is noteworthy that the *tad/cpa* clusters do not encode orthologs of the retraction ATPase from canonical T4Ps, although a recent report shows that *C. crescentus* pili retract [31••]. Moreover, *tad/cpa* does not encode a recognizable PG hydrolase that could clear the PG locally to permit the assembly of a trans-envelope structure [32], suggesting that a trans-encoded hydrolase executes this task (see below).

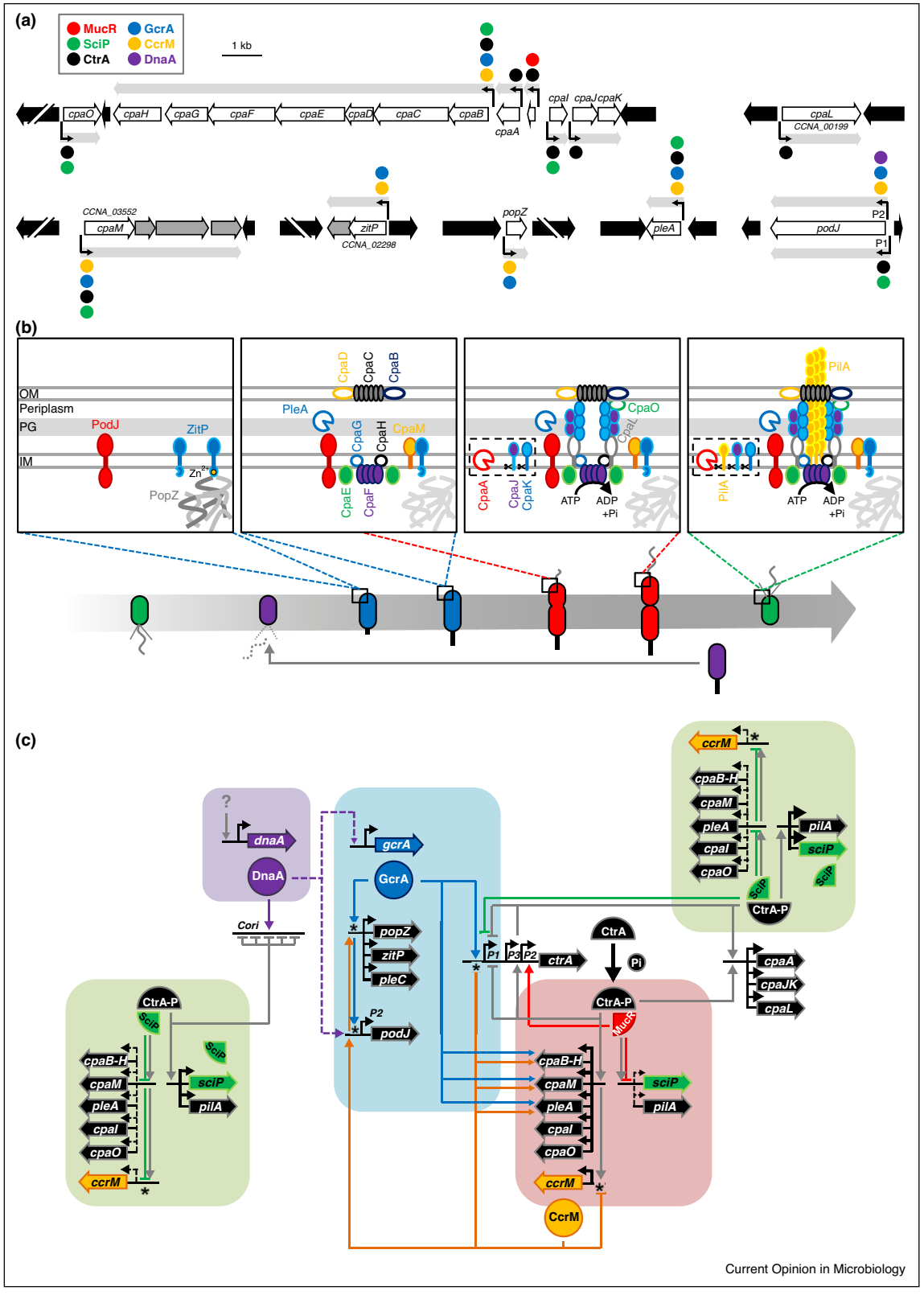
Two polar localization pathways for pilus biogenesis

C. crescentus pili are the principal receptor sites for the virulent caulophage CbK (φCbK) [8,33•] (Figure 1d), a characteristic that was exploited in genetic screens for pilus mutants by selection for survival in the presence of φCbK. These mutants not only revealed the *tad/cpa* gene cluster, but also the genes for trans-encoded spatio-temporal regulators of piliation [8,29•,34•,35–39] (Figure 2a). Subsequent mutational analyses showed the existence of at least two pathways directing the assembly of the polar RcpA/CpaC secretion channel (Figure 2b).

PodJ, a bitopic and bifunctional membrane protein that features an N-terminal (cytoplasmic) coiled-coil domain and a predicted (periplasmic) PG-binding domain at the C-terminus, is required for the early stages of pilus assembly [35]. PodJ is synthesized and accumulates in

(Figure 1 Legend Continued) replicative stalked cell (ST) for entry into S-phase and to proceed to division. During the G1 → S transition, SW cells shed their flagellum and retract their pili before they elaborate a stalk appendage tipped by holdfast from the denuded pole. **(b)** Predictive structure and function of the Tad/Cpa secretion system. This model is adapted from [16] and based on homologues of Tad proteins with those in other bacterial and archaeal systems. Tad/Rcp and equivalent Cpa names are indicated. IM, inner (cytoplasmic) membrane; OM, outer membrane; PG, peptidoglycan. **(c)** The phylogenetic tree (adapted from [12,64]) shows the presence (coloured boxes) or absence (open boxes) of genes predicted to encode orthologs for pilus structural components (PilA and CpaA-L/O; linear violet stain), polar determinants (CpaM, ZitP, PodJ and PopZ; radial gold stain), and master cell regulators (CtrA and GcrA; red-green and dark blue, respectively). BLASTP searches were used to determine the distribution of all proteins in the same selected α-proteobacterial dataset. The corresponding Cpa and Tad nomenclatures and predicted functions are indicated. **(d)** Indirect visualization of polar pili in *C. crescentus* wild-type cells exposed to the pilus-specific bacteriophage φCbK and imaged by transmission electron microscopy (TEM) following uranyl acetate staining [15]. Open arrows point to tufts of polar phage, filled arrows to a single phage particle. Flagella are marked by small filled arrowheads.

Figure 2



The *C. crescentus* cell cycle transcriptional regulatory network orchestrates Cpa machinery synthesis and assembly. **(a)** Operon structure of the genes implicated in assembly or polar localization of the Cpa secretion system. Bent and grey arrows indicate identified promoters and operon structures, respectively [52,65]. Promoter occupancy by the regulators is depicted by a coloured circle based on published ChIP-Seq data (GcrA

early S-phase [25,40,41,42•]. It localizes to the pole opposite the stalk [25,40] where it subsequently recruits the TadZ/CpaE cytoplasmic factor (whose synthesis occurs after PodJ) [25] (Figure 2b). In the absence of PodJ, CpaE remains dispersed and since polar CpaE is required for the assembly of the polar CpaC secretion channel, PodJ mutants also cannot assemble pili and a polar CpaC complex [25]. Simultaneously, PodJ also directs the assembly of the secretion machinery for the adhesive holdfast at the newborn pole [43].

Recently, a second pilus localization pathway converging on CpaC was elucidated [34•]. In this pathway the bifunctional ZitP protein acts via the CpaM protein [34•] (Figure 2b) to orchestrate polar pilus biogenesis. ZitP is a bitopic membrane protein with an N-terminal zinc-finger domain and a C-terminal Domain of Unknown Function (DUF3426) that is required for pilus assembly [34•]. Expression of ZitP is genetically coordinated with PodJ in early S-phase (Figure 2b,c). ZitP is polarly localized and required to recruit the polysaccharide deacetylase homolog CpaM into a complex at the newborn pole [34•]. CpaM, in turn, is synthesized after ZitP, but concomitantly with CpaE (Figure 2c) [34•]. Cells lacking either CpaM or ZitP are unable to export the PilA/Flp pilin and do not assemble a proper CpaC/RcpA complex [34•]. Thus, two polarity proteins synthesized in early S-phase, ZitP and PodJ, are each required for the localization of two downstream pilus assembly factors, CpaM and CpaE, that are expressed later in the cell cycle and that are required for the formation of a multimeric CpaC channel (Figure 2b,c). Interestingly, while the two pathways converge on CpaC, they do not appear to affect each other's localization [34•], that is, CpaM is still polarly localized in the absence of PodJ and, conversely, CpaE is still polarized in cells lacking ZitP.

Many of the genera encoding a near complete Tad/Cpa gene cluster also have a *cpaM* ortholog (Figure 1c), suggesting that the function of CpaM in Tad assembly is also a feature of other Tad systems. Since CpaM resembles sugar deacetylases, it is conceivable that its function is to locally deacetylate a structure in the periplasm (for example PG, lipids or protein) to permit the assembly of the CpaC channel exclusively at the newborn pole. Interestingly, a sugar deacetylase is known to affect the adhesive properties of the *C. crescentus* holdfast [44] and de-acetylation of PG can influence the susceptibility to different PG hydrolases [45].

It is unknown if additional control mechanisms act on the ZitP-CpaM system, but dedicated localization determinants exist. Structural analysis by NMR revealed that the cytoplasmic N-terminal region of ZitP folds into a zinc-finger domain (ZnR) fold [46••] and that this zinc-bound ZnR complex ($ZnR \bullet Zn^{2+}$) interacts directly with the conserved polar matrix protein PopZ [47,48]. While the integrity of the zinc-binding architecture is required for the interaction with PopZ, it is dispensable for pilus assembly and polar localization of CpaM [34•,46••]. It is evident that the global polarity network of PopZ that affects diverse traits of polarity such as stalk biogenesis [47,48], also extends to the ZitP pilus assembly factor. Nevertheless, PopZ is not necessary for pilus biogenesis in *C. crescentus* and it is also dispensable for the polar recruitment of CpaM [34•].

How PodJ and ZitP affect CpaC assembly is unknown. Since the functional domains in PodJ and ZitP that are required for pilus biogenesis reside in the periplasm, it is possible that they influence the assembly of the pilus secretion channel, possibly via the putative PG hydrolase PleA [49] (Figure 2a,b). Although PleA is required for the biogenesis of pili and the flagellum [36,50], it is unknown whether it has PG hydrolase activity *in vitro* or whether it can function as activator of PG hydrolases [51]. A possible role of PleA as PG hydrolase is plausible since it harbours a Lytic Transglycosylase (LT) and Goose Egg White Lysozyme (GEWL) domain and since it is required for the assembly of the CpaC channel at the cell pole as well as for external flagellar structures [49]. Moreover, PleA accumulation is restricted to late S-phase at the same time when CpaC and CpaM synthesis occurs [42•,49].

(Figure 2 Legend Continued) [53••,56•]; CtrA, SciP and MucR [52,57••,58•]) or *in vitro* electrophoretic mobility shift assays (DnaA [66]). CcrM (m6A-methylation) data are determined by ChIP-Seq analysis [54•] and global methylome analysis [67•]. **(b)** Sequence of pilus protein localization and pilus assembly during the cell cycle. The scheme below the panel shows the corresponding time in the cell cycle when the target promoters or each module fire. The fluctuation of transcripts from these target genes during the cell cycle as determined by microarray and RNA-seq analysis [13,52,68,69]. IM, inner membrane; OM, outer membrane; PG, peptidoglycan. **(c)** Representation of the genetic regulatory modular network controlling cell cycle transcription in *C. crescentus* (adapted from [12]) and focused on the pilus localization and architectural genes. GcrA/CcrM (boxed in blue; * indicates m6A methylation mark introduced by CcrM at GANTC motifs), CtrA/SciP (boxed in green; P- represents the phosphorylation activation of CtrA by the CckA/ChpT phosphorelay) and CtrA/MucR (boxed in red) transcriptional modules control sequential waves of transcription in S-phase, G2-phase and G1-phase, respectively. For each module, a list of validated target genes was generated from the same ChIP-seq, microarray and RNA-seq datasets. This genetic regulatory network is also synchronized with the upper scheme in panel in **(b)**. DnaA control at the *gcrA* and *podJ* promoters is depicted as dotted lines because the binding of DnaA at these promoters has only been demonstrated *in vitro*, but not yet *in vivo* [66]. Based on recent *cpaM*, *cpaB-H* and *pleA* expression data [56•], it is possible that a first pulse in promoter activation is provided by GcrA/CcrM and then maximally reinforced by CtrA-dependent activation. Such two-step activation might explain why the first expression occurs coincident with GcrA but peaks later, typical of CtrA-dependent transcriptional activation.

Cell cycle-dependent synthesis of pilus proteins

The synchronizability of *C. crescentus* provides an excellent system to explore the temporal control of piliation as a function of the bacterial cell cycle (Figure 2b,c). Cell cycle-controlled transcription is executed by at least three conserved transcriptional modules in which pairs of antagonistic regulatory proteins activate sequentially the promoters of S-phase, G2-phase and G1-phase genes [12]. These six DNA-binding global regulators also target various promoters of the *cpa* genes and/or the aforementioned trans-encoded pilus factors [13,52,42•] (Figure 2c).

Concomitant with the G1 → S transition and the initiation of DNA replication by the DnaA initiator protein, the GcrA transcription factor accumulates in early ST (S-phase) cells [39] and activates transcription from many S-phase promoters such as *popZ*, *podJ* and *zitP*. The expression of these polar landmark factors by GcrA [13,34•,42•,52,53••,54•] triggers an early polar differentiation cascade that is required to install pili at this site [25,40]. Interestingly, GcrA-dependent activation also requires the CcrM adenine methyltransferase [53••,54•,55,56•] that introduces methylation marks at the N6-position of adenine (m6A) in the context of 5'-GANTC-3' sequences during the previous cell cycle (G2-phase). These m6A-marked GANTC sequences overlap the site in the promoter that GcrA binds to and enhance GcrA's affinity for target promoters [53••,54•,55,56•]. In the absence of GcrA, CcrM has a negative effect on the activity of these promoters [53••], presumably through its association with GANTC sequences at these promoters.

Further illustrating the inter-connectivity between the cell cycle transcriptional modules, the GcrA-CcrM regulatory pair also concurrently activates the *P1* promoter of the CtrA-encoding gene. CtrA is an OmpR-like response regulator that is activated by phosphorylation and that targets the promoters of G2-phase and G1-phase genes [13,42•,57••]. CtrA is also a negative regulator of replication initiation, ensuring that chromosome replication does not occur prematurely in G1-phase daughter cells [11,37,58]. Interestingly, CtrA binds and activates the promoter regions of *cpa* genes (*pilA*, *cpaA*, *cpaB-H*, *cpaI*, *cpaJ/K* and *cpaO*) (Figure 2a) as well as the promoters of the trans-encoded *pleA* and *cpaM* genes [8,57••,59•]. Although CtrA binds these promoters, they do not all fire at the same time presumably because different negative regulators (either SciP or MucR, see below, Figure 2a,c) act on them. Indeed, global mRNA steady-state analyses revealed that these transcripts peak at different times in the cell cycle [13,57••]. The *cpaM* and *pleA* transcripts as well as the *cpaB-H* operon peak first (late S-phase), followed by the *cpaA*, *cpaI*, *cpaJ/K* and *cpaO* transcripts in late S-/G2-phase [42•] before cell division has been completed. Finally, as the predivisional cell has been compartmentalized, the *pilA* transcript accumulates and is maintained in the G1-phase progeny [8,14,42•,57••].

The negative regulator SciP, a helix–turn–helix domain protein that is only present in G1-phase [57••,60,61], targets the CtrA-activated promoters of G2-phase genes. *In vivo*, SciP associates with the promoters of *cpaA*, *cpaI*, *cpaO*, as well as with the promoter regions of *cpaB-H*, *cpaM* and *pleA* (Figure 2a) [57••]. At the end of the G2-phase, the pilus secretion apparatus is fully assembled at the future piliated pole, while awaiting the accumulation of the PilA pilin [8,9]. For the induction of *pilA* transcription in G1-phase, the direct negative regulation by the MucR (mucoidy regulator) repressor that keeps this and other promoters in the inactive state must be overcome [57••,62]. How this occurs is unclear [62], but it may involve local changes in promoter architecture or differential activities in CtrA/MucR during the cell cycle. In fact, the histidine kinases PleC and DivJ or other factors such as the bifunctional regulator KidO can modulate the level of CtrA phosphorylation [15]. In contrast to SciP (whose expression is directly repressed by MucR [57••]), MucR abundance does not fluctuate during the cell cycle [57••]. The fact that premature assembly of pili in predivisional cells can be induced simply by transcribing *pilA* from a constitutive promoter (P_{tac}) [8] or by inactivating KidO [15] attests the strict temporal control of PilA expression and pilus assembly.

Concluding remarks

Apart from the obligate intracellular rickettsial lineage, the *tad/cpa* pilus system is broadly conserved in α-proteobacterial genomes (Figure 1c) and was also shown to be required for piliation in the rhizobial lineage [18•]. Moreover, transcriptional analysis in synchronized *Sinorhizobium meliloti* cells revealed that at least one out of the three redundant *tad/cpa* systems (*cpaA1-D1/F1*) is also cell cycle-regulated [63••]. The fact that many of the trans-encoded piliation factors are also conserved raises the possibility that their spatiotemporal action on the Cpa/Tad system is not restricted to the *Caulobacter* genus.

It is unclear why two independent and converging pathways are required — PodJ-CpaE and ZitP-CpaM — to promote the assembly of the CpaC (RcpA) pilus secretion channel and the subsequent pilin (PilA/Flp) export at the newborn cell pole. It suggests that CpaC assembly is a major milestone in pilus biogenesis that must be carefully regulated, perhaps because an outer membrane channel that is prematurely active is potentially dangerous. Interestingly, there are also many different checkpoints that operate in the flagellar assembly pathway to ensure the sequential construction of individual substructures of a complex nanomachine [1]. Since PodJ and ZitP are also bifunctional proteins that control other aspects of morphogenesis and/or the *C. crescentus* developmental cycle, the PodJ/CpaE and ZitP/CpaM pathways allow for

coordination of pilus assembly with other cell cycle-controlled events. Such coordination is further ensured by the genetically wired transcriptional control circuitry (Figure 2c). Future work is thus aimed at understanding how the transcriptional switches are triggered and how the new pole is identified by polarity systems such as PodJ-CpaE and ZitP-CpaM systems to enable the subsequent localized assembly of the pilus secretion machinery.

Conflict of interest

The authors declare no conflict of interest.

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