# Second messenger mediated spatiotemporal control of cell cycle and development

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**Anna Dürig**

aus Krauchthal, Schweiz

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auf Antrag von

- Prof. Dr. Urs Jenal
- Prof. Dr. Bernhard Erni

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Prof. Dr. Eberhard Parlow

# Abstract

During the biphasic life cycle of *Caulobacter crescentus* motile, free-living swarmer cells differentiate into sessile, surface attached stalked cells. The swarmer cell is replication inert and is unable to divide. During the swarmer-to-stalked cell differentiation, degradation of CtrA, a master regulator that blocks replication initiation, leads the onset of chromosome replication. After this obligate cell differentiation step, which is mainly regulated by the degradation of the master cell cycle regulator CtrA, stalked cells immediately initiate their chromosome replication. Recently, dynamic co-localization of CtrA and its protease ClpXP to cell pole was proposed as a timing mechanism for cell cycle-dependent CtrA degradation.

We have identified the response regulator PopA as an essential regulator for CtrA sequestration to the incipient stalked cell pole and for subsequent CtrA degradation by the nearby ClpXP protease complex. Time laps fluorescence microscopy of PopA-GFP showed that PopA itself dynamically sequesters to the cell poles during the *C. crescentus* cell cycle. While PopA sequestration to the flagellated pole depends on PodJ, a swarmer pole specificity factor, localization to the incipient stalked pole depends on the C-terminal GGDEF output domain of PopA. We demonstrate that in contrast to most GGDEF domain proteins, PopA lacks diguanylate cyclase activity. Instead, PopA functions as cyclic di-GMP effector protein, which specifically binds the bacterial second messenger at a conserved binding site (I-site) within the GGDEF domain. An intact PopA I-site is required for PopA sequestration to the incipient stalked pole as well as for CtrA degradation during the cell cycle. PopA directs CtrA to the ClpXP occupied cell pole via a direct interaction with an adaptor protein, RcdA. Based on this we postulate that c-di-GMP bound PopA facilitates the dynamic distribution of CtrA to the cell pole where it s degraded by ClpXP. This is the first report that links c-di-GMP to protein dynamics and cell cycle control in bacteria.

In addition to its prominent role in cell cycle control, PopA was identified as novel component of the complex regulatory network that orchestrates polar development in *C. crescentus*. PopA, together with PleD and DgcB, two active diguanylate cyclases, controls cell motility, holdfast formation and surface attachment. Our data suggest that PopA interferes with PleD and DgcB to coordinate cell motility, stalk biogenesis, holdfast formation and finally surface attachment. Based on this, we propose that

PopA is a bifunctional protein, involved in control and coordination of *C. crescentus* cell cycle and development.

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# 1 Introduction

## 1.1 Bacterial cells have a highly organized three-dimensional structure

Bacteria are ubiquitous and are the most widespread organisms on earth. Over several billion years of evolution, they managed to adapt to almost every habitat or specific biological niche on this planet. Under the selection pressure for fast growth and high reproduction rate, they evolved simple cell morphologies and small genomes with high coding densities. For many years, bacteria were considered as a primitive and unstructured cellular state compared to the highly organized eukaryotic cells. But technological advances, mainly in fluorescence light microscopy, allowed a deeper insight into the organization of bacteria. Recent studies have shown that bacteria make use of different mechanisms to actively and dynamically control cell cycle progression and morphological differentiation.

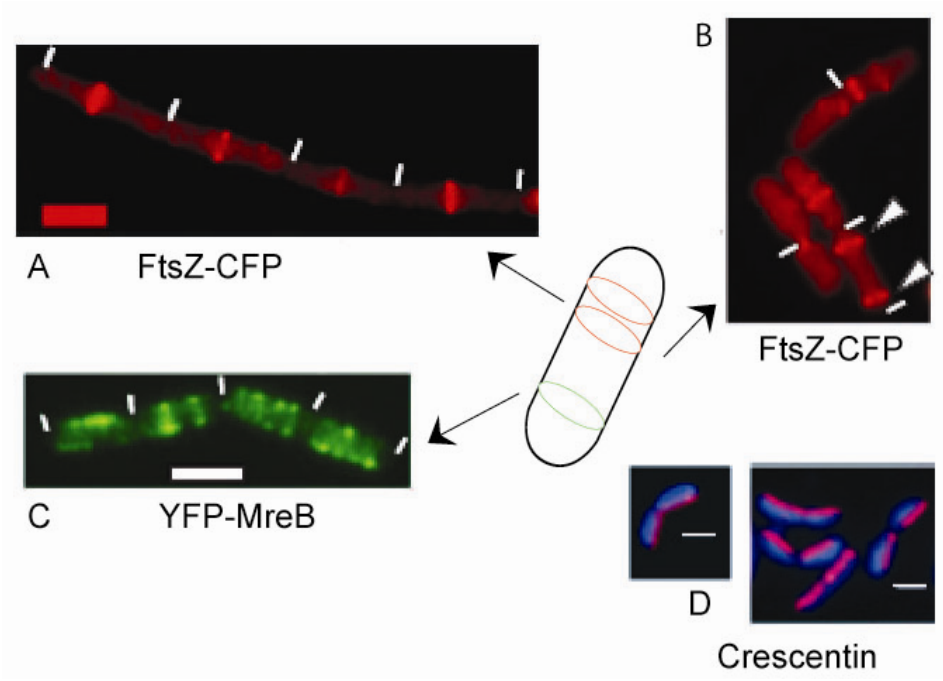


**Figure 1.1**

Fluorescence microscopy of cytoskeletal elements in bacteria. Horizontal bars are 2 µm. A) and B) Fluorescence microscopy of FtsZ-CFP in *B. subtilis*. FtsZ forms a ring in the middle of the cell (Z-ring are indicated by white arrows) C) *B. subtilis* cells expressing YFP-MreB. MreB forms helical filaments underneath the cell membrane. D) Immunofluorescence with anti-crescentin antibody (pink), cells are stained with DAPI (blue). Illustration adapted from (Graumann, 2004).

Bacteria show dynamic cytoskeletal elements and distinct compartmentalization with proteins localizing to specific subcellular sites.
Lately it was shown, that all known eukaryotic cytoskeletal elements, tubulin, actin and intermediate filaments (IFs), are also present in bacteria (Figure 1.1). Bacterial tubulin (e.g. FtsZ), actin (e.g. MreB) and intermediate filaments (e.g. crescentin in *Caulobacter crescentus*) homologues are key regulatory players controlling cell division, cell shape, bacterial DNA segregation, and possibly maintenance of cell polarity. The highly dynamic behavior of the bacterial cytoskeleton might play an important role in the transport of macromolecules and in protein localization to distinct subcellular sites within the cell (reviewed in (Graumann, 2004; Thanbichler and Shapiro, 2008).

### 1.1.1 Protein localization in bacteria

Over the past years, a major breakthrough was the observation that many proteins and even larger protein complexes are distributed to specific subcellular sites in the bacterial cell. Growing evidence indicated that the highly dynamic localization of structural and regulatory proteins, including signal transduction proteins, chromosome partition proteins and proteases, contribute to the coordination of cell division, cell differentiation and to the bacterial cell fate. In *C. crescentus* every asymmetric cell division gives raise to two different cell types, to swarmer cells and surface attached stalked cells (see Chapter 1.2.1). The asymmetric distribution of cell-fate determinants already in the incipient progeny is required and responsible for the generation of daughter cells exhibiting different morphological features and performing diverse functions (Jacobs and Shapiro, 1998; Shapiro and Losick, 1997). Protein localization, e.g. to the cell poles, the incipient division plane or the septum, allows the bacterial cell to express unique functions at distinct subcellular sites, e.g. the sequestration of the replication complex to midcell and to generate subcellular organelles, including pili or flagella (reviewed in (Collier and Shapiro, 2007; Ebersbach and Jacobs-Wagner, 2007).
The dynamic three-dimensional organization in bacteria is a key regulatory mechanism, which controls different cellular events, including cell cycle and cell development. But how is cell polarity maintained and transmitted to the progeny? How

are proteins sequestered to specific subcellular sites, such as the cell poles, and retained there?

In contrast to eukaryotes, bacteria lack the endoplasmatic reticulum, the golgi apparatus and transport vesicles, but they can still target and deliver specific proteins to distinct sites within the cell. In *Escherichia coli* the cell division protein FtsZ is very precisely localized to the future division plane (see below). FtsZ is only one example of protein localization to one distinct subcellular site, but so far, many more examples are characterized. However, only little is known about how proteins localize to cell poles or to other specific sites in the cell. How do they reach their correct destination? So far, several models for membrane protein localization on one hand and a couple of possibilities for the sequestration of soluble proteins on the other hand are described.

The targeted-insertion and the diffusion-and-capture model represent two alternative ways to explain the localization of integral-membrane and membrane-associated proteins to specific positions in the membrane. For the sequestration of soluble proteins different models were proposed, including protein targeting through the formation of dynamic gradients (see below) or localization via the interaction of preexisting receptor structures.

*The targeted-insertion model*

The targeted-insertion model describes a process, in which a newly synthesized protein is delivered to a specific subcellular site, where it is translocated directly to its destination site in the membrane. The targeted-insertion model is discussed on IcsA in *Shigella flexneri* (Charles et al., 2001). IcsA, an outer membrane protein that is required for intracellular motility and virulence is localized to the old cell pole, where it controls the assembly of an actin tail inside the host cell. Studies on IcsA localization suggest that the unipolar localization of IcsA results from direct and selective targeting to the pole (Steinhauer et al., 1999). More recent fluorescence microscopy studies, propose that IcsA localization happens prior to its secretion across the cytoplasmic membrane by the Sec apparatus (Brandon et al., 2003; Charles et al., 2001). This is in agreement with the observation that IcsA localization to the cell pole is independent of a signal peptide sequence (Charles et al., 2001). Based on these findings it has been postulated that IcsA first recognizes a prelocalized receptor structure in the

cytoplasmic membrane, which targets IcsA secretion to the cell pole mediated by the Sec pathway (Brandon et al., 2003; Charles et al., 2001).
However, targeted-insertion is not the only way to achieve membrane protein localization.

*The diffusion-and-capture model*

An alternative possibility to localize membrane proteins is described by the diffusion-and-capture model. Newly synthesized proteins are inserted randomly into the membrane and diffuse in the membrane until they are captured by a previously localized receptor.
SpoIVFB, a polytopic membrane protein, which is involved in the late stages of sporulation in *Bacillus subtilis* is synthesized in the mother cell and targeted to the septal membrane. In vegetatively growing cells, expressing SpoIVFB-GFP from an inducible promoter, SpoIVFB-GFP is randomly distributed in the cytoplasmic membrane. However, during the initiation of sporulation and in the absence of inducer SpoIVFB-GFP accumulates at the septum. This finding indicates that SpoIVFB randomly inserts into the cytoplasmic membrane, diffuses to the septal membrane and is captured at the septal membrane (Rudner et al., 2002). Another example for the diffusion-and-capture mechanism is the localization of PleC, a membrane bound histidine kinase that localizes to the flagellated pole of the *C. crescentus* predivisional cells (Wheeler and Shapiro, 1999). Single-molecule fluorescence microscopy provided indirect evidence for PleC localization by the diffusion-and-capture mechanism. One fraction of the YFP tagged PleC molecules is localized to the cell pole and the residual PleC molecules are moving randomly throughout the cell membrane. No directed or biased motion could be detected for the PleC molecules suggesting that the molecules are freely diffusing and captured at the pole by some sort of receptor (Deich et al., 2004).
However, the targeted-insertion model as well as the diffusion-and-capture model is based on a determinant or receptor, which captures the membrane protein at the correct subcellular site. In all cases the identification of the receptor structure is a critical issue.

*Landmark proteins tag cell poles and position polar organelles*

Correct cell pole development, including the positioning of polar appendages like flagellum and pili, is one big challenge in bacteria. But, what directs the synthesis of new polar organelles to specific sites? Recently, TipN, a membrane-bound coiled-coil rich protein, was identified as a landmark protein, which marks the two new cell poles and ensures cell polarity of the daughter cells after cell division in *C. crescentus* (Huitema et al., 2006; Lam et al., 2006). The Δ*tipN* mutant exhibits multiple cell polarity defects, including inaccurate placement of the division plane toward the new cell pole and reversed cell polarity (Lam et al., 2006). In addition, TipN marks the site for new flagellum assembly through the polar localization of the EAL domain protein TipF. Polar localized TipF is required for the early steps of flagellar assembly (Huitema et al., 2006). Moreover, TipN also determines the polar positioning of the histidine kinase PleC, which is required for polar pili biogenesis (Viollier et al., 2002b).
PodJ is a second general polar localization factor (Hinz et al., 2002; Viollier et al., 2002a). The full-length PodJ protein ($PodJ_L$) is sequestered to the incipient flagellated pole where it mediates the positioning of the histidine kinase PleC and the pilus assembly factor CpaE to the same pole. After cytokinesis the full-length PodJ protein is proteolytically cleaved to the shorter $PodJ_S$ form, which regulates chemotaxis and holdfast formation.
TipN and PodJ are examples for general localization factors, which are required for the targeting of proteins that regulate the positioning of polar organelles. So far, the mechanism how the cell decodes the positional information of TipN and PodJ is not known.

*Protein targeting through the formation of dynamic gradients*

In contrast to the targeted-insertion and diffusion-and-capture model, is the MinCDE system in *Escherichia coli* a self-contained oscillatory system, which is independent of any receptor structures. The MinCDE system controls cytokinesis in *E. coli* by the correct positioning of the Z-ring to midcell. FtsZ, a bacterial tubulin homologue, is essential for cell division and assembles into the cytoskeletal Z-ring exactly in the middle of the cell. The Z-ring acts as a recruitment factor for at least a dozen proteins, which are required for correct cytokinesis in E. coli. The three proteins MinC, MinD and

MinE regulate the placement of the division site by establishing a gradient of negative regulators of Z-ring assembly (Figure 1.2).

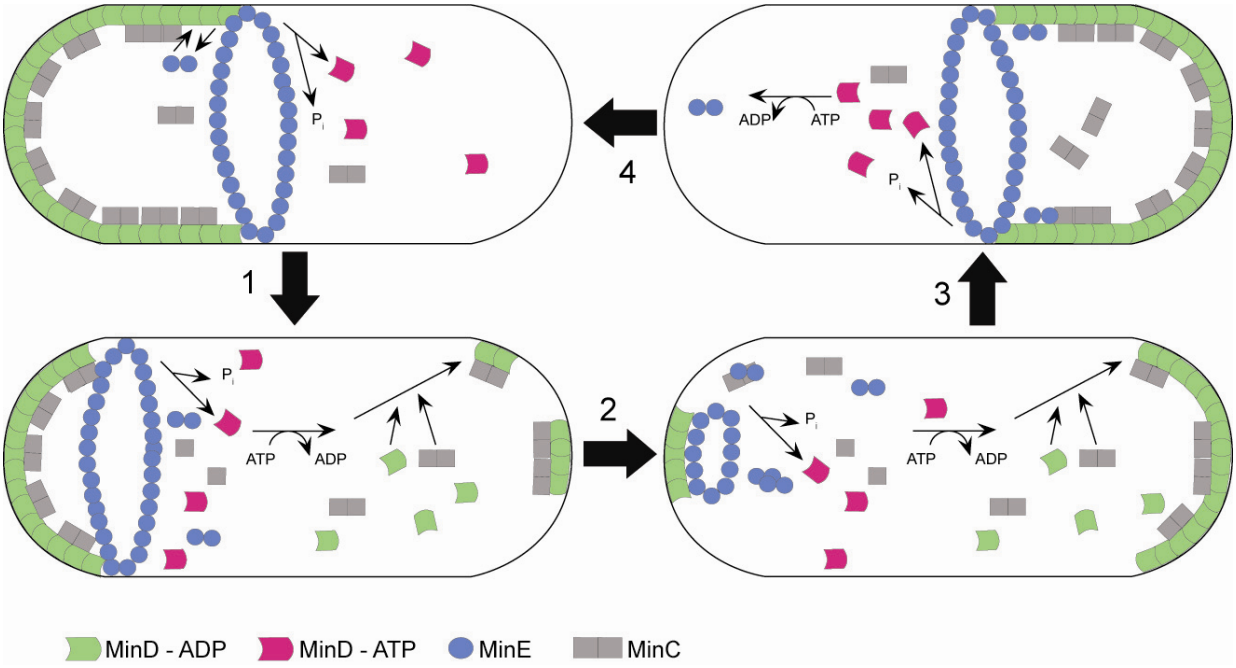


**Figure 1.2**

The MinCDE oscillatory system in *E. coli.* MinD-ATP binds to the membrane and assembles together with MinC forming clusters at one cell pole. The MinE (E-ring) is formed at the edge of the polar MinCD clusters forcing MinC and MinD to disassemble. When the polar MinCD cluster disassembles, the E-ring shrinks back to the pole, which leads to the release of MinE. From the rapid pole-to-pole oscillation evolves a zone of division inhibition close to the cell poles. Illustration according to (Lutkenhaus, 2007)

MinD is an ATPase, which belongs to the WACA (Walker A cytoskeletal ATPase) family, and binds in its ATP-bound form to the cytoplasmic membrane and its ATPase activity is stimulated by MinE (Hu et al., 2002). In addition to the possibility to form dimers, MinD is able to interact with MinC and MinE. MinC is the effector protein and responsible for the inhibition of the cell division by interacting with FtsZ. Despite being the division inhibitor MinC lacks site specificity and is only a passenger in the oscillating system following MinD (Hu et al., 1999). But how is this oscillatory system regulated? MinD-ATP binds to the membrane and attracts MinC, which leads to the inhibition of FtsZ-ring formation by MinCD clusters (Figure 1.2). MinE follows the

MinCD clusters and displaces MinC by stimulating the ATPase activity of MinD, which ultimately induces the detachment of both proteins. According to the model proposed by Huang et al. MinD-ATP concentrations are lower in the vicinity of the old pole, because MinD-ATP binding to the membrane is favored by already bound MinD (Huang et al., 2003b). This allows the increase of MinD-ATP at the other pole and as the concentration rises, it eventually binds to the membrane and forms a new polar zone of MinCD clusters. Now, MinE is released from the old cell pole and starts to stimulate the ATPase activity of MinD at the new pole. Therefore, the concentration of division inhibitor MinC is maintained high near the cell poles and low near midcell, which results in the Z-ring formation at midcell. The ability of MinE to stimulate MinD ATPase correlates with its ability to stimulate the oscillation of the Min system (Hu and Lutkenhaus, 2001) MinD and MinE are both required for oscillations. (reviewed in (Lutkenhaus, 2007; Rothfield et al., 2005)).

But what does ensure correct cell division if bacteria lack a functional MinCDE system? The nucleoid occlusion was discovered as a fail-safe mechanism in mutants that are impaired in the MinCDE system, to ensure proper cell division under conditions of unbalanced growth. Cell division never occurs at regions in the cell, which contain chromosomal DNA (Yu and Margolin, 1999). Recent studies identified two unrelated proteins, NocA from *B. subtilis* (Wu and Errington, 2004) and SlmA from *E. coli* (Bernhardt and de Boer, 2005), which mediate this nucleoid occlusion effect . Both proteins harboring a helix-turn-helix DNA-binding motif interact nonspecifically with chromosomal DNA and therefore colocalize with the nucleoid. Based on the observation that SlmA recruits FtsZ to the nucleoid preventing Z-ring formation, one can speculate that SlmA is a inhibitor of Z-ring assembly (Bernhardt and de Boer, 2005). However, the exact mechanisms of nucleoid occlusion is still unclear.

## 1.2 *Caulobacter crescentus* – a model organism for cell cycle control and bacterial development

### 1.2.1 The Caulobacter crescentus life cycle

*Caulobacter crescentus* is a rod-shaped bacterium and belongs to the α-proteobacteria. *C. crescentus* lives in freshwater environments, including streams and lakes (Poindexter, 1981). The unique life cycle of *C. crescentus* includes a characteristic asymmetric cell division, which gives raise to two genetically identical, but morphologically and physiologically different daughter cells, the free living swarmer (SW) cell and the surface attached stalked (ST) cell (Figure 1.3). The SW cell possesses one polar flagellum, polar pili, is motile and is able to perform chemotaxis. However, the SW cell is not able to initiate chromosme replication. Before the SW cell initiates replication, it goes through an obligate cell differentiation step, during which the polar chemotaxis apparatus is lost, pili are retracted, and the flagellum is ejected and replaced by an adhesive holdfast and the stalk structure (Figure 1.3). The holdfast at the tip of the stalk mediates adhesion and is essential for the irreversible surface attachment of the ST cell (Merker and Smit, 1988). In contrast to the SW cell, the differentiated ST cell is able to initiate chromosome replication. As the ST cell develops into a predivisional (PD) cell, the pili secretion apparatus, a new flagellum and the chemotaxis machinery is synthesized and assembled at the pole opposite the stalk. After the cell division, the newborn ST cell immediately reinitiates DNA replication. In contrast to many other more rapidly growing bacteria, chromosome replication is initiated only once per cell cycle in *C. crescentus* (Marczynski, 1999).

The *C. crescentus* cell cycle can be divided into three distinct phases, a pre-synthesis gap (G1-phase), a DNA synthesis phase (S-phase) and a division phase (G2-phase). The possibility to synchronize the *C. crescentus* cell cycle and to isolate newborn swarmer cells by density gradient centrifugation has made *C. crescentus* to one of the preferred model systems to analyze cell cycle progression. In addition, the integration of bacterial cell differentiation into cell division and the possibility to distinguish morphologically SW (G1 phase) and ST cells (S-phase) made *C. crescentus* not only to a model system to study cell cycle control but also for bacterial cell biology.

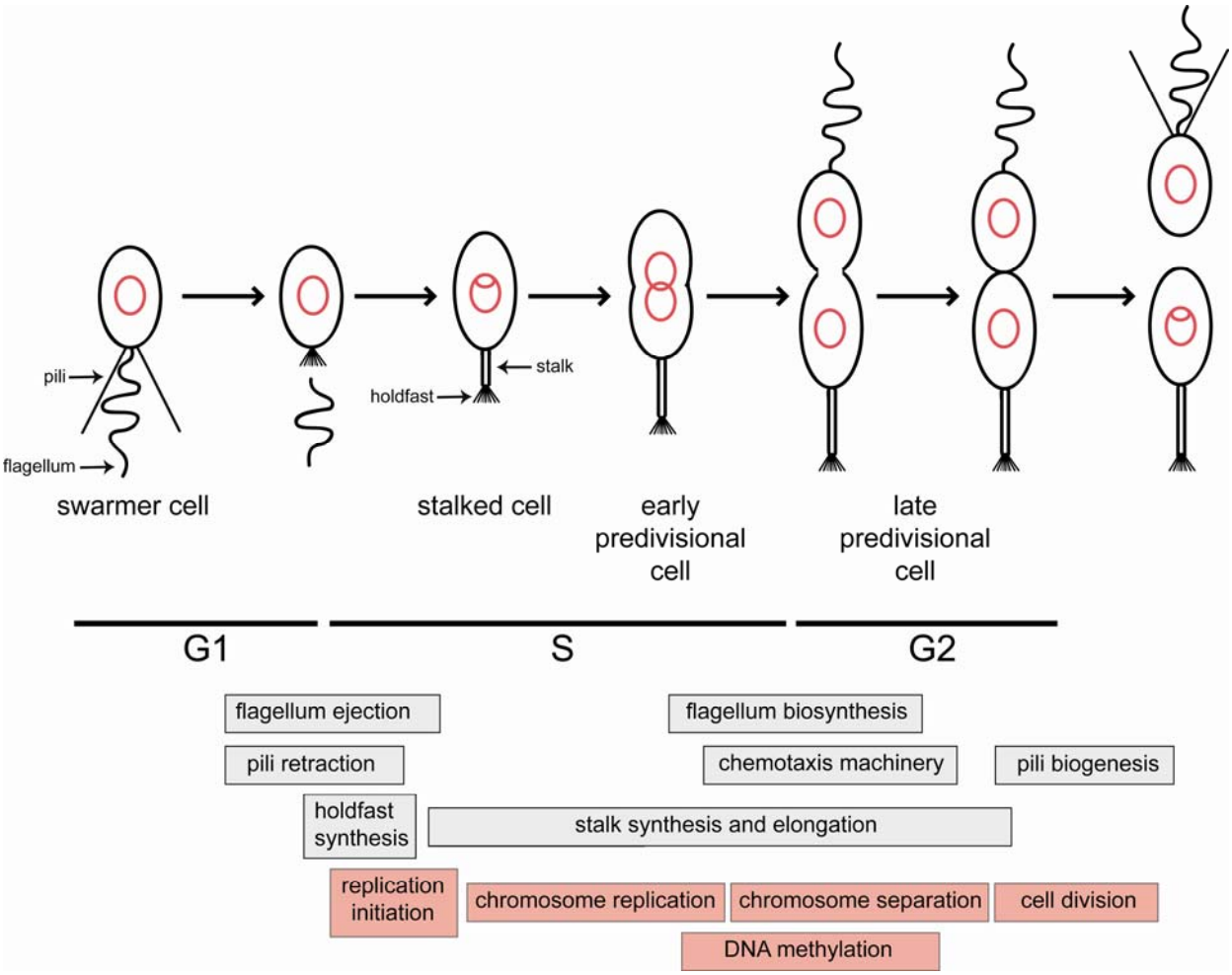


**Figure 1.3**

Schematic of the *Caulobacter crescentus* life cycle. The motile, piliated swarmer cell undergoes an obligate cell differentiation step, called G1-to-S phase or swarmer-to-stalked cell transition, to differentiate into a sessile, surface attached stalked cell. During the transition pili are retracted, the flagellum is ejected and replaced by the stalk and holdfast structure. The stalked cell initiates chromosome replication. Quiescent chromosomes are represented by circles and replicating chromosomes are indicated by "θ" structures. Morphogenetic events are indicated in grey boxes, cell cycle events in red boxes. This figure was adopted from (Jacobs-Wagner, 2004).

### 1.2.2 Cell cycle control in *Caulobacter crescentus* by three master regulators

Cell cycle progression in *C. crescentus* is tightly controlled by the synthesis and degradation of different master regulators at specific checkpoints during the cell cycle. This ensures the correct chromosome replication and cell division. Cell cycle progression is controlled by a cyclical genetic circuit of the three master regulators, CtrA, GcrA and DnaA. Periodic fluctuations of these three proteins are accomplished by the combination of timed synthesis and degradation (Collier et al., 2006). 19% of all genes in *C. crescentus* are cell cycle regulated and at least 200 genes are directly or indirectly controlled by CtrA, GcrA or DnaA (Collier et al., 2006; Holtzendorff et al., 2004; Hottes et al., 2005; Laub et al., 2002; Laub et al., 2000). The complex genetic circuit for cell cycle progression in *Caulobacter* is simplified and schematically summarized in figure 1.4 (illustration according to (Collier et al., 2006)).

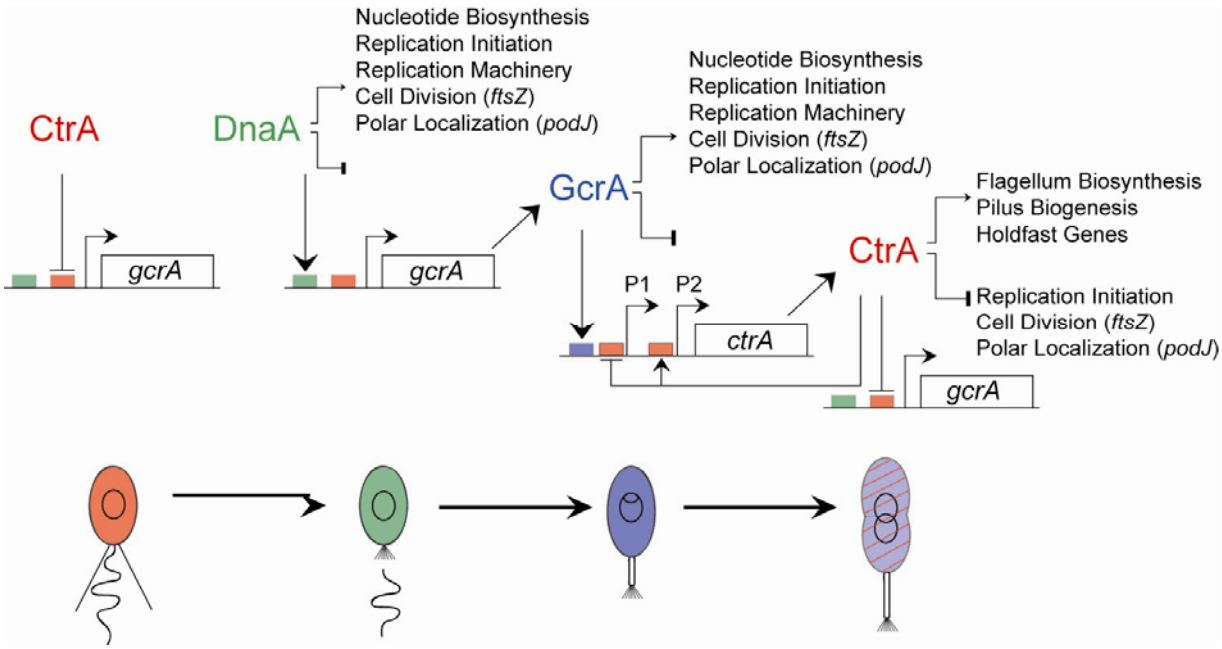


**Figure 1.4**

Schematic of cell cycle control in *C. crescentus* through periodic accumulation of CtrA, DnaA and GcrA. The presence of CtrA during the cell cycle is indicated in red, DnaA in green and GcrA in blue (Collier et al., 2006). Cellular functions are shown.

In SW cells, CtrA represses the transcription of *gcrA*. During the G1-to-S phase transition CtrA is degraded und the *gcrA* promoter is derepressed (Holtzendorff et al., 2004). In addition, DnaA, which is essential for DNA replication initiation (Zweiger and Shapiro, 1994), is synthesized, binds to the DnaA binding box of the *gcrA* promoter and activates *gcrA* transcription. This leads to a burst of GcrA in stalked cells and at the same time DnaA synthesis is stopped and DnaA is removed by degradation through the ClpP protease (Gorbatyuk and Marczynski, 2005). During initiation of cell division GcrA is more stable and efficiently accumulates, which turns on CtrA transcription in predivisional cells. In late predivisional cells accumulated CtrA, together with the disappearance of DnaA, shuts off *gcrA* transcription (Collier et al., 2006). The precise timing, teamwork and the correct sequential expression of CtrA, GcrA and DnaA is essential for cell cycle progression.

A main characteristic of *C. crescentus* is that the cyclical genetic circuit of CtrA, GcrA and DnaA coordinates chromosome replication and polar morphogenesis (Figure 1.3, Figure 1.4). In swarmer cells CtrA represses the expression of the polarity factor PodJ, which is required for the assembly of the pili-specific secretion appartus, the holdfast formation and the chemotaxis machinery (Crymes et al., 1999; Hinz et al., 2002; Viollier et al., 2002a). At the same time the activating effect on this gene is removed by the proteolysis of DnaA (Gorbatyuk and Marczynski, 2005). During the G1-to-S phase transition, accumulating levels of DnaA activate the transcription of GcrA, PodJ and FtsZ, which is ensures proper cell division. At the same time, GcrA, directly or indirectly, positively activates the expression of PleC and PodJ, both required for polar morphogenesis. All three master regulators converge on PodJ, a general localization factor responsible for the correct positioning of polar organelles, regulating expression either positively (GcrA, DnaA) or negatively (CtrA).

Furthermore, the expression of PilA, the structural subunit of the pilus filament, is stimulated by CtrA at cell division. In addition, CtrA and GcrA, directly or indirectly, control the expression of flagellar genes. CtrA~P activates the expression of early structural and regulatory flagella genes. The expression of *fljMNO* requires GcrA (Laub et al., 2002; Messer and Weigel, 2003)

### 1.2.3 CtrA controls cell cycle progression in Caulobacter crescentus

CtrA (cell cycle transcriptional regulator) is the best characterized master cell cycle regulator in *C. crescentus*. CtrA is an essential member of the response regulator family of the two component systems and contains a DNA binding output domain. CtrA controls cell cycle, including the control of chromosome replication initiation, chromosome methylation, cell division, as well as developmental events, as the initiation of flagellar and pili biosynthesis (Laub et al., 2002; Quon et al., 1996). CtrA directly regulates the transcription of 96 genes, which are organized in 55 operons (Laub et al., 2002). CtrA is activated by phosphorylation of the conserved aspartate residue $D_{51}$ of the receiver domain (Domian et al., 1997). The active phosphorylated form of CtrA, CtrA~P, binds to a specific 9-mere DNA sequence motif, the so called CtrA binding box (TTAA-N7-TTAA) (Marczynski and Shapiro, 1992). In addition, active CtrA~P binds directly to five different sites within the chromosome replication origin (OriC) blocking replication initiation exclusively in SW cells and in the swarmer cell compartment of predivisional cells. Active CtrA~P is essential and sufficient to block DNA replication initiation *in vivo*. In agreement with this, mutations in the CtrA binding boxes of the OriC lead to increased levels of transcription resulting in cells with multiple chromosomes (Quon et al., 1998).

*CtrA activity is redundantly controlled during the cell cycle by transcription, phosphorylation and proteolysis*

Because CtrA function and control is crucial for correct cell cycle progression, CtrA activity is redundantly controlled by multiple mechanisms, including transcription, phosphorylation and degradation (Domian et al., 1997). CtrA transcription is tightly regulated by positive and negative feed-back loops and is under the control of two temporally controlled promoters, P1 and P2 (Figure 1.4). P1, a weak promoter, is only active in stalked cells and early predivisional cells when CtrA is absent as it is negatively controlled by CtrA. This mechanism allows re-synthesis of CtrA after it has been cleared from the cell by proteolysis (see below). In contrast, P2 is a strong promoter, active in late predivisional cells and swarmer cells, and under positive feedback control. Accumulation of active CtrA in late PD cells leads to the activation of the P2 promoter, which results in a burst of CtrA (~22.000 molecules per cell). This

burst of CtrA in late PD cells and in SW cells is responsible and required for the repression of chromosome replication initiation in these cell types (Domian et al., 1999).

In addition to the transcriptional control, correct phosphorylation and degradation of CtrA is essential for cell cycle progression (Domian et al., 1997). In SW and PD cells high CtrA~P levels repress chromosome replication initiation by directly blocking the OriC (Quon et al., 1998). It has been demonstrated that the histidine kinase CckA is required for CtrA phosphorylation *in vivo* (Jacobs et al., 1999). Recently, extensive *in vitro* biochemical analysis showed that CtrA is not directly phosphorylated by CckA, but through the additional phosphotransfer protein ChpT (Biondi et al., 2006). The CckA-ChpT-CtrA phosphorelay controls the initiation of chromosome replication. In order to initiate chromosome replication active CtrA~P needs to be removed during the G1-to-S phase transition through dephosphorylation and controlled degradation (Domian et al., 1997). Cell cycle-dependent degradation of CtrA requires the ClpXP protease (Chien et al., 2007; Jenal and Fuchs, 1998). Genetic data has indicated that a block of CtrA dephosphorylation and CtrA degradation at the same time leads to a G1-cell cycle arrest. In contrast, blocking either dephosphorylation or degradation has no impact on cell cycle progression (Domian et al., 1997). Similarly, active CtrA~P must be removed from the stalked compartment of PD cells to ensure that after cell division the newborn ST cell is able to immediately initiate chromosome replication.

*Spatial control of CtrA degradation*

Spatial control of signaling molecules is a conserved mechanism to establish cell polarity (Shapiro, 1992). The redundant regulatory pathways, which control CtrA activity, involve the subcellular localization and spatial organization (Figure 1.5).

The replication block by CtrA~P needs to be removed from SW cells or the SW cell compartment of late PD cells to allow chromosome replication. Coincident with its clearance from the cell, CtrA localizes to the incipient stalked pole of the differentiating and newborn ST cell (Figure 1.5). Polar localization of CtrA to the cell pole is strictly linked to its degradation (Ryan et al., 2004; Ryan et al., 2002). Interestingly, the protease complex ClpXP, which is essential for CtrA degradation, co-localizes and interacts with CtrA at the incipient stalked pole (Jenal and Fuchs, 1998; McGrath et al.,

2006). Together this suggests that the correct timing of CtrA degradation during the cell cycle is mediated by the sequestration of the CtrA substrate and its cognate protease ClpXP to the same subcellular site during the G1-to-S phase transition (Figure 1.5, Figure 1.6).

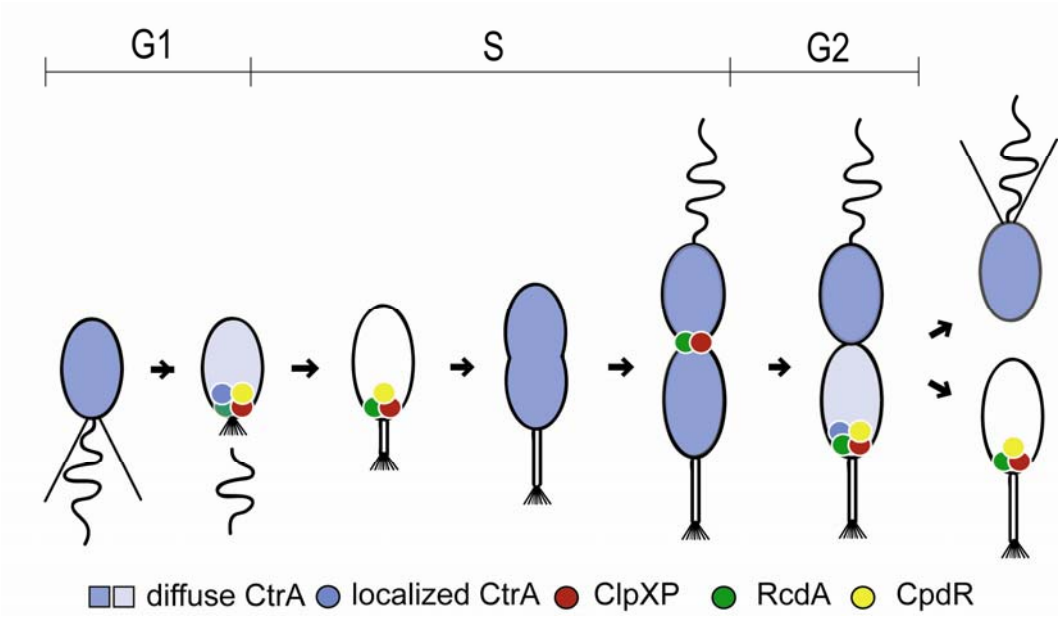


**Figure 1.5**
Polar localization of the components involved in CtrA degradation during the cell cycle.

What are the factors and signals for CtrA and ClpXP recruitment to the cell pole and how is the timing of CtrA and ClpXP localization to the incipient stalked pole regulated? RcdA, a small protein of unknown function, has been identified as a recruitment factor for CtrA targeting to the cell pole (McGrath et al., 2006). In accordance with its role as recruitment factor for CtrA, RcdA also localizes to the same pole and is required for CtrA degradation (Figure 1.5). In the absence of RcdA is CtrA delocalized and as a consequence CtrA is not degraded during the G1-to-S phase transition (McGrath et al., 2006). However, ClpXP localization is not dependent on RcdA. Recently, CpdR, a single domain response regulator, has been identified to be required for ClpXP localization to the incipient stalked pole and for subsequent CtrA degradation (Figure 1.5, Figure 1.6). Interestingly, CpdR itself localizes to the incipient stalked pole as a function of its phosphorylation state; genetic data indicated that non-phosphorylated

CpdR is sequestered to the pole, while CpdR~P remains dispersed (Iniesta et al., 2006). CpdR phosphorylation is controlled by the CckA-ChpT phosphorelay. The CckA-ChpT pathway not only regulates phosphorylation of CpdR, but also controls the phosphorylation of CtrA (see above). Phosphate flux through the CckA-ChpT pathway thus leads to active CtrA~P blocking chromosome replication and at the same time turns off the CpdR-ClpXP localization cascade preventing CtrA from being degraded. In contrast, downregulation of CckA would lead to an increase of non-phosphorylated CtrA and at the same time to CtrA degradation mediated by CpdR-dependent ClpX recruitment to the cell pole (Biondi et al., 2006) (Figure 1.6).

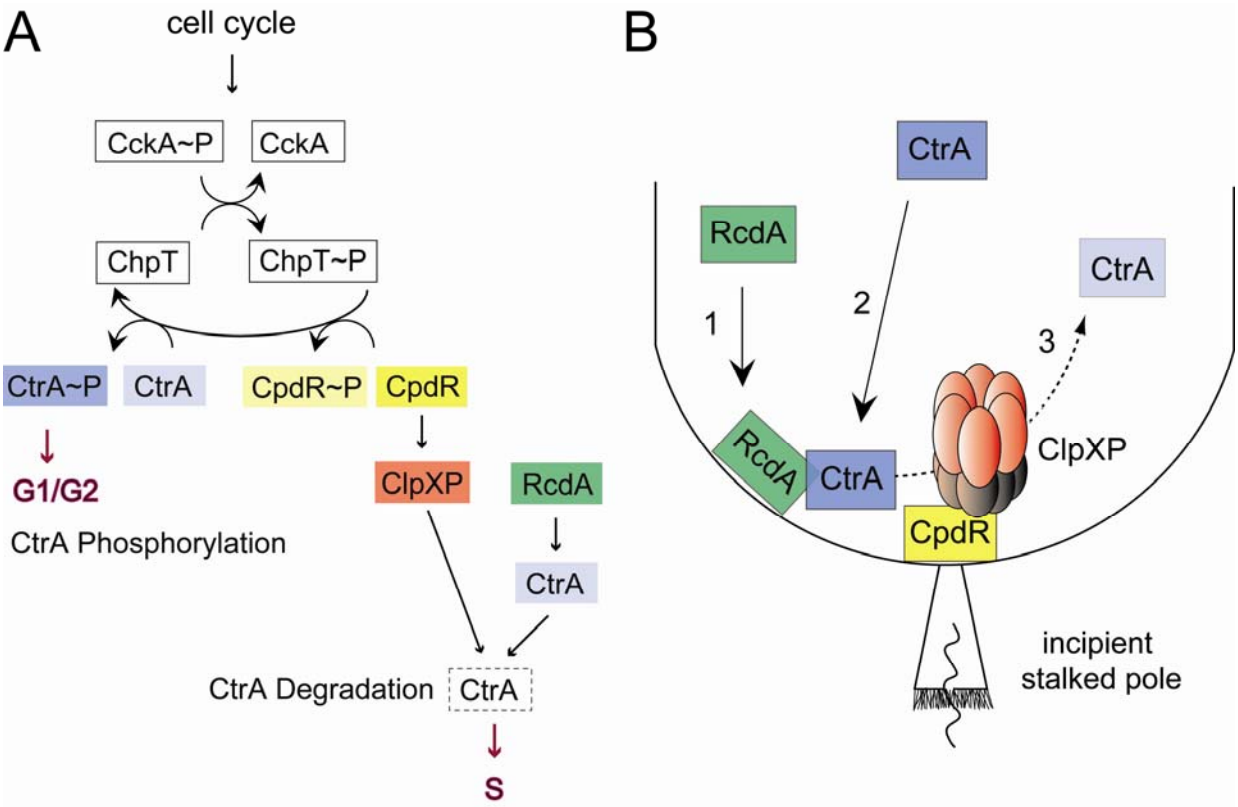


**Figure 1.6**

A) The CckA-ChpT phosphorelay controls CtrA and CpdR phosphorylation. B) Model for CtrA degradation at the incipient stalked pole. 1) RcdA localizes to the incipient stalked pole. 2) Polar RcdA acts as recruitment factor and targets CtrA to the incipient stalked pole. 3) Localized CpdR delivers ClpXP to the pole where the local pool of CtrA gets degraded by the ClpXP protease complex.

CtrA degradation is an excellent example for the importance of spatial control for bacterial cell physiology. All data available indicate that degradation exclusively takes place at the incipient stalked pole during a short time window. Apparently, to ensure correct CtrA degradation, both the protease ClpXP and the CtrA substrate have to be present at the pole at the same time. Whereas, the localization of ClpX is controlled by the CckA-ChpT phosphorelay, it is not clear how RcdA-mediated CtrA localization is controlled. What are the internal or external signals that lead to CtrA localization? The model for CtrA degradation at the incipient stalked pole is shown in Figure 1.6.

## 1.3 Cyclic-di-GMP – a novel bacterial second messenger

### 1.3.1 A brief historical outline

About 20 years ago, the ubiquitous bacterial second messenger (3'-5')-cyclic-di-guanosine monophosphate (c-di-GMP) was discovered as a specific positive effector of the cellulose synthase in *Gluconacetobacter xylinum* (Aloni et al., 1983; Ross et al., 1985; Ross et al., 1987). Biochemical analysis followed by reverse genetics indentified two classes of enzymes controlling cellulose production in *G. xylinum*, diguanylate cyclases (DGCs) and specific phosphodiesterases (PDEs). The diguanylate cyclases (DGCs) which contain the conserved GGDEF domain convert two molecules of GTP to c-di-GMP. Later, biochemical analysis of PleD, a response regulator with a C-terminal GGDEF domain in *C. crescentus*, showed that PleD is a DGC and that this enzymatic activity resides in its GGDEF domain (Paul et al., 2004). Specific phosphodiesterases (PDEs) degrade c-di-GMP to the linear product pGpG (Tal et al., 1998). The PDE activity was confined to the EAL (Christen et al., 2005; Schmidt et al., 2005; Tamayo et al., 2005) and the HD-GYP domain (Ryan et al., 2006). Intracellular c-di-GMP levels are antagonistically controlled by the catalytic activity of DGCs and PDEs (Figure 1.7). The observation, that GGDEF and EAL domain proteins also exist in organisms, which are not producing cellulose, raised the speculation for additional c-di-GMP mediated cellular functions.

### 1.3.2 C-di-GMP controls motility, biofilm formation and virulence in bacteria

Growing evidence suggests that c-di-GMP is one major component, which activates biofilm formation while inhibiting cell motility, thus regulating the switch between the motile and sessile lifestyles (Figure 1.7). Furthermore, c-di-GMP-mediated biofilm formation and motility play an important role in the ability of many pathogens to cause disease. Recently, c-di-GMP has been attributed to the regulation of virulence factors in different pathogenic species (reviewed in (D'Argenio and Miller, 2004; Jenal and Malone, 2006; Kolter and Greenberg, 2006; Romling and Amikam, 2006; Tamayo et al., 2007)

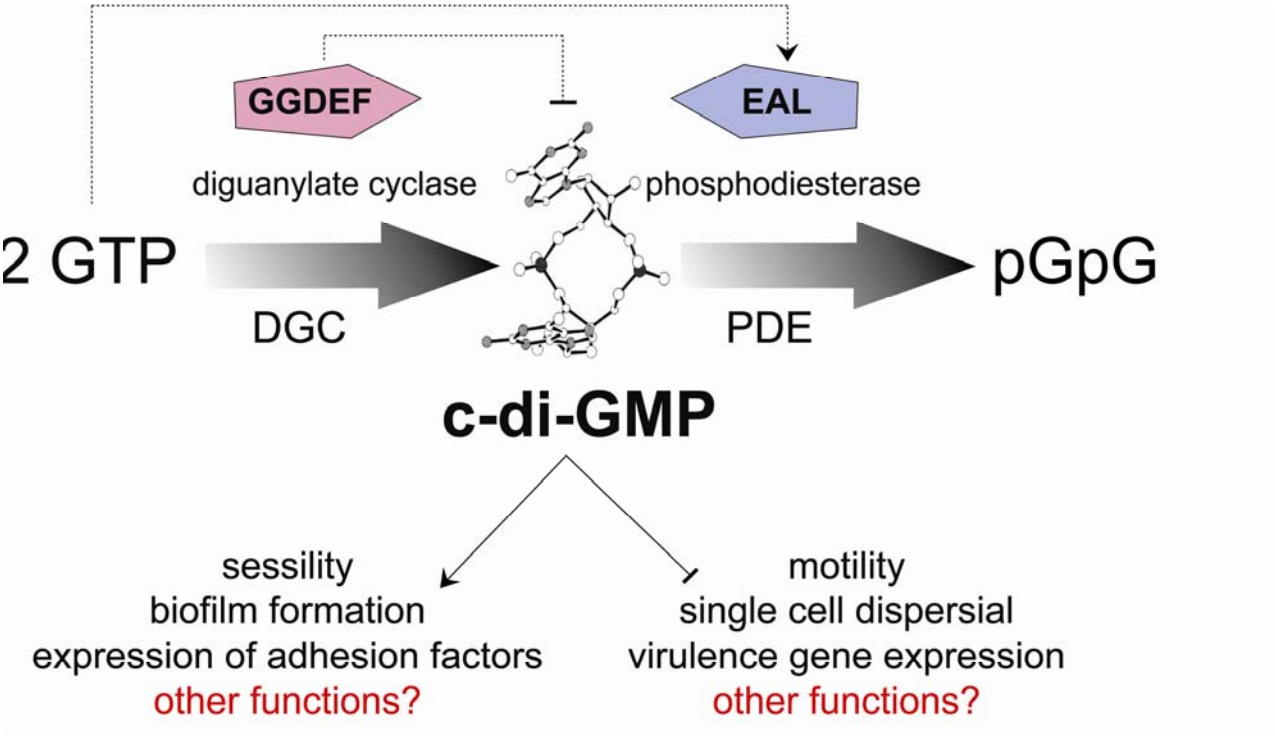


**Figure 1.7**

Schematic of synthesis and hydrolysis of c-di-GMP. The GGDEF domain (pink) is required for the synthesis of c-di-GMP and converts 2 GTP to c-di-GMP. The EAL domain (blue) catalyzes the degradation of c-di-GMP into the linear product pGpG. Cellular functions of c-di-GMP are shown.

*C-di-GMP controls cell motility in bacteria*

An increasing number of studies has implicated GGDEF and EAL domain proteins in the control of cell motility, including swimming, swarming and twitching motility (reviewed in (Jenal and Malone, 2006; Tamayo et al., 2007)). E.g. in *Pseudomonas aeruginosa*, twitching motility is regulated by the GGDEF-EAL composite domain protein FimX (Huang et al., 2003a). FimX, an active PDE, localizes to the cell pole and is required for the surface assembly of type IV pili, which mediates twitching motility in *P. aeruginosa* (Huang et al., 2003a; Kazmierczak et al., 2006). In addition, twitching motility in *P. aeruginosa* is also regulated by the Wsp chemosensory system including WspR, an active DGC, and WspF, a methylesterase with homology to CheB. Based on the observation that a *wspF* mutation leads to decreased swimming and twitching motility, they postulated that a *wspF* mutation causes constitutive activation of the

DGC WspR. As a consequence elevated c-di-GMP levels promote sessility and activate biofilm formation (D'Argenio et al., 2002; Hickman et al., 2005).
In addition to twitching motility, increased levels of c-di-GMP downregulate flagellar motility (Beyhan et al., 2006; Simm et al., 2004; Tischler and Camilli, 2004). In *Salmonella enterica* serovar Typhimurium the DGC AdrA and the PDE YhjH antagonistically control swimming and swarming motility (Simm et al., 2004). In *Vibrio cholerae* equivalent experiments have shown that overexpression of the DGC VCA0956 abolishes swimming, whereas expression of the PDE *vieA* enhanced swimming motility (Tischler and Camilli, 2004). In accordance with this finding, a whole-genome transcriptome analysis in *V. cholerae* clearly shows a strict correlation between ectopically increased c-di-GMP levels and the repression of genes involved in the biosynthesis of the flagellum, motility and chemotaxis (Beyhan et al., 2006).
In bacterial pathogens motility often plays a critical role in the early steps of colonization of the host. Therefore, c-di-GMP mediated motility is important in bacterial pathogenesis. Consistent with its role in controlling twitching motility in *P. aeruginosa*, the *fimX* mutant exhibits dramatically decreased cytotoxicity towards tissue culture cells (Huang et al., 2003a; Kazmierczak et al., 2006).

*Biofilm formation is regulated by c-di-GMP*

In addition to their increasingly well-studied role in the regulation of cell motility, GGDEF and EAL domain protein are also implicated in the production of extracellular polysaccharides (EPS) and the formation of biofilms. Biofilms are bacterial communities consisting of one or more species, which are usually attached to a surface and embedded by a matrix of exopolysaccharides, proteins and nucleic acids (Branda et al., 2005; Kolter and Greenberg, 2006). C-di-GMP activates biofilm formation in a variety of different bacterial species, including *P. aeruginosa*, *Salmonella* Typhimurium, *Vibrio* ssp. and *Y. pestis* (Garcia et al., 2004; Hickman et al., 2005; Kirillina et al., 2004; Simm et al., 2004; Tischler and Camilli, 2004).
In *V. cholerae* the biosynthesis of *Vibrio* exopolysaccharides (VPS), one kind of exopolysaccharides, is encoded by two operons, which are under the control of the two transcriptional activators *vspR* and *vspT* (Casper-Lindley and Yildiz, 2004). A mutation in the PDE gene *vieA* dramatically induces the expression of the *vps* genes, which

results in increased biofilm formation (Tischler and Camilli, 2004). Similarly, while the overexpression of the DGC gene *vca0957* results in increased biofilm formation, the overexpression of the PDE gene *vieA* leads to decreased biofilm formation (Beyhan et al., 2006). In addition, MbaA, a GGDEF-EAL composite domain protein and presumable DGC, is involved in maintenance of the three-dimensional biofilm architecture (Bomchil et al., 2003).

Importantly, for optimal attachment to surfaces and the initial steps of biofilm formation, *V. cholerae* uses in addition to VPS the characteristic of twitching motility, which encoded by mannose-sensitive hemagglutinin Type IV pili (Watnick et al., 1999; Watnick and Kolter, 1999). In agreement with this observation, it was shown that the precise timing of assembly and loss of polar organelles, including pili, flagellum and holdfast, is critical for optimal surface attachment in *C. crescentus* (see in Section 1.3.3) (Levi and Jenal, 2006).

Extensive studies on the opportunistic pathogen *P. aeruginosa*, which is often associated with cystic fibrosis lung infections, showed that this organism uses a variety of c-di-GMP-mediated processes, including the production of different EPS, chemotaxis (see in above in “c-di-GMP controls cell motility in bacteria”) and twitching motility to regulate biofilm formation (Friedman and Kolter, 2004; Hickman et al., 2005; Jackson et al., 2004; Kazmierczak et al., 2006; Lee et al., 2007; Merighi et al., 2007). Recently, it has been demonstrated that c-di-GMP binding to the receptor protein Alg44 is essential for alginate production in *P. aeruginosa*, a component of the EPS matrix (Merighi et al., 2007). Similarly, the regulation of the PEL polysaccharide is mediated by c-di-GMP and activated by c-di-GMP binding to the receptor protein PelD (Lee et al., 2007).

Consistent with the finding that biofilm formation is activated by c-di-GMP while motility is decreased, one can propose, that c-di-GMP controls the switch between a motile and a sessile, biofilm-like lifestyle in bacteria (Figure 1.7).

*C-di-GMP modulates virulence properties in bacteria*

Interestingly, in addition to its role in controlling the switch between motile and sessile lifestyles, c-di-GMP can directly modulate virulence in bacteria. In *V. cholerae*, the causative agent of cholera, the *vieSAB* operon was implicated in the regulation of the

transcription of the *ctxAB* cholera-toxin (CT) (Tischler et al., 2002). The response regulator VieA, which contains a C-terminal HTH DNA-binding domain and an EAL domain, shows PDE activity and is required for virulence in mouse and for virulence gene expression *in vitro* (Tamayo et al., 2005; Tischler and Camilli, 2004; Tischler and Camilli, 2005; Tischler et al., 2002). The VieA PDE activity is critical for the positive regulation of *ctxAB* and the transcriptional activator *toxT*, which is required for toxin-coregulated pili, the major colonization factor (Higgins et al., 1992; Tischler and Camilli, 2004). The PDE VieA plays a central role in the c-di-GMP-mediated transition between an environmental (biofilm) and a host (virulent) lifestyle (reviewed in (Jenal and Malone, 2006; Tamayo et al., 2007).

To summarize, until now, c-di-GMP signaling is involved in regulation of a variety of different cellular processes, including motility, virulence gene expression, sessility, biofilm formation and the expression of adhesion factors. However, the speculation stays if c-di-GMP signaling controls additional, still undiscovered pathways (Figure 1.7).

### 1.3.3 C-di-GMP controls pole development in *Caulobacter crescentus*

*Caulobacter crescentus* with its unique life cycle can be used as a model organism to study the c-di-GMP mediated transition from motility to sessility. The obligate switch between the motile and sessile life style is an integral part of the *C. crescentus* life cycle and is coupled to a constant remodeling of the cell poles. This includes the correct assembly and function of a single polar flagellum and adhesive pili in swarmer cells. These polar organelles are subsequently replaced by an adhesive holdfast and the stalk during the swarmer-to-stalked cell transition. The precise timing of assembly and loss of polar organelles, including the ejection of the flagellum and holdfast biogenesis, is critical for optimal surface attachment during the *C.crescentus* division cycle (Levi and Jenal, 2006). Several reports have linked c-di-GMP signaling proteins with *C. crescentus* pole morphogenesis (Aldridge and Jenal, 1999; Aldridge et al., 2003; Huitema et al., 2006; Paul et al., 2004). In particular, PleD and TipF have been identified as major players in *C. crescentus* pole remodeling.

TipF (CC0710), an EAL domain protein, is required for the correct assembly and positioning of the flagellum during maturation of the swarmer cell pole. In addition, in

the *tipF* mutant pili were less abundant compared to wild-type. Although it is not clear yet if TipF is a PDE, its EAL domain is crucial for the *in vivo* function of the protein (Huitema et al., 2006).

PleD (CC2462), a well characterized diguanylate cyclase controls remodeling of the stalked cell pole. A Δ*pleD* mutant fails to efficiently eject the flagellum during the swarmer-to-stalked cell transition resulting in hypermotile cells. In contrast, expression of a constitutively active *pleD* mutant causes a paralyzed flagellum and non-motile cells (Aldridge et al., 2003; Paul et al., 2004). In addition to controlling the flagellum, PleD is also required for correct timing of holdfast biosynthesis and proper elongation of the stalk. However, the observation that both stalk formation and holdfast biogenesis are only partially affected in the Δ*pleD* mutant suggested that additional and possibly redundant c-di-GMP signaling components are involved in coordinating pole differentiation in *C. crescentus*. Also so far, a counteractive PDE that would keep c-di-GMP concentrations low in the motile SW cells has not been identified.

### 1.3.4 The “paradox” of multiple paralogous GGDEF and EAL domain proteins

Interestingly, c-di-GMP signaling proteins are found throughout the bacterial kingdom. The number of GGDEF and EAL domain proteins is highly variable and differs from organism to organism. Some species like *Helicobacter pylori*, completely lack GGDEF and EAL proteins, but most bacteria encode an intermediate number of GGDEF and EAL domain proteins in their genome. E.g. the chromosome of *Escherichia coli* codes for 19 GGDEF and 17 EAL domain proteins. However, a few organisms, like *Vibrio vulnificus*, encode for over 100 of these proteins. *C. crescentus* has four GGDEF, three EAL and seven GGDEF-EAL composite domain proteins, which are shown in Figure 1.8.

Strikingly, most of the GGDEF and EAL domain proteins do not stand alone. They are associated with known or hypothetical signal input domains (Jenal, 2004; Jenal and Malone, 2006) and approximately one third harbors membrane spanning domains. The large number of different input domains suggests a wide variety of environmental signals, which are perceived and transmitted by the c-di-GMP network.

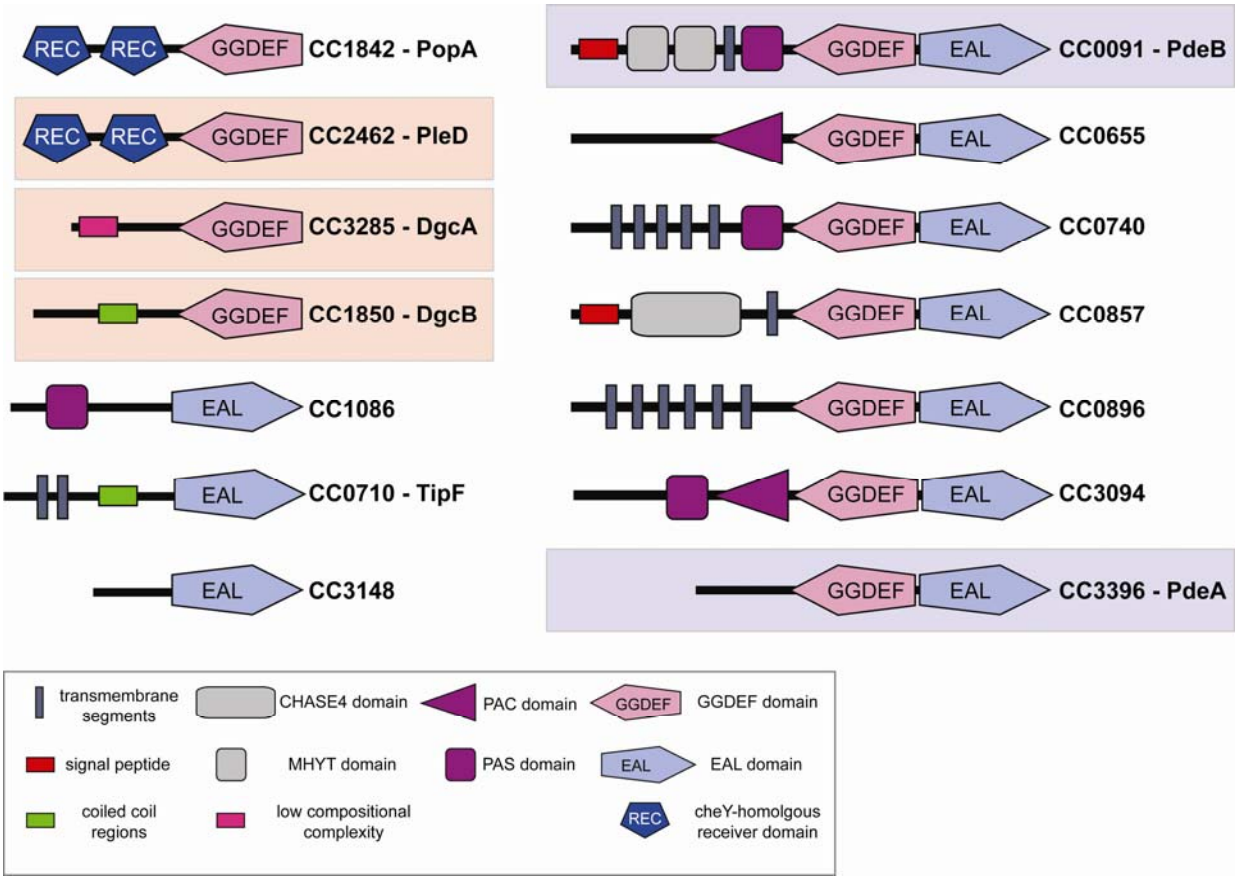


**Figure 1.8**

GGDEF and EAL domain proteins in *Caulobacter crescentus*. Catalytic active DGCs are highlighted in red, active PDEs are shown in blue. Additional domains are explained in the legend.

*C. crescentus* has a total of 11 GGDEF domain proteins, which might all act as DGCs responding to different internal and external signals. But given the fact that c-di-GMP can freely diffuse within the cell, how can one individual DGC or PDE specifically affect one c-di-GMP-mediated cellular function? How can this signaling paradox be explained? One possible explanation might be through a combination of spatial and temporal control. This would argue for distinct c-di-GMP circuits, which are separated in time, through differential expression, and/or in space, through compartmentalization of the signaling proteins, possibly in a complex with its downstream targets (Jenal, 2004; Jenal and Malone, 2006; Ross et al., 1991). Originally, the idea of spatial control of DGCs and PDEs and c-di-GMP as “local pacemakers” came from work on *G. xylinum* by M. Benziman and coworkers. They postulated that in *G. xylinum* DGCs and PDEs together are located close to the cellulose synthase, their regulatory target. C-di-

GMP, which allosterically activates the cellulose synthase, could act as “local pacemaker” to regulate spatially adjacent cellulose synthesizing units (Ross et al., 1991). This is consistent with the recent observation that BcsA, one of the subunits of the cellulose synthase contains a c-di-GMP binding domain (Amikam and Galperin, 2006).

Evidence for localized activity of DGCS and the existence of c-di-GMP microcompartments was provided by extensive studies on the DGC PleD. PleD is targeted to the stalked pole during the swarmer-to-stalked cell transition as a function of its phosphorylation state (Figure 1.9); only phosphorylated and active PleD is localized to the stalked pole, this suggests that this protein very specifically acts at this subcellular site by activating targets localized “next door” (Paul et al., 2007; Paul et al., 2004). Recently, it was shown, that the GGDEF domain protein YdaM and the GGDEF-EAL composite domain protein YciR antagonistically control curli fimbriae in *E. coli*. Together they regulate CsgD, which activates the *dcgBAC* curli operon (Weber et al., 2006).

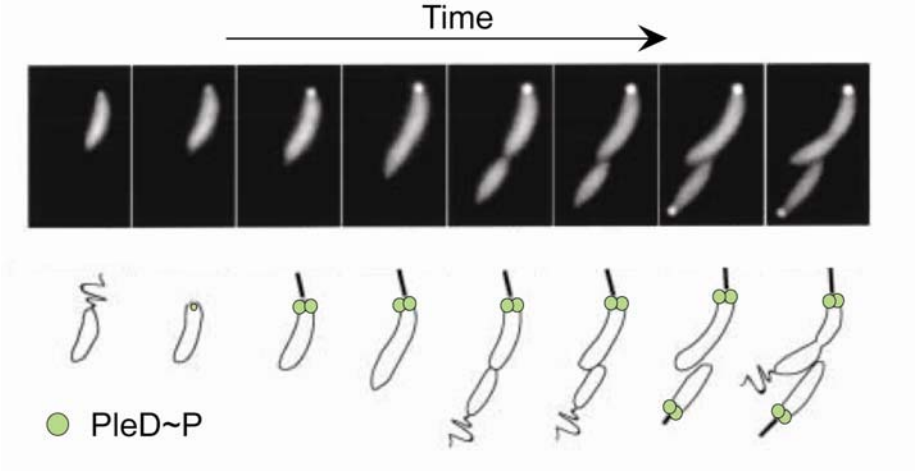


**Figure 1.9**

Polar localization of PleD-GFP to the stalked cell pole during *C. crescentus* cell differentiation. Progression of the cell cycle and positioning of PleD-GFP are indicated schematically. Picture adapted from (Paul et al., 2004).

Spatial control of c-di-GMP signaling molecules might be a universal regulatory mechanism to ensure that individual DGCs and PDEs selectively affect distinct c-di-GMP mediated pathways in microcompartments. Most probably, additional control mechanisms, including tight temporal control of synthesis and/or proteolysis of c-di-GMP signaling components, are needed to avoid cross-talk between different c-di-GMP-dependent regulatory pathways.

# 2 Aim of the thesis

GGDEF and EAL domain proteins are widespread thorough the bacterial kingdom and most species have an average number of 20 to 40 GGDEF and EAL domain proteins. The genome of *Caulobacter crescentus* encodes 14 GGDEF and EAL domain proteins, which might all act as DGCs and PDEs. Apart form the well-characterized *bona fide* DGC PleD, most of these GGDEF and EAL domain proteins have been poorly characterized in regard to their cellular function.

The main aim of this thesis was to analyze possible cellular functions of the 11 GGDEF and GGDEF-EAL composite proteins by a global systematical approach. Single and multiple GGDEF domain deletion mutants were generated and screened for *in vivo* phenotypes in respect to cell cycle control and polar development. This task becomes even more interesting considering the aspect of c-di-GMP as a small molecule, which can freely diffuse within the cell. How is specificity generated? Are there dedicated pairs or groups of specific DGCs and PDEs controlling together one specific downstream pathway? What kind of downstream pathways does c-di-GMP regulate apart from flagellar ejection and holdfast formation during the swarmer-to-stalked cell transition, which is regulated by the DGC PleD?

In a second part, the question of how one individual DGC or PDE specifically can affect one specific c-di-GMP-mediated regulatory function was addressed. To test a possible role of spatial and temporal control, proteins, which showed a phenotype *in vivo*, were analyzed during the cell cycle by means of fluorescence microscopy.

# 3 Results

## 3.1 Second messenger mediated spatiotemporal control of protein degradation during the bacterial cell cycle

# Second messenger mediated spatiotemporal control of protein degradation during the bacterial cell cycle

Anna Dürig, Marc Folcher, Sören Abel, Torsten Schwede, Nicolas Amiot[1], Bernd Giese[1], and Urs Jenal[2]

Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland,
[1]Department of Chemistry, University of Basel, St. Johanns-Ring 19, 4056 Basel, Switzerland

[2]For correspondence: Phone: ++41 (0)61 267 2135; Fax: ++41 (0)61 267 2118; e-mail: urs.jenal@unibas.ch

Short title: c-di-GMP mediated protein degradation control

Key Words: PopA, cyclic di-GMP, CtrA, protein degradation, Caulobacter crescentus, cell cycle, polar localization, second messenger

**Summary:**

Second messengers control a wide range of important cellular functions in eukaryotes and prokaryotes. Here we show that cyclic di-GMP, a global bacterial second messenger promotes cell cycle progression in *Caulobacter crescentus* by mediating the degradation of the replication initiation inhibitor CtrA. During the G1-to-S phase transition both CtrA and its cognate protease ClpXP dynamically localize to the old cell pole where CtrA is degraded. Sequestration of CtrA to the cell pole depends on PopA, a newly identified c-di-GMP effector protein. PopA itself dynamically localizes to cell pole and directs CtrA to this subcellular site via interaction with the RcdA adaptor protein. PopA mutants that are unable to bind c-di-GMP fail to sequester to the cell pole and, as a consequence, to promote CtrA degradation. Thus, c-di-GMP facilitates CtrA degradation during the cell cycle by controlling the dynamic sequestration of the PopA recruitment factor to the cell pole. Finally, we present evidence that CtrA degradation and G1-to-S cell cycle progression rely on converging pathways responsible for substrate and protease localization to the old cell pole. This is the first report that links c-di-GMP to protein dynamics and cell cycle control in bacteria.

## Introduction

Regulated proteolysis has a major impact on cellular physiology as it plays a primordial role in cell cycle control, stress response, and cell differentiation in both pro- and eukaryotes. To avoid unwanted protein destruction eukaryotic cells largely restrict proteolysis to specific cellular compartments. In bacteria several energy-dependent cytoplasmic proteases and their associated factors are responsible for the rapid degradation of a number of key cellular regulators {Gottesman, 2003}{Jenal, 2003}. These so called self-compartmentalizing proteases select their substrates through an ATPase complex, which gates the access to the proteolytic active site. To specifically select target proteins destined for degradation bacteria have evolved a series of regulatory mechanisms, including trans-translation {Keiler, 1996}, interference of small effector molecules {Zhu, 2001}, pre-processing {Alba, 2002}{Kanehara, 2002}, protein association {Gonzalez, 2000}{Johansson, 1999}, or the use of specific targeting factors {Turgay, 1998}. The recent observation that in *Caulobacter crescentus* the master cell cycle regulator CtrA dynamically sequesters to the old cell pole, where it is degraded by the polarly localized ClpXP protease complex, suggested that a spatial concurrence might also play a role in protein degradation control in bacteria {McGrath, 2006}{Iniesta, 2006}.

In *C. crescentus* protein degradation plays a significant role in controlling cell cycle progression {Domian, 1997}{Grünenfelder, 2001}. *Caulobacter* cells divide asymmetrically to produce two distinct daughter cells, a smaller motile swarmer cell and a larger surface adherent stalked cell. Whereas the newborn stalked cell enters S-phase and reinitiates chromosome replication immediately, the chromosome of the swarmer cell

remains quiescent for an extended period, equivalent to the G1-phase of eukaryotic cells. Concurrent with the morphological transformation of the swarmer cell into a stalked cell, the replication block is suspended and cells proceed into S-phase. Differential activity of the essential response regulator CtrA is critical to control the *Caulobacter* G1-to-S phase transition. Phosphorylated CtrA, CtrA~P, blocks the initiation of replication by directly binding to five sites in the chromosomal OriC region where it apparently restricts access of replication initiation factors {Quon, 1998}. The activity of CtrA is redundantly controlled at the levels of expression, phosphorylation, and degradation {Domian, 1997}{Domian, 1999}. Importantly, to initiate chromosome replication activated CtrA~P is eliminated from the cell by two redundant mechanisms, temporally controlled dephosphorylation and proteolysis {Domian, 1997}.

*In vivo* and *in vitro* experiments have demonstrated that the essential ClpXP protease complex degrades CtrA during G1-to-S transition {Jenal, 1998} {Chien, 2007}. The observation that ClpXP rapidly degrades CtrA *in vitro* without the requirement for additional stimulatory factors indicated that control of CtrA degradation might involve an inhibitory mechanism {Chien, 2007}. Moreover, recent findings suggested that cell cycle-dependent degradation of CtrA involves spatial control. Intriguingly, the ClpXP protease complex transiently sequesters to the incipient stalked cell pole during the G1-to-S transition {McGrath, 2006}. At the same time CtrA transiently localizes to the same pole where it is degraded by ClpXP {Ryan, 2004}{McGrath, 2006} (Fig. 1A). Two distinct factors, which themselves sequester to the stalked cell pole, are responsible for the dynamic localization of the protease and its substrate. RcdA, a protein that interacts with CtrA *in vivo* helps to localize CtrA to the pole {McGrath, 2006}. Similarly, polarly

localized CpdR tags ClpXP to the incipient stalked cell pole {Iniesta, 2006}. CpdR is a member of the response regulator family of two-component signal transduction systems that lacks a dedicated output domain and consists of a receiver domain module with a conserved Asp51 phosphoryl acceptor residue. Phosphorylation controls CpdR localization to the cell pole and by that the cellular dynamics of ClpXP {Iniesta, 2006} {Biondi, 2006}. The observation that phosphorylation and localization of CpdR inversely correlate during the cell cycle together with the finding that a CpdRD51A mutant almost exclusively localizes to the pole, suggested that CpdR sequestration and ClpXP recruitment are negatively controlled by phosphorylation {Iniesta, 2006}. Strikingly, phosphorylation of CtrA and CdpR are catalyzed by the same cell cycle phosphorelay comprising the CckA sensor kinase and the ChpT phosphotransferase {Biondi, 2006}. Under conditions where the CckA-ChpT pathway is active, CtrA is activated by phosphorylation and concomitantly appears to be stabilized through the phosphorylation of CdpR and delocalization of ClpXP. Inversely, CckA downregulation would prevent the phosphorylation of CtrA and CpdR and, as a consequence, would lead to ClpXP localization and CtrA degradation {Biondi, 2006}.

These studies suggested that the timing of CtrA degradation during the G1-to-S transition is intimately linked to its dynamic localization to the cell pole. But what are the molecular mechanisms that mediate RcdA and CtrA localization to this subcellular site and how is this event temporally controlled during the cell cycle? Here we propose that the second messenger c-di-GMP critically contributes to temporal and spatial control of CtrA degradation during the *C. crescentus* cell cycle. C-di-GMP has recently been recognized as ubiquitous second messenger in bacteria controlling the transition between

a motile, single-cell state and a sessile, surface-attached biofilm mode in a wide range of organisms {Jenal, 2006}{Tamayo, 2007}. Two opposing enzyme activities, diguanylate cyclase (DGC) and phosphodiesterase (PDE), control the cellular level of c-di-GMP. The DGC and PDE activities are contained within the highly conserved GGDEF and EAL domains, respectively {Paul, 2004} {Christen, 2005}. GGDEF and EAL domains are often associated with sensory input domains and it is assumed that these regulatory proteins serve to directly couple environmental or internal stimuli to a specific cellular response through the synthesis or degradation of c-di-GMP. But how these two enzyme classes are regulated is still largely unclear. DGCs are activated through dimerization of two GGDEF protomers {Paul, 2007} {Wassmann, 2007}. In addition, many DGCs are tightly controlled by product inhibition through the binding of c-di-GMP to an allosteric I-site, which is distinct from the catalytic active A-site {Chan, 2004} {Christen, 2006}.

We have recently shown that *C. crescentus* pole morphogenesis during the swarmer-to-stalked cell transition is controlled by the DGC PleD {Aldridge, 2003} {Paul, 2004} {Levi, 2006}. PleD is an unorthodox member of the response regulator family of two-component signal transduction systems with two receiver domains arranged in tandem fused to a GGDEF output domain {Chan, 2004}. During development PleD is activated by phosphorylation and in response is sequestered to the differentiating pole {Paul, 2004} {Paul, 2007}. The observation that phosphorylation-mediated dimerization not only leads to DGC activation but also to PleD polar localization, suggested a coupling of these two events and a spatially confinement of PleD mediated c-di-GMP signaling to the old cell pole {Paul, 2004} {Paul, 2007}. Here we have analyzed the role of PopA, a PleD paralog with identical Rec1-Rec2-GGDEF domain structure, in *C. crescentus*

development and cell cycle progression. Similar to PleD, PopA is sequestered to the old cell pole. But in contrast to PleD, PopA localization does not require phosphorylation but depends on c-di-GMP binding to the conserved I-site of its GGDEF output domain. We demonstrate that PopA directly interacts with RcdA and helps to recruit both RcdA and CtrA to the cell pole. Based on our data we postulate that upon c-di-GMP binding PopA dynamically sequesters to the old cell pole where it helps to recruit the machinery responsible for cell cycle-dependent degradation of CtrA. This establishes the GGDEF domain as bona fide c-di-GMP effector module and discovers a novel role for c-di-GMP in interfering with the central machinery driving cell proliferation.

## RESULTS

### PopA is a structural homolog of the PleD diguanylate cyclase

In the course of the functional characterization of *C. crescentus* proteins involved in c-di-GMP turnover, we analyzed open reading frame CC1842. This gene codes for a response regulator with two receiver domains and a GGDEF output domain (Fig. 1B). Because of its homology to the diguanylate cyclase PleD {Aldridge, 2003} {Paul, 2004} {Chan, 2004} {Paul, 2007}, CC1842 was renamed *popA* (paralog of *pleD*). Based on this homology relationship, the overall fold of the receiver domains and the GGDEF domain can be expected to be conserved, and a 3-D model of the PopA structure was build using the crystal structure of PleD {Chan, 2004} as template (23% identity; Fig. S1). Despite the low sequence conservation, the modeled PopA structure was similar to PleD (Fig. 1C), suggesting that the overall fold of the receiver domains and the GGDEF domain is conserved. Sequence comparison of PleD and PopA revealed that the phosphoryl acceptor site (Asp55; P-site) of the first receiver domain and the I-site motif (RVED) of the GGDEF domain were conserved, while the catalytic A-site motif was degenerate (Fig. 1B).

### PopA is required for cell cycle dependent degradation of CtrA

A chromosomal *popA* in-frame deletion mutant was generated and analyzed for a number of morphological and cell cycle-associated markers. The mutant strain showed a significantly reduced motility on semisolid agar plates compared to wild type (data not shown), suggesting a specific defect in motor function or in timing of motility during the cell cycle. Moreover, the Δ*popA* mutant failed to degrade the cell cycle regulator CtrA

upon entry into S-phase (Fig. 2A). Cell cycle-dependent degradation of the chemoreceptor McpA, another ClpX substrate {Tsai, 2001}, was not affected (Fig. 2A). Thus, PopA appears to be specifically required for CtrA degradation during the cell cycle. To test if PopA phosphorylation or the GGDEF output domain are required for CtrA degradation, we generated mutations in the conserved P- (D55N) and I-site (R357G), and in the degenerate A-site motif (E368Q) (Fig. 1C). Analysis of CtrA turnover in the $popA_{D55N}$, $popA_{R357G}$, or $popA_{E368Q}$ mutant strains revealed that CtrA was degraded normally in the P- and A-site mutants but stabilized in the I-site mutant (Fig. 2A).

To confirm that PopA interferes with CtrA stability, wild type and Δ*popA* mutant strains were engineered that expressed the *YFP-CtrARD+15* allele from the xylose-inducible promoter $P_{xylX}$. The YFP-CtrARD+15 fusion protein is a fluorescent CtrA derivative, which contains the minimal requirements for cell cycle-regulated proteolysis and polar sequestration {Ryan, 2002}. Cells grown in the presence of xylose were synchronized and released into fresh minimal medium lacking xylose. In wild-type cells both full-length CtrA and YFP-CtrARD+15 were degraded normally during the G1-to-S transition (Fig. 2B, Fig. S2). However, synthesis of the fusion protein did not resume after cells had entered S-phase, confirming that the *yfp-ctrARD+15* allele was not expressed under these conditions (Fig. 2B). In the Δ*popA* mutant the YFP-CtrARD+15 fusion protein was stabilized (Fig. 2B, Fig. S2).

Mutants that are unable to remove active CtrA during the cell cycle show a distinct G1 arrest and cell division block {Domian, 1997}. To test if *popA* mutants display a similar cell cycle arrest, plasmid-borne copies of *ctrA* wild-type and *ctrAD51E*, which codes for a constitutive active form of the regulator {Domian, 1997 #3653}, were

expressed from the xylose inducible promoter. While the expression of *ctrA* or *ctrAD51E* had no effect in *C. crescentus* wild-type cells, the Δ*popA* mutant showed a pronounced cell division block upon induction by xylose (Fig. 2C).

To conclude, these data suggest that PopA is required for the cell cycle-dependent degradation of the CtrA master regulator and that PopA is required to promote the G1-to-S phase transition in *C. crescentus*.

**PopA is required for CtrA and RcdA sequestration to the cell pole**

To analyze at which level PopA interferes with CtrA degradation we first examined the cellular position of the YFP-CtrARD+15 fusion protein in the Δ*popA* mutant. Whereas YFP-CtrARD+15 transiently localizes to the cell poles in *C. crescentus* wild type cells, CtrA foci were not present in stalked or predivisional cells of the Δ*popA* mutant (Fig. 3A). Instead, diffuse fluorescence was observed in all Δ*popA* mutant cells, indicative of a stabilized CtrA fusion protein. These results indicated that PopA is involved in polar sequestration of CtrA. We then asked if PopA was required for the localization of RcdA. As shown in Fig. 3B an RcdA-GFP fusion localizes to the old pole in wild type cells, but fails to sequester to the pole in the Δ*popA* mutant. Based on these results we propose that PopA is positioned upstream of RcdA in the signal transduction cascade leading to cell cycle-dependent degradation of CtrA and that PopA directs CtrA to the cell pole via the localization of RcdA.

Because RcdA interacts with CtrA and ClpX *in vivo* {McGrath, 2006} we examined if PopA also interacts with any of these factors. For this we used the bacterial adenylate cyclase two-hybrid (BACTH) system, which is based on the interaction-mediated reconstruction of a cyclic AMP (cAMP) signaling cascade {Karimova, 1998}. Fusions

between PopA, RcdA, CpdR, CtrA, ClpX, or ClpP and two complementary fragments, T25 and T18, that constitute the catalytic domain of *Bordetella pertussis* adenylate cyclase, were generated in all possible combinations and assayed for cAMP production on maltose MacConkey agar plates. Strong signals indicating interaction were obtained for the following protein pairs: ClpX/ClpX, ClpP/ClpP, ClpX/CpdR, and CpdR/CpdR (data not shown). This is in agreement with earlier results demonstrating ClpX-CpdR interaction by co-immunoprecipitation {Iniesta, 2006} or with ClpX and ClpP forming oligomeric complexes {Wang, 1998} {Kim, 2003}. The observation that CpdR also strongly interacts with itself suggests that this protein is able to form oligomers. In addition, we obtained a strong positive signal for the interaction between PopA and RcdA (Fig. 3C). Interaction with RcdA as measured by the two-hybrid system did not require an intact PopA I-site or A-site (Fig. 3C). Relatively weak but reproducible interaction signals were obtained for PopA/PopA. No interactions were detected between PopA and CtrA (data not shown). This result adds PopA to the protein-protein interaction map of the CtrA degradation machinery as outlined in Fig. S3.

To measure the interaction between PopA and RcdA *in vivo*, we used fluorescence resonance energy transfer (FRET), which relies on the distance-dependent transfer of energy from an excited donor fluorophore to an acceptor fluorophore {Miyawaki, 2000}{Selvin, 2000}{Sourjik, 2002}. We engineered two *C. crescentus* strains containing plasmid-born copies of either *popA-cfp* and *rcdA-yfp* or *popA-yfp* and *rcdA-cfp*. Both strains were used to perform FRET measurements by quantifying the difference of the CFP donor fluorescence before and after specific bleaching of the YFP acceptor (see experimental procedures) {Sourjik, 2002}. For both strains clear differences in CFP

fluorescence intensity were measured, indicative of a direct interaction between the two partners (Fig. 3D). To conclude, we propose that PopA directly interacts with RcdA and by mediating RcdA localization to the old cell pole directs CtrA to this subcellular site during the G1-to-S transition.

**PopA localizes to the new and old cell pole in an RcdA- and ClpX-independent manner**

The observation that PopA directly interacts with RcdA and directs this small protein to the cell pole prompted us to test if PopA itself is sequestered to the *C. crescentus* cell poles where it could act as pole specificity factor for RcdA. To observe the dynamic intracellular position of PopA, we constructed a *popA-egfp* fusion expressed from its own promoter on a low copy number plasmid. The PopA-eGfp fusion protein was fully functional as Δ*popA* mutant cells carrying a plasmid-borne *popA-egfp* allele showed a wild type motility and CtrA turnover phenotype (data not shown). As shown in Figs. 4A and S4, PopA-eGFP localizes to the cell poles throughout the cell cycle. A single focus appeared at one pole of the incipient swarmer cell. Because predivisional cells show a focus at both the stalked and the flagellated pole, we presume that the focus observed in newborn swarmer cells occupies the old flagellated pole. During the G1-to-S transition PopA-eGFP dynamically positions to the new cell pole resulting in a bipolar distribution pattern in stalked and predivisional cells (Figs. 4A, S4, movies S10). It is important to note that in stalked and predivisional cells the fluorescence intensity is different at the two cell poles with stronger foci normally marking the old stalked cell pole. After cell division PopA-eGFP asymmetrically positions to the new pole of the daughter stalked

cell, while the new pole inherited by the swarmer progeny remains unoccupied during most of the G1 phase (Fig. 4A, S4).

Because polar positioning of RcdA requires both ClpX {McGrath, 2006} and PopA (Fig. 3B) we wanted to test if PopA localization also requires one of these factors. As shown in Figs. 4B and 4C, PopA-eGFP localization was unaltered in mutants either lacking RcdA or being depleted for ClpX. Based on this we propose that PopA is at the top of the recruitment and degradation hierarchy for CtrA and that this factor is primarily responsible for the spatiotemporal behavior associated with CtrA degradation during the cell cycle.

**PopA localization to the old cell pole requires an intact c-di-GMP binding site**

If PopA alone is responsible for the temporal and spatial control of RcdA and CtrA upon entry into S-phase one would expect that the dynamic sequestration of the three proteins to the cell pole more or less coincides. However, PopA localizes to the new pole long before RcdA and CtrA are sequestered to the same subcellular site. It is possible that PopA works in conjunction with (an) additional factor(s) responsible for temporal control of CtrA degradation. Alternatively, the cellular dynamics and specificities of PopA might be more complex and thus not apparent by analyzing its overall distribution in wild type cells. PopA is a bifunctional protein involved in motility and cell cycle-dependent degradation of CtrA and could for instance have different function-specific addresses in the cell. To test this possibility we analyzed the molecular basis of PopA sequestration to the cell poles. The observation that in the $popA_{R357G}$ I-site mutant RcdA failed to mediate CtrA degradation even though $PopA_{R357G}$ was still able to interact with RcdA, suggested

that an intact I-site might be required for polar localization of PopA rather than for the subsequent recruitment of RcdA. To test this hypothesis, low copy number plasmids containing the mutant alleles *popA*$_{D55N}$-*egfp* (P-site), *popA*$_{R357G}$-*egfp* (I-site), and *popA*$_{E368Q}$-*egfp* (A-site) were constructed and were introduced into the *C. crescentus* wild type and Δ*popA* mutant strains. In accordance with their wild type-like CtrA degradation behavior, both PopA P- and A-site mutants showed a localization pattern indistinguishable from PopA wild type (Figs. 5, S5). In contrast, PopA$_{R357G}$-eGfp showed a characteristic unipolar localization pattern (Figs. 5, S5B). Noticeably, the PopA I-site mutant failed to localize to the stalked cell pole but was still able to sequester to the opposite pole of the cell. As a result of PopA$_{R357G}$-eGfp loss from the stalked cell pole an increased diffuse fluorescence was observed in all cells. Because of the asymmetric positioning of PopA$_{R357G}$-eGfp in predivisional cells, newborn swarmer cells inherited a fluorescent focus at the old flagellated pole. During the G1-to-S transition PopA$_{R357G}$-eGfp was rapidly lost from the old pole after 20 to 40 minutes and appeared at the opposite new pole after 40 to 60 minutes (Fig. S5B). The relatively high number of cells with no detectable polar PopA$_{R357G}$-eGfp focus at the beginning of S-phase coincides with the fading of the fluorescent signal at the old pole and the subsequent appearance of a fluorescent focus at the new pole (Fig. S5B). However, as polar signals are relatively weak at this stage of the cell cycle and can easily be missed by selecting the wrong focal plane during data acquisition, the number of cells without polar foci is most likely overestimated.

In summary, the PopA$_{R357G}$-eGfp mutant appears to specifically recognize the new pole of the cell but disappears from this site as cells undergo the G1-to-S transition at a

time corresponding to RcdA and CtrA recruitment to the old pole. Based on this we propose that I-site specific binding of c-di-GMP is required for spatiotemporal control of PopA during the cell cycle and that ligand binding either sequesters PopA to the old stalked pole or retains pre-localized protein at this subcellular site during cell differentiation. In agreement with this, the C-terminal GGDEF domain is required for polar localization of PopA as both a Rec1-Rec2-eGfp and a Rec1-eGfp fusion failed to localize to the cell pole (Fig. S6, Tab. S1).

The localization pattern of $PopA_{R357G}$-eGfp suggested that an additional mechanism is required to sequester PopA to the new cell pole. Because the motility defect of the *ΔpopA* mutant is similar to the phenotype described for a *podJ* mutant {Wang, 1993 #2360} and because PodJ functions as a swarmer pole-specific protein localization factor {Viollier, 2002 #4465}, we next analyzed if PopA localization to the new cell pole was dependent on PodJ. Similar to a PopA I-site mutant, PopA wild type showed a unipolar pattern in a Δ*podJ* mutant. But unlike $PopA_{R357G}$-eGfp, PopA-eGFP primarily localized to the pole opposite the stalk under these conditions (Fig. 5, Tab. S2). Moreover, when the I-site mutant $PopA_{R357G}$-eGfp was analyzed in the Δ*podJ* mutant the polar foci were replaced by a strong diffuse fluorescence throughout the cell (Fig. 5, Tab. S2). Based on these results we conclude that PodJ is responsible for PopA recruitment to the new cell pole where it might engage in motility-specific functions. Together these data suggested that PopA has two function-specific addresses in the cell. While PodJ directs the protein to the new cell pole, binding of c-di-GMP to the PopA I-site is responsible for PopA recruitment to or retention at the ClpXP-occupied old pole during the G1-to-S transition.

**PopA lacks DGC activity but binds c-di-GMP specifically and with high affinity**

The phenotype of the *popA*$_{R357G}$ mutant indicated that the conserved I-site plays an important role in temporal and spatial control of PopA. More specifically, these data suggested that PopA specifically binds c-di-GMP at the I-site and, in response, alter its dynamic cellular behavior. Because the I-site was originally identified as an allosteric binding site of the GGDEF domain that regulates diguanylate cyclase activity {Chan, 2004} {Christen, 2006} {Wassmann, 2007}, we first analyzed if PopA, despite its degenerate A-site, shows enzymatic activity. A hexahistidine-tagged version of PopA was purified and used for DGC *in vitro* activity assays {Paul, 2004} {Christen, 2006}. Because DGCs are active as a dimers, which are able to form spontaneously at high protein concentrations ($K_d$ of 100 μM) {Wassmann, 2007} {Paul, 2007} we assayed PopA at increasing concentrations. However, PopA failed to show DGC activity even at the highest protein concentrations used (data not shown). This is in agreement with the observation that most amino acid changes in the highly conserved GGDEF signature motif abolished enzyme activity of an active DGC {Malone, 2006}.

Next we used a UV crosslink assay {Christen, 2006} with radiolabeled c-di-GMP to assay ligand binding of PopA. The following proteins were purified and analyzed: PopA wild type, PopA$_{E368Q}$ (A-site mutant), and PopA$_{D357G}$ (I-site mutant). While both PopA wild type and PopA$_{E368Q}$ bound c-di-GMP, ligand binding was abolished in the I-site mutant protein PopA$_{D357G}$ (Fig. 6A). Binding of radiolabeled ligand was then assayed in the presence of increasing concentrations of non-labeled c-di-GMP to determine binding affinity. As shown in Figs. 6B and 6C, PopA binds c-di-GMP with a $K_d$ of about 2 μM. Other nucleotides like GTP or GDP were not able to chase radiolabeled c-di-GMP

suggesting that binding of c-di-GMP to PopA is highly specific (data not shown). Together this suggested that PopA is a *bona fide* c-di-GMP binding protein and that it exploits the conserved I-site to modulate its own activity and cellular behavior in response to fluctuating levels of c-di-GMP.

**CpdR and PopA pathways converge leading to cell cycle-dependent degradation of CtrA.**

To analyze if PopA, in addition to its role in RcdA and CtrA sequestration, is also involved in the recruitment of the ClpXP protease to the cell pole, we assayed ClpX localization during the *C. crescentus* cell cycle. As reported previously {Iniesta, 2006 #4951}, in *C. crescentus* wild type ClpX localizes to the old cell pole during the G1-to-S transition, coinciding with CtrA degradation (Figs. 7, 7S; note that the cell timing in Figs. 7 and 7S is identical to the experiments shown in Fig. 2A). ClpX localization, although not completely abolished, was significantly impaired in a Δ*popA* mutant (Figs. 7, 7S). Importantly, a *popA* P-site mutant (*popAD55N*) showed an equally impaired ClpX localization (Figs. 7, 7S) arguing that PopA is being phosphorylated *in vivo* and that phosphorylation of PopA, directly or indirectly, influences ClpX localization. Reduction of ClpX localization in *popA* mutants was associated with a similar reduction in the localization of the ClpXP targeting factor CpdR (Tab. 1). In contrast, localization of $CpdR_{D51A}$, a constitutively active mutant that can no longer be phosphorylated, is not reduced in *popA* mutants (Fig. 8A, B). Thus, we conclude that PopA interferes with the polar recruitment of the ClpXP protease by stimulating polar sequestration of CpdR in a phosphorylation-dependent manner.

The observation that the ClpXP protease and its substrate CtrA have distinct targeting factors raised the questions if these two polar recruitment pathways converge and if they are ultimately responsible for the timing of CtrA degradation during the G1-to-S transition. To test this we analyzed CtrA stability in *popA* mutants that also carried a *cpdR*$_{D51A}$-YFP mutant allele. The CpdR$_{D51A}$ mutant more effectively localizes to the cell pole as compared to wild type CpdR and to cause an increased CtrA turnover via a more efficient polar recruitment of the ClpXP protease {Iniesta, 2006}. In agreement with this, we find severely reduced levels of CtrA in cells expressing the *cpdR*$_{D51A}$ (Fig. 8C). This was due to increased degradation of CtrA, as normal CtrA levels were restored in cells co-expressing the stable variant CtrA::Ω (Fig. 8C). Surprisingly, while cells carrying the *cpdR*$_{D51A}$ allele showed a severe filamentation and bulging phenotype in a *popA* wild type background, they had a normal morphology in a Δ*popA* mutant (Fig. 8A, B). This effect was not due to reduced localization of CpdR$_{D51A}$-YFP in the *ΔpopA* mutant (Fig. 8B). Strikingly, in the Δ*popA* null mutant or the *popA* I-site mutant (R357G) normal CtrA levels and cell morphology was restored even when the expression of the *cpdR*$_{D51A}$ allele was induced (Fig. 8C).

In summary, these data provide evidence that the CpdR-ClpXP and PopA-RcdA-CtrA localization pathways converge and that CtrA degradation is mediated through the concomitant dynamic localization of these factors to the old cell pole during the G1-to-S transition.

## DISCUSSION

In *C. crescentus* G1-to-S cell cycle progression is mediated by the irreversible destruction of the master regulator CtrA. To understand how this event is regulated and to elucidate

the general control mechanisms operating during the bacterial cell cycle we have examined the spatiotemporal behavior of CtrA degradation. CtrA is degraded by the ClpXP protease complex {Jenal, 1998} {Chien, 2007}, which dynamically positions to the old cell pole coincident with CtrA turnover {McGrath, 2006}. The observation that CtrA itself sequesters to the same pole before being degraded suggested that the timing of CtrA degradation might be dictated by a dynamic spatial convergence of substrate and protease at this subcellular site {Ryan, 2004}{McGrath, 2006}. Here we present evidence that the timing of CtrA degradation is ultimately mediated by the bacterial second messenger c-di-GMP via the dynamic polar localization of a c-di-GMP specific binding protein, PopA. Our data suggest that PopA, in its c-di-GMP ligated form, is sequestered to the cell pole where it acts as polar recruitment factor for CtrA. This is the first report that links c-di-GMP to the dynamic spatiotemporal control of the bacterial cell cycle and is reminiscent of the function of eukaryotic second messengers in cell polarity and behavior {Janetopoulos, 2005} {Insall, 2007} {Evans, 2007 }.

**PopA is responsible for polar recruitment of CtrA during the G1-to-S transition**

CtrA localization to the cell pole is mediated by RcdA, a small stalked pole-specific protein that interacts with CtrA and ClpXP *in vivo* {McGrath, 2006 #4883}. We show here that RcdA also interacts with PopA, a GGDEF domain protein required for CtrA degradation and RcdA localization. The strong positive signals observed with the bacterial two-hybrid system and by FRET analysis, indicated that PopA and RcdA interaction is direct. Furthermore, epistasis experiments positioned PopA upstream of RcdA. Thus, we propose that PopA directs CtrA to the cell pole via its interaction with

RcdA, which in turn might play an intermediary role between CtrA and PopA (Fig. 9A). It is important to note that *in vitro* experiments have argued against the idea that RcdA enhances CtrA degradation by tethering the substrate to its protease {Chien, 2007}. However, RcdA could contribute to the timing of CtrA polar localization and/or confer substrate specificity to the cellular machinery that recruits proteins destined for degradation to the ClpXP tagged cell pole.

But if PopA is at the top of the cascade that determines CtrA sequestration and degradation, what controls its activity during the cell cycle? We found that PopA, like the other components involved in CtrA degradation, dynamically localizes to the *C. crescentus* cell poles. PopA localization control appears to be complex in that the protein is sequestered to both the new and the old cell poles at distinct times of the cell cycle. Remarkably, PopA localization to these two subcellular sites relies on distinct mechanisms and might serve two distinct cellular functions. PopA localization to the incipient swarmer pole requires PodJ, a cell polarity determinant that also recruits the PleC histidine kinase/phosphatase and components of the pili assembly machinery to the flagellated pole {Viollier, 2002}{Hinz, 2003}{Lawler, 2006}. Upon cell division full-length PodJ, $PodJ_L$, is processed into a truncated form, $PodJ_S$, which is needed for chemotaxis of the newborn swarmer cell {Wang, 1993}{Viollier, 2002}{Lawler, 2006}. Localization of PopA to the swarmer pole requires the cytoplasmic portion of $PodJ_S$ (A. Moser and U. Jenal, unpublished). Hence, $PodJ_S$ mediated targeting of PopA to the flagellated pole might be important for the proper functioning of the flagellar motor. Although PodJ is cleared from the cell pole during the G1-to-S transition {Chen, 2006 #4809}, PopA persists at the incipient stalked pole. This strongly suggested the existence

of a second, PodJ-independent polar localization mechanism for PopA, which is specific for the incipient stalked cell pole and for the CtrA degradation pathway. In support of this, a PopA I-site mutant ($PopA_{D357G}$) was able to localize to the swarmer pole but failed to localize to the stalked cell pole independent of the presence or absence of PodJ.

Several experiments suggested that for CtrA sequestration and degradation the important functional element of the PopA GGDEF output domain is not the catalytic active A-site, but rather the conserved I-site. First, PopA sequestration to the incipient stalked cell pole required an intact I-site, but not the P- or A-site. Second, PopA lacks the highly conserved GGDEF signature motif and biochemistry experiments failed to detect PopA DGC activity, even when high protein concentrations were used. This is consistent with the finding that the catalytic activity of DGCs requires a highly conserved GGDEF active site {Malone, 2006}{Wassmann, 2007}, and argues that PopA is not involved in the synthesis of c-di-GMP. Third, PopA is a c-di-GMP binding protein. Binding studies with PopA wild type and mutant proteins demonstrated that the protein is able to specifically bind c-di-GMP with high affinity and that an intact I-site, but not the A-site, is required for binding. The binding affinity ($K_d$ 2 μM) is similar to the affinities determined for the allosteric I-sites of two enzymatically active DGCs {Chan, 2004} {Christen, 2006} {Wassmann, 2007}. It has been proposed that product inhibition of DGCs represents a major control element for c-di-GMP signaling establishing threshold levels of the second messenger in the cell {Christen, 2006}. From this it can be inferred that PopA binds c-di-GMP in a physiologically relevant concentration range {Christen, 2006}. The observation that an intact I-site is required for PopA sequestration to the stalked cell pole, but not for protein-protein interaction with RcdA, argues that c-di-GMP

binding specifically influences the timing of PopA sequestration to the old cell pole, rather than its interaction with downstream components.

If a transient increase of c-di-GMP during the G1-to-S transition is responsible for the timing of PopA, RcdA and CtrA localization, cell cycle control must be mediated by one or several DGCs and/or PDEs. We have recently shown that c-di-GMP levels peak during the G1-to-S transition and that the PleD diguanylate cyclase is mainly responsible for this fluctuation (R. Paul and U. Jenal, unpublished). However, PopA localization was unaltered in a *pleD* mutant. Also, PopA localization was not affected in mutants lacking any of the other 12 *C. crescentus* proteins harboring a GGDEF or EAL domain (S. Abel and U. Jenal, unpublished). This leaves the possibility that several DGCs redundantly contribute to the c-di-GMP pool required for PopA activation. It is also possible that cell cycle timing of c-di-GMP levels and PopA localization to the stalked cell pole might ultimately be determined by a c-di-GMP specific phosphodiesterase, which specifically reduces c-di-GMP levels in G1.

**A possible mechanism for c-di-GMP mediated PopA localization to the cell pole**

PopA and the diguanylate cyclase PleD show a similar dynamic localization to the incipient stalked cell pole. But do they also take advantage of a similar localization mechanism? PleD localization and activation during the swarmer-to-stalked cell transition requires phosphorylation-mediated dimerization {Paul, 2004} {Paul, 2007} {Wassmann, 2007}. Based on the finding that the ability to dimerize is critical for PleD

activation and polar localization we have proposed a simple model for the coupling of PleD DGC activity to its subcellular distribution {Paul, 2007}. The model predicts that the timing of PleD polar localization during the cell cycle is determined by phosphorylation-dependent dimerization. Despite of its conserved phosphoryl acceptor site, PopA phosphorylation does not appear to be required for polar localization. PopA and PleD share the same Rec1-Rec2-GGDEF domain structure but are only 23% identical. Intriguingly, the amino residues that contribute to the Rec1-Rec2 interdomain interface in activated PleD dimers {Wassmann, 2007} are strictly conserved in PopA (Fig. S1). *In vivo* interaction (Fig. S3) and biochemical experiments (A. Moser and U. Jenal, unpublished) suggested that PopA can oligomerize. This raises the possibility that PopA oligomerization also influences its dynamic cellular behavior. But how would c-di-GMP binding affect PopA oligomerization? Atomic simulations of ligated and unligated PleD have suggested reduced flexibility of all three domains upon c-di-GMP binding to the I-site. Strikingly, simulations found stronger correlations between D1 and D2 for unligated PleD, which may affect the dimerization rate {Schmid, 2007}. Consistent with the idea that I-site occupancy negatively influences PleD dimerization, mutation of two residues of the Rec2 domain involved in c-di-GMP binding displayed a 20-fold higher DGC activity compared with wild-type PleD {Christen, 2006}. Hence, it is possible that binding of c-di-GMP to the I-site affects the oligomerization behavior of PleD and PopA in a similar manner and by that influences the dynamic positioning of these proteins during the cell cycle.

**Converging localization pathways for substrate and protease mediate cell cycle-dependent degradation of CtrA**

Two response regulators, CpdR and PopA, are involved in directing the protease ClpXP and its substrate CtrA to the emerging stalked cell pole. Whereas PopA is responsible for the transient localization of CtrA, CpdR controls ClpX localization. CpdR and ClpX polar recruitment depends on the phosphorylation state of CpdR {Iniesta, 2006}. In particular, in the presence of $CpdR_{D51A}$, a mutant that can no longer be phosphorylated, the proportion of cells with ClpX and CpdR at the cell pole is dramatically increased and, as a result, cellular levels of CtrA are severely reduced {Iniesta, 2006} (Fig. 8). Expression of $CpdR_{D51A}$ also results in a severe cell morphology and cell growth phenotype. The finding that cell morphology, viability, and CtrA levels are restored in *popA* mutant cells expressing $cpdR_{D51A}$ argues that this phenotype is a direct consequence of reduced levels of CtrA and possibly additional ClpXP substrates. The latter can be inferred from the observation that the effect of $cpdR_{D51A}$ is not completely abolished in cells expressing a stable CtrA variant. In contrast, the expression of a PopA I-site mutant that fails to localize to the stalked cell pole fully suppresses the $cpdR_{D51A}$ phenotype. Together this argues that the two pathways responsible for the polar localization of substrate and protease converge and together are responsible for the accurate cell cycle timing of CtrA degradation (Fig. 9A). In addition, the convergent CpdR and PopA pathways might be interlinked. We found that PopA also contributes to CpdR and ClpX localization. In *ΔpopA* mutants CpdR and ClpX frequently mislocalize. This effect is most pronounced during G1-to-S transition, when CtrA is being degraded. Intriguingly, a PopA mutant lacking the conserved phosphoryl acceptor site Asp55 showed the same localization defect for CdpR and ClpX arguing that this effect is somehow mediated

through PopA phosphorylation. Although we failed to provide evidence for a direct interaction between PopA and CpdR, we cannot exclude that PopA is part of a macromolecular complex at the stalked cell pole thereby contributing to CpdR and ClpX localization. Alternatively, mislocalisation of CdpR and ClpX in *popA* mutants could stem from increased phosphorylation of CpdR under these conditions. The observation that $CpdR_{D51A}$ localizes normally to the stalked cell pole even when PopA is absent (Fig. 8) is in line with this hypothesis and argues against a direct involvement of PopA in CpdR localization. Thus, we propose that PopA activity and polar localization, in addition to being stimulated by c-di-GMP binding to the I-site of its output domain, is modulated negatively by phosphorylation of the first receiver domain. Such a mechanism would allow PopA to integrate distinct signals from the cell cycle via phosphorylation and c-di-GMP binding. Recently, the CckA-ChpT phosphorelay was shown to phosphorylate both CtrA and CpdR in response to cell cycle cues {Biondi, 2006}. This elegant mechanism allows the cells to inversely control CtrA activation and degradation during the cell cycle. It is possible that the CckA-ChpT pathway controls CtrA stability not only by turning off CpdR and preventing ClpXP localization, but in parallel downregulates CtrA recruitment through the phosphorylation of PopA.

## EXPERIMENTAL PROCEDURES

*Strains, plasmids, and media*

The bacterial strains and plasmids used in this study are listed in Table S1. *Caulobacter crescentus* strains were either grown in peptone yeast extract (PYE), in minimal glucose

media (M2G, (Ely, 1991), or minimal xylose media (M2X) at 30$^{o}$C, unless stated otherwise. Where necessary, growth medium was supplemented with D-xylose varying from 0.1-0.3%. Newborn swarmer cells (SW cells) were isolated by Ludox gradient centrifugation (Jenal and Shapiro, 1996), and released into the appropriate minimal medium. Plasmids were introduced into *C. crescentus* either by conjugation or electroporation.

*E. coli* strains were grown in Luria Broth (LB) media. Antibiotics for selection were added to the media where necessary. The exact procedure of strain and plasmid construction is available on request.

*Microscopy*

For fluorescence imaging cells were placed on a microscope slide layered with a pad of 1% agarose dissolved in water or in PYE for time laps microscopy. An Olympus IX71 microscope equipped with an UPlanSApo 100x/1.40 Oil objective (Olympus, Germany) and a coolSNAP HQ (Photometrics, AZ, United States) CCD camera was used to take differential interference contrast (DIC) and fluorescence photomicrographs. For GFP fluorescence FITC filter sets (Ex 490/20 nm, Em 528/38 nm), for YFP (Ex 500/20 nm, Em 535/30 nm) and for CFP (Ex436/10, Em 470/30 nm) were used with an exposure time of 1.0 sec. Images were processed with softWoRx v3.3.6 (Applied Precision, WA, United States) and Photoshop CS2 (Adobe, CA, United States) softwares.

*Bacterial Two-Hybrid Analysis*

Proteins of interest were fused in frame to the 3'end of the T25 fragment (pKT25) and to the 3' end (pUT18C) or 5' end (pUT18) of the T18 fragment of the *B. pertussis* adenylate cyclase {Karimova, 1998 #4940}. pKT25-zip and pUT18C-zip were used as positive controls. The adenylate cyclase deficient *E. coli* strain MM337 was used to screen for positive interactions. pKT25 derivatives were transformed together with pUT18 or pUT18C derivatives into MM337 and the transformants selected on LB with ampicillin (100 ug/ml) and kanamycin (50 ug/ml). To screen for protein-protein interaction single colonies were streaked on McConkey Agar Base supplemented with maltose (1%), ampicillin (100 μg/ml) and kanamycin (50 μg/ml).

*Protein Expression and Purification*

Expression plasmids (pET21C, Novagen) were transformed into *E. coli* BL21 (DE3) ArcticExpress (Stratagen). The strains were grown in LB with ampicilin 100 μg/l at 17°C. Expression was induced with IPTG (0.1 mM) over night. Cells were collected by centrifugation, resuspended in cold sonication buffer SB (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM imidazole). Cells were disrupted by sonication using a Branson Sonifier and cell debris were removed by centrifugation. The clear lysate was incubated for 1 h with 1 ml Ni-NTA agarose (Qiagen). The matrix was washed with SB containing 250 mM NaCl and proteins were eluted with SB buffer containing 50 mM and 250 mM imidazole, respectively. Proteins were dialyzed against 20 mM Tris pH 8, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 % glycerol and dialyzed fractions were concentrated using Amicon ultrafiltration cell. The concentrated proteins were further purified by gel filtration using a Superdex 75 PC 3.2 /30 column on Smart system (GE

healthcare) equilibrated with 20 mM Tris pH 8, 250 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. The monomer fractions were collected and used for UV cross-linking assay.

*Antibody Production and Immunoblots*

PopA fused to a C-terminal hexa-histidine tag was purified as described above and injected into rabbits for polyclonal antibody production (Laboratoire d'Hormonologie, Marloie, Belgium). For immunoblots anti-PopA serum was diluted 1:5'000. Antibodies against CtrA and McpA were used as described (Domian et al., 1997; Tsai and Alley, 2000).

*Fluorescence Resonance Energy Transfer*

Cultures of strains UJ4329 and UJ4330 were grown in PYE supplemented with 2.5 μg/ml tetracycline and 2.5 μg/ml gentamycin until they reached an OD660 of 0.3. 5 ml of this culture were harvested by centrifugation, washed with and resuspended in 50 μl tethering buffer (10mM potassium phosphate, 0.1mM EDTA, 1 mM L-methionine, 10mM sodium lactate, pH 7). For FRET analysis the cell suspension was placed on thin agarose pads (1 % agarose in tethering buffer) on microscopy slides and allowed to immobilise for 5–10 min. The agarose pads were then covered with cover slips and were sealed with an Apiezon grease. Fluorescence of 300-500 cells was monitored in each experiment. Fluorescence signals in cyan and yellow channels were detected using two photon-counting photomultipliers (H7421-40, Hamamatsu,Bridgewater, NJ) whose outputs were converted to analog signals by ratemeters (RIS-375, Rowland Institute).

*UV Cross-linking with [$^{33}$P] c-di-GMP*

The $^{33}$P-labeled c-di-GMP was enzymatically produced using [$^{33}$P]GTP (300 Ci/mmol) and purified as described (Christen et al., 2006). Purified protein samples were incubated 10 min on ice in reaction buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 10 mM $MgCl_2$, and 5 mM β-mercaptoethanol) together with 1 μM c-di-GMP and $^{33}$P-radiolabeled c-di-GMP (0.75 μCi, 6000 Ci/mmol). Samples were then UV irradiated and analyzed as previously described (Christen et al., 2006). The c-di-GMP binding constant of PopA was determined as described previously (Christen et al., 2006).

*Comparative Modeling of PopA*

A comparative three-dimensional model of *Caulobacter crescentus* PopA was built based on the crystal structure of the response regulator PleD in complex with c-di-GMP (PDB: 1W25; (Chan et al., 2004)). Template identification and alignment was performed by scanning the PDB database {Berman, 2000 #4973} for suitable template structures using a PSI-BLAST (Altschul et al., 1997) sequence profile for the target based on the NCBI non-redundant protein sequence database {Wheeler, 2007 #4974}. Model coordinates were generated in Swiss-Model Workspace following visual assessment of placements of insertions and deletions in the alignment {Schwede, 2003 #4975}. The orientation of c-di-GMP in the model was inferred from the PleD - c-di-GMP template structure complex.

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## FIGURE LEGENDS

**Figure 1: Dynamic protein localization and CtrA degradation during the cell cycle.**

(A) Schematic of CtrA, ClpXP, RcdA, and CpdR localization during the *C. crescentus* cell cycle. (B) Sequence alignment of the PleD and PopA paralogs. The amino acid sequence flanking the phosphoryl acceptor site (P-site), I-site, and A-site are shown with the conserved residues colored in red and the signature motifs boxed. (C) Comparison of the 3-D structure of the GGDEF domains of PleD (as determined by x-ray crystallography (Chan et al., 2004)) and PopA (as determined by modeling). A- and I-sites are marked and the position of a dimer of c-di-GMP bound to the I-site is indicated. In the PleD crystal structure a c-di-GMP monomer is found in the A-site (Chan et al., 2004).

**Figure 2: Cell cycle dependent degradation of CtrA requires PopA.**

(A) Immunoblots of synchronized cultures of *C. crescentus* wild type and *popA* mutant strains. The upper panels show immunoblots stained with anti-CtrA antibodies, immunoblots shown in the lower panels were stained with anti-McpA antibodies. (B) Synchronized swarmer cells of strains expressing *yfp-ctrARD+15* from the xylose-inducible promoter $P_{xyl}$ were released into M2G minimal glucose medium and monitored throughout the cell cycle. Samples of *C. crescentus* wild type (upper panel) and *popA* mutant (lower panel) were analyzed by immunoblots using anti-CtrA antibodies. The YFP-CtrARD+15 fusion protein and wild type CtrA are marked. (C) Morphology of *C. crescentus* wild type and Δ*popA* mutant expressing *ctrA* (left panels) or $ctrA_{D51E}$ (right

panels) from the xylose inducible promoter $P_{xyl}$. Cells were harvested under inducing (xylose) or non-inducing (glucose) conditions and analyzed by light microscopy.

**Figure 3: PopA mediates polar localization of RcdA and CtrA.**

(A) CtrA localization to the cell pole requires PopA. Wild-type and Δ*popA* mutant cells expressing *yfp-ctrARD+15* were analyzed by DIC and fluorescence microscopy. Polar foci of Yfp-CtrARD+15 are marked by arrows and shown schematically in the right panel. (B) RcdA localization to the cell pole requires PopA. Wild type and Δ*popA* mutant cells expressing *rcdA-yfp* were analyzed by DIC and fluorescence microscopy. Polar foci of RcdA-Yfp are shown schematically in the right panel. (C) PopA directly interacts with RcdA. The red color on McConkey agar base maltose plates is an indicator for protein-protein interaction. 1) pT18-zip + pT25-zip (positive control); 2) pT25-PopA + pT18-RcdA; 3) pT25-PopA + pT18; 4) pT25-$PopA_{E368Q}$ (A-site mutant) + pT18-RcdA; 5) pT25-$PopA_{E368Q}$ (A-site mutant) + pT18; 6) pT25-$PopA_{R357G}$ (I-site mutant) + pT18-RcdA; 7) pT25-$PopA_{R357G}$ (I-site mutant) + pT18, 8) pT25 + pT18-RcdA. (D) In *vivo* FRET analysis demonstrating direct protein-protein interaction between PopA and RcdA. *C. crescentus* cells expressing *rcdA-ecfp* and *popA-eyfp* (left panel) or *popA-ecfp* and *rcdA-eyfp* (right panel) were analyzed. The intensity of the CFP channel was recorded before and after YFP-specific bleaching. The increase of signal intensity of the CFP channel after specific bleaching of the YFP channel is a measure of the FRET efficiency (%).

**Figure 4: PopA localizes to the old and new cell poles.**

(A) PopA dynamically localizes to the old and new cell pole during the *C. crescentus* cell cycle. Representative time-laps experiment with *C. crescentus* wild-type expressing *popA-egfp*. DIC images (top), fluorescent images (middle), and a schematic representation (bottom) are shown. (B) RcdA is dispensable for PopA localization to the cell poles. Mixed cultures of *C. crescentus* wild type and Δ*rcdA* mutant cells expressing *popA-egfp* were analyzed by DIC and fluorescence microscopy. The polar localization pattern of PopA-eGFP was indistinguishable in the two strains. (C) ClpX is dispensable for PopA localization to the cell poles. Mixed cultures of the *C. crescentus* conditional *clpX* mutant strain UJ271 expressing *popA-egfp* were analyzed by DIC and fluorescence microscopy under permissive (PYEX) and restrictive conditions (PYEG). The polar localization pattern of PopA-eGFP was indistinguishable under these conditions.

**Figure 5: Distinct mechanisms mediate PopA localization to the new and old pole.**

*C. crescentus* wild type and *podJ* mutant expressing GFP fusion proteins to PopA wild type and the following PopA mutants were analyzed by DIC and fluorescence microscopy: $PopA_{D55N}$ (P-site mutant), $PopA_{E368Q}$ (A-site mutant), $PopA_{R357G}$ (I-site mutant). Polar localization is indicated schematically in the panels on the right.

**Figure 6: PopA specifically binds c-di-GMP at the conserved I-site of the GGDEF domain.**

(A) UV crosslink experiment of purified hexahistidine-tagged PopA with [$^{33}$P] labeled c-di-GMP. The following proteins were used: PopA, PopA$_{E368D}$ (A-site mutant), PopA$_{R357G}$ (I-site mutant). The Coomassie blue stained gel (left) and the autoradiograph (right) are shown. (B) UV crosslinking of purified PopA with [$^{33}$P] labeled c-di-GMP and increasing concentrations of non-labeled c-di-GMP (0 – 80 μM). Coomassie blue-stained gel (top panel) and Autoradiograph (bottom panel) are shown.

**Figure 7: PopA is required for proper localization of ClpX to the cell pole.**

Cultures of *C. crescentus* wild type and *popA* mutants expressing a *clpX-egfp* were synchronized and cells were analyzed by DIC and fluorescence microscopy as they progressed through the cell cycle. At intervals, cells were scored for polar localization of ClpX. The upper panel shows the ratio of cells with a polar ClpX-GFP focus. The lower panel shows the ratio of cells with mis- or delocalized ClpX-GFP. The timing of cell cycle progression is equivalent to the experiments shown in Fig. 2A.

**Figure 8: CpdR and PopA constitute two converging pathways leading to cell cycle-dependent degradation of CtrA.**

Cultures of *C. crescentus DcpdR* single (A) and *DcpdRDpopA* double mutants (B) expressing *cpdR$_{D51A}$-yfp* under the control of the xylose-inducible promoter P$_{xyl}$, were grown in the presence of xylose and analyzed microscopically. (C) Cultures of *C. crescentus DcpdR* single (left panels) and *DcpdRDpopA* double mutants (right panels)

expressing *cpdR*$_{D51A}$*-yfp* under the control of the xylose-inducible promoter $P_{xyl}$, were grown in the presence (PYEX) or absence of xylose (PYEG) and analyzed by immunoblots using anti-CtrA (upper panels) and anti-CC1850 (lower panels) antibodies, respectively.

**Figure 9: Model for the role of PopA in cell cycle-dependent degradation of CtrA.**

(A) Converging pathways involved in polar sequestration of ClpXP and its substrate CtrA. The CckA-ChpT phosphorelay inversely regulates CtrA activity and stability through the phosphorylation of CtrA and CpdR. A possible link of the CckA phosphorelay with PopA activity is indicated. Sensor histidine kinase and phosphotransfer protein are shown in blue, response regulators are highlighted green. The model proposes that cell cycle-dependent localization of PopA to the stalked cell pole involves the timed synthesis and/or hydrolysis of c-di-GMP by one or several as yet unidentified DGCs or PDEs. Upon binding of c-di-GMP PopA sequesters to the cell pole, where it recruits RcdA and CtrA (B).

**SUPPLEMENTAL MATERIAL:**

**Figure S1: Sequence comparison of the diguanylate cyclase PleD and the c-di-GMP binding protein PopA.**

A sequence alignment of PopA (Query) and PleD (Sbjct) is shown. The three domains are indicated by different colors. Conserved residues of Rec1 known to be important for intra-molecular signaling are boxed green, conserved residues of the putative dimerization interface are boxed red, and residues important for GGDEF domain function are highlighted in purple.

**Figure S2: CtrA is stabilized in a Δ*popA* mutant.**

Cell cycle time lapse experiment with wild-type and Δ*popA* mutant cells expressing *yfp-ctrARD+15* from the xylose inducible promoter $P_{xyl}$. Cells grown in the presence of the inducer xylose were synchronized and released into fresh M2G minimal glucose medium lacking the inducer. Samples were removed at intervals and analyzed by DIC and fluorescence microscopy.

**Fig. S3: Protein – protein interaction map of the *C. crescentus* CtrA degradation machinery.**

Arrows indicate interactions between proteins. Interactions shown by co-immunoprecipitation are in blue, interactions shown by the bacterial two-hybrid system (BACTH) are in red. Stippled arrows indicate weak interactions.

**Fig. S4: Polar localization of PopA-eGFP during the *C. crescentus* cell cycle.**

(A) *C. crescentus* wild-type cells expressing *popA-egfp* were synchronized and samples removed at 20 minute intervals were analyzed by fluorescence microscopy. Cell cycle progression is shown schematically. (B) Statistics of PopA-eGFP localization as shown in (A). Cells with no focus, with a PopA-eGFP focus at one pole, and with a bipolar pattern were scored throughout the cell cycle as indicated.

**Fig. S5: Polar localization of PopA I- and A-site mutants during the *C. crescentus* cell cycle.**

*C. crescentus popA* mutant cells expressing either $popA_{E368Q}$-*egfp* (A) or $popA_{D357G}$-*egfp* (B) were synchronized and samples removed at 20 minute intervals were analyzed by DIC and fluorescence microscopy. The statistics of $PopA_{E368Q}$-eGFP and $PopA_{D357G}$-eGFP localization is shown underneath the microscopy panels. Cells with no focus, with a focus at one pole, and with a bipolar pattern were scored throughout the cell cycle as indicated.

**Fig. S6: The GGDEF domain is required for efficient localization of PopA to the pole.**

*C. crescentus popA* mutant cells expressing *popA-rec1-egfp* (receiver domain 1 of PopA), *popA-rec1-rec2-egfp* (receiver domains 1 and 2 of PopA), or *popA-egfp* (full-length PopA) were analyzed by DIC (left) and fluorescence microscopy (middle). The localization patterns are indicated on the right.

**Fig. S7: PopA is required for proper localization of ClpX to the cell pole.**

Cultures of *C. crescentus* wild type and *popA* mutants expressing a *clpX-egfp* were synchronized and cells were analyzed by DIC and fluorescence microscopy as they progressed through the cell cycle. Polar localization of ClpX is shown schematically in the middle panel. Progression of cells through the cell cycle is indicated on the left.
The timing of cell cycle progression is equivalent to the experiments shown in Fig. 2A.

**Fig. S8: CpdR is not required for localization of PopA to the cell pole.**

Cultures of *C. crescentus* wild type and *DcpdR*mutant expressing *popA-egfp* were analyzed by DIC (middle) and fluorescence microscopy (left). The localization patterns are indicated on the right.

**Fig. S9: PopA is required for proper CpdR localization to the cell pole.**

Cultures of *C. crescentus DcpdR* (upper panels) and *DcpdRDpopA* (lower panels) mutants expressing *cpdR-yfp* were synchronized and cells were analyzed by DIC and fluorescence microscopy throughout the cell cycle. The localization patterns are shown schematically.

**Table S1: Strains and plasmids**

*C. crescentus* strains

| Name | Description | Source or Reference |
|---|---|---|
| NA1000 | Synchronizable laboratory strain of CB15 | (Evinger and Agabian, 1977) |
| UJ271 | NA1000clpX::Ω::pUJ168 | (Jenal and Fuchs, 1998) |
| UJ2765 | NA1000Δ*podJ* and plasmid pAD5 | This study |
| UJ2796 | NA1000 and plasmid pAD5 | This study |
| UJ2827 | NA1000Δ*popA* | This study |
| UJ3125 | NA1000 and plasmid pEJ146 | This study |
| UJ3127 | NA1000Δ*popA* and plasmid pEJ146 | This study |
| UJ3159 | NA1000Δ*popA* and plasmid pAD19 | This study |
| UJ3563 | NA1000Δ*popA* and plasmid pAD32 | This study |
| UJ3565 | NA1000Δ*popA* and plasmid pAD30 | This study |
| UJ3640 | NA1000Δ*popA*Δ*podJ* | This study |
| UJ3665 | NA1000Δ*popA*Δ*rcdA* and plasmid pAD5 | This study |
| UJ3666 | NA1000Δ*popA*Δ*podJ* and plasmid pAD5 | This study |
| UJ3672 | NA1000Δ*popA*Δ*podJ* and plasmid pAD30 | This study |
| UJ3742 | NA1000Δ*popA rcdA*::prcdA-egfp | This study |
| UJ3743 | NA1000Δ*popA* xylX::pX-clpX-egfp | This study |
| UJ3966 | NA1000 and plasmid pID42 | This study |
| UJ3967 | NA1000 and plasmid pIDC42 | This study |
| UJ3969 | NA1000Δ*popA* and plasmid pID42 | This study |
| UJ3970 | NA1000Δ*popA* and plasmid pIDC42 | This study |
| UJ3972 | NA1000clpX::Ω::pUJ168 and plasmid pAD5 | This study |
| UJ4329 | NA1000 and plasmids pAD105, pAD82 | This study |
| UJ4330 | NA1000 and plasmids pAD106, pAD83 | This study |
| UJ4331 | NA1000Δ*popA* and plasmid pAD128 | This study |
| UJ4333 | NA1000Δ*popA* and plasmid pAD130 | This study |
| UJ4374 | Δ*cpdR* and plasmid pAD147 | This study |
| UJ4417 | NA1000Δ*popA* xylX::pX-clpX-egfp and plasmid pAD150 | This study |
| UJ4401 | NA1000Δ*popA*Δ*cpdR* | This study |
| UJ4434 | Δ*cpdR* and plasmid p*cpdR-yfp* | This study |
| UJ4435 | NA1000Δ*popA*Δ*cpdR* and plasmid p*cpdR-yfp* | This study |
| UJ4471 | NA1000Δ*popA*Δ*cpdR* and plasmid pX-cpdRD51A-yfp | This study |

| | | |
|---|---|---|
| UJ4473 | Δ*cpdR* and plasmid pX-cpdRD51A-yfp | This study |
| LS4183 | NA1000 xylX::pX-clpX-egfp | (McGrath et al., 2006) |
| LS4191 | NA1000 *rcdA*::prcdA-egfp | (McGrath et al., 2006) |
| NA1000Δ*cpdR* | Deletion of *cpdR* ($tet^R$) | (Skerker et al., 2005) |
| NA1000Δ*podJ* | Deletion of *podJ* | (Viollier et al., 2002c) |

## *E. coli* strains

| Name | Description | Source or Ref. |
|---|---|---|
| MM337 | E. coli K-12 *araD139 flbB5301* ptsF25 rbsR relA1 rpsL150 -(argF-lac)U169 -cya | M. Manson |
| DH10B | $F^-$*mcrA* D(*mrr*$^-$hsd RMS$^-$*mcr*BC) f80d*lacZ*DM15D*lacX*74 endA1 recA1deoR D(*ara, leu*)7697 *araD139 galU galK nupG rpsL thi pro* $^-$ *hsd*$^+$ *recA RP4-2-Tc*::*Mu-Tn7* | Simon et al. 1983 |
| S17 | F-, lambda (-), thi, pro,recA, restriction (-) modification (+), RP4 derivative integrated into the chromosome with Tet::Mu, Km::Tn7 | Simon et al. 1983 |
| DH5a | DH5a (F-) F` endA1 hsdR17 (rK-mK plus) glnV44 thi1 recA1 gyr delta(Nalr) relA1 delta(lacIZYA-argF)U169 deoR (F80dlac delta (lacZ) M15) | Woodcock et al. 1989 |
| $Arctic^R$ | BL21 (DE3) arctic express strain for protein expression | Stratagene |

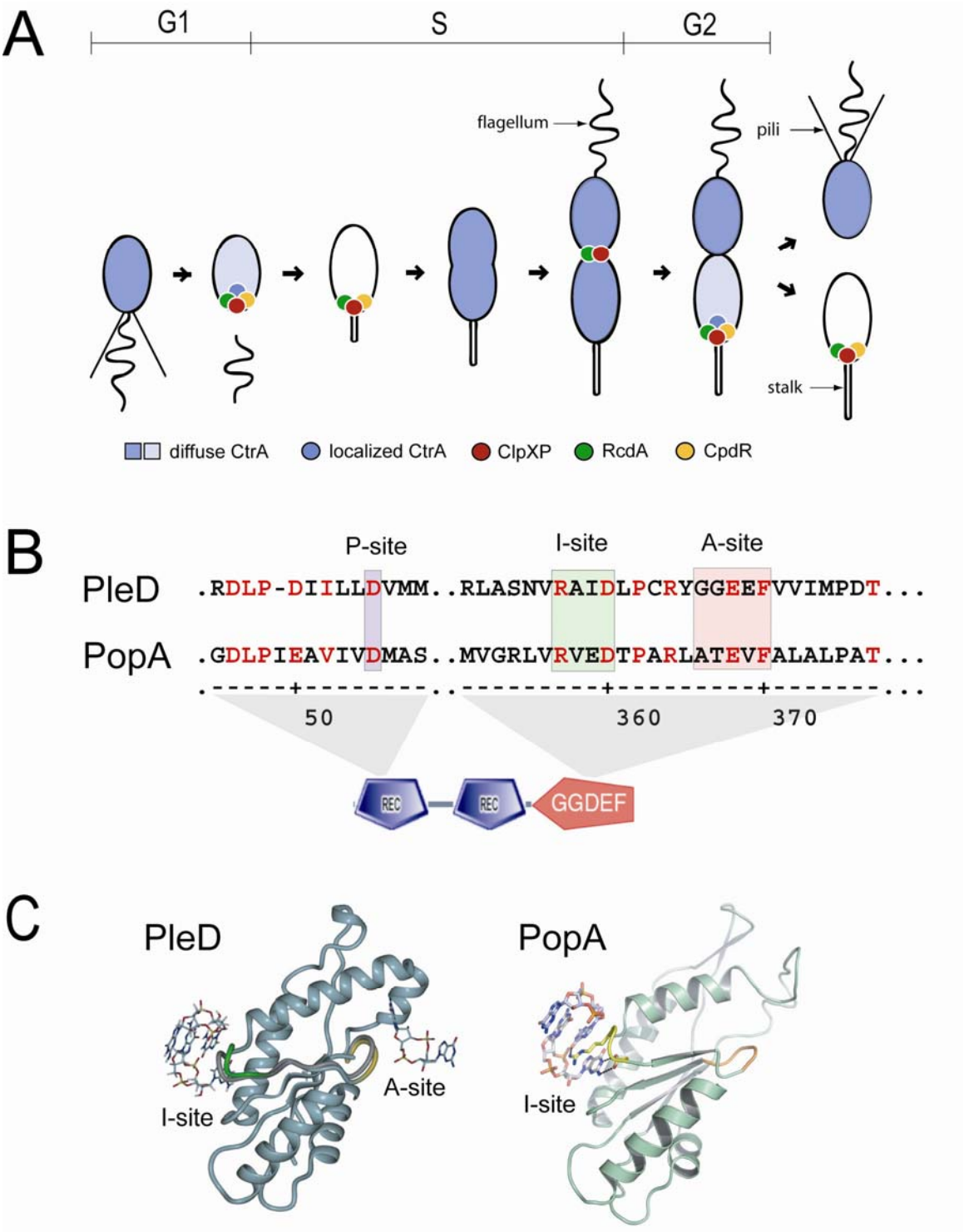
**Plasmids**

| Name | Description | Source or Ref. |
|---|---|---|
| pAD5 | pMR20; *popA-eGfp* under control of *popA* promoter | This study |
| pAD8 | pNPTS138; used for clean deletion of *popA* | This study |
| pAD19 | pMR20; $popA_{D55N}$-*eGfp* under control of *popA* promoter | This study |
| pAD30 | pMR20; $popA_{R357G}$-*eGfp* under control of *popA* promoter | This study |
| pAD32 | pMR20; $popA_{E368Q}$-*eGfp* under control of *popA* promoter | This study |
| pAD33 | pET21C; $popA_{R357G}$ C-terminal $His_6$ tag | This study |
| pAD34 | pET21C; $popA_{E368Q}$ C-terminal $His_6$ tag | This study |
| pAD44 | pUT18C; *clpX* C-terminal fused to the T18 fragment | This study |

| | | |
|---|---|---|
| pAD45 | pKT25; *clpX* C-terminal fused to the T25 fragment | This study |
| pAD47 | pUT18C; *clpP* C-terminal fused to the T18 fragment | This study |
| pAD48 | pKT25; *clpP* C-terminal fused to the T25 fragment | This study |
| pAD50 | pUT18C; *rcdA* C-terminal fused to the T18 fragment | This study |
| pAD51 | pKT25; *rcdA* C-terminal fused to the T25 fragment | This study |
| pAD53 | pUT18C; *cpdR* C-terminal fused to the T18 fragment | This study |
| pAD54 | pKT25; *cpdR* C-terminal fused to the T25 fragment | This study |
| pAD56 | pUT18; *rcdA* N-terminal fused to the T18 fragment | This study |
| pAD58 | pUT18; *clpX* N-terminal fused to the T18 fragment | This study |
| pAD60 | pUT18; *clpP* N-terminal fused to the T18 fragment | This study |
| pAD62 | pUT18; *cpdR* N-terminal fused to the T18 fragment | This study |
| pAD65 | pUT18; *ctrA* N-terminal fused to the T18 fragment | This study |
| pAD67 | pUT18; *popA* N-terminal fused to the T18 fragment | This study |
| pAD82 | pMR20; *popA-eYfp* under control of *popA* promoter | This study |
| pAD83 | pMR10; *popA-eCfp* under control of *popA* promoter | This study |
| pAD90 | pKT25; $popA_{R357G}$ C-terminal fused to the T25 fragment | This study |
| pAD91 | pKT25; $popA_{E368Q}$ C-terminal fused to the T25 fragment | This study |
| pAD105 | pBBR-MCS-5; *rcdA-eCfp* under the control of *rcdA* promoter | This study |
| pAD106 | pBBR-MCS-5; *rcdA-eYfp* under the control of *rcdA* promoter | This study |
| pAD128 | pMR20; *popA-Rec1-eGfp* under the control of *popA* promoter | This study |
| pAD130 | pMR20; *popA-Rec1Rec2-eGfp* under the control of *popA* promoter | This study |
| pAD140 | pKT25; *ctrA* C-terminal fused to the T25 fragment | This study |
| pAD141 | pKT25; *popA* C-terminal fused to the T25 fragment | This study |
| pAD142 | pUT18C; *ctrA* C-terminal fused to the T18C fragment | This study |
| pAD143 | pUT18C; *popA* C-terminal fused to the T18C fragment | This study |
| pAD147 | pMR10; *popA-eGfp* under control of *popA* promoter | This study |
| pAD150 | pMR20; $popA_{D55N}$ under the control of *popA* promoter, chloramphenicol cassette integrated | This study |
| pAD153 | pMR20; $popA_{R357G}$ under the control of *popA* promoter, kanamycin cassette integrated | This study |
| pBBR1MCS-5 | $Gent^R$, broad host range cloning vector | Kovach et al. 1995 |
| p*cpdR-yfp* | pMR11; *cpdR* 3' fused to *eyfp* with *cpdR* promoter | Iniesta et al. 2007 |
| pECFP | $Amp^R$ vector for creation of eCFP-fusion proteins | Clontech |
| pEGFP | $Amp^R$ vector for creation of eGFP-fusion proteins | Clontech |
| pEJ146 | pMR10-Pxyl::YFP-CtrA RD+15 | Ryan et al. 2002 |
| pET21C | $Amp^R$ expression vector, high copy number | Novagen |
| pET21C::PopA | pET21C; *popA* C-terminal $His_6$ tag | This study |

| | | |
|---|---|---|
| pEYFP | $Amp^R$ vector for creation of eYFP-fusion proteins | Clontech |
| pID42 | pJS14; *Pxyl::CtrA* | Domian et al. 1997 |
| pIDC42 | pJS14; *Pxyl::CtrAD51E* | Domian et al. 1997 |
| pJS14 | $Chlor^R$ high copy number expression vector | J. Skerker |
| pKT25 | pSU40 derivative with T25 fragment of CyaA | Karimova et al. 2001 |
| pKT25-zip | pKT25 derivative with leucine zipper of GCN4 | Karimova et al. 2001 |
| pMR10 | $Kan^R$ low copy number and broad host range vector | Roberts et al. 1996 |
| pMR20 | $Tet^R$ low copy number and broad host range vector | Roberts et al. 1996 |
| pNPTS138 | $Kan^R$, suicide vector with *sacB* gene and *oriT* | D. Alley |
| prcdA-egfp | pXGFP4 with *xylX* promoter replaced with last 300 bp of rcdA | McGrath et al. 2006 |
| pUT18C-zip | pUT18C derivative with leucine zipper of GCN4 | Karimova et al. 2001 |
| pUT18C | pUC19 derivative with T18 fragment of CyaA. C-terminal fusions | Karimova et al. 2001 |
| pUT18 | pUC19 derivative with T18 fragment of CyaA. N-terminal fusions | Karimova et al. 2001 |
| pX-clpX-egfp | pXGFP4 with *clpX* | McGrath et al. 2006 |
| pX-cpdRD51A-yfp | pMR31; *cpdRD51A* 3' fused to *eyfp* with *xylX* promoter | Iniesta et al. 2007 |

**Figure 1**


A
G1
S
G2
flagellum
pili
stalk
diffuse CtrA
localized CtrA
ClpXP
RcdA
CpdR
B
P-site
I-site
A-site
PleD .RDLP-DIILLDVMM..RLASNVRAIDLPCRYGGEEFVVIMPDT...
PopA .GDLPIEAVIVDMAS..MVGRLVRVEDTPARLATEVFALALPAT...
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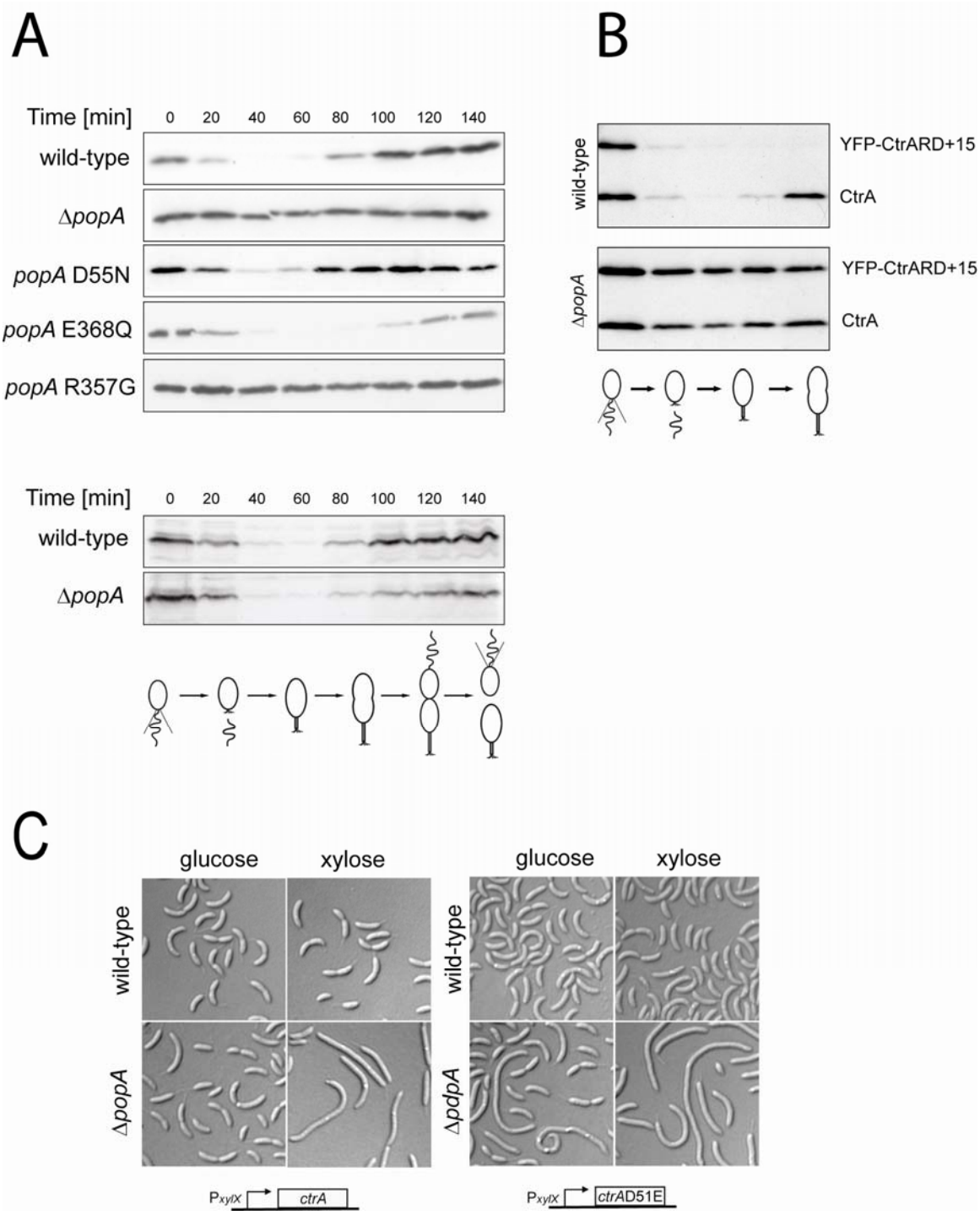
360
370
REC
REC
GGDEF
C
PleD
A-site
I-site
PopA
I-site

**Figure 2**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Time [min]
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20
40
60
80
100
120
140
wild-type
ΔpopA
popA D55N
popA E368Q
popA R357G
Time [min]
0
20
40
60
80
100
120
140
wild-type
ΔpopA
B
wild-type
YFP-CtrARD+15
CtrA
ΔpopA
YFP-CtrARD+15
CtrA
C
glucose
xylose
glucose
xylose
wild-type
ΔpopA
wild-type
ΔpdpA
PxylX
ctrA
PxylX
ctrAD51E

**Figure 3**

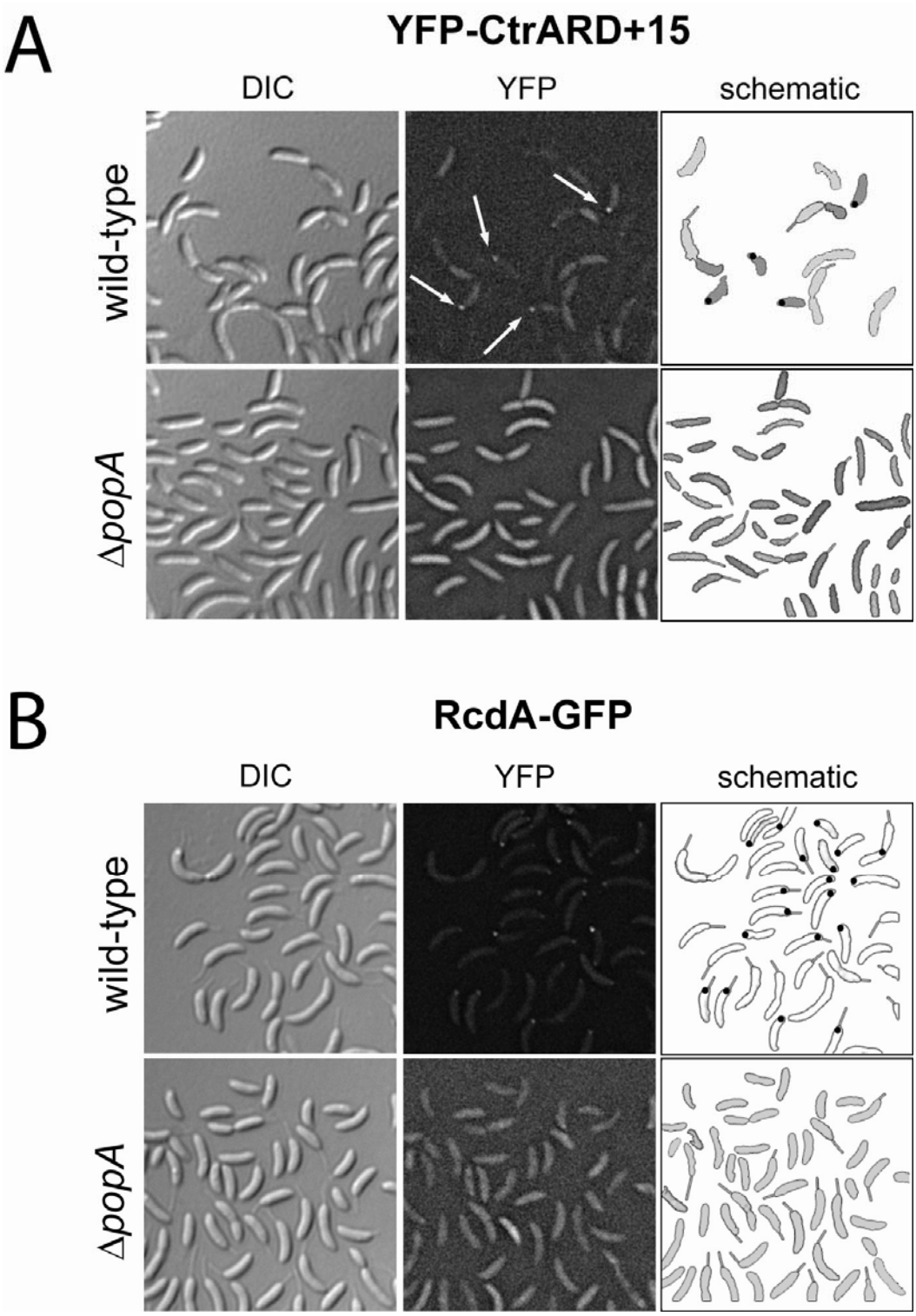

A
YFP-CtrARD+15
DIC
YFP
schematic
wild-type
ΔpopA
B
RcdA-GFP
DIC
YFP
schematic
wild-type
ΔpopA

**Figure 3**

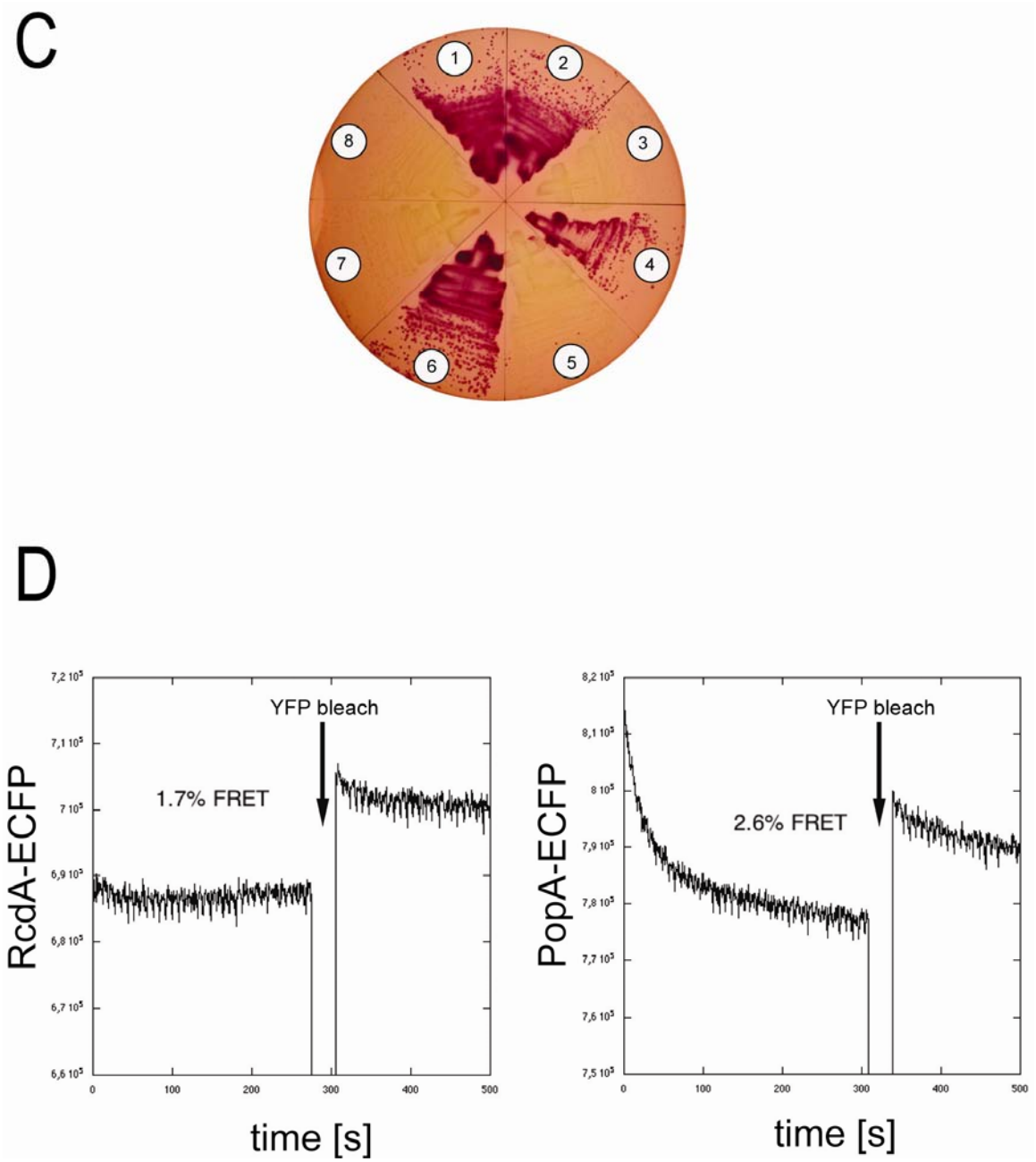

C
1
2
3
4
5
6
7
8
D
RcdA-ECFP
YFP bleach
1.7% FRET
time [s]
PopA-ECFP
YFP bleach
2.6% FRET
time [s]

**Figure 4**

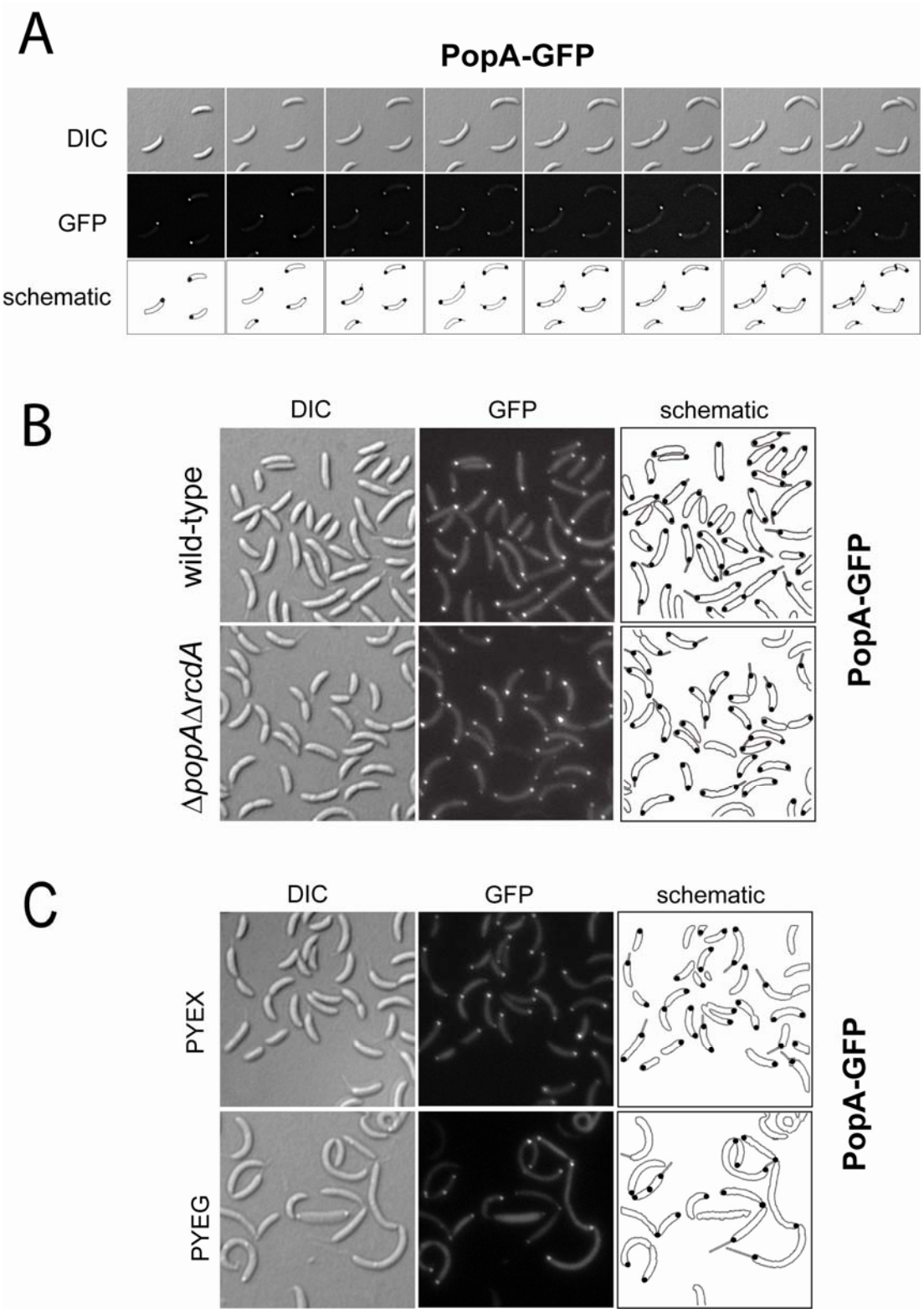

A
PopA-GFP
DIC
GFP
schematic
B
DIC
GFP
schematic
wild-type
ΔpopAΔrcdA
PopA-GFP
C
DIC
GFP
schematic
PYEX
PYEG
PopA-GFP

**Figure 5**

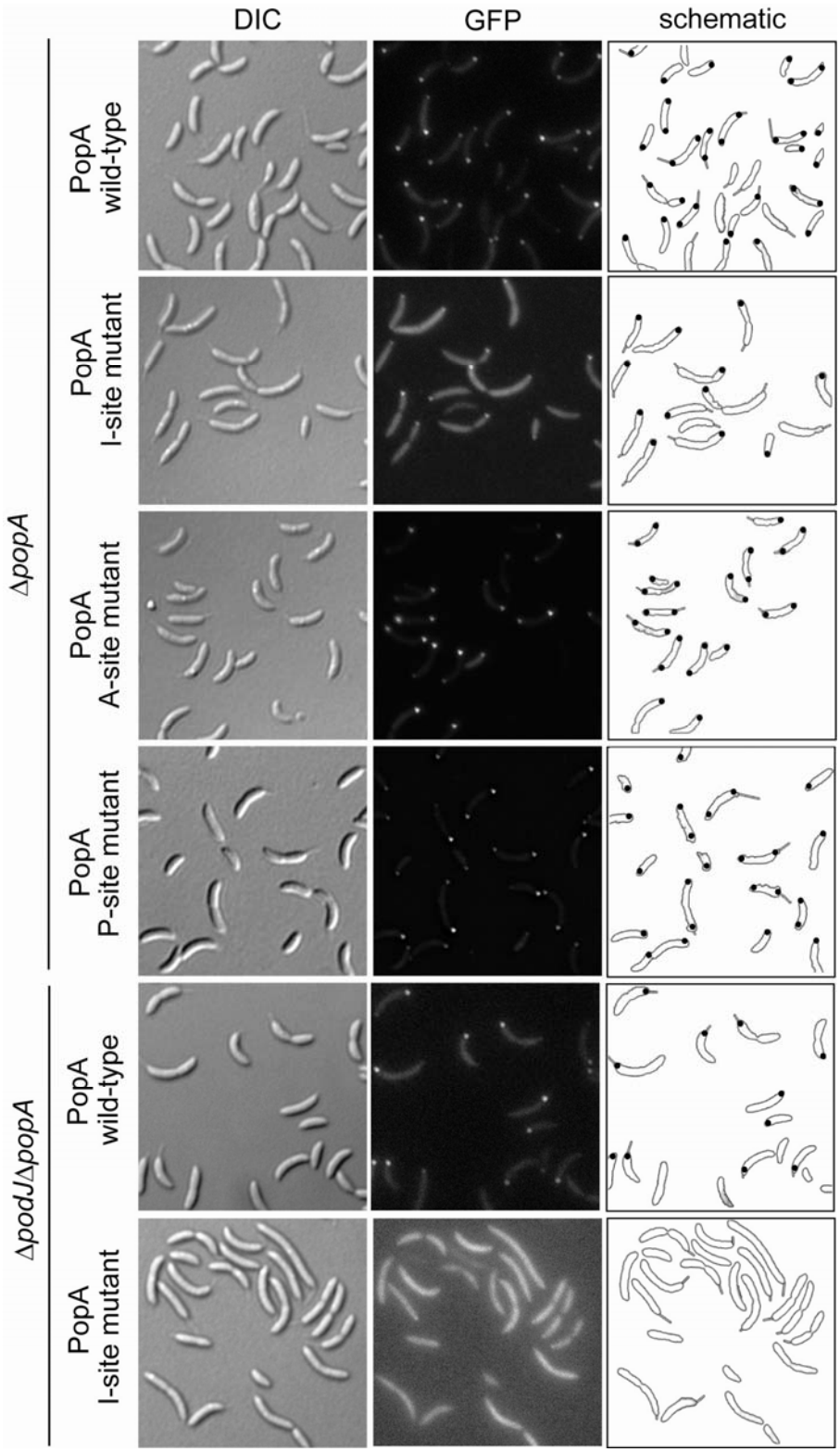

DIC
GFP
schematic
ΔpopA
ΔpodJΔpopA
PopA wild-type
PopA I-site mutant
PopA A-site mutant
PopA P-site mutant
PopA wild-type
PopA I-site mutant

**Figure 6**

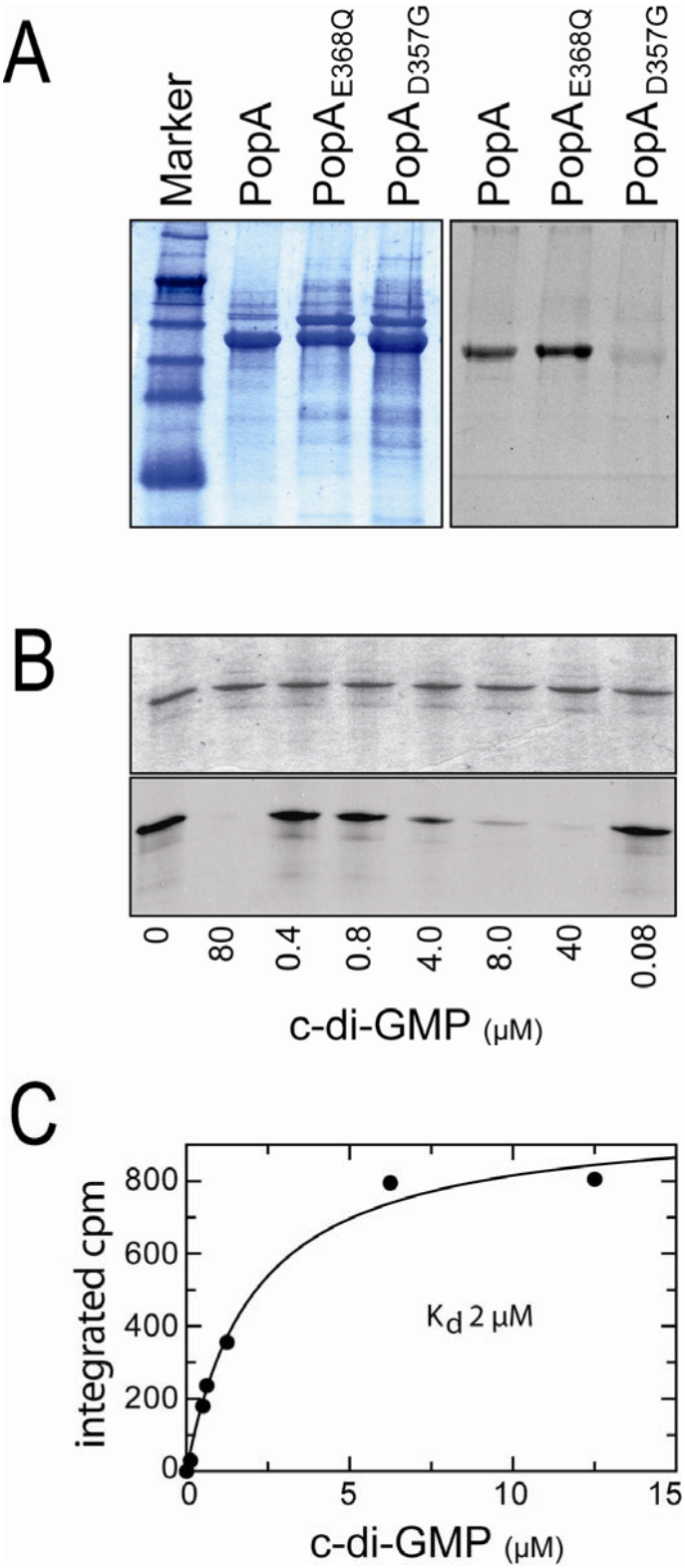

A
Marker
PopA
PopA E368Q
PopA D357G
PopA
PopA E368Q
PopA D357G
B
0
80
0.4
0.8
4.0
8.0
40
0.08
c-di-GMP (μM)
C
integrated cpm
800
600
400
200
0
0
5
10
15
Kd 2 μM
c-di-GMP (μM)

**Figure 7**

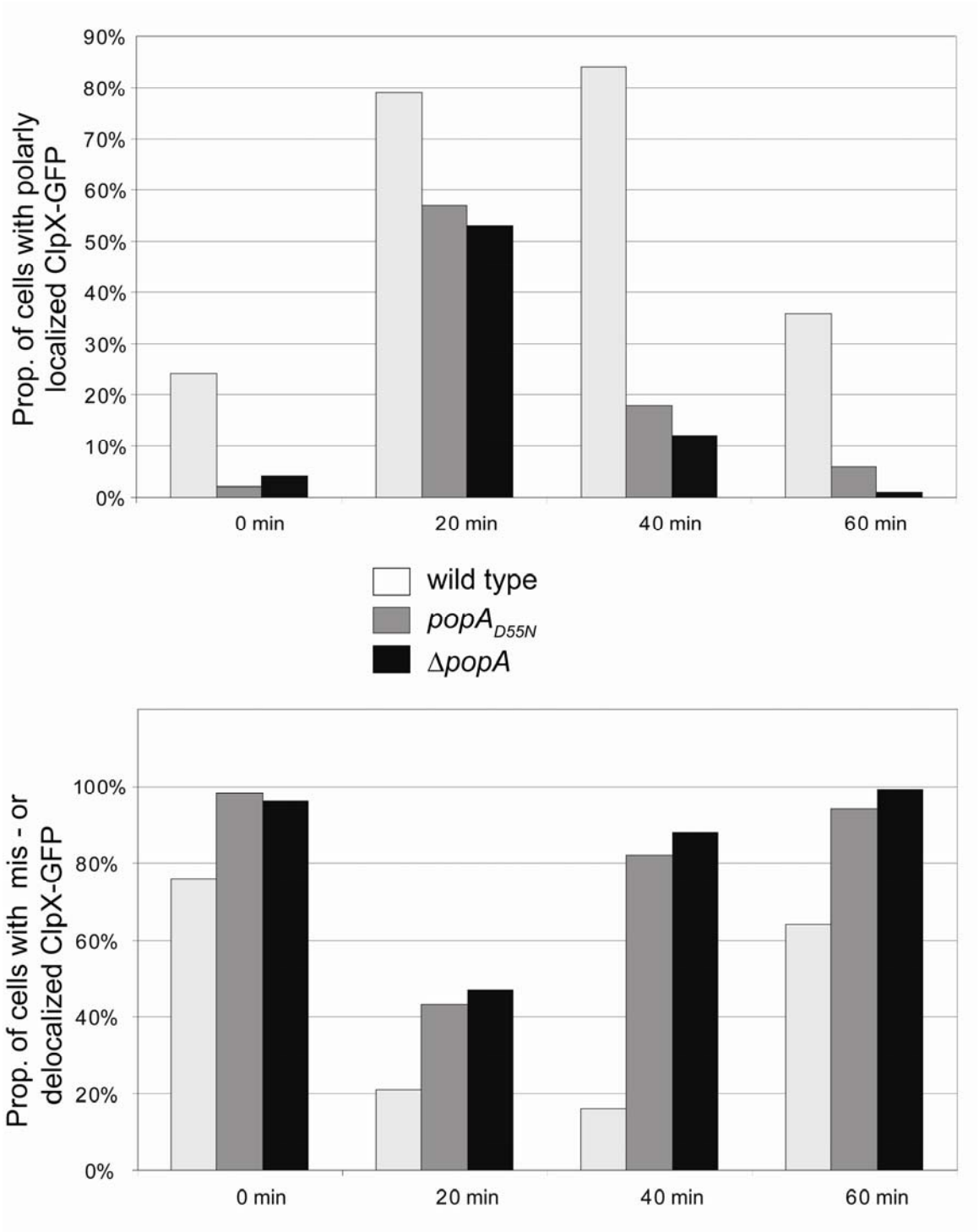

Prop. of cells with polarly localized ClpX-GFP
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0 min
20 min
40 min
60 min
wild type
popA D55N
ΔpopA
Prop. of cells with mis - or delocalized ClpX-GFP
100%
80%
60%
40%
20%
0%
0 min
20 min
40 min
60 min

**Figure 8**

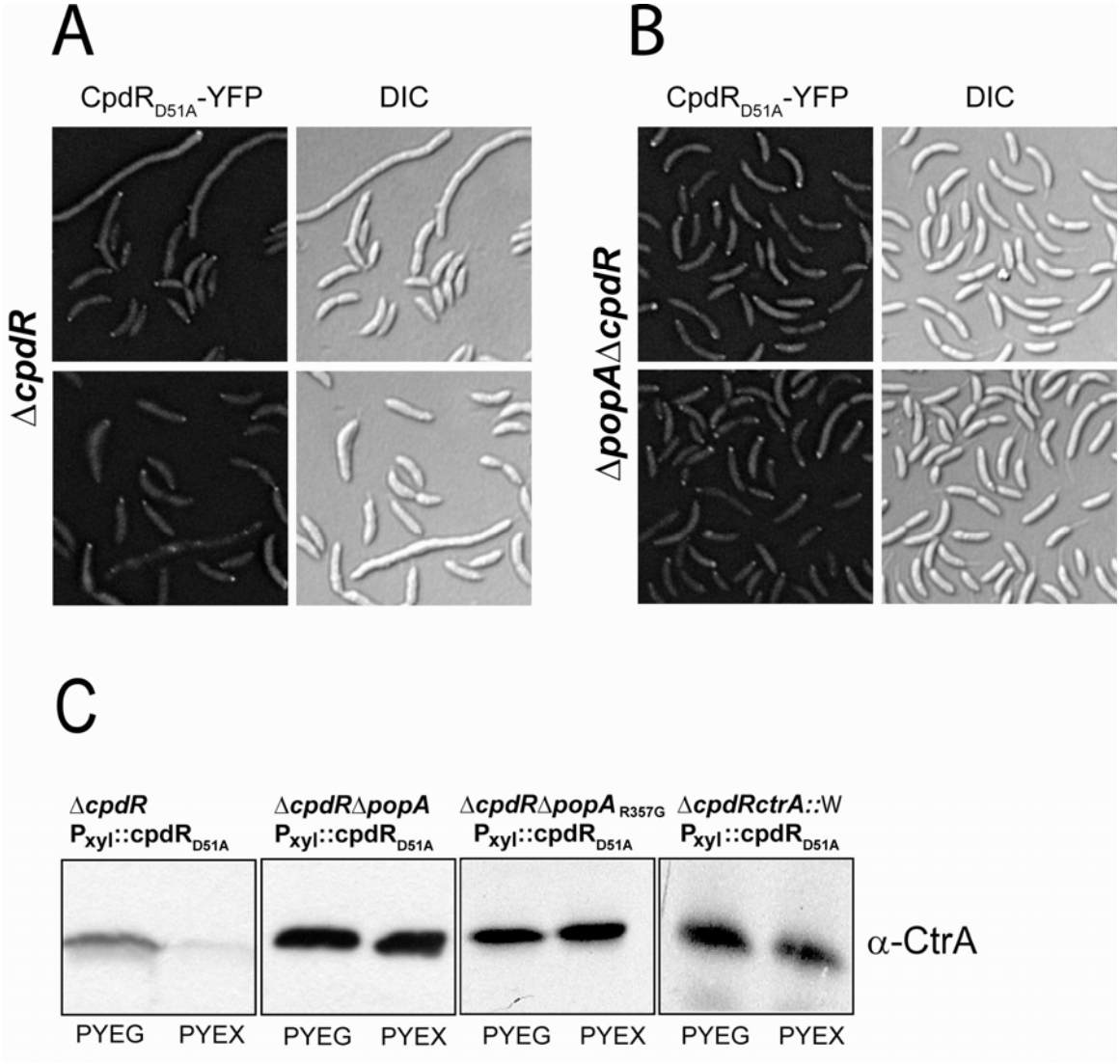

A
CpdR$_{D51A}$-YFP
DIC
Δ*cpdR*
B
CpdR$_{D51A}$-YFP
DIC
Δ*popA*Δ*cpdR*
C
Δ*cpdR*
P$_{xyl}$::cpdR$_{D51A}$
Δ*cpdR*Δ*popA*
P$_{xyl}$::cpdR$_{D51A}$
Δ*cpdR*Δ*popA* $_{R357G}$
P$_{xyl}$::cpdR$_{D51A}$
Δ*cpdRctrA::*W
P$_{xyl}$::cpdR$_{D51A}$
α-CtrA
PYEG PYEX PYEG PYEX PYEG PYEX PYEG PYEX

**Figure 9**

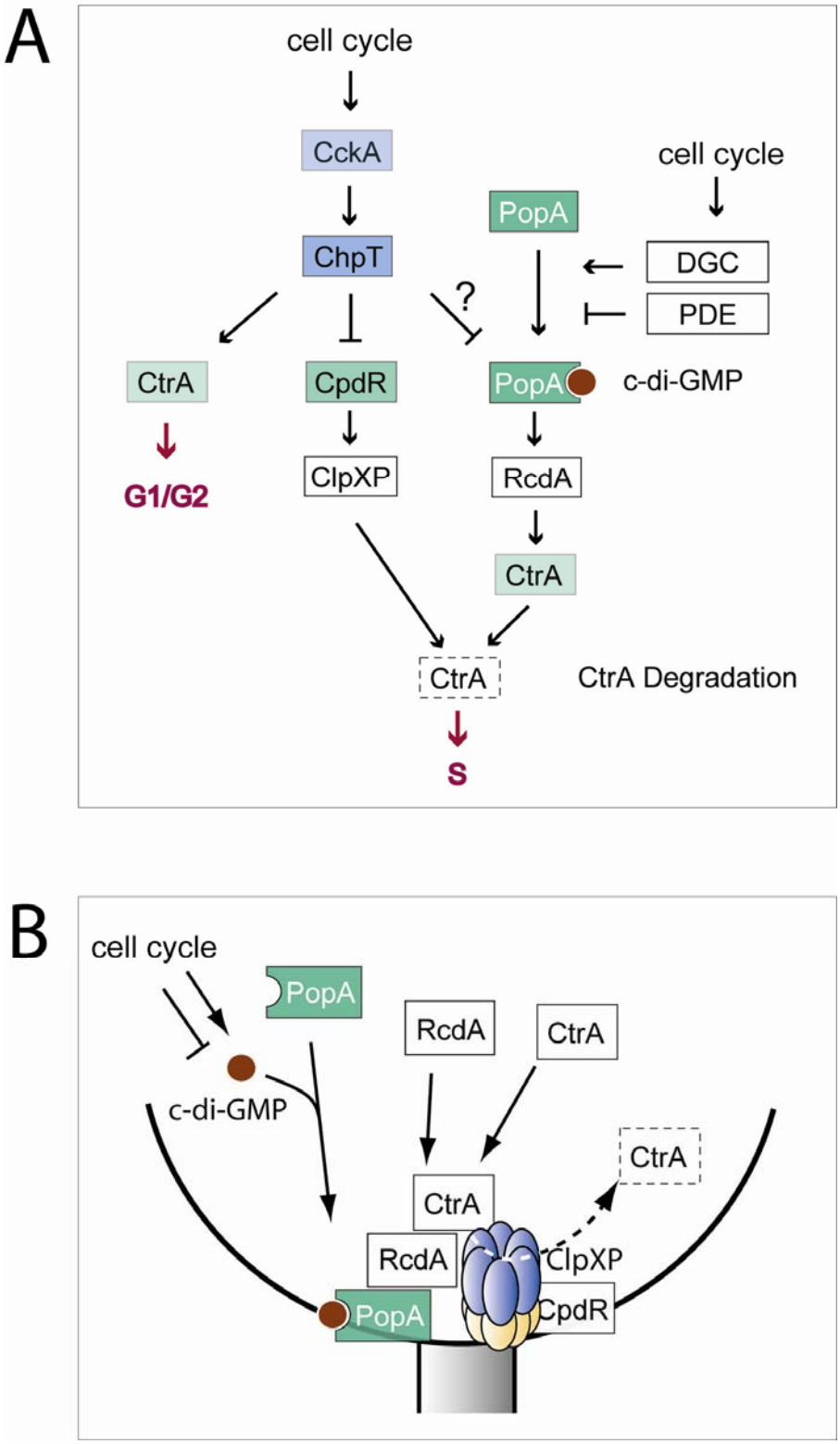

A
cell cycle
CckA
ChpT
CtrA
G1/G2
CpdR
ClpXP
?
PopA
cell cycle
DGC
PDE
PopA
c-di-GMP
RcdA
CtrA
CtrA
CtrA Degradation
S
B
cell cycle
PopA
c-di-GMP
RcdA
CtrA
CtrA
RcdA
ClpXP
CtrA
PopA
CpdR

**Figure S1**

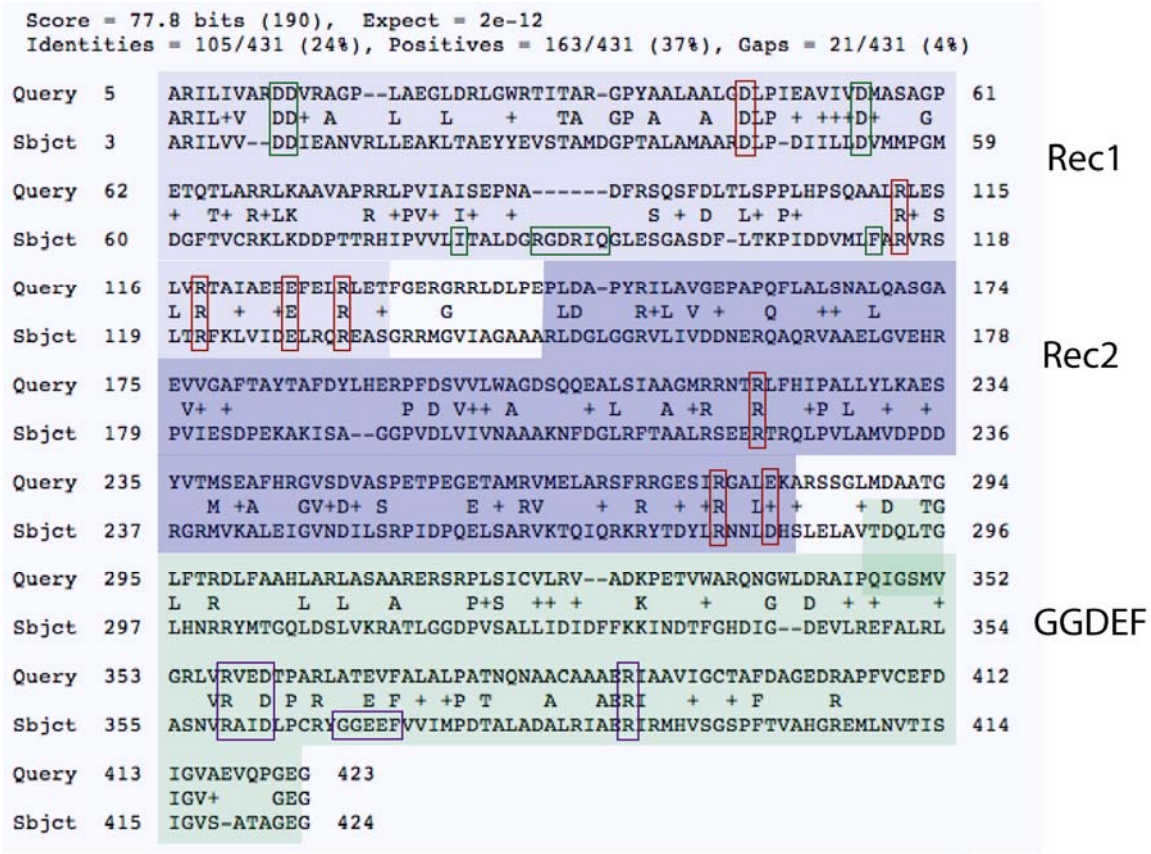

Score = 77.8 bits (190), Expect = 2e-12
Identities = 105/431 (24%), Positives = 163/431 (37%), Gaps = 21/431 (4%)
Query 5 ARILIVARDDVRAGP--LAEGLDRLGWRTITAR-GPYAALAALGDLPIEAVIVDMASAGP 61
ARIL+V DD+ A L L + TA GP A A DLP + +++D+ G
Sbjct 3 ARILVV--DDIEANVRLLEAKLTAEYYEVSTAMDGPTALAMAARDLP-DIILLDVMMPGM 59
Query 62 ETQTLARRLKAAVAPRRLPVIAISEPNA------DFRSQSFDLTLSPPLHPSQAALRLES 115
+ T+ R+LK R +PV+ I+ + S + D L+ P+ R+ S
Sbjct 60 DGFTVCRKLKDDPTTRHIPVVLITALDGRGDRIQGLESGASDF-LTKPIDDVMLFARVRS 118
Query 116 LVRTAIAEEEFELRLETFGERGRRLDLPEPLDA-PYRILAVGEPAPQFLALSNALQASGA 174
L R + +E R + G LD R+L V + Q ++ L
Sbjct 119 LTRFKLVIDELRQREASGRRMGVIAGAAARLDGLGGRVLIVDDNERQAQRVAAELGVEHR 178
Query 175 EVVGAFTAYTAFDYLHERPFDSVVLWAGDSQQEALSIAAGMRRNTRLFHIPALLYLKAES 234
V+ + P D V++ A + L A +R R +P L + +
Sbjct 179 PVIESDPEKAKISA--GGPVDLVIVNAAAKNFDGLRFTAALRSEERTRQLPVLAMVDPDD 236
Query 235 YVTMSEAFHRGVSDVASPETPEGETAMRVMELARSFRRGESIRGAIEKARSSGLMDAATG 294
M +A GV+D+ S E + RV + R + +R L+ + + D TG
Sbjct 237 RGRMVKALEIGVNDILSRPIDPQELSARVKTQIQRKRYTDYLRNNLDHSLELAVTDQLTG 296
Query 295 LFTRDLFAAHLARLASAARERSRPLSICVLRV--ADKPETVWARQNGWLDRAIPQIGSMV 352
L R L L A P+S ++ + K + G D + + +
Sbjct 297 LHNRRYMTGQLDSLVKRATLGGDPVSALLIDIDFFKKINDTFGHDIG--DEVLREFALRL 354
Query 353 GRLVRVEDTPARLATEVFALALPATNQNAACAAAERIAAVIGCTAFDAGEDRAPFVCEFD 412
VR D P R E F + +P T A AERI + + F R
Sbjct 355 ASNVRAIDLPCRYGGEEFVVIMPDTALADALRIAERIRMHVSGSPFTVAHGREMLNVTIS 414
Query 413 IGVAEVQPGEG 423
IGV+ GEG
Sbjct 415 IGVS-ATAGEG 424
Rec1
Rec2
GGDEF

**Figure S2**

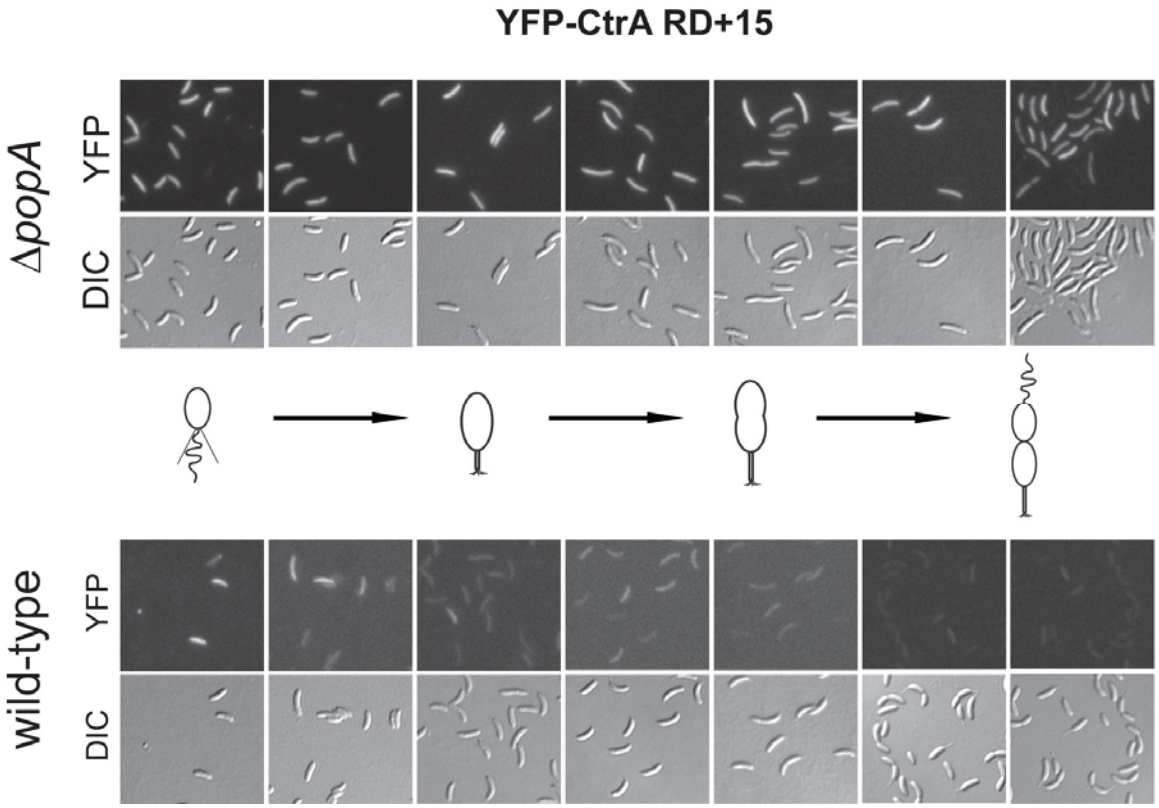


**Figure S3**

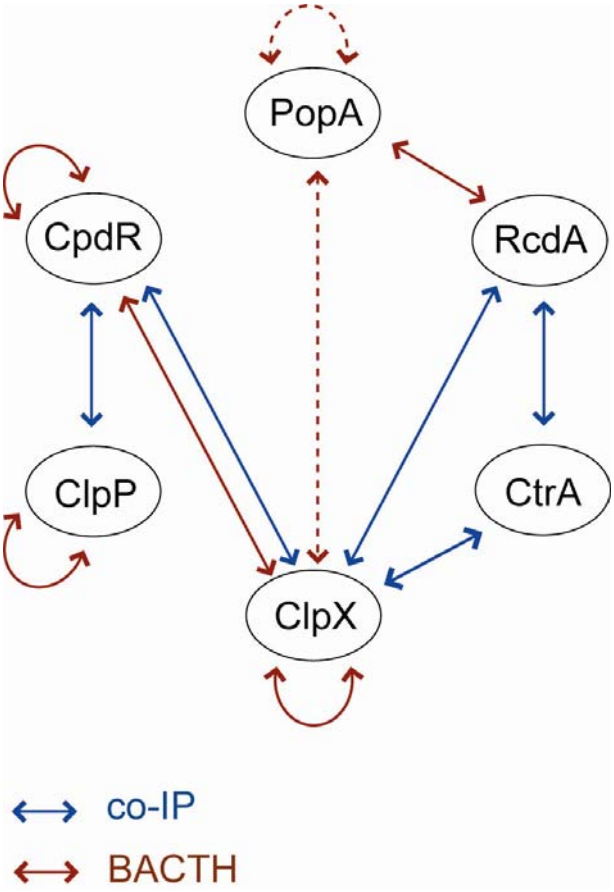


**Figure S4**

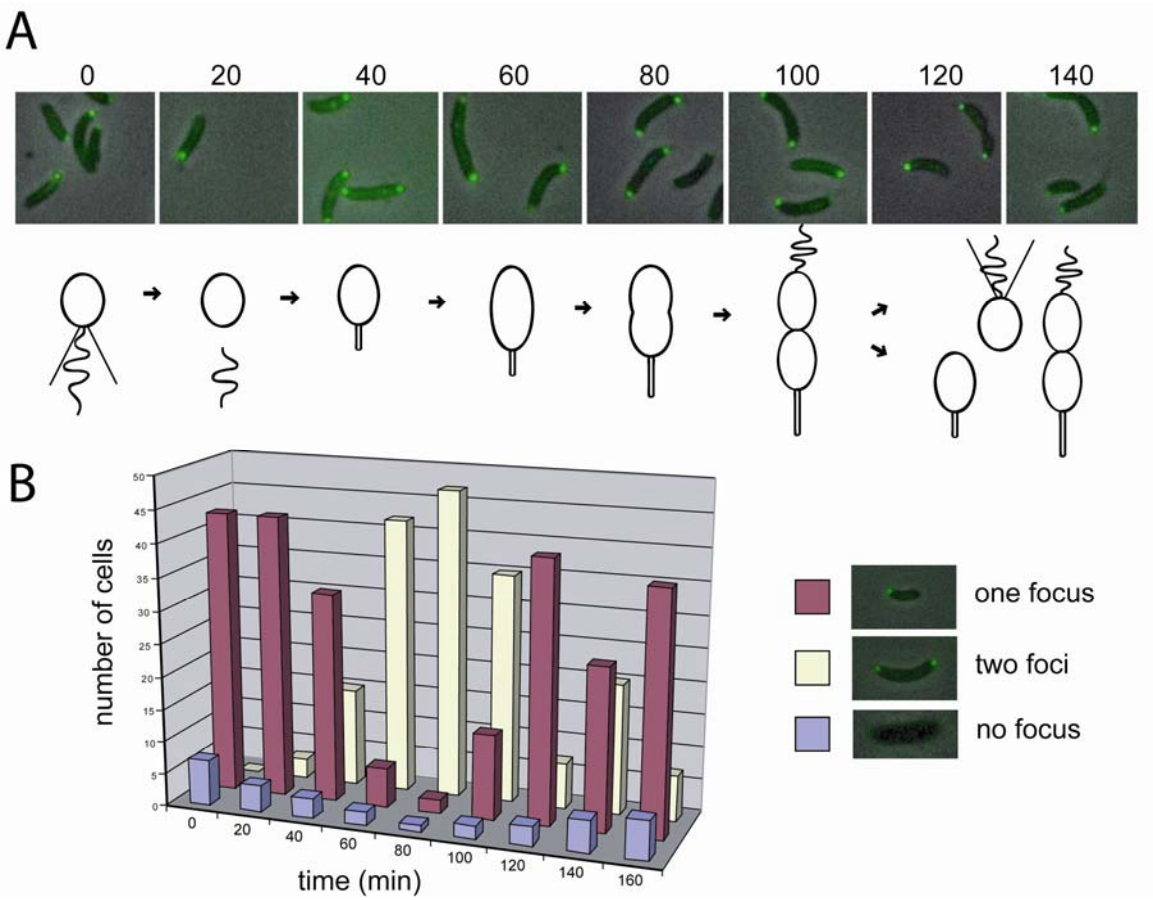

A
0
20
40
60
80
100
120
140
B
number of cells
time (min)
0
20
40
60
80
100
120
140
160
one focus
two foci
no focus

**Figure S5**

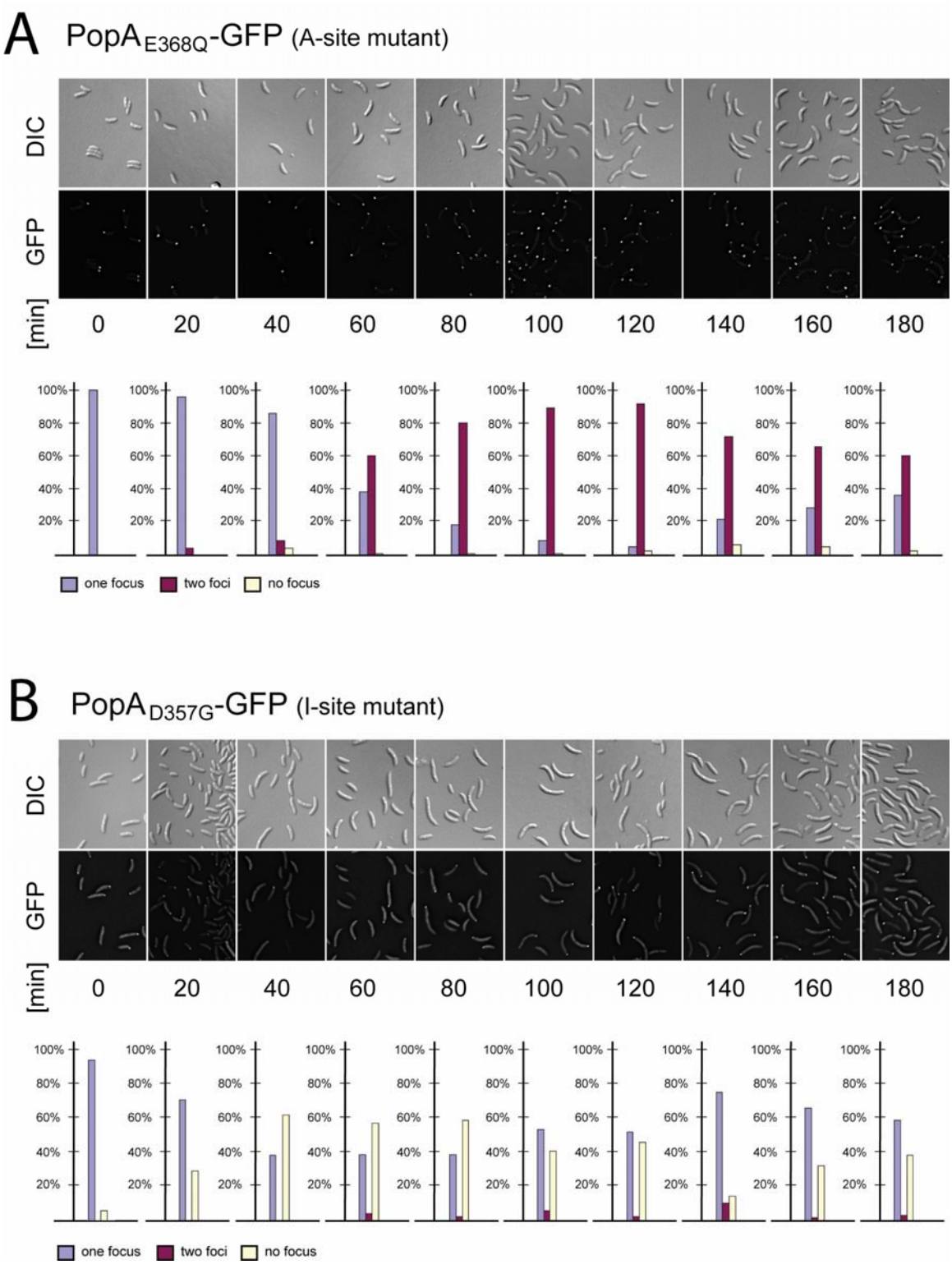

A
PopA E368Q-GFP (A-site mutant)
DIC
GFP
[min]
0
20
40
60
80
100
120
140
160
180
100%
80%
60%
40%
20%
one focus
two foci
no focus
B
PopA D357G-GFP (I-site mutant)
DIC
GFP
[min]
0
20
40
60
80
100
120
140
160
180
100%
80%
60%
40%
20%
one focus
two foci
no focus

**Figure S6**

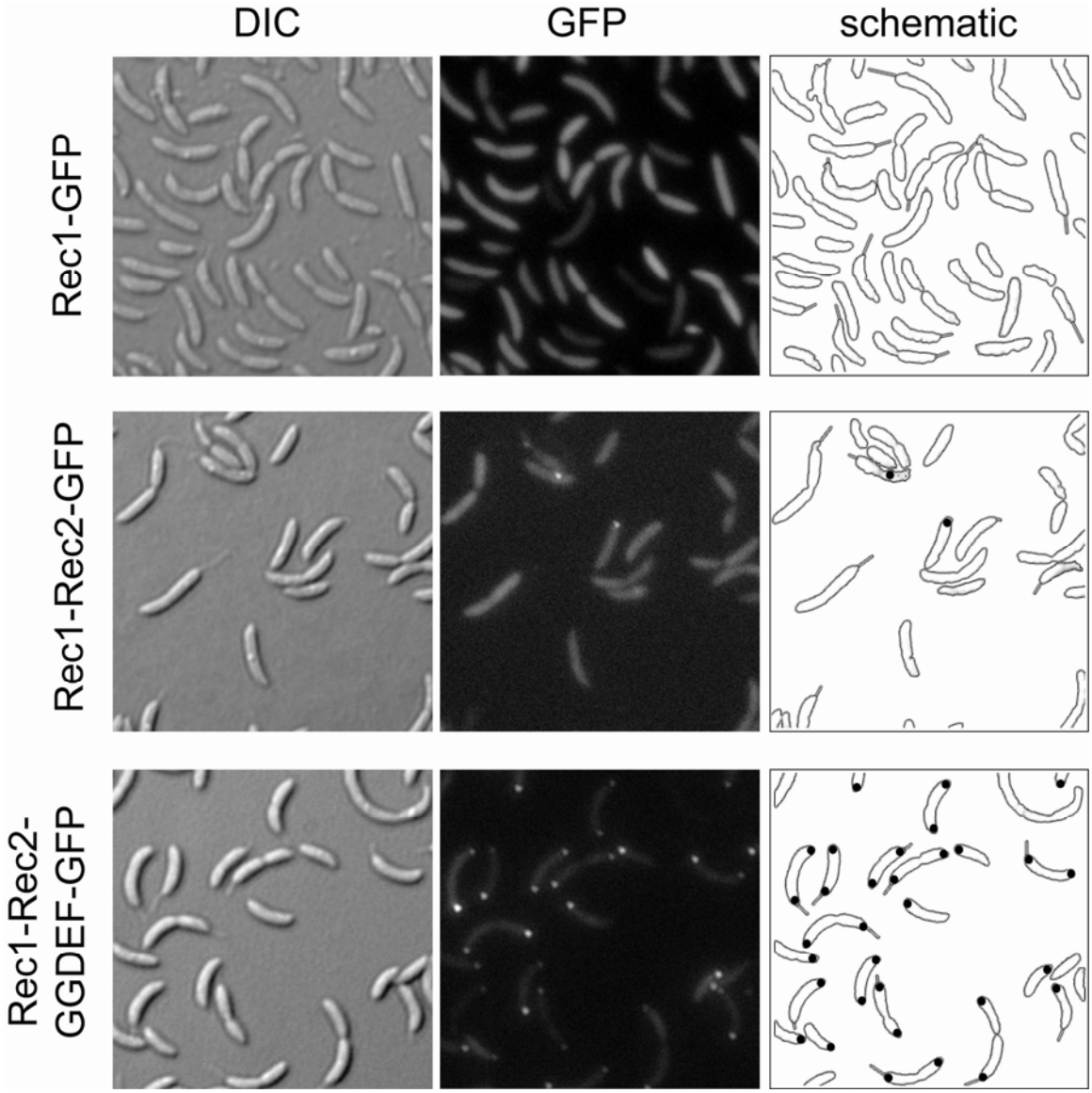


**Figure S7**

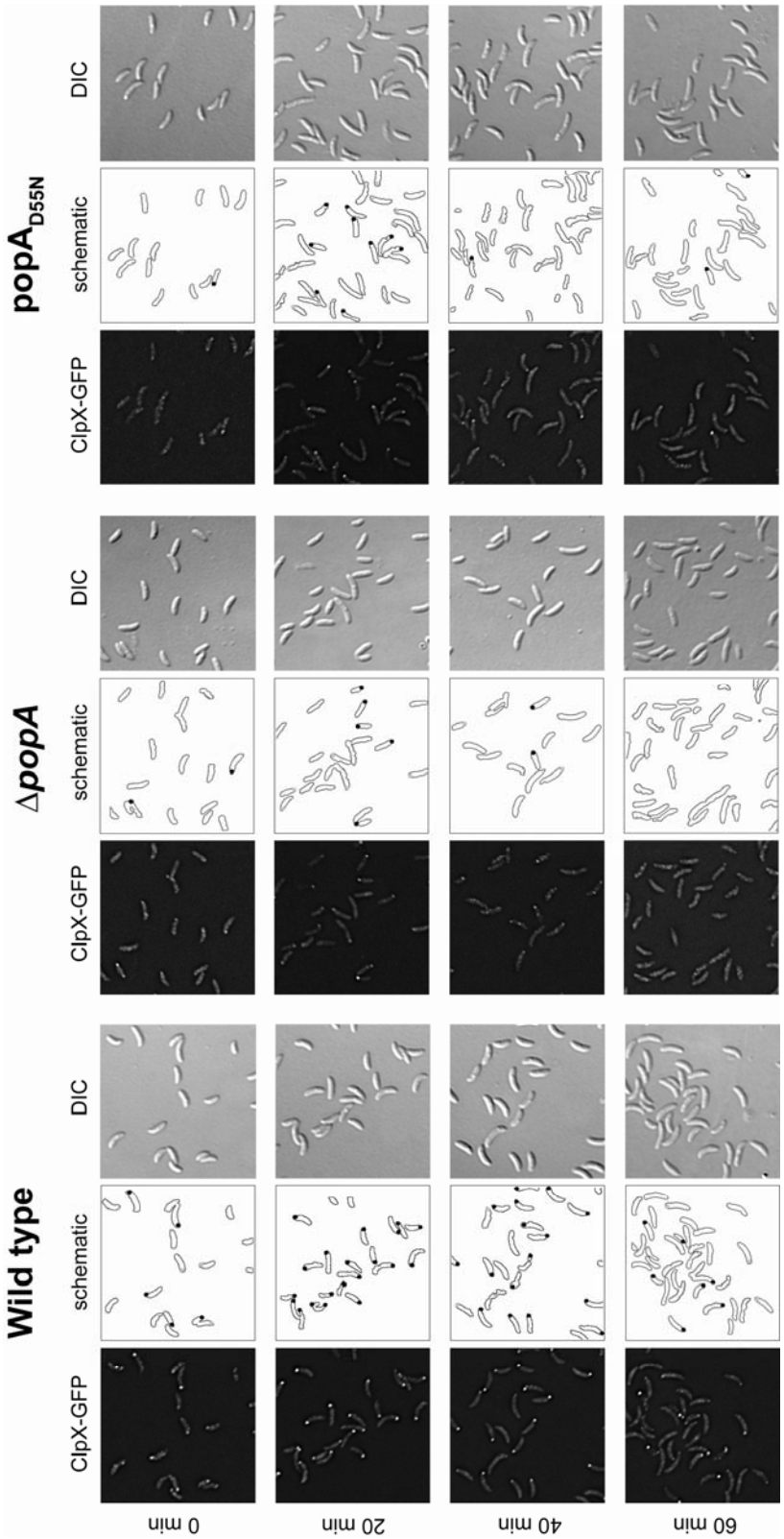

Wild type
ΔpopA
popA D55N
ClpX-GFP
schematic
DIC
0 min
20 min
40 min
60 min

**Figure S8**

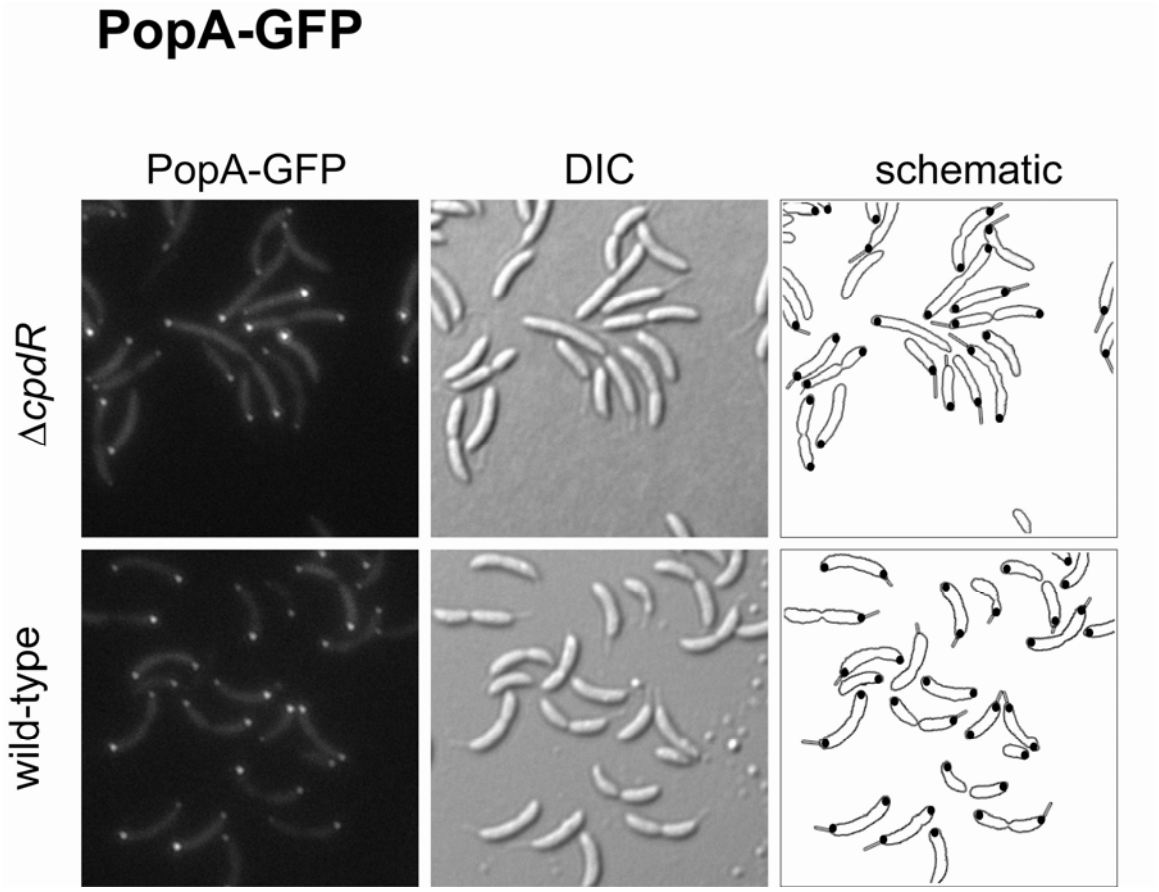


**Figure S9**

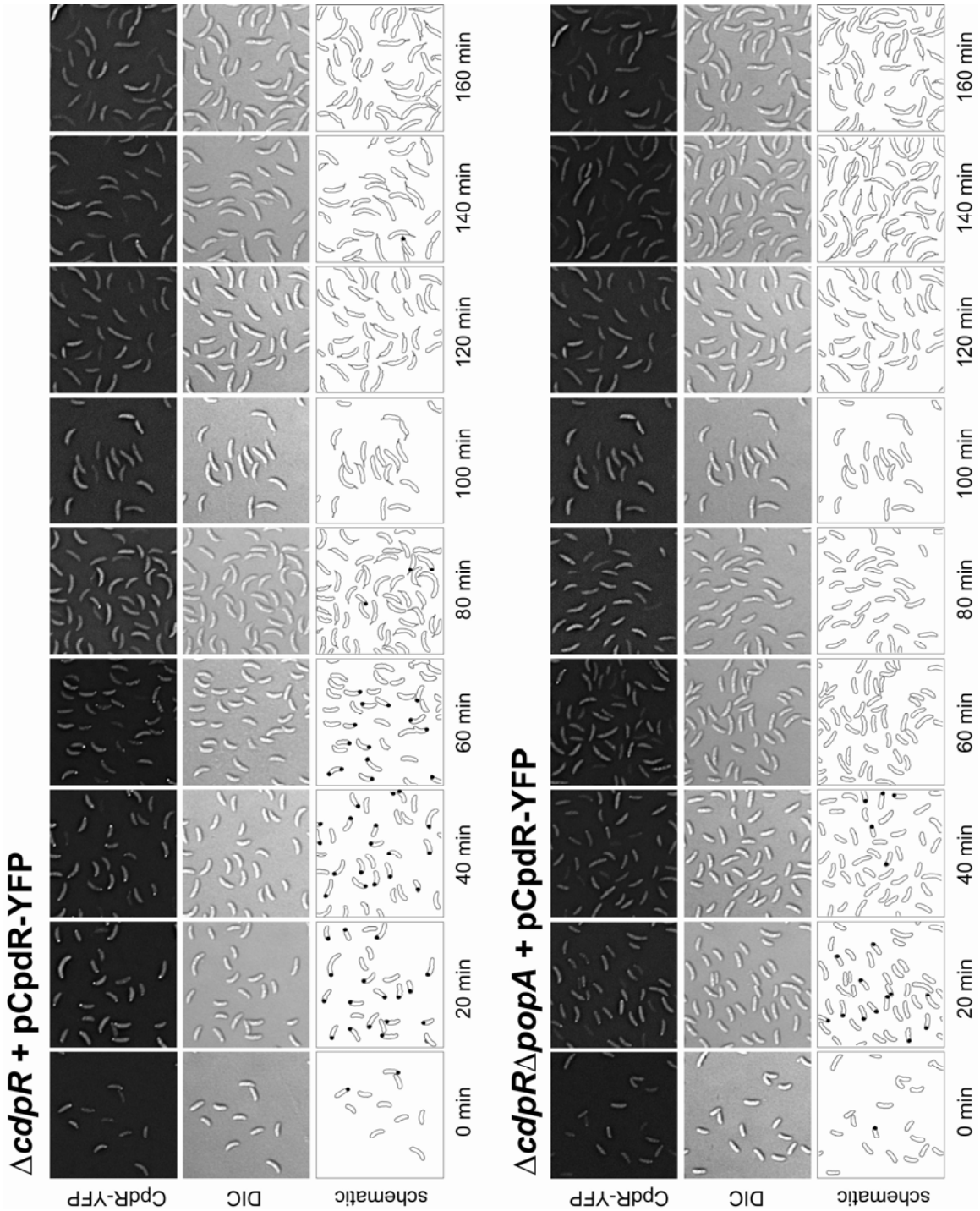

Δcdpr + pCpdR-YFP
ΔcdpRΔpopA + pCpdR-YFP
CpdR-YFP
DIC
schematic
0 min
20 min
40 min
60 min
80 min
100 min
120 min
140 min
160 min

**Table 1**

| | CdpR-YFP localized  | | CdpR-YFP delocalized  | |
|---|---|---|---|---|
| | Δ*cpdR* | Δ*cpdR*Δ*popA* | Δ*cpdR* | Δ*cpdR*Δ*popA* |
| 0 min | 57% | 36% | 43% | 64% |
| 20 min | 75% | 38% | 25% | 62% |
| 40 min | 68% | 13% | 32% | 87% |
| 60 min | 36% | 4% | 64% | 96% |
| 80 min | 4% | 1% | 96% | 99% |

## 3.2 Additional results

### 3.2.1 "$R_{357}$" of the "RXXD" I-site motif is required for PopA localization and CtrA degradation during the cell cycle

In addition to the *popA*$_{R357G}$ I-site mutant, other eight *popA* I-site mutants were generated and analyzed for its localization and for CtrA degradation during the cell cycle.
In a first experiment, every single residue of the "RXXD" motif was replaced by alanine. In a second approach, the amino acid exchanges in the "RXXD" motif were based on the study about the feedback inhibition of DgcA under the assumption that both proteins are controlled by c-di-GMP in a similar way. For DGC DgcA it has been shown that the "RNRD" and "RGQD" mutations had no effect on feedback inhibtion, but the "GVGD" as well as the "RESE" abolished feedback inhibition by c-di-GMP, but these mutants retained their DGC activity (Christen et al., 2006).

*Material and Methods*

***C. crescentus and E. coli strains***

| Name | Description | Source or Reference |
|---|---|---|
| UJ4248 | UJ2827 + plasmid pAD115 | This study |
| UJ4249 | UJ2827 + plasmid pAD116 | This study |
| UJ4250 | UJ2827 + plasmid pAD117 | This study |
| UJ4251 | UJ2827 + plasmid pAD118 | This study |
| UJ4252 | UJ2827 + plasmid pAD119 | This study |
| UJ4253 | UJ2827 + plasmid pAD120 | This study |
| UJ4254 | UJ2827 + plasmid pAD121 | This study |
| UJ4255 | UJ2827 + plasmid pAD122 | This study |

**Plasmids**

| Name | Description | Source or Reference |
|---|---|---|
| pAD115 | pMR20; PopA I-site "AVED" mutant fused to GFP; own promoter | This study |
| pAD116 | pMR20; PopA I-site "RAED" mutant fused to GFP; own promoter | This study |

| | | |
|---|---|---|
| pAD117 | pMR20; PopA I-site "RVAD" mutant fused to GFP; own promoter | This study |
| pAD118 | pMR20; PopA I-site "RVEA" mutant fused to GFP; own promoter | This study |
| pAD119 | pMR20; PopA I-site "RNRD" mutant fused to GFP; own promoter | This study |
| pAD120 | pMR20; PopA I-site "RGQD" mutant fused to GFP; own promoter | This study |
| pAD121 | pMR20; PopA I-site "GVGD" mutant fused to GFP; own promoter | This study |
| pAD122 | pMR20; PopA I-site "RESE" mutant fused to GFP; own promoter | This study |

**Table 3.1**
Strains and plasmid used in this section.

*Results and Discussion*

The arginine residue R357 of the I-site motif “RXXD” is absolutely required for PopA localization and CtrA degradation. The analysis of the localization pattern (localization to the incipient stalked pole) of *popA*$_{D360X}$ shows a partial localization defect. Despite the partial localization defect, the *popA*$_{D360X}$ mutants still degrade CtrA during the G1-to-S phase transition. This suggests that only the arginine residue R357 of the “RXXD” motif is strictly required for CtrA localization and degradation, but not the aspartic acid D360.
One explanation for the phenotype of the *popA*$_{D360X}$ mutants might be, that they still bind c-di-GMP, but less efficient than wild-type, and this residual c-di-GMP binding is sufficient for CtrA degradation. This explanation also supports the observation of the partial localization defect in the *popA*$_{D360X}$ mutants. To elaborate the exact role of residue D360 more experiments, including statistical and biochemical analysis should be carried out.

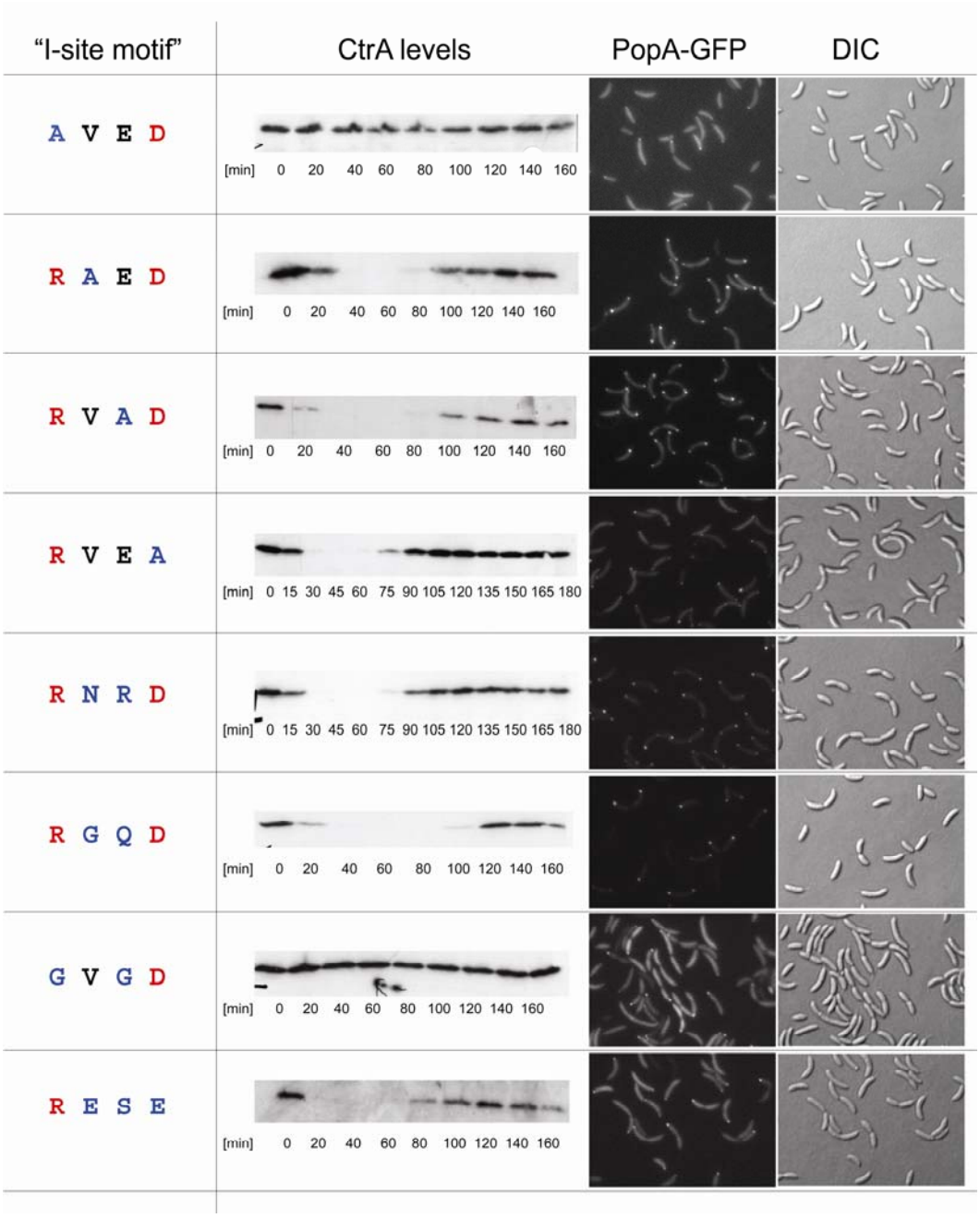



**Figure 3.1**

The amino acid exchanges are indicated in blue and the conserved residues are highlighted in red. CtrA levels are measured during cell cycle using immunoblot analysis (α-CtrA). Localization of the each I-site mutant is analyzed by fluorescence microscopy.

### 3.2.2 PopA localization to the cell poles is redundantly controlled by several GGDEF and EAL domain proteins

Previous experiments showed that PopA localization to the incipient stalked pole, at which CtrA is degraded by the ClpXP protease complex, is dependent on an intact "RXXD" I-site motif (see Chapter 3.1). In parallel, *in vitro* biochemical analysis demonstrated, that c-di-GMP binding to PopA occurs at the I-site (see Chapter 3.1). So far, there only exist a correlation between c-di-GMP binding to the I-site and polar PopA localization. To find evidence that PopA localization to the incipient stalked pole is directly linked to c-di-GMP binding, the localization of PopA-GFP was analyzed in different DGC and PDE mutant strains.

#### *Material and Methods*

***C. crescentus and E. coli strains***

| Name | Description | Source or Reference |
|---|---|---|
| UJ2895 | NA1000Δ*popA*Δ*pleD* + pAD5 | This study |
| | NA1000 +pBBR-MCS5-CC3396 + pAD5 | This study |

**Plasmids**

| Name | Description | Source or Reference |
|---|---|---|
| pAD5 | pMR20; PopA fused to GFP; own promoter | This study |
| pBBR-MCS5-CC3396 | pBBR; overexpression of *pdeA* | Jake Malone |

**Table 3.2**
Strains and plasmid used in this section.

#### *Results and Discussion*

In the Δ*pleD*Δ*dgcB* mutant the PopA polar localization pattern was not changed compared to wild-type. This suggests that other and/or additional diguanylate cyclases contribute to the c-di-GMP pool that controls PopA localization.

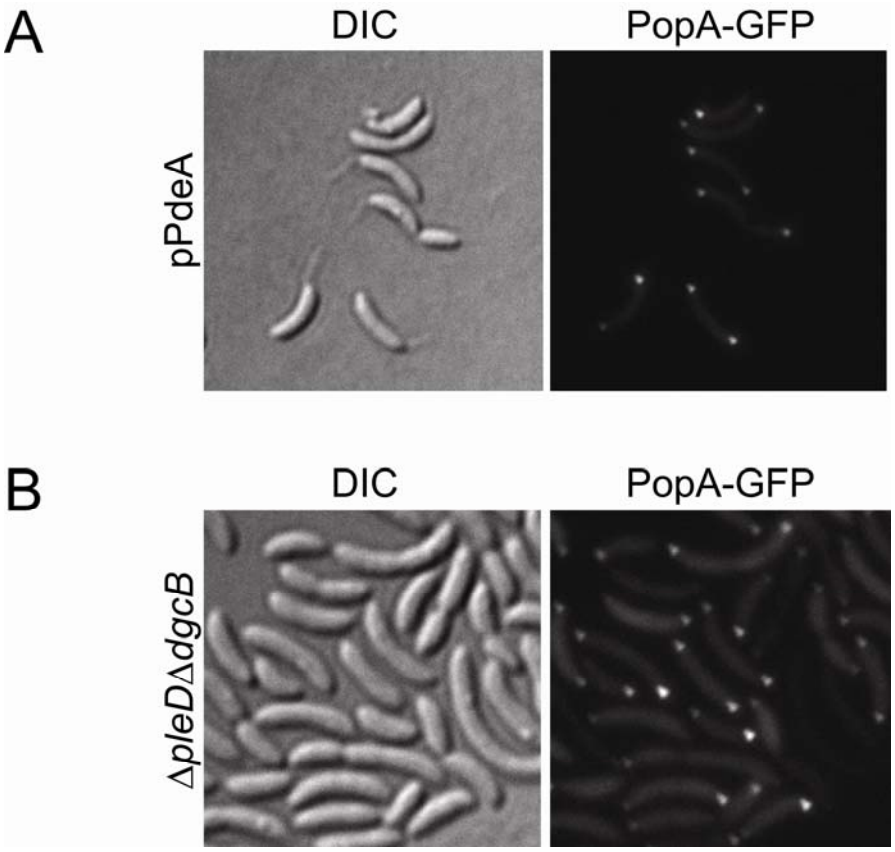



**Figure 3.2**

A) PopA-GFP localization in NA1000 overexpressing the PDE *pdeA* from medium copy plasmid. B) PopA-GPF localization in NA100Δ*pleD*Δ*dgcB*

Overexpression of *pdeA*, a phosphodiesterase, had no effect on PopA localization to the incipient stalked pole. One possible explanation for unchanged PopA sequestration might be that PdeA levels are proteolytically controlled during the cell cycle and therefore overexpression of *pdeA* has no effect (S. Abel, unpublished). Decreasing the c-di-GMP concentration by overexpression an active PDE is still an attractive model to test PopA sequestration to the incipient stalked pole. However, for prospective experiments it would be sensible to choose a phosphodiesterase from another organism or only a fragment to avoid possible regulation.

### 3.2.3 PopA is not required for PleD and PleC localization to the cell pole

PopA is a polar targeting factor for CtrA localization to the cell pole. Is PopA required for the polar sequestration of additional proteins? Previous studies showed PleD (Paul et al., 2004) and PleC (Wheeler and Shapiro, 1999) polar localization to the cell pole. To test if PopA acts as more general polar targeting factor, PleD-GFP and PleC-GFP localization was analyzed in wild-type and in the Δ*popA* mutant.

#### *Material and Methods*

***C. crescentus and E. coli strains***

| Name | Description | Source or Reference |
|---|---|---|
| UJ2724 | UJ1267 + plasmid pSA41 | Sören Abel |
| UJ3544 | UJ1267 + plasmid pPleC-EGFP | Ralf Paul |
| UJ4087 | UJ2827 + plasmid pPleC-EGFP | This study |
| UJ4088 | UJ2827 + plasmid pSA41 | This study |

**Plasmids**

| Name | Description | Source or Reference |
|---|---|---|
| pSA41 | pBBR; PleD-GFP under endogenous promoter | Sören Abel |
| pPleC-EGFP | pMR20; PleC-EGFP | (Wheeler and Shapiro, 1999) |

**Table 3.3**
Strains and plasmid used in this section.

#### *Results and Discussion*

PleC and PleD localization to the cell poles is not dependent on PopA. Based these initial data one can suggests that PopA is neither a recruitment factor nor a receptor for PleC and PleD at the pole.

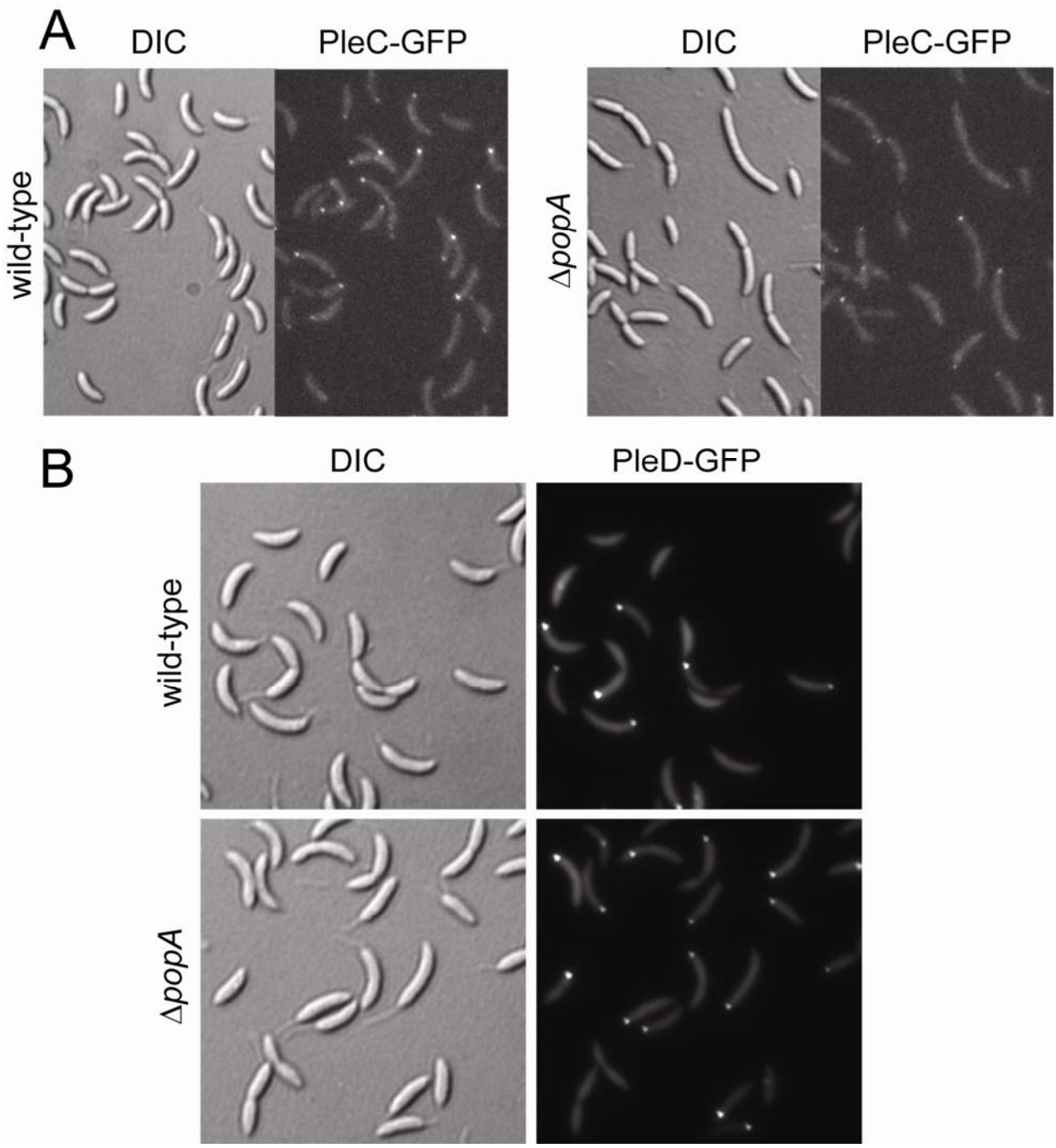



**Figure 3.1**

A) PleC-eGFP localization in NA1000 wild-type (left) and in the Δ*popA* mutant background (right). B) PleD-eGFP localization in NA1000 wild-type (top) and in the Δ*popA* mutant background (bottom)

### 3.2.4 CtrA localization varies from *Caulobacter crescentus* wild-type to wild-type strain

CtrA localization to the incipient stalked pole is dependent on PopA in the NA1000 wild-type (UJ1267) background. NA1000 is a stable lab wild-type strain that lost the ability to build a holdfast and fails to attach to surfaces. So far, it is not known, which mutation or deletion led to the NA1000 phenotype. In contrast to NA1000, the ATCC strain CB15 (#19089) is able to form holdfast and to attach to surfaces. All different CB15 wild-type and NA1000 wild-ype strains from our lab were tested for CtrA localization to the incipient stalked pole. In parallel, PopA levels were measured by immunoblot analysis using $\alpha$-CtrA.

*Material and Methods*

***C. crescentus and E. coli strains***

| Name | Description | Source or Reference |
|---|---|---|
| UJ1267 | NA1000 wild-type strain | (Evinger and Agabian, 1977) |
| LS1250 | CB15 wild-type strain | L. Shapiro |
| UJ1597 | CB15 wild-type strain | A. Newton |
| UJ2827 | NA1000Δ*popA* (UJ1267 background) | This study |
| UJ3125 | NA1000 (UJ1267) + plasmid pEJ146 | This study |
| UJ3127 | UJ2827 + plasmid pEJ146 | This study |
| UJ3128 | CB15 (UJ1597) + plasmid pEJ146 | This study |
| UJ3129 | CB15 (LS1250) + plasmid pEJ146 | This study |

**Plasmids**

| Name | Description | Source or Reference |
|---|---|---|
| pEJ146 | pMR10-Pxyl::YFP-CtrA RD+15 | (Ryan et al., 2002) |

**Table 3.4**
Strains and plasmid used in this section.

*Results and Discussion*

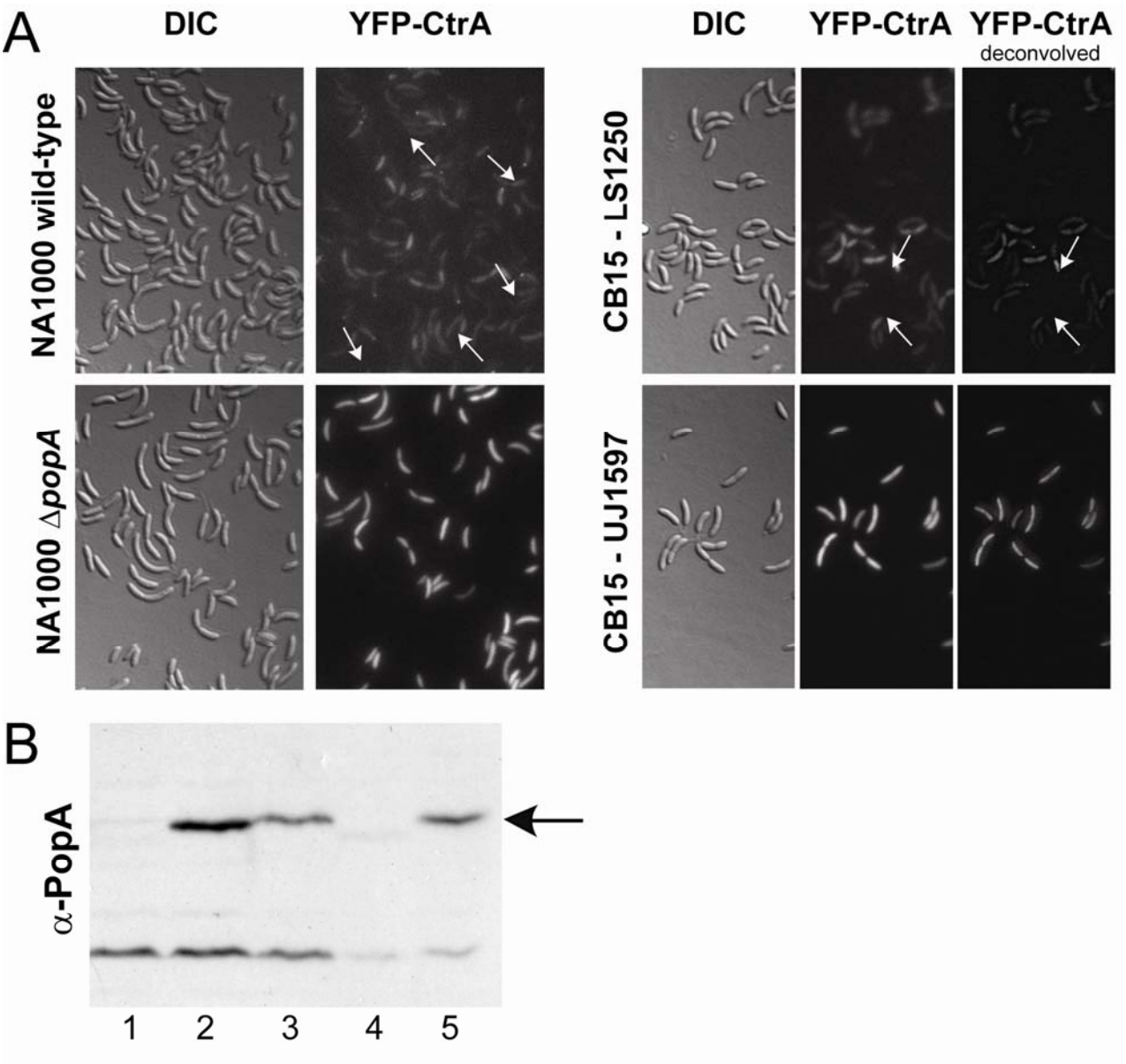



**Figure 3.4**

A) DIC and fluorescence (deconvolved) microscopy. YFP-CtrA localization in NA1000 wild-type, LS1250 ("CB15 wild-type"), UJ1597 ("CB15 wild-type) and NA1000Δ*popA* (control). CtrA localization to the incipient stalked pole is marked by arrows. B) PopA levels (indicated by arrow) measured by immunoblot analysis (α-PopA) of 1) UJ1597, exponential growth phase, 2) UJ1267, exponential growth phase, 3) LS1250, exponential growth phase, 4) UJ1597, stationary growth phase, 5) UJ1267, stationary growth phase. The exact PopA levels of the different strains cannot be compared due to varying amount of proteins loaded.

As expected, in UJ1267 (NA1000 wild-type) and in LS1250 (“CB15 wild-type”) is CtrA sequestered to the incipient stalked pole. In contrast, in UJ1597 (“CB15 wild-type”) CtrA fails to localize to the incipient stalked pole. NA1000Δ*popA* was used as a negative control. Interestingly, the loss of CtrA localization in UJ1597 correlates with no detectable PopA protein using western blot analysis. This suggests that PopA is required for CtrA degradation in UJ1597. However, it is still unclear if PopA is sufficient or if additional factors are required for CtrA localization in UJ1597. To answer this question, UJ1597 should be complemented by bringing back *popA* in trans and analyzed for CtrA localization. Further, it would be interesting to test CtrA degradation in UJ1597 and to correlate the data with the levels of PopA.

### 3.2.5 Blocking CtrA dephosphorlyation and degradation causes a G1 cell cycle arrest

Previous studies showed that the activity of CtrA is redundantly controlled at the levels of expression, phosphorylation and degradation (Domian et al., 1997; Domian et al., 1999). To initiate DNA replication the swarmer cell has to remove active CtrA~P during the G1-to-S phase transition by dephosphorylation and controlled degradation. Mutants that are impaired in dephosphorylation and degradation of active CtrA during the cell cycle show a G1 cell cycle arrest and a division block (Domian et al., 1997). It has previously been shown that PopA is required for CtrA degradation during the G1-to-S phase transition (see Chapter 3.1). The induction of transcriptional fusions of *ctrA* wild-type ($P_{xyl}$::*ctrA*) or $ctrA_{D51E}$ ($P_{xyl}$::$ctrA_{D51E}$, constitutive active *ctrA* allele (Domian et al., 1997)) resulted in a cell division block in the Δ*popA* mutant (see Chapter 3.1).

In addition, translational *ctrA* ($P_{xyl}$::*xylX'Φ ctrA)* and $ctrA_{D51E}$ fusions ($P_{xyl}$::*xylX'Φ* $ctrA_{D51E}$*)* to the *xylX* gene were generated, and analyzed in wild-type and Δ*popA* mutant backgrounds. Cell morphology and CtrA levels in wild-type and in the Δ*popA* mutant strain were investigated upon induction of the transcriptional or the translational *ctrA* fusions.

To prove a possible G1-cell cycle arrest in Δ*popA* upon overexpression of *ctrA* or $CtrA_{D51E}$, the mating frequency was calculated, when a medium copy plasmid carrying either *ctrA* or $ctrA_{D51E}$ was introduced into the Δ*popA* mutant or into the wild-type strain.

## *Material and Methods*

### *C. crescentus and E. coli strains*

| Name | Description | Source or Reference |
|---|---|---|
| UJ1267 | NA1000 wild-type | This study |
| UJ2827 | NA1000Δ*popA* | This study |
| UJ3962 | NA1000 wild-type + plasmid pAD41 | This study |
| UJ3963 | NA1000 wild-type + plasmid pAD42 | This study |
| UJ3964 | NA1000Δ*popA* + plasmid pAD41 | This study |
| UJ3965 | NA1000Δ*popA* + plasmid pAD42 | This study |
| UJ3966 | NA1000 + plasmid pID42 | This study |
| UJ3967 | NA1000 + plasmid pIDC42 | This study |
| UJ3969 | NA1000Δ*popA* + plasmid pID42 | This study |
| UJ3970 | NA1000Δ*popA* + plasmid pIDC42 | This study |
| | NA1000 wild-type + plasmid pB2C51E | This study |
| | NA1000 wild-type + plasmid pB2CWT | This study |
| | NA1000Δ*popA* + plasmid pB2C51E | This study |
| | NA1000Δ*popA* + plasmid pB2CWT | This study |

### Plasmids

| Name | Description | Source or Reference |
|---|---|---|
| pAD41 | pUJ142; translational fusion of *ctrA*; $P_{xyl}$::*xylX'Φ ctrA* | This study |
| pAD42 | pUJ142; translational fusion of $ctrA_{D51E}$; $P_{xyl}$::*xylX'Φ* $ctrA_{D51E}$ | This study |
| pID42 | pJS14; transcriptional fusion of *ctrA*; $P_{xyl}$::*ctrA* | (Domian et al., 1997) |
| pIDC42 | pJS14; transcriptional fusion of $ctrA_{D51E}$; $P_{xyl}$::$ctrA_{D51E}$ | (Domian et al., 1997) |
| pB2C51E | pBBR1-MCS-2; $ctrA_{D51E}$ on medium copy plasmid | A. Stotz |
| pB2CWT | pBBR1-MCS-2; *ctrA* wild-type on medium copy plasmid | A. Stotz |

**Table 3.5**

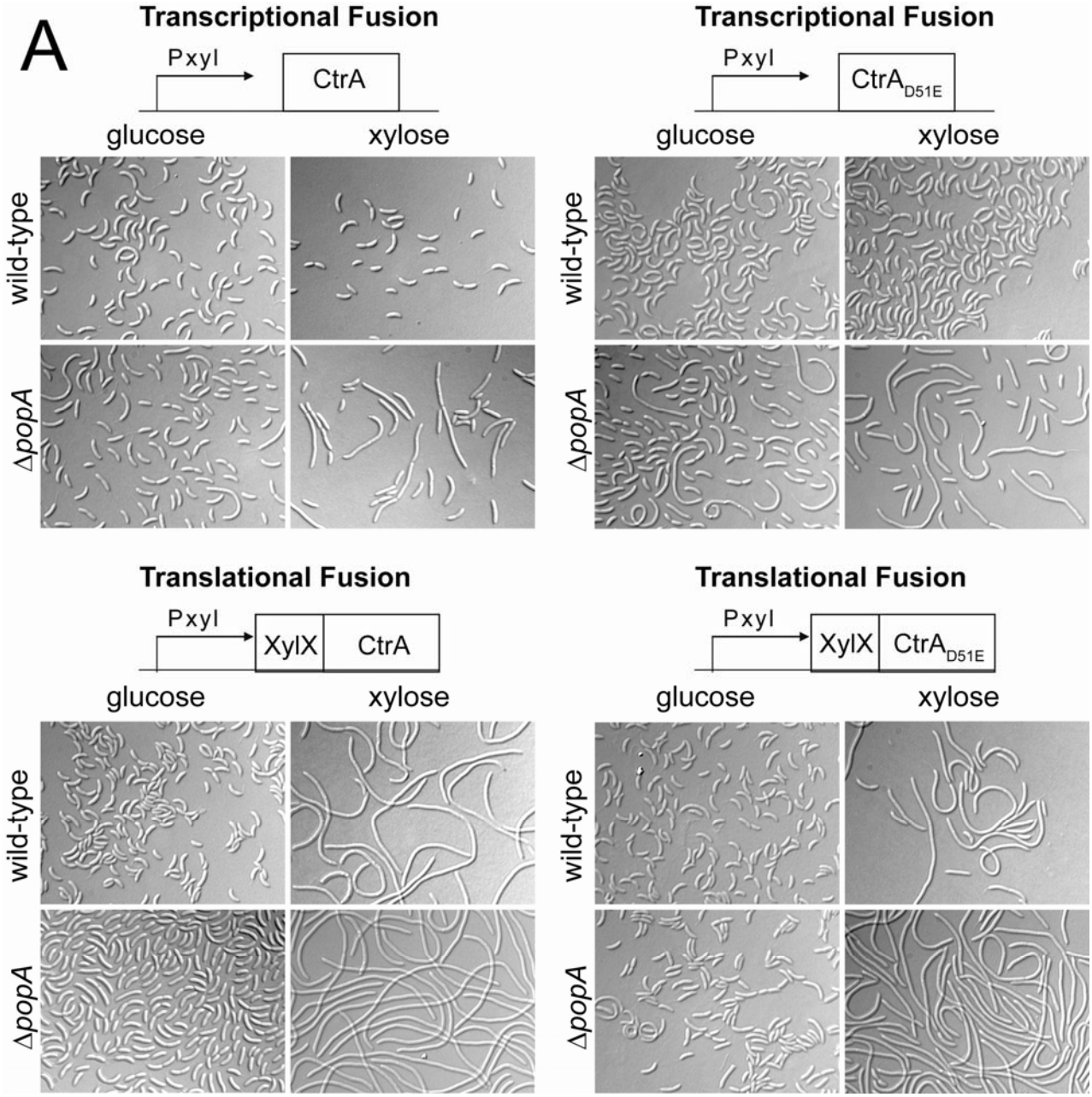
Strains and plasmid used in this section.

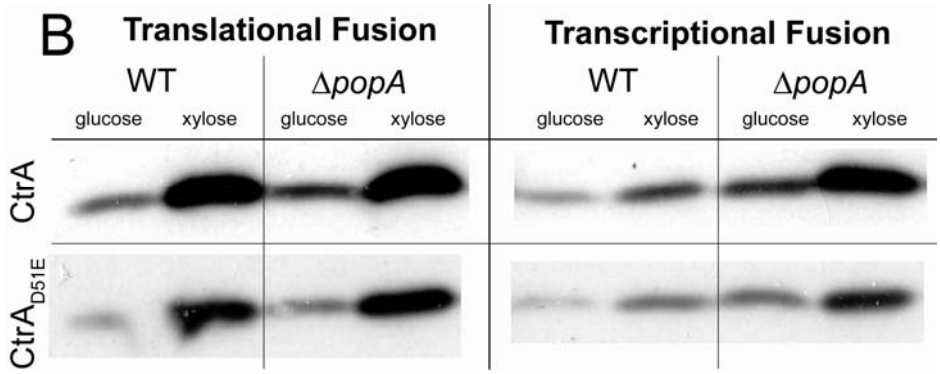
*Results and Discussion*

While the induction of the transcriptional c*trA* and $ctrA_{D51E}$ fusions in wild-type had no effect, it resulted in the Δ*popA* mutant in filamentous cells suggesting a cell division block (see Chapter 3.1). However, induction of the translational *ctrA* and $ctrA_{D51E}$ fusions caused extremely filamentous cells in wild-type as well as in the Δ*popA* mutant.

In parallel CtrA levels of non-induced and induced wild-type and Δ*popA* cells were measured using immunoblot analysis. Induction of the translational *ctrA,* and $ctrA_{D51E}$ fusions led to higher CtrA levels than the induction of the transcriptional fusions. This suggests that in addition to the phosphorylation and degradation control, the amount of CtrA is crucial for cell viability. Even wild-type cells loose viability, when *ctrA* (translational fusion) is overexpressed despite functional dephosphorylation and degradation control mechanisms. The N-terminal fusion of CtrA to the first six amino acids of XylX protein seems to stabilize CtrA which results in higher CtrA levels in the cells.

Introducing *ctrA* wild-type and $ctrA_{D51E}$ on a medium copy number plasmid into the Δ*popA* mutant results in a dramatically reduced mating efficiency compared to wild-type cells. After the mating of *ctrA* or $ctrA_{D51E}$ into the Δ*popA* mutant only a few colonies are growing compared to wild-type (bacterial lawn), which are most likely suppressors. This is an additional piece of data which supports the observation, that overexpression of *ctrA* wild-type and $ctrA_{D51E}$ results in a G1 cell cycle arrest in Δ*popA*.


A
Transcriptional Fusion
PxyI
CtrA
glucose
xylose
wild-type
ΔpopA
Transcriptional Fusion
PxyI
CtrA D51E
glucose
xylose
wild-type
ΔpopA
Translational Fusion
PxyI
XylX
CtrA
glucose
xylose
wild-type
ΔpopA
Translational Fusion
PxyI
XylX
CtrA D51E
glucose
xylose
wild-type
ΔpopA


B
Translational Fusion
Transcriptional Fusion
WT
ΔpopA
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glucose
xylose
glucose
xylose
glucose
xylose
glucose
xylose
CtrA
CtrA D51E

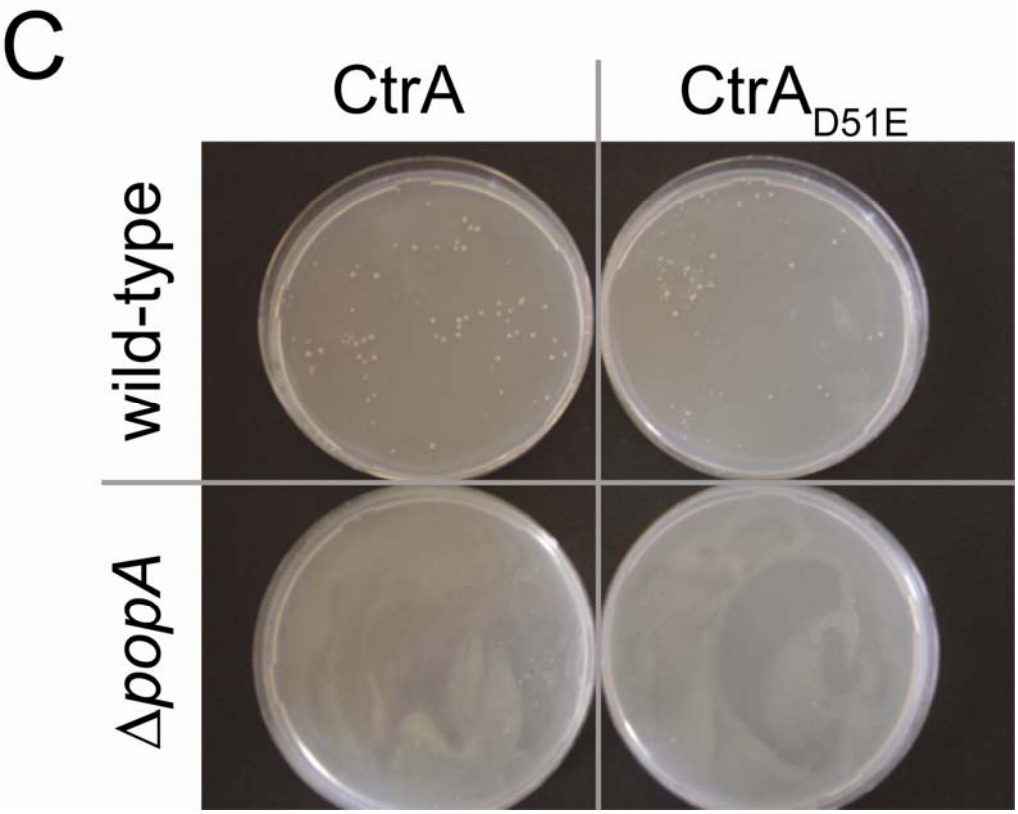



**Figure 3.5**

A) Light microscopy of wild-type and Δ*popA* cells expressing either transcriptional *ctrA* and $ctrA_{D51E}$ fusions ($P_{xyl}$::*ctrA* or $P_{xyl}$::$ctrA_{D51E}$) or translational *ctrA* and $ctrA_{D51E}$ fusions ($P_{xyl}$::*xylX'Φ ctrA* or $P_{xyl}$::*xylX'Φ* $ctrA_{D51E}$) under non-induced and induced conditions. B) CtrA levels of wild-type and Δ*popA* cells expressing either transcriptional *ctrA* and $ctrA_{D51E}$ fusions or translational *ctrA* and $ctrA_{D51E}$ fusions under non-induced and induced conditions using immunoblot analysis (α-CtrA). C) Mating of $pCtrA_{D51E}$ (pB2C51E) and pCtrA (pB2CWT) into wild-type and the Δ*popA* mutant. To calculate the mating efficiency equal amount of cells of each strain were plated on PYE.

### 3.2.6 The interactom of factors controlling CtrA degradation

To analyze possible protein-protein interactions between the components involved in CtrA degradation, ClpX, ClpP, RcdA, CpdR, CtrA and PopA were tested for direct interaction by the bacterial two hybrid analysis. All possible combinations of the different proteins were generated and checked for c-AMP production on McConkey plates. Positive protein-protein interactions are displayed by red colonies, negative protein-protein interactions by white colonies on McConkey agar base maltose plates. In chapter 3.1 (Figure S3) all positive protein-protein interactions are schematically summarized.

*Material and Methods*

***E. coli strains***

| Name | Description | Source or Reference |
|---|---|---|
| MM337 | K-12<br>araD139 flbB5301 ptsF25 rbsR relA1 rpsL150-(argF-lac)U169 -cya | M. Manson<br>(A. Boehm) |

**Plasmids**

| Name | Description | Source or Reference |
|---|---|---|
| pKT25 | pSU40 derivative with T25 fragment of CyaA | (Karimova et al. 2001) |
| pKT25-zip | pKT25 derivative with leucine zipper of GCN4 | (Karimova et al. 2001) |
| pUT18 | pUC19 derivative with T18 fragment of CyaA. N-terminal fusion | (Karimova et al. 2001) |
| pUT18C | pUC19 derivative with T18 fragment of CyaA. C-terminal fusions | (Karimova et al. 2001) |
| pUT18C-zip | pUT18C derivative with leucine zipper of GCN4 | (Karimova et al. 2001) |
| pAD44 | pUT18C; *clpX* C-terminal fused to the T18 fragment | This study |
| pAD45 | pKT25; *clpX* C-terminal fused to the T25 fragment | This study |
| pAD47 | pUT18C; *clpP* C-terminal fused to the T18 fragment | This study |
| pAD48 | pKT25; *clpP* C-terminal fused to the T25 fragment | This study |
| pAD50 | pUT18C; *rcdA* C-terminal fused to the T18 fragment | This study |
| pAD51 | pKT25; *rcdA* C-terminal fused to the T25 fragment | This study |
| pAD53 | pUT18C; *cpdR* C-terminal fused to the T18 fragment | This study |

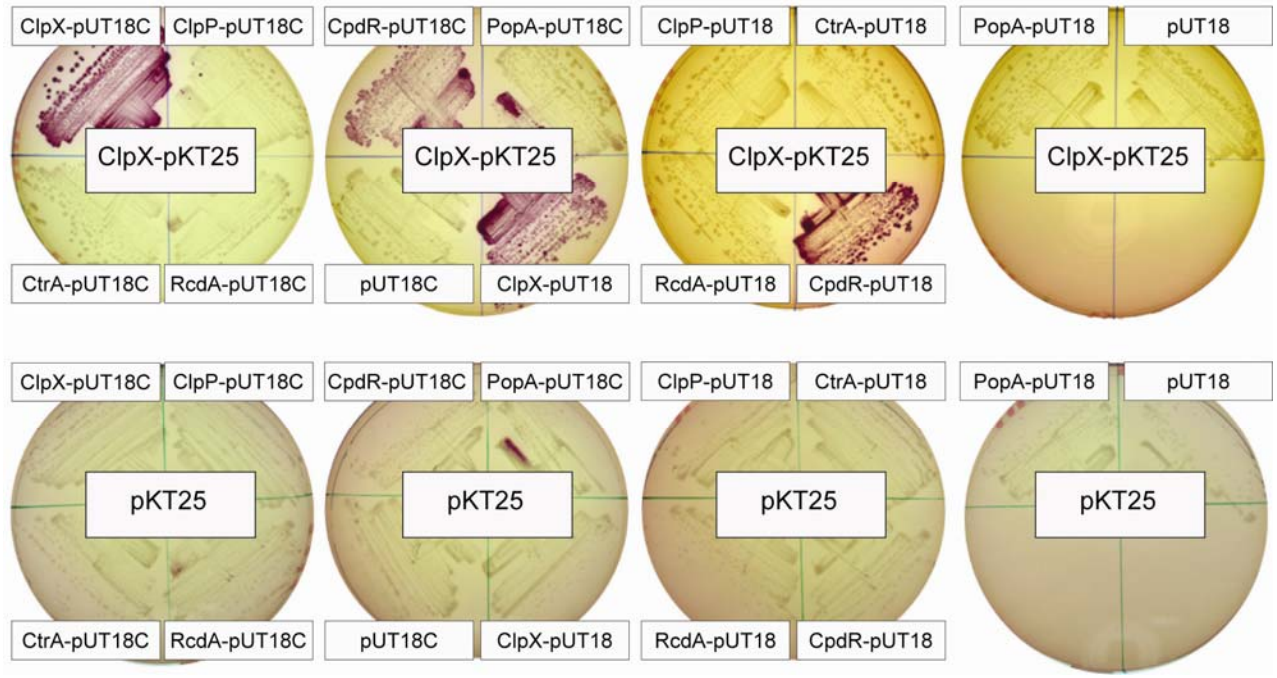
| | | |
|---|---|---|
| pAD54 | pKT25; *cpdR* C-terminal fused to the T25 fragment | This study |
| pAD56 | pUT18; *rcdA* N-terminal fused to the T18 fragment | This study |
| pAD58 | pUT18; *clpX* N-terminal fused to the T18 fragment | This study |
| pAD60 | pUT18; *clpP* N-terminal fused to the T18 fragment | This study |
| AD62 | pUT18; *cpdR* N-terminal fused to the T18 fragment | This study |
| pAD65 | pUT18; *ctrA* N-terminal fused to the T18 fragment | This study |
| pAD67 | pUT18; *popA* N-terminal fused to the T18 fragment | This study |
| pAD140 | pKT25; *ctrA* C-terminal fused to the T25 fragment | This study |
| pAD141 | pKT25; *popA* C-terminal fused to the T25 fragment | This study |
| pAD142 | pUT18C; *ctrA* C-terminal fused to the T18 fragment | This study |
| pAD143 | pUT18C; *popA* C-terminal fused to the T18 fragment | This study |

**Table 3.6**
Strains and plasmid used in this section.

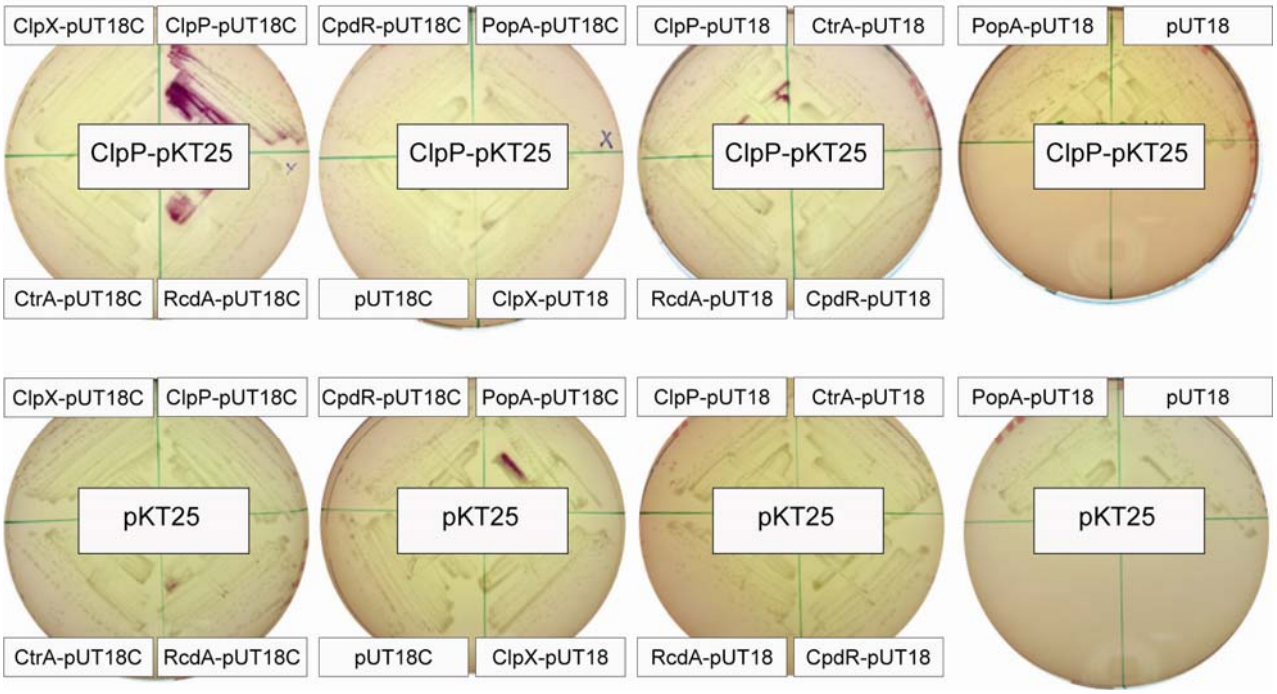
### *Results and Discussion*

Strong signals indicating interaction were obtained for the following protein pairs: ClpX/ClpX, ClpP/ClpP and ClpX/CpdR. This is in agreement with previous studies (Iniesta et al., 2006). In addition, the following protein-protein interactions were found: CpdR/CpdR (strong signal), PopA/RcdA (strong signal), PopA/PopA (weak signal), CpdR/RcdA (weak signal) and ClpP/RcdA (very weak signal). Importantly, the fusion RcdA-pKT25 showed positive interaction with the empty plasmids pUT18 and pUT18C, which suggests false positive interactions. As a consequence all the results using the RcdA-pKT25 fusion are ignored.

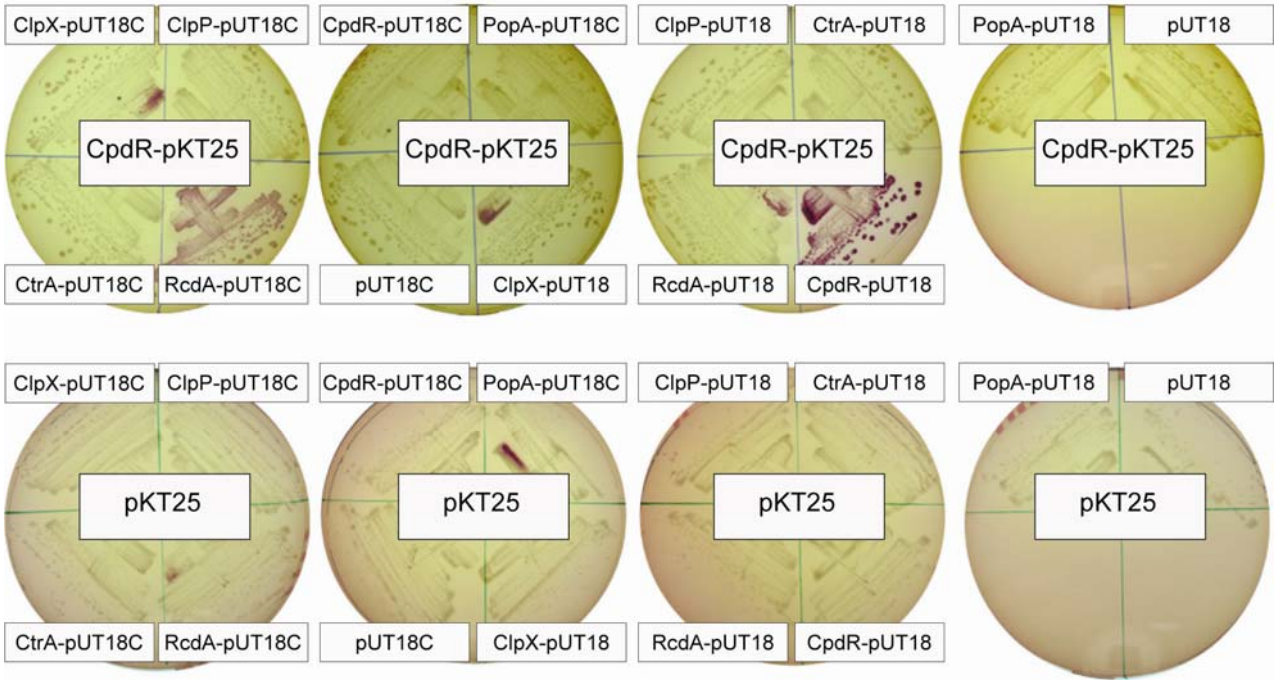
## ClpX - Interactions



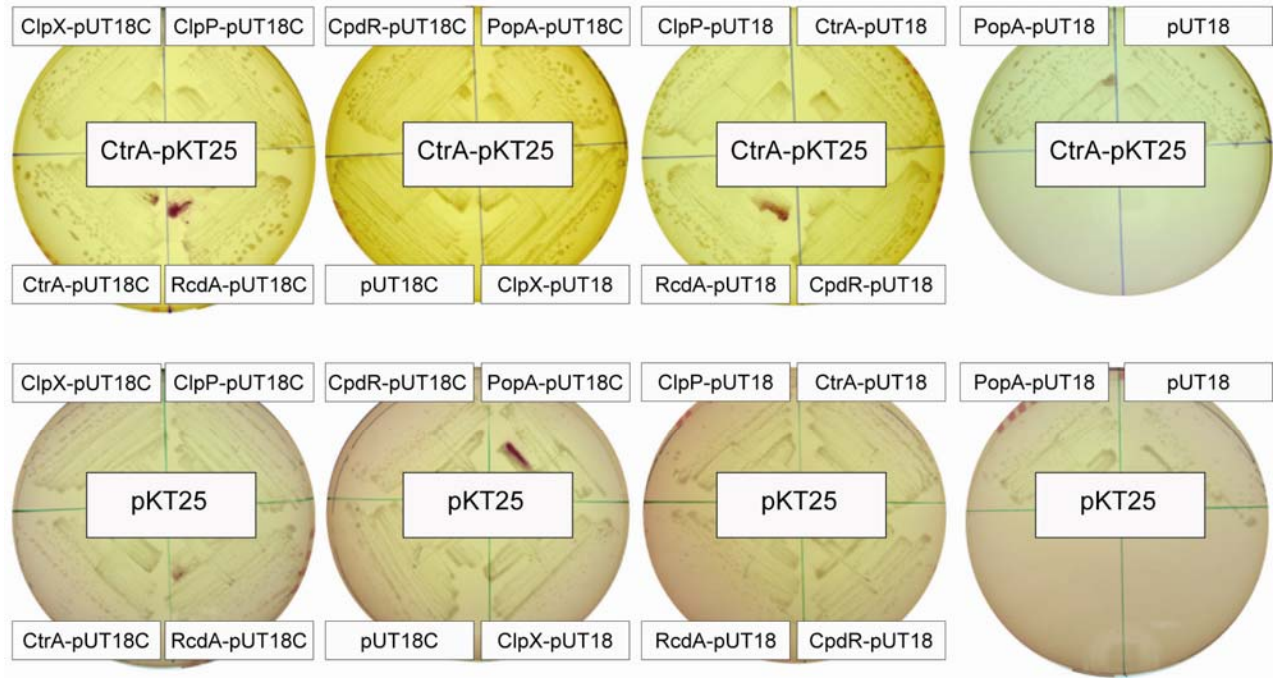

## ClpP - Interactions



## CpdR - Interactions




## CtrA - Interactions



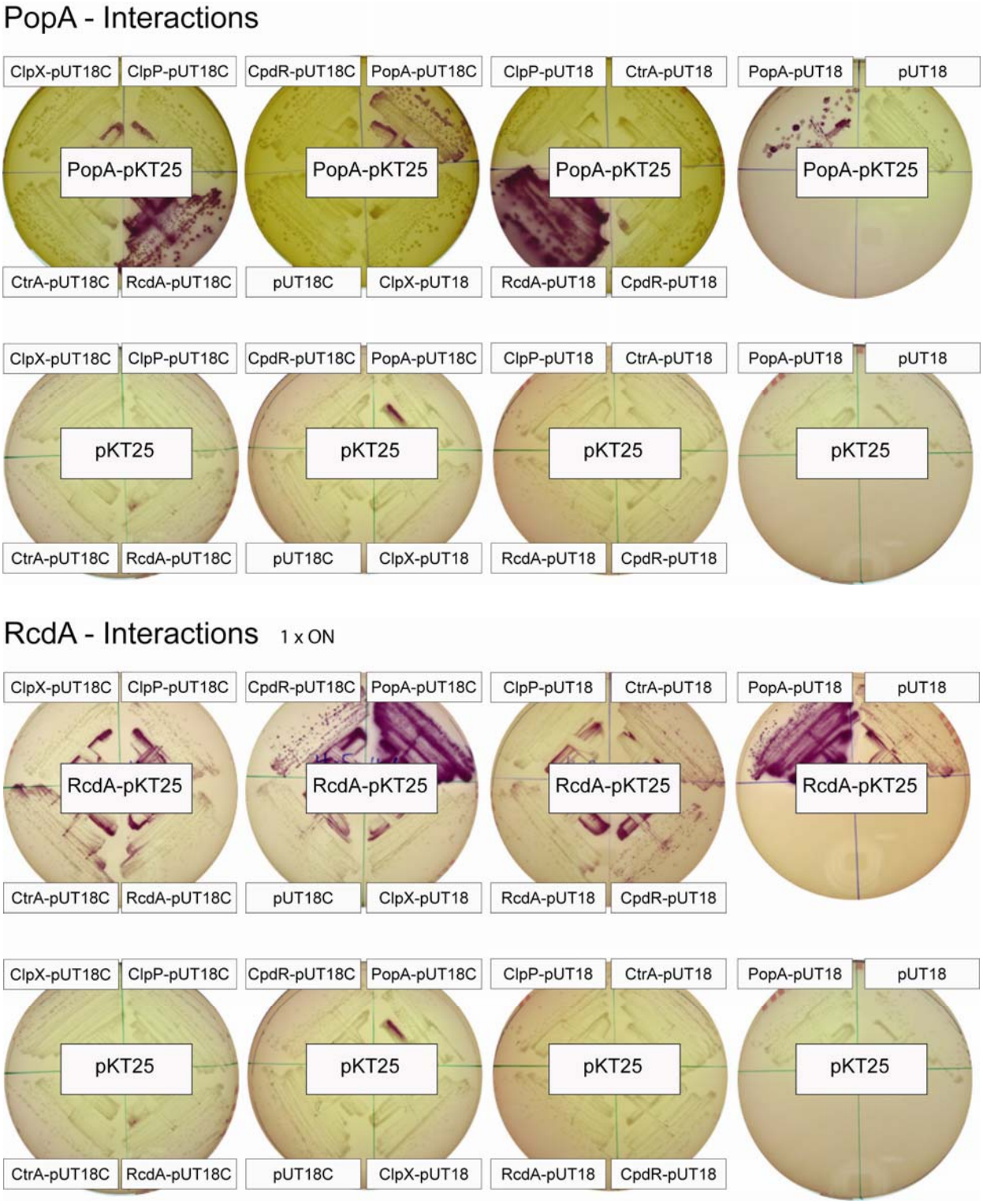



**Figure 3.6**

Protein-protein interactions are analyzed on McConkey agar base (+ maltose) plates. Positive interaction is shown in red, negative in white.

### 3.2.7 PopA forms oligomers and the first receiver domain of PopA interacts with RcdA

To analyze the PopA/PopA and PopA/RcdA interaction in more detail, single domains of PopA were fused to the T18 and T25 fragment of the adenylate cyclase and assayed for interaction with RcdA and single domains of PopA as well as full-length PopA on McConkey agar base plates (+maltose).

*Material and Methods*

***E. coli strains***

| Name | Description | Source or Reference |
|---|---|---|
| MM337 | K-12<br>araD139 flbB5301 ptsF25 rbsR relA1 rpsL150-(argF-lac)U169 -cya | M. Manson<br>(A. Boehm) |

**Plasmids**

| Name | Description | Source or Reference |
|---|---|---|
| pKT25 | pSU40 derivative with T25 fragment of CyaA | (Karimova et al. 2001) |
| pKT25-zip | pKT25 derivative with leucine zipper of GCN4 | (Karimova et al. 2001) |
| pUT18 | pUC19 derivative with T18 fragment of CyaA. N-terminal fusion | (Karimova et al. 2001) |
| pUT18C | pUC19 derivative with T18 fragment of CyaA. C-terminal fusions | (Karimova et al. 2001) |
| pUT18C-zip | pUT18C derivative with leucine zipper of GCN4 | (Karimova et al. 2001) |
| pAD50 | pUT18C; *rcdA* C-terminal fused to the T18 fragment | This study |
| pAD51 | pKT25; *rcdA* C-terminal fused to the T25 fragment | This study |
| pAD67 | pUT18; *popA* N-terminal fused to the T18 fragment | This study |
| pAD143 | pUT18C; *popA* C-terminal fused to the T18 fragment | This study |
| pAD156 | pUT18C; *popA-Rec1* C-terminal fused to the T18 fragment | This study |
| pAD157 | pUT18C; *popA-Rec2* C-terminal fused to the T18 fragment | This study |
| pAD158 | pUT18C; *popA-Rec1+Rec2* C-terminal fused to the T18 fragment | This study |
| pAD159 | pUT18C; *popA-GGDEF* C-terminal fused to the T18 fragment | This study |
| pAD160 | pKT25; *popA-Rec1* C-terminal fused to the T25 fragment | This study |

| | | |
|---|---|---|
| pAD161 | pKT25; *popA-Rec2* C-terminal fused to the T25 fragment | This study |
| pAD162 | pKT25; *popA-Rec1+Rec2* C-terminal fused to the T25 fragment | This study |
| pAD163 | pKT25; *GGDEF* C-terminal fused to the T25 fragment | This study |

**Table 3.7**
Strains and plasmid used in this section.

*Results and Discussion*

Unfortunately, popA-Rec1 fused to pUT18C as well as popA fused to pUT18 show positive interaction with the empty plasmid pKT25. This is an indication for false positives and therefore all results using popA-Rec1 fused to pUT18C and popA fused to pUT18 are ignored. However, a positive interaction of the Rec1 domain of PopA with RcdA could be demonstrated. This suggests that the Rec1 domain of PopA is required for the interaction with RcdA. Negative interactions of single domains might also be due to the instability of the fusion proteins and these negative results should be taken with caution.
Furthermore, the comprehensive protein-protein interaction study revealed direct interaction of PopA with itself (see Chapter 3.6, Figure 3.6). This argues that PopA forms oligomers. In addition, a weak interaction of the PopA-Rec1Rec2 fragment to full-length PopA could be observed. This might be first evidence that the GGDEF domain of PopA is not required for PopA oligomerization.

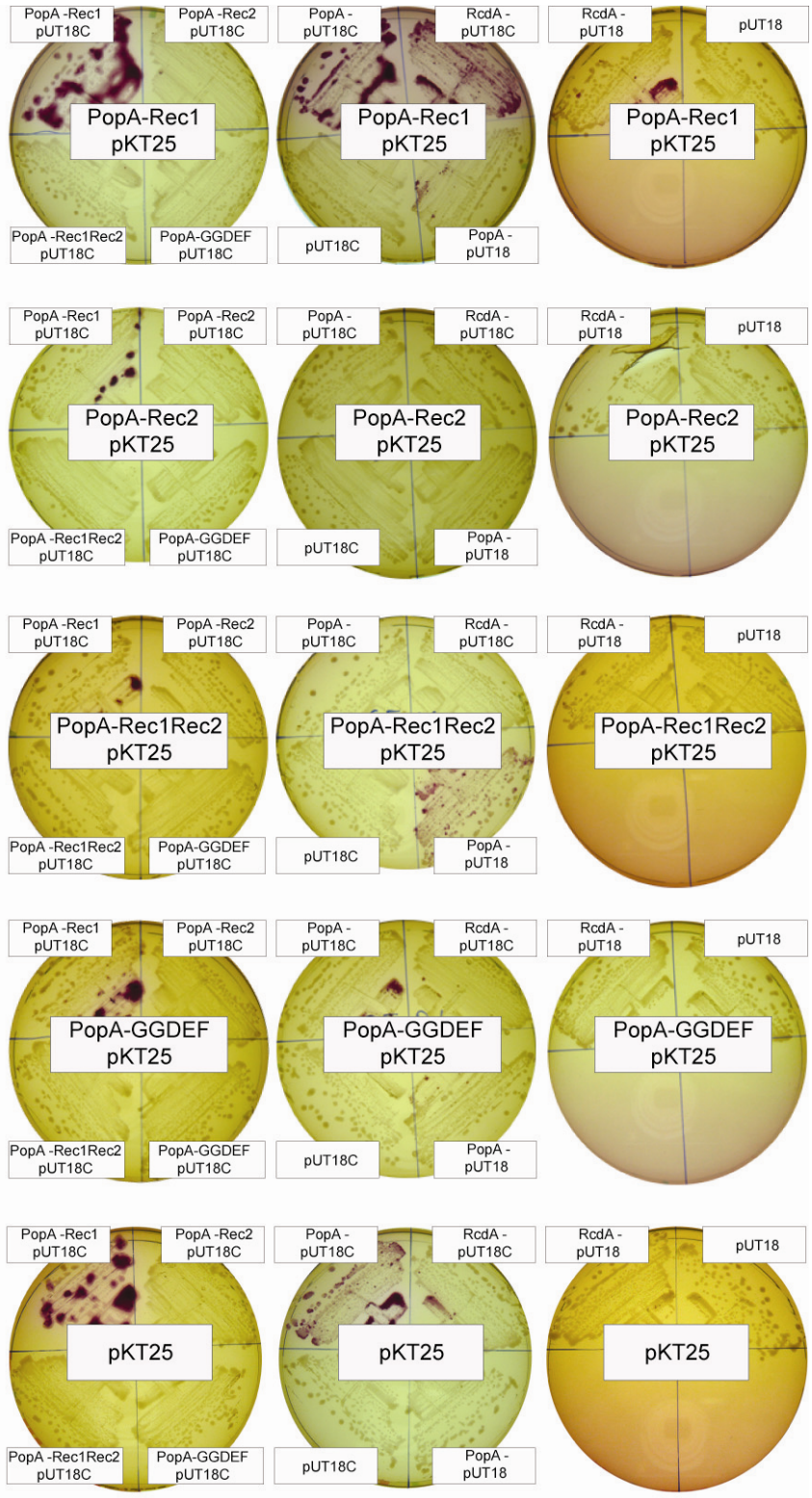



**Figure 3.7**

Protein-protein interactions are analyzed on McConkey agar base (+ maltose) plates. Positive interaction is shown in red, negative in white.

### 3.2.8 PopA controls cell motility and attachment

So far, it has been shown that the c-di-GMP effector protein PopA is required for CtrA sequestration to the incipient stalked pole and for CtrA degradation during the G1-to-S phase transition. Furthermore, it has been demonstrated that PopA-GFP dynamically localizes to the cell poles during the cell cycle. PopA sequestration to the incipient stalked pole is dependent on an intact I-site. The *popA* I-site mutant fails to localize to the incipient stalked pole. This is in agreement with the observation that the *popA* I-site mutant, which lacks c-di-GMP binding *in vitro*, fails to degrade CtrA during the G1-to-S phase transition *in vivo*. Surprisingly, the *popA* I-site mutant is still sequestered to the newborn swarmer cell pole. This finding suggests that PopA might act as a multifunctional protein that in addition to its cell cycle function is also part of the complex regulatory network, which controls cell motility and surface attachment during the *C. crescentus* life cycle. In the following section, PopA was analyzed in respect to cell motility and surface attachment in the context of a regulatory network consisting of multiple GGDEF domain proteins.

#### Materials and Methods

*Strains, plasmids, and media*

All bacterial strains and plasmids used in this study are summarized in Table 3.8 *C. crescentus* strains were either grown in peptone yeast extract (PYE), Hutner base–imidazole-buffered–glucose–glutamate (HIGG), or M2G minimal medium (Poindexter, 1978) at 30$^{o}$C. Plasmids were introduced into *C. crescentus* either by conjugation or electroporation.
*E. coli* strains were grown in Luria Broth (LB) media. Antibiotics for selection were added to the media where necessary.

***C. crescentus and E. coli strains***

| Name | Description | Source or Reference |
|---|---|---|
| NA1000 | Synchronizable laboratory strain of CB15 | (Evinger, 1977) |

| | | |
|---|---|---|
| CB15 | wild-type strain, LS1250 | L. Shapiro |
| UJ284 | NA1000Δ*pleD* | (Aldridge and Jenal, 1999) |
| UJ730 | CB15Δ*pleD* | (Levi and Jenal, 2006) |
| UJ2821 | NA1000Δ*dgcB* | This study |
| UJ2827 | NA1000Δ*popA* | This study |
| UJ2874 | NA1000Δ*popA*Δ*pleD* | This study |
| UJ2876 | NA1000Δ*popA*Δ*dgcB* | Chapter 3.1 |
| UJ2878 | NA1000Δ*pleD*Δ*dgcB* | This study |
| UJ3123 | NA1000Δ*pleD*Δ*dgcB* | This study |
| UJ3124 | CB15Δ*popA*Δ*pleD* | This study |
| UJ3154 | CB15Δ*dgcB* | This study |
| UJ3155 | CB15Δ*popA* | This study |
| UJ3159 | NA1000Δ*popA* and plasmid pAD19 | Chapter 3.1 |
| UJ3160 | NA1000Δ*popA* and plasmid pAD5 | Chapter 3.1 |
| UJ3239 | NA1000Δ*popA* and empty vector pMR20 | This study |
| UJ3563 | NA1000Δ*popA* and plasmid pAD32 | Chapter 3.1 |
| UJ3565 | NA1000Δ*popA* and plasmid pAD30 | Chapter 3.1 |
| UJ3642 | CB15Δ*popA*Δ*dgcB* | This study |

***E. coli* strains**

| **Name** | **Description** | **Source or Reference** |
|---|---|---|
| DH10B | F$^-$*mcrA* D(*mrr*$^-$hsd RMS$^-$*mcr*BC) f80d*lacZ*DM15D*lacX*74 endA1 recA1deoR D(*ara, leu*)7697 *araD139 galU galK nupG rpsL thi pro* $^-$ *hsd*$^+$ *recA RP4-2-Tc*::*Mu-Tn7* | (Simon et al., 1983) |
| S17 | F-, lambda (-), thi, pro,recA, restriction (-) modification (+), RP4 derivative integrated into the chromosome with Tet::Mu,Km::Tn7 | (Simon et al., 1983) |
| DH5α | DH5α (F-) F` endA1 hsdR17 (rK-mK plus) glnV44 thi1 recA1 gyr delta(Nalr) relA1 delta(lacIZYA-argF)U169 deoR (Φ80dlac delta (lacZ) M15) | (Woodcock et al., 1989) |

**Plasmids**

| Name | Description | Source or Reference |
|---|---|---|
| pAD5 | pMR20; *popA-eGfp* under control of *popA* promoter | Chapter 3.1 |
| pAD7 | pNPTS138; used for clean deletion of *dgcB* | Chapter 3.1 |
| pAD8 | pNPTS138; used for clean deletion of *popA* | (Aldridge and Jenal, 1999) |
| pAD19 | pMR20; $popA_{D55N}$-*eGfp* under control of *popA* promoter | (Levi and Jenal, 2006) |
| pAD30 | pMR20; $popA_{R357G}$-*eGfp* under control of *popA* promoter | This study |
| pAD32 | pMR20; $popA_{E368G}$-*eGfp* under control of *popA* promoter | This study |
| pMR20 | $Tet^R$ low copy number and broad host range vector | (Roberts et al., 1996) |
| pNPTS138 | $Kan^R$, suicide vector with *sacB* gene and *oriT* | D. Alley |
| pPA24 | pNPTS138; used for clean deletion of *pleD* | (Aldridge and Jenal, 1999) |

**Table 3.8**
Strains and plasmid used in this section.

*Attachment Assay*

The surface attachment assay for *C. crescentus* is based on a 96 well polystyrene microtiter plate assay (O'Toole and Kolter, 1998). Single colonies were inoculated into 180 µl fresh PYE supplemented with appropriate antibiotic, and incubated under shaking conditions (200 rpm) at 30ºC for 24h. Afterwards the cell suspension was discarded and the wells were washed 3 times under a stream of distilled water to remove unattached cells. After the 96 well plates were dried, 200 µl of 0.3% crystal violet (CV) staining solution were added to each well.
The 96 well plates were incubated with shaking for 15 min and subsequently washed again several times with water. The CV staining was dissolved in 20% acetic acid and the color intensity was measured with a microtiterplate reader spectrophotometer at 600 nm.

*Holdfast Staining*

200 µl exponentially growing cells were mixed with 2 µl FITC-conjugated wheat germ agglutinin (stock 5 mg/ml) mixed and incubated for 20 min in the dark. Afterward the

reaction mixture was washed by adding 1 ml water and by gently inverting the tube several times. The reaction mixture was centrifuged at high speed for 1 minute. The cell pellet was resuspended in 20-30 µl water and analyzed by fluorescence microscopy using the FITC filter set.

*Microscopy*

2-3 µl cell culture was dropped on a 1-2% agarose pad on a microscope slide. An Olympus IX71 microscope equipped with an UPlanSApo 100x/1.40 Oil objective (Olympus, Germany) and a coolSNAP HQ (Photometrics, AZ, United States) CCD camera was used to take differential interference contrast (DIC) and fluorescence photomicrographs. For FITC-WGA stained cells the FITC filter set (Ex 490/20 nm, Em 528/38 nm) was used with an exposure time of 1.0 sec. Images were processed with softWoRx v3.3.6 (Applied Precision, WA, United States) and Photoshop CS2 (Adobe, CA, United States) softwares.

*Motility Assay – Semi-solid agar plates*

Semi-solid agar plates containing 0.3% agar (DIFCO®) were inoculated with single colonies, incubated for 72h at 30°C. Afterwards the semi-solid agar plates were scanned and analyzed by Photoshop CS2 (Adobe, CA, United States) software.

*Hobson BacTracker*

The Hobson BacTracker (Hobson Tracking System, Sheffield, UK) allows precise measurements of individual motility parameters. Curvilinear velocity (CLV), straight-line velocity (SLV), motility or the swimming direction (TL%) were recorded and analyzed. The system consists of a normal phase contrast microscope connected to a video camera. The video camera records motile bacterial tracks on a screen and the result is either displayed as a table or as graphical trail draws (track).

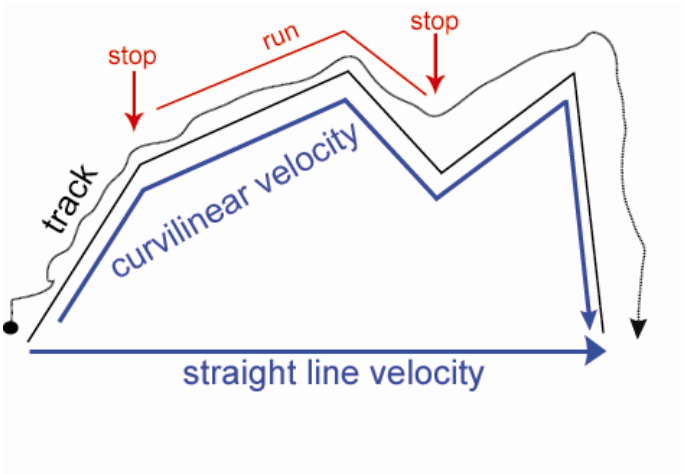



**Figure 3.8**

Graphical display of a motile cell track from the point of detection until the cell disappears from the detection field. The actual distance of the track which the cell passed is highlighted by the dashed black line and the track measured by the computer software is displayed by the black solid line. A run (shown in red) is the distance between two stops where the swimming speed drops below a certain threshold value. From the travelled distance and the time the curvilinear and the straight line velocity can be calculated (shown in blue).

| | |
|---|---|
| *Track* | Path traveled by a motile cell. It is measured from the point of detection until the cell disappears. |
| *Curvilinear velocity (CLV)* | The actual length of a complete track divided by the total time for the travelled track [µm/s]). |
| *Straight line velocity (SLV)* | The straight line distance between the start and end point of a track divided by the time taken for the track [µm/s]. |
| *Track linearity percentage (TL%)* | The ratio of SLV to CLV. The TL% value gives some information on how straight or curvilinear bacteria are swimming. The TL% value is 100% for bacteria which are swimming completely straight and 0% for bacteria which are spinning. |
| *Motility* | Number of motile cells which were detected in the video frame divided by the recording time [motile cells/s]. |

For every experiment 40 µl of a cell culture ($OD_{660}$ = 0.1) were spotted on a microscope slide. CLV, SLV and motility were measured at five different spots on the slide each for 2 min. Every strain was at least measured in triplicates.

## Results and Discussion

*Mutants lacking PopA show reduced motility*

A Δ*popA* mutant was analyzed on semisolid agar plates to score cell motility. Surprisingly, the Δ*popA* mutant (UJ3239) was clearly impaired in motility. In comparison, Δ*popA* complemented with a fully functional *popA-egfp* fusion on a low copy number plasmid (UJ3160) showed normal motility (Figure 3.9). No difference between wild-type and Δ*popA* complemented with *popA-egfp* could be observed (data not shown).

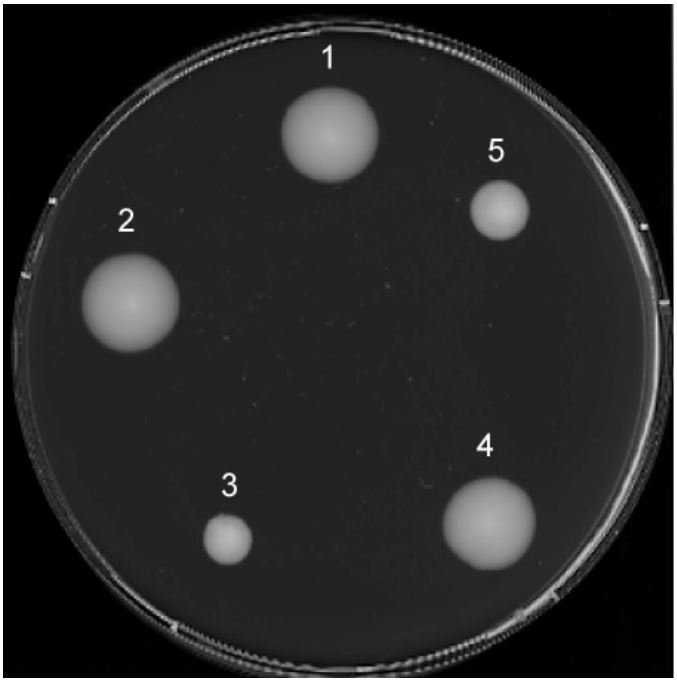



**Figure 3.9**

Cell motility as measured on semi-solid agar plates. 1) Δ*popA* + p*popA*$_{D55N}$-*egfp* (UJ3159), 2) Δ*popA* + p*popA-egfp* (UJ3160), 3) Δ*popA* + empty vector (UJ3239), 4) Δ*popA* + p*popA*$_{E368Q}$-*egfp* (UJ3563), 5) Δ*popA* + p*popA*$_{R357G}$-*egfp* (UJ3565)

To test if PopA phosphorylation, or elements of the GGDEF domain are required for cell motility the Δ*popA* mutant was complemented with low copy number plasmids carrying *popA*$_{D55N}$-*egfp* (P-site mutant; UJ3159), *popA*$_{R357G}$-*egfp* (I-site mutant; UJ3565) or *popA*$_{E368Q}$-*egpf* (A-site mutant; UJ3563) and analyzed on semi solid agar plates. The reduced motility phenotype of Δ*popA* was fully restored upon complementation with *popA*$_{D55N}$-*egfp* and *popA*$_{E368Q}$-*egfp*, but not with *popA*$_{R357G}$-*egfp*. To conclude, PopA and an intact PopA I-site are required to sustain normal cell motility (Figure 3.9).

To rule out the possibility that the Δ*popA* mutant phenotype was due to an impaired or misplaced flagellum, electron microscopy (EM) of Δ*popA* mutant and wild-type was performed. The EM pictures for wild-type as well as for the Δ*popA* mutant clearly showed the presence of a single intact polar flagellum at the swarmer pole (Figure 3.10). But reduced flagellar protein levels or, reduced overall flagellation can not be ruled out.

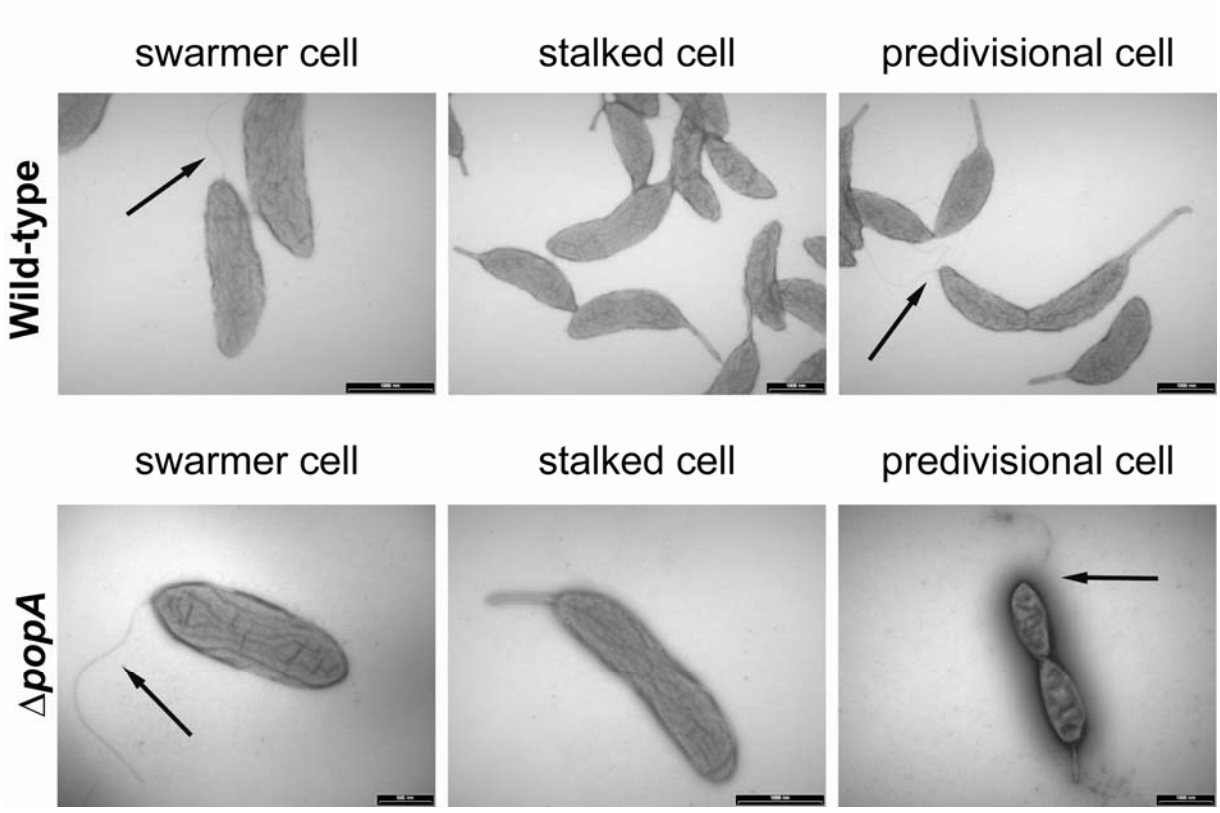



**Figure 3.10**

Electron micrographs of swarmer cells (left), stalked cells (middle) and predivisional cells (right) of wild-type NA1000 (upper panel) and Δ*popA* mutant cells (lower panel). Flagella are indicated by arrows.

To more carefully analyze the motility defect, the Δ*popA* mutant was analyzed by means of the Hobson BacTracker system. The Hobson BacTracker software detects motile particles and records precisely the individual tracks of each motile cell, giving a more detailed analysis of the swimming behavior (Karim et al., 1998; Sourjik and Schmitt, 1996). The straight line velocity (SLV), the curvilinear velocity (CLV) and the tracks made by bacteria were determined, from which the number of motile cells could be calculated (see Material and Methods).The Δ*popA* mutant shows significantly reduced CLV and SLV values compared to wild-type cells (Table 3.9). In addition, the number of running tracks was measured for the Δ*popA* mutant and was used to estimate the overall motility. The computer software recorded all motile cells that were swimming through the detection field in a defined time period and displayed their tracks schematically (see Material and methods). An exponentially growing culture of Δ*popA* showed half as much motile cells as compared to a wild-type culture (Figure 3.12, Table 3.9). To summarize, these studies indicate that PopA interferes with the function and/or the assembly of the *C. crescentus* flagellar motor.

*A network comprising several GGDEF domain proteins controls cell motility and holdfast-mediated attachment*

The finding that PopA and PleD contribute to the regulation of cell motility in *C. crescentus* let us to analyze a possible interference of these two factors. In addition, we also tested if DgcB (CC1850), a *bona fide* DGC, is involved in cell motility control.

As shown in Figure 3.11, mutations in *popA*, *pleD*, and *dgcB* all had distinct effects on motility on semi-solid agar plates. While the *ΔdgcB* mutant formed colonies of increased swarm size, indicating more efficient motility, mutants lacking either *popA* or *pleD* formed smaller swarm colonies.

In an attempt to unravel the effective contributions of PopA, DgcB, and PleD to cell motility, all possible mutant combinations were generated and assayed for motility on semi-solid agar plates. The motility phenotype of a Δ*popA*Δ*pleD* double mutant was further reduced as compared to the corresponding single mutants (Figure 3.11).

Surprisingly, in contrast to the Δ*popA* and Δ*pleD* single mutants, spontaneous motile suppressors were readily observed for the Δ*popA*Δ*pleD* double mutant (Figure 3.11). Combining the Δ*popA* with the *dgcB* mutation resulted in an intermediate motility

phenotype. The same intermediate swarm pattern could also be observed for Δ*pleD*Δ*dgcB* (Figure 3.11).

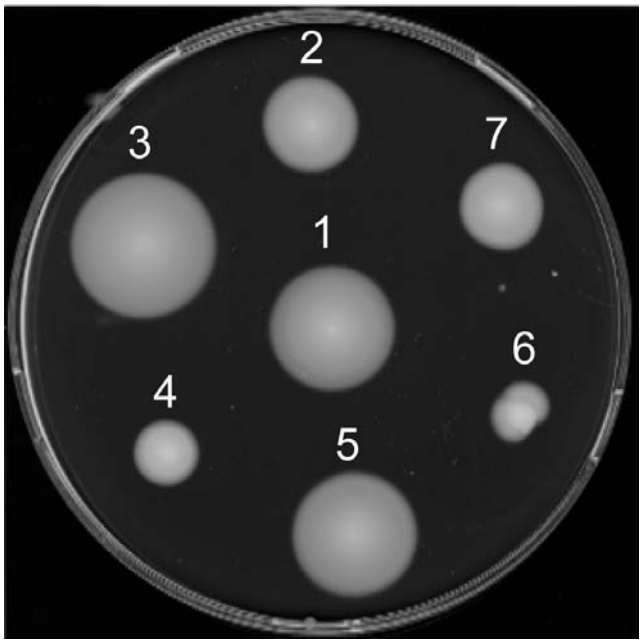



**Figure 3.11**

Semi-solid agar plate incubated for 72 hours at 30°C. 1) wild-type CB15 (LS1250), 2) ΔpleD (UJ730), 3) Δ*dgcB* UJ3154), 4) Δ*popA* (UJ3155), 5) Δ*pleD*Δ*dgcB* (UJ3123), 6) Δ*popA*Δ*pleD* (UJ3124), 7) Δ*popA*Δ*dgcB* (UJ3642)

To distinguish between the different roles of DgcB, PopA and PleD in cell motility and to more carefully analyze the respective phenotypes, the corresponding single and double mutants were analyzed by means of the Hobson BacTracker system. Exponentially growing wild-type cells, ∆*popA*, Δ*dgcB*, Δ*pleD*, Δ*popA*Δ*pleD*, Δ*popA*Δ*dgcB* and Δ*pleD*Δ*dgcB* mutant cells were measured for SLV, CLV and motility.

Both, Δ*pleD* and Δ*popA* cells show significantly reduced CLV and SLV values compared to wild-type cells (Table 3.9). Despite this reduction in swimming speed, the TL% value (gives an indication of the straightness or the curvilinearity of the swimming behavior) of both mutants was indistinguishable from wild-type. The CLV and SLV values for the Δ*dgcB* mutant were higher than for Δ*pleD* and Δ*popA*. The Δ*dgcB* mutant also swims faster than wild-type, although its TL% value remains unchanged (Table 3.9). The

Δ*popA*Δ*pleD* mutant showed only slightly reduced CLV and SLV compared to the corresponding single mutants but drastically reduced values compared to wild-type cells (Table 3.9). Importantly, the track linearity percentage value (TL%) of the Δ*popA*Δ*pleD* mutant is reduced, suggesting a more curvilinear swimming behavior for this mutant. The Δ*popA*Δ*dgcB* and the Δ*pleD*Δ*dgcB* mutants showed intermediate CLV and SLV values compared to the corresponding single mutants (Table 3.9).

| | CLV [μm/s] | SLV [μm/s] | TL% [SLV/CLV] | Motility [cells/s] |
|---|---|---|---|---|
| wild-type | 36.20 ± 2.54 | 23.06 ± 2.47 | 63 ± 3 | 1.18 ± 0.27 |
| Δ*popA* | 28.87 ± 3.39 | 17.65 ± 4.24 | 60 ± 8 | 0.44 ± 0.14 |
| Δ*pleD* | 25.49 ± 0.85 | 15.67 ± 0.93 | 62 ± 2 | 3.62 ± 0.55 |
| Δ*dgcB* | 40.28 ± 2.53 | 26.57 ± 2.66 | 66 ± 3 | 1.56 ± 0.35 |
| Δ*popA*Δ*dgcB* | 34.39 ± 2.21 | 22.75 ± 2.46 | 66 ± 2 | 0.73 ± 0.13 |
| Δ*pleD*Δ*dgcB* | 29.31 ± 0.8 | 19.04 ± 0.91 | 65 ± 1 | 3.7 ± 0.52 |
| Δ*popA*Δ*pleD* | 24.23 ± 1.12 | 10.41 ± 2.48 | 44 ± 2 | 0.25 ± 0.11 |

**Table 3.9**

Curvilinear velocity, straight line, track linear percentage and the motility (motile cells/s) were measured for wild-type, the indicated mutant strains.

In addition, the overall motility of each mutant strain was measured. As expected, an exponentially growing culture of Δ*pleD* showed three times more motile cells as compared to a wild-type culture (Figure 3.12, Table 3.9). While this is in agreement with previously published results (Aldridge and Jenal, 1999), it contrasts with the behavior of

this mutant strain on semi-solid agar plates (Figure 3.11). The reason for this is not clear, but it has been speculated that *pleD* mutants have a chemotaxis defect (Burton et al. 1997). In contrast, for the Δ*popA* mutant less motile cells were detected compared to wild-type (Figure 3.12, Table 3.9). While this corresponds well with the behavior of this mutant on semisolid agar plates, the effect is even more dramatic in a Δ*popA*Δ*pleD* double. Not only was the number of motile cells reduced by a factor of five as compared to wild-type and by a factor of 14 compared to the Δ*pleD* single mutant (Table 3.9), but the length of the runs was significantly shortened in this mutant strain (Figure 3.12).

In contrast to the Δ*popA*Δ*pleD* mutant, the hypermotility phenotype of Δ*pleD* was not influenced by a deletion of *dgcB*. The Δ*pleD*Δ*dgcB* mutant showed the same hypermotility phenotype as the single Δ*pleD* mutant (Figure 3.12, Table 3.9). Finally, the motility behavior of the Δ*popA*Δ*dgcB* double mutant is reflected by the average motility values of the respective single mutants (Figure 3.12, Table 3.9).

To conclude, these data indicate that the diguanylate cyclase DgcB has a mild negative effect on both swimming speed and overall numbers of motile cells. It remains to be shown if DgcB contributes to the timing of motility during the *C. crescentus* cell cycle and/or affects motor function directly. In contrast, PopA is required for optimal swimming speed and overall motility. The observation that the mutant phenotype of *popA* is most dramatic in a *pleD* mutant background argues for regulatory interference of the PleD DGC and the PopA effector protein to control *C. crescentus* cell motility.

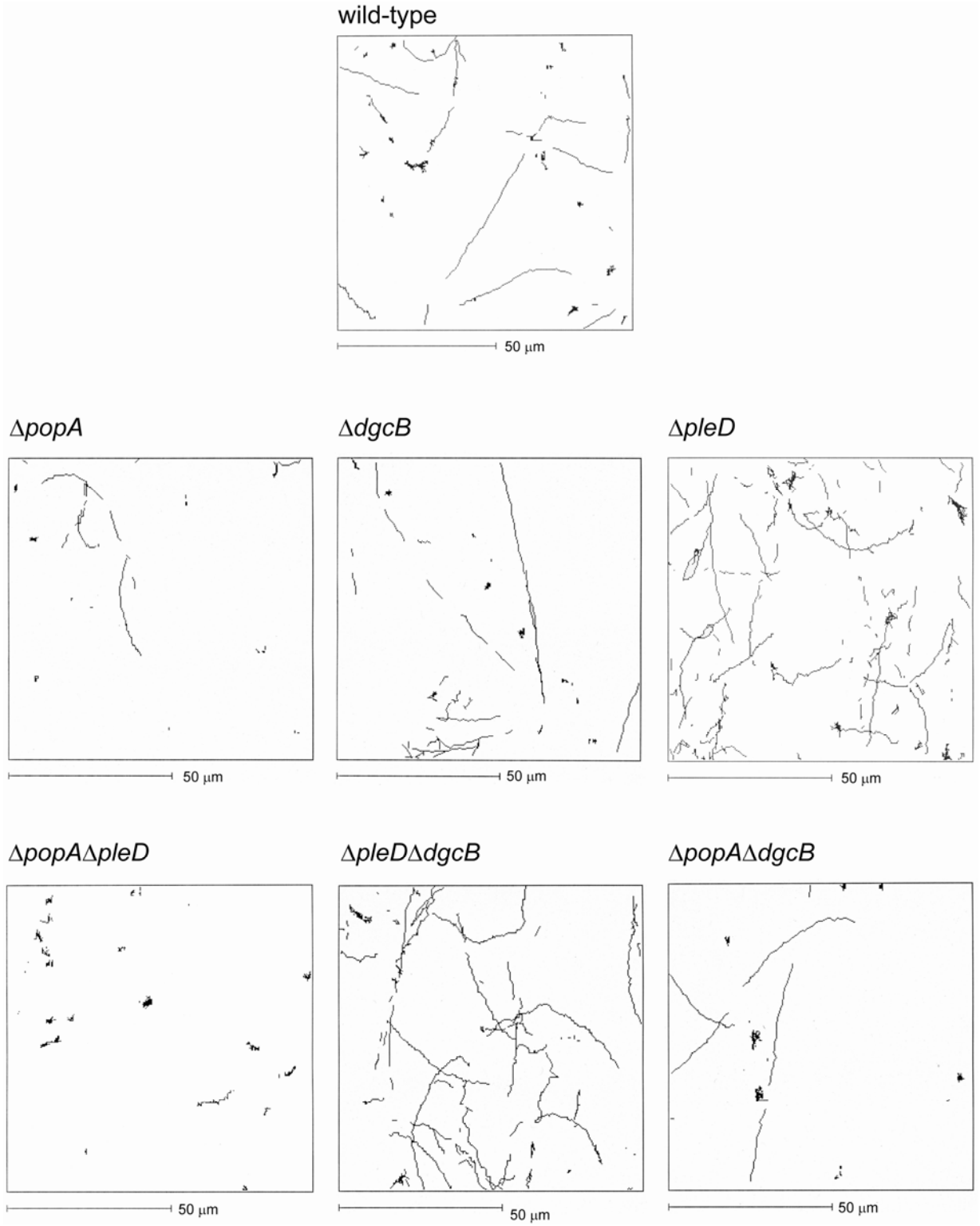



**Figure 3.12**

Representative bacterial tracks of wild-type and the indicated single and double mutants were recorded for two seconds and are schematically illustrated.

We also tested a possible contribution of PopA to holdfast formation and surface attachment. As shown in Figures 3.13 and 3.14 holdfast formation and attachment to polystyrene surfaces of the Δ*popA* mutant was similar to wild type. In contrast, the mutant lacking PleD showed the expected reduction in attachment (~40%) and holdfast formation (Levi and Jenal, 2006) (Figure 3.13, Figure 3.14). Similarly, a *dgcB* mutant strain had reduced holdfast levels and lower surface attachment as compared to wild type. Analysis of the Δ*popA*Δ*pleD*, Δ*popA*Δ*dgcB*, and Δ*pleD*Δ*dgcB* double mutants revealed several interesting findings. A strain lacking both DgcB and PleD was unable to form holdfast and attach to surfaces, arguing that the two diguanylate cyclases together control holdfast biogenesis during the *C. crescentus* cell cycle. This result is also consistent with the observation that PleD and DgcB redundantly control cell motility (see above). A *popA dgcB* double mutant showed attachment levels similar to the *dgcB* single mutant. Together with the results obtained for the *popA* single mutant, this suggests that PopA apparently does not contribute to cell attachment. However, the Δ*popA*Δ*pleD* mutant was strongly impaired in attachment and holdfast formation (Figure 3.13, Figure 3.14). In fact, attachment levels were as low as in the mutant lacking both diguanylate cyclases. This is reminiscent of the unexpected effect of the Δ*popA*Δ*pleD* double mutant on motility. Importantly, the growth rates of all of the mutant strains were not affected (data not shown).

In summary, these findings indicate that PopA, in addition to its central role in cell cycle progression, is also involved in regulating in *C. crescentus* motility and surface attachment. The correlation between attachment phenotypes and holdfast formation of all mutants analyzed suggests that PopA primarily controls surface adherence by controlling holdfast biogenesis.

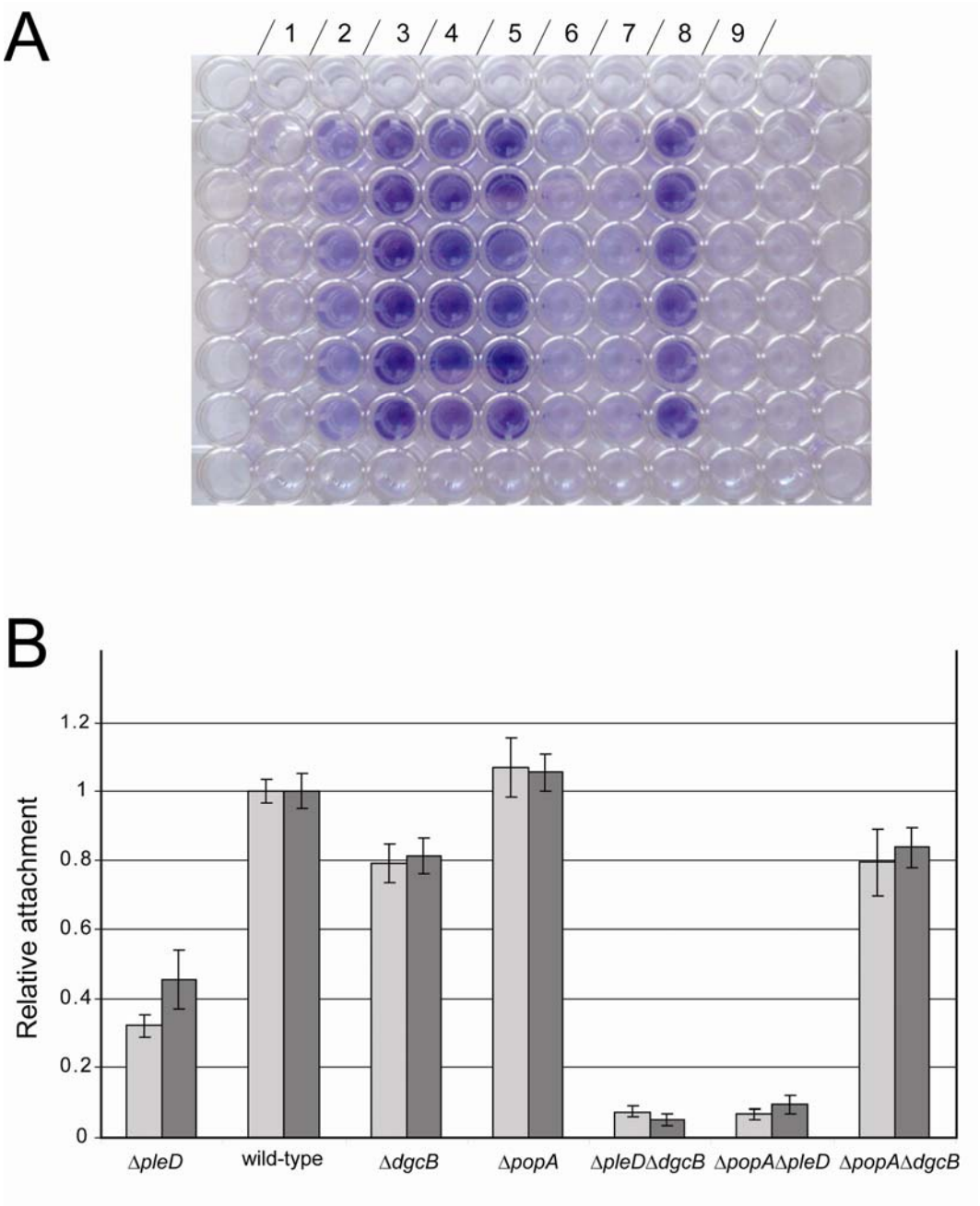



**Figure 3.13**

A) Crystal violet attachment assay. 1) NA1000 (negative control lacking holdfast, UJ1267), 2) CB15 *ΔpleD* (UJ739), 3) CB15 wild-type (LS1250), 4) CB15 *ΔdgcB* (UJ3154), 5) CB15 *ΔpopA* (UJ3155), 6) CB15 *ΔpleDΔdgcB* (UJ3123), 7) CB15 *ΔpopAΔpleD* (UJ3124), 8) CB15 *ΔpopAΔdgcB*, 9) no cell control.

B) Quantification of cell attachment shown in A). Light and dark grey represent two independent experiments.

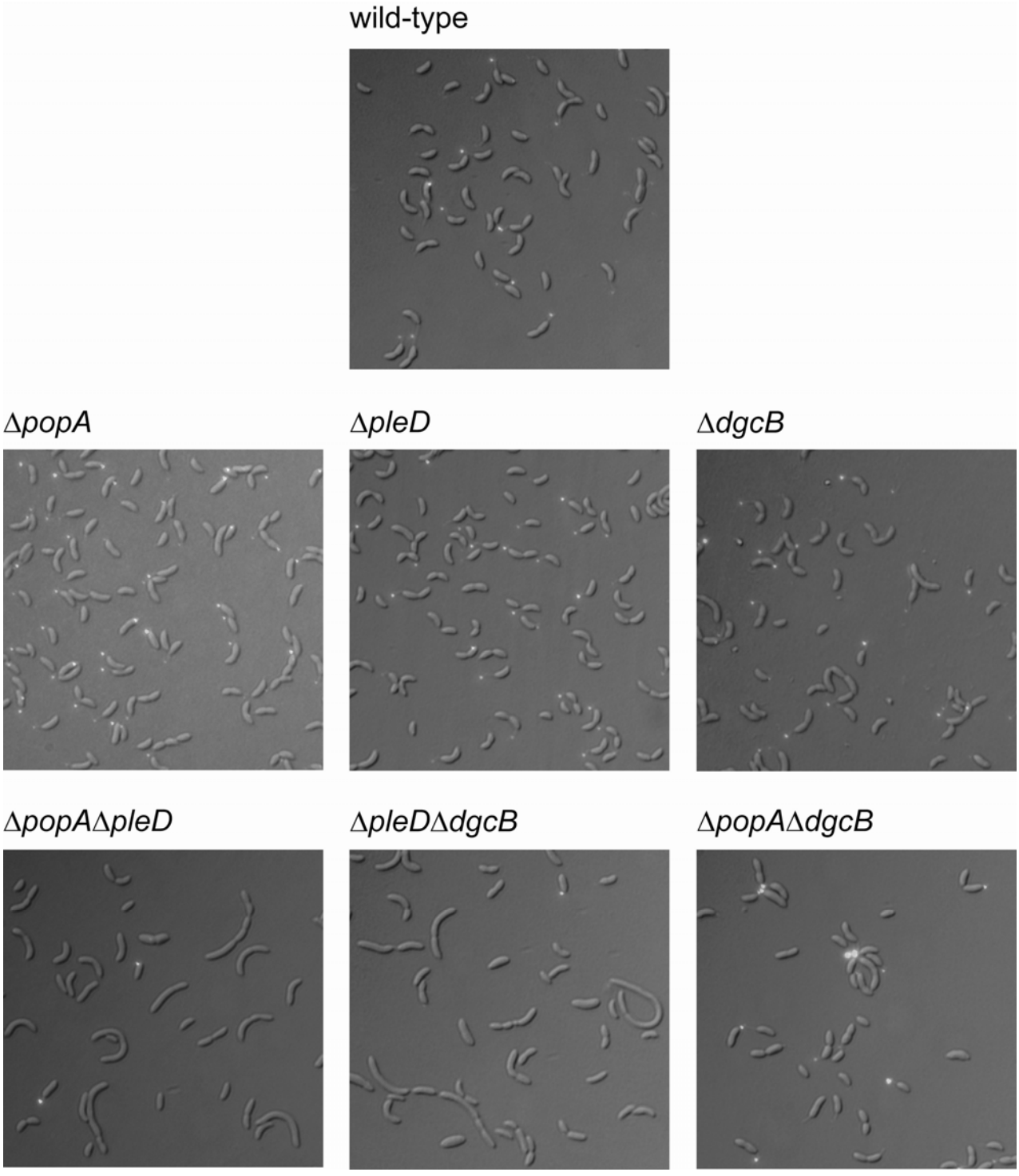



**Figure 3.14**

Exponentially growing cells were stained with oregon green-conjugated wheat-germ agglutinin and examined by fluorescence microscopy using the FITC filters.

*The ΔpopAΔpleD and ΔpleDΔdgcB double mutants show a cell division defect*

The cell morphologies of the Δ*popA*, Δ*pleD*, and Δ*dgcB*, single as well as the Δ*popA*Δ*pleD*, Δ*popA*Δ*dgcB,* and Δ*pleD*Δ*dgcB* double mutant strains were analyzed by light microscopy (DIC microscopy) and by electron microscopy (EM). Morphologies of Δ*popA*, Δ*dgcB* and Δ*popA*Δ*dgcB* were normal and comparable to NA1000 wild-type cells. However, in growing populations of the Δ*popA*Δ*pleD* and the Δ*pleD*Δ*dgcB* mutants many cells were elongated and filamentous (Figure 3.15). In addition, many cells although showing constriction, failed to separate properly, resulting in strings of connected cells. The cell division defect was most pronounced in the Δ*popA*Δ*pleD* double mutant and could be fully restored with either a plasmid-borne copy of *popA* or *pleD* (data not shown).

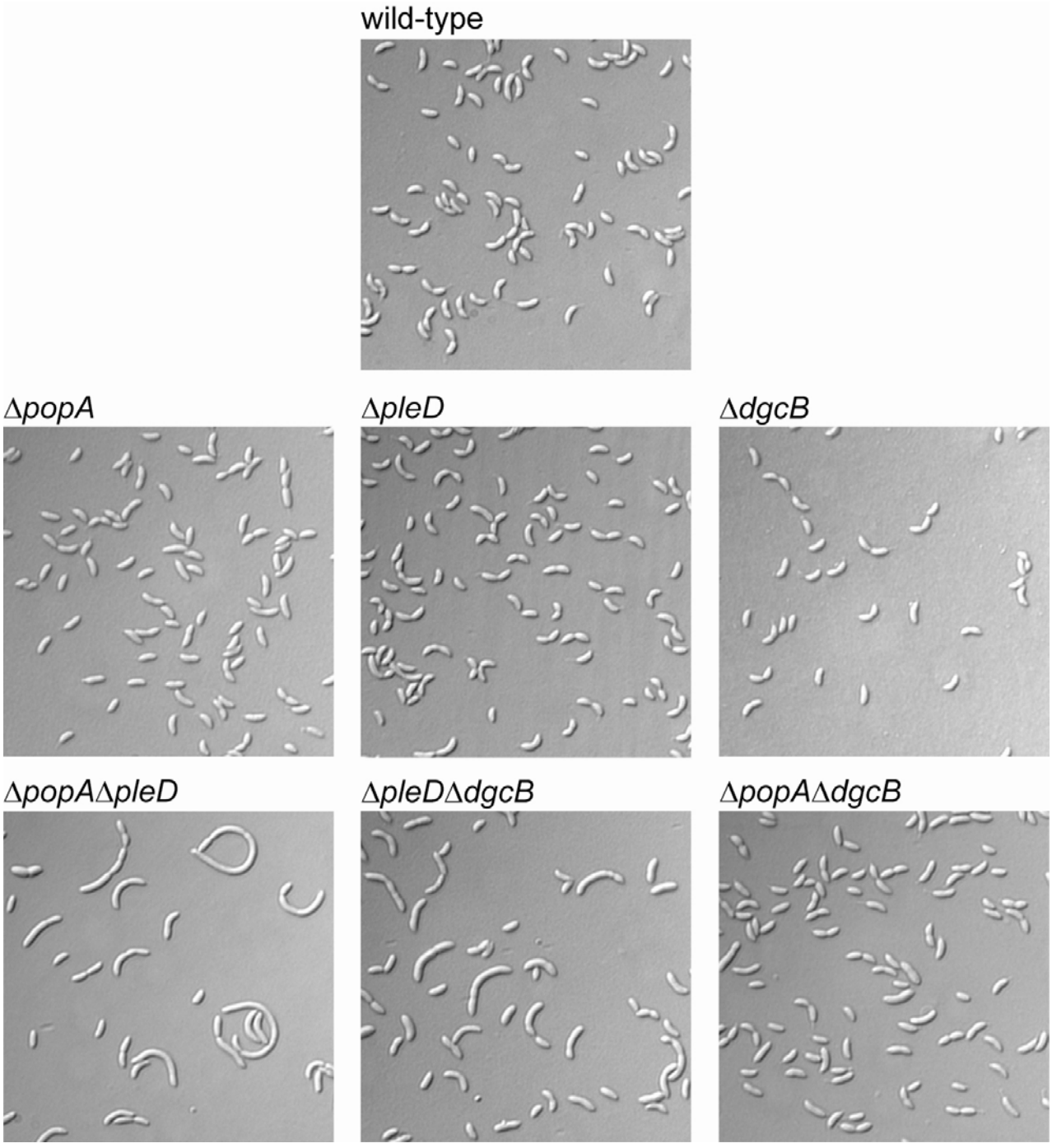



**Figure 3.15**

Light microscopy (DIC) pictures of exponentially growing CB15 wild-type (LS1250), Δ*popA* (UJ3155), Δ*pleD* (UJ730), Δ*dgcB* (UJ3154), Δ*popA*Δ*pleD* (UJ3124), Δ*popA*Δ*dgcB* (UJ3642) and Δ*pleD*Δ*dgcB* (UJ3123) cells.

Statistical analysis revealed that 10% of the Δ*popA*Δ*pleD* double mutant cells were impaired in daughter cell separation, 22% of the cells were filamentous with one shorter attached daughter cell at one end, and 3% of the cells had stalks that were misplaced at lateral positions (Figure 3.16).

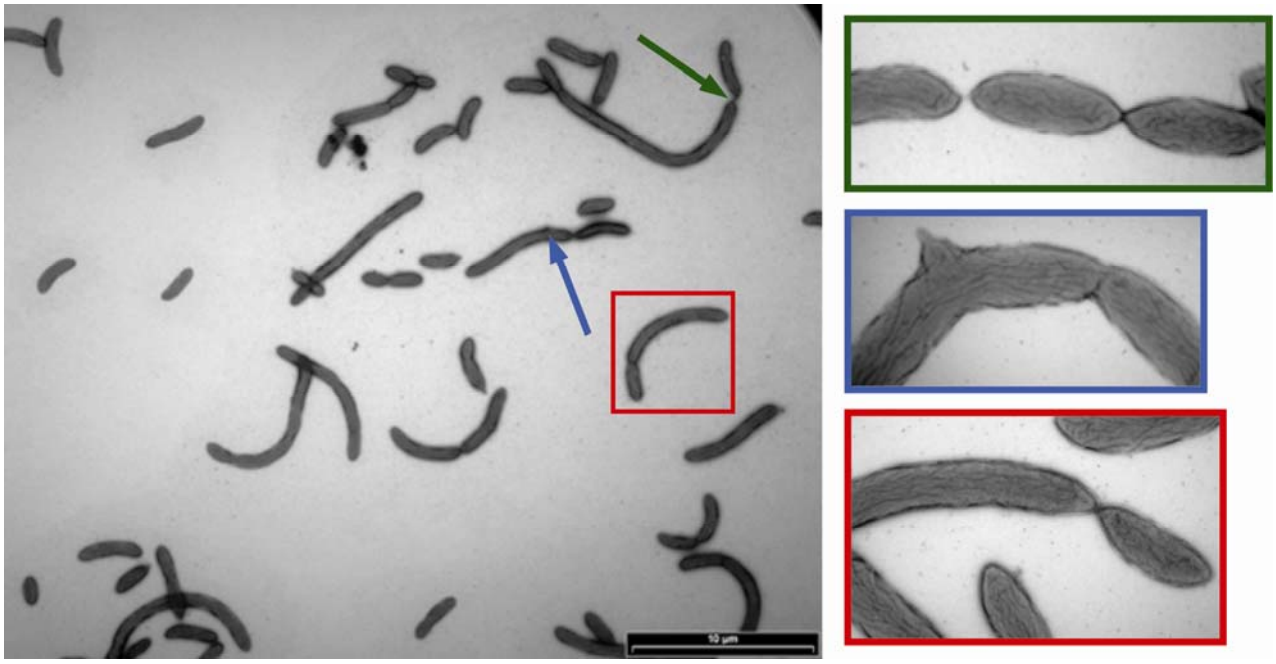


**Figure 3.16**

EM picture of Δ*popA*Δ*pleD* (UJ2874). An overview of exponentially growing Δ*popA*Δ*pleD* is shown on the left and typical examples of morphological abnormalities are highlighted by different colors on the right. A green) cells that are not properly separated B blue) side stalks C red) cells not dividing properly.

Consistent with their cell division phenotype Δ*popA*Δ*pleD* and Δ*pleD*Δ*dgcB* mutants showed a severe defect in stalk elongation (Figure 3.15, Figure 3.16). While the Δ*popA* and Δ*dgcB* single mutants showed normal-length stalks, Δ*pleD* had shorter but visible stalks as reported before (Aldridge et al. 1999) (Figure 3.15). The Δ*popA*Δ*dgcB* double mutant not only showed normal cell morphology, but was unaffected with respect to stalk formation (Figure 3.15). As stalk elongation is known to be controlled by phosphate availability (Gonin et al., 2000) we tested if stalk formation in the stalk mutant strains was restored at low $PO_4$ concentrations.

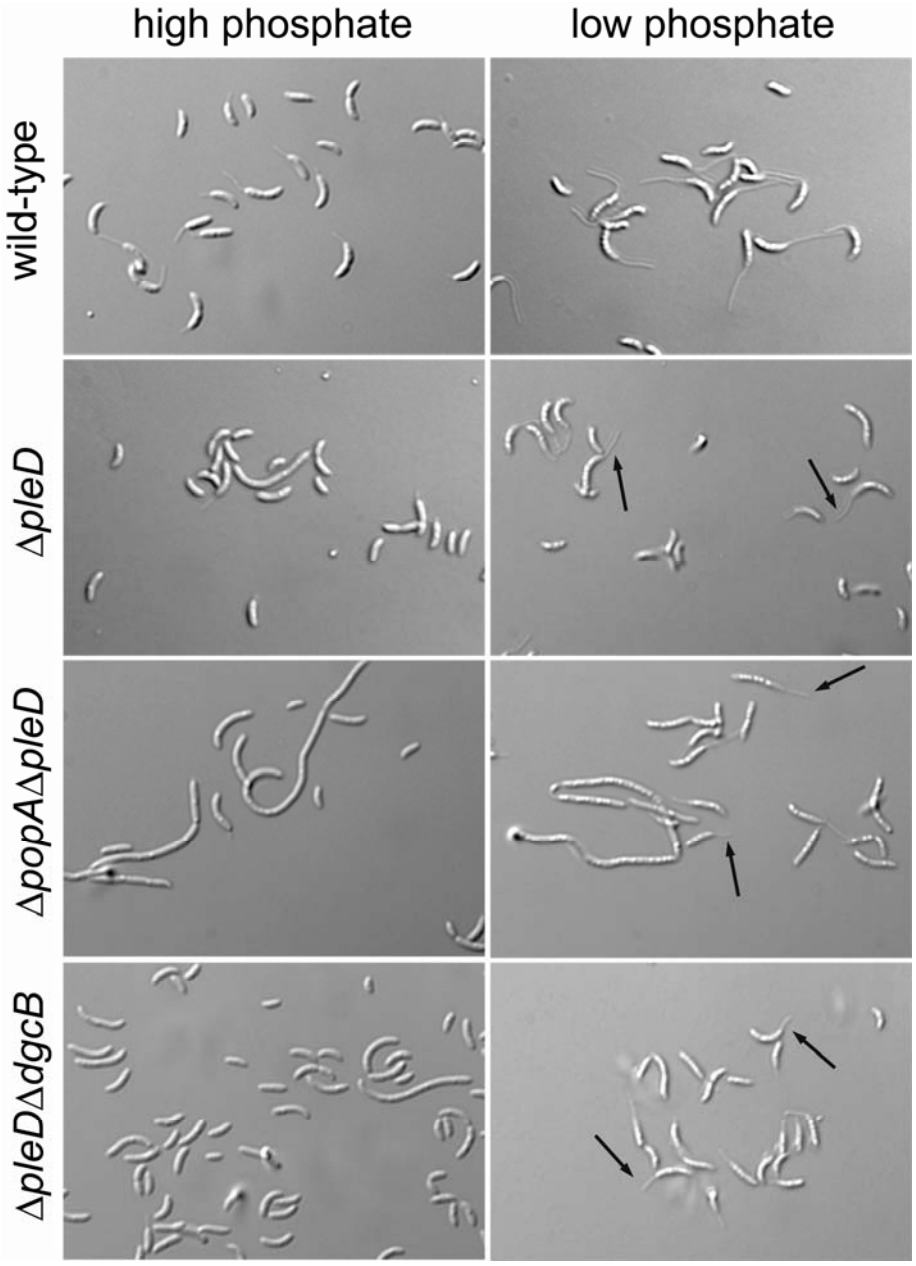



**Figure 3.17**

Wild-type, Δ*pleD*, Δ*pleD*Δ*dgcB* or Δ*popA*Δ*pleD* cells were grown in HIGG medium under high phosphate conditions (left) or in HIGG medium under low phosphate conditions (right).

As shown in Figure 3.17, stalk formation was indeed restored in all three mutants under low phosphate conditions.

Together this adds stalk formation to the list of cellular functions, which are controlled by c-di-GMP and indicates that the diguanylate cyclases PleD and DgcB are the main

regulators that contribute c-di-GMP to induce holdfast formation and stalk elongation in *C. crescentus*. The observation that stalks are absent in a *popApleD* double mutant, suggests that PopA is also part of the regulatory pathway(s) controlling stalk formation. The prominent phenotype of a *popApleD* double mutant with respect to motility, holdfast biogenesis, attachment, and stalk formation, further suggests a regulatory interaction between PopA and PleD (see Discussion 4.4).

# 4 Discussion

In recent years, an increasing number of genetic and biochemical studies established a global role for c-di-GMP signaling mediated by GGDEF and EAL domain proteins in bacteria. Most studies could link c-di-GMP signaling to motility or to some form of community behavior establishing a paradigm that associates low levels of c-di-GMP with a free-living, planktonic form and high levels of c-di-GMP with sessility and biofilm formation. Despite this clear functional assignment for c-di-GMP, the question remained if this signaling compound harbors additional functions apart form controlling the switch between motility and sessility.

The genomes of most bacterial species encode on average 20 to 40 GGDEF and EAL domain proteins. In the light of this abundance one major issue is to figure out how DGCs and PDEs activities are coordinated, to what kind of signals they respond to, to what downstream effector molecules they are talking to, and how specificity of these pathways is achieved.

We identified PopA as a novel c-di-GMP effector protein, which controls the sequestration and degradation of the master cell cycle regulator CtrA. The dynamic localization of PopA to the incipient stalked pole and CtrA degradation during the cell cycle require an intact PopA c-di-GMP binding site. Based on our results, we postulate a new function for the second messenger c-di-GMP in controlling cell cycle progression in *Caulobacter crescentus*. Furthermore, the discovery that the GGDEF domain itself can act as c-di-GMP effector protein might help to explain the abundance of these proteins in certain bacterial species.

## 4.1 C-di-GMP controls cell cycle progression in *Caulobacter crescentus*

So far, c-di-GMP signaling has been exclusively attributed to the control and the regulation of cell development in *Caulobacter crescentus*. The GGDEF and EAL domain proteins PleD and TipF have been shown to orchestrate the remodeling of the cell poles during the cell cycle (Aldridge and Jenal, 1999; Aldridge et al., 2003; Huitema et al., 2006; Paul et al., 2004). In contrast, PopA, a c-di-GMP effector protein, controls the polar sequestration and degradation of the master cell cycle regulator CtrA. Based on these

results we propose a new function for the second messenger c-di-GMP in controlling the cell cycle progression in *C. crescentus*. PopA is the first example of a GGDEF domain protein controlling chromosome replication, the core mechanism of each bacterium.

The observation that c-di-GMP touches the core mechanism for bacterial propagation raises immediately the question if c-di-GMP is essential for bacterial growth and survival. E.g. is a c-di-GMP gutted *C. crescentus* strain (all GGDEF domain proteins deleted) still viable? It is important to note that a Δ*pleD*Δ*dcgB* mutant strain, in which two of the 11 GGDEF domain proteins are deleted, already shows a slight cell division defect, including elongated, filamentous and not properly separated cells (see Figure 3.15). Based on this observation one can speculate that mutation of additional GGDEF domain proteins would lead to accumulating problems in cell growth and proliferation. Preliminary data show that overexpression of a PDE from *Pseudomonas aeruginosa* leads to a severe cell division defect represented by elongated, filamentous and not properly separated cells (M. Nicollier and A. Dürig, unpublished). This finding provides additional evidence that c-di-GMP might be essential in *C. crescentus*.

PopA localization to the incipient stalked pole and CtrA degradation require an intact I-site. But what are the possible DGCs and PDEs, which are contributing to the c-di-GMP pool which regulates c-di-GMP-mediated PopA localization in a cell cycle-dependent manner? PleD would have been the ideal DGC candidate, because PleD co-localizes with PopA to the same subcellular site during the same time window of the cell cycle. However, PopA localization is unaffected in the Δ*pleD* mutant as well as in a Δ*dgcB* single and a Δ*pleD*Δ*dgcB* double mutant (see Section 3.2.2). Based on these results, one can conclude that additional DGCs contribute to PopA localization possibly in a redundant manner or that a so far unidentified DGC very specifically activates PopA cellular dynamics. Alternatively, one could speculate that a specific phosphodiesterase tightly controls the c-di-GMP levels during the cell cycle and ensures PopA sequestration to the incipient stalked pole. Preliminary results overexpressing a PDE from *P. aeruginosa* show partially delocalized PopA-GFP during the cell cycle and a partial stabilization of CtrA, which argues for decreased c-di-GMP levels in the cell (M. Nicollier, A. Dürig, unpublished). This finding suggests that PopA sequestration to the incipient stalked pole and CtrA degradation are dependent on c-di-GMP binding.

To summarize, growing evidence indicate distinct c-di-GMP pathways which are orchestrated by the spatial control of DGC and PDE together with the corresponding c-di-GMP effector proteins.

Is the principle of c-di-GMP controlling cell cycle progression of general importance or is it a peculiarity of *C. crescentus*? In any case, we should keep in mind that c-di-GMP-mediated cell cycle control in *C. crescentus* might be a special case, because cell development, the switch between sessility and motility, is an integral part of the *C. crescentus* cell cycle. There exists the possibility that *C. crescentus* has recruited the c-di-GMP control system, that was already in place to control the motile-sessile switch, to drive its cell cycle, or to coordinate and link development and cell cycle, including chromosome replication. Furthermore, several components required for cell cycle progression, including CtrA, RcdA and CpdR, are restricted to familiy of $\alpha$-proteobacteria. To gain more evidence for a general and global role of c-di-GMP-mediated cell cycle control, PopA homologs in different bacterial species in the $\alpha$-purple branch should be analyzed in respect to their cell cycle control. But if PopA is *C. crescentus* specific this would argue against a general role of c-di-GMP in controlling cell cycle.

## 4.2 PopA constitutes the first member of a novel class of c-di-GMP effector proteins

So far, the GGDEF domain has been implicated primarily in the catalysis of c-di-GMP formation from two GTP molecules. Extensive biochemical and structural studies on PleD, a *bona fide* DGC, suggest that the active site (A-site) is formed by the highly conserved GGDEF loop and is essential for DGC activity (Figure 4.2) (Chan et al., 2004; Paul et al., 2004; Wassmann et al., 2007). Two $Mg^{++}$ ions and the GTP substrate are coordinated by conserved residues of the active site (Wassmann et al., 2007). Mutations in any of these residues abolished activity of PleD *in vitro* and *in vivo* (Paul et al., 2007; Paul et al., 2004; Wassmann et al., 2007). Similarly, mutational analysis of WspR, a GGDEF domain protein from *P. fluorescens*, has shown that form a collection of 75 single amino acid substitutions in the GGDEF motif, all mutations abolished DGC activity, except for the GGEEF to GGDEF mutation (Malone et al., 2006). Based on these

findings, one can argue that a conserved GGDEF sequence motif is critical for DGC activity. Interestingly, some DGCs contain an additional conserved sequence motif, the I-site, which is separated exactly by five amino acids from the A-site (Figure 4.2) (Christen et al., 2006). The finding, that some GGDEF domains lack this allosteric c-di-GMP binding site suggested that two different classes of DGCs might exist: Low-activity DGCs ($A^+I^-$) lacking product inhibition and high-activity DGCs ($A^+I^+$) with built-in negative feedback regulation (Figure 4.1), reviewed in (Jenal and Malone, 2006)).

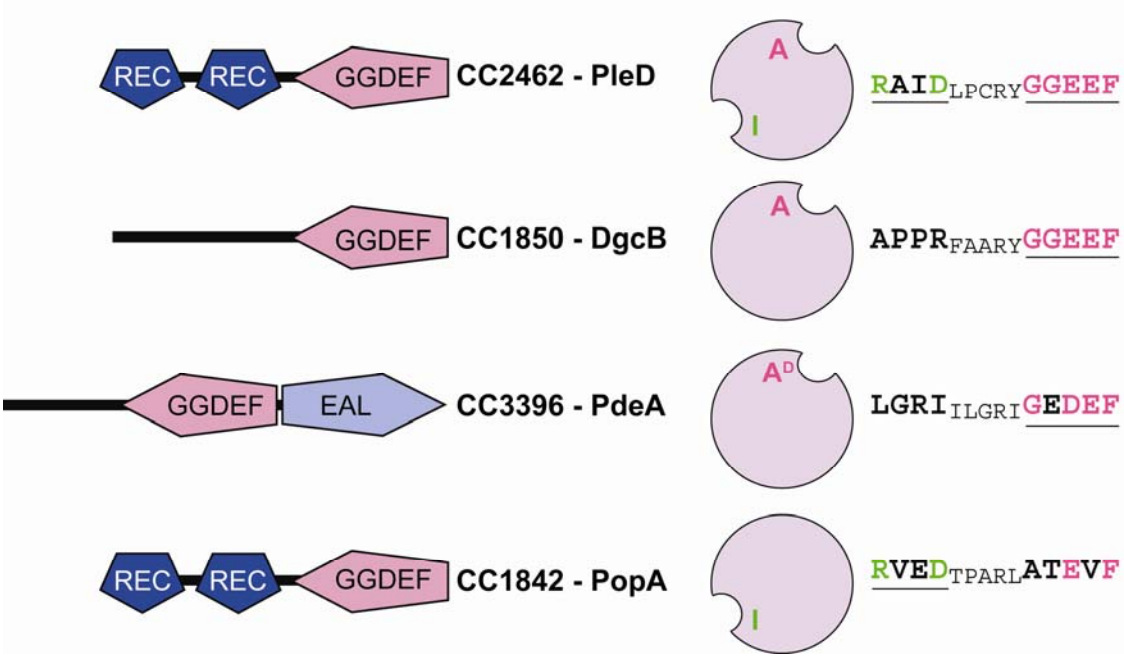



**Figure 4.1**

GGDEF domains with distinct functions in *C. crescentus*. The domain organization of four representative GGDEF domain proteins form *C. crescentus* is illustrated on the left. Schematic representation of each GGDEF domain, indicating A-site and/or I-site is described on the middle. The amino acid sequences of the I- (green) and A-site (pink) motifs are shown on the right. Functional sites are underlined. The GGDEF domain of PdeA, CC3396, lost its catalytic activity ($A^D$) (Figure according to (Jenal and Malone, 2006)).

However, only 90% of the GGDEF and 62% of the GGDEF-EAL composite proteins show a conserved A-site motif (Galperin et al., 2001). Consistent with this, all GGDEF domain proteins, for which DGC activity could be demonstrated, have a conserved A-site motif. But what is the physiological role of the GGDEF domain proteins with a degenerate A-site

motif? Initial studies on PdeA, a phosphodiesterase GGDEF-EAL composite protein with a GGDEF domain harboring a degenerate A-site, proposed an adopted allosterical role for the associated GGDEF domain (Figure 4.1). Interestingly, the degenerate GGDEF domain of PdeA apparently retained its ability to bind GTP, and in response activates the neighboring PDE domain (Christen et al., 2005). Consistent with its function as an allosteric domain binding GTP to the GGDEF domain, PdeA lacks the conserved I-site residues (Figure 4.1).

Based on the recent studies on PopA we postulate the existence of a new class of functionally distinct GGDEF domains harboring a conserved I-site but a degenerate A-site ($A^-$ $I^+$). As expected from the completely degenerate A-site motif ("ATEVF"), PopA apparently lacks enzymatic activity. However, in agreement with the conserved I-site residues, PopA is able to bind c-di-GMP specifically and with a high affinity (Figure 4.1).

Consistent with the biochemical analysis of PopA, the overall modeled three-dimensional structure of the GGDEF domain of PopA looks very similar to the one of PleD, but compared to PleD, PopA misses the A-site, but retained the I-site (Figure 4.2).

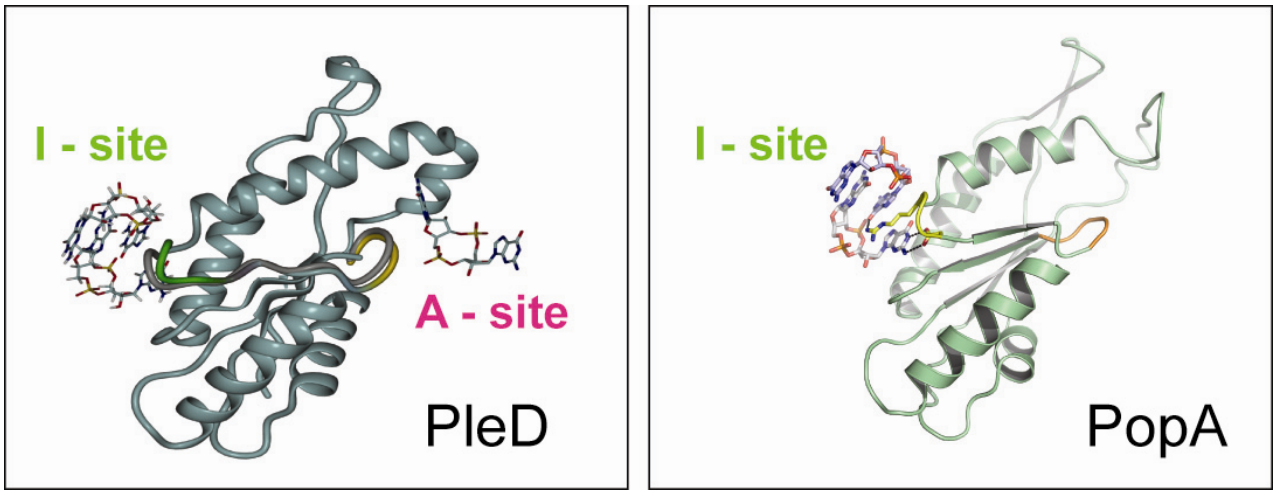



**Figure 4.2**

Three-dimensional crystal structure of the GGDEF domain of PleD (left) and the modeled three-dimensional structure of the GGDEF domain of PopA (right). The I-site is labeled in green, the A-site is labeled in pink (in PleD).

Because PopA activity seems to be dependent on the presence of a conserved I-site, we postulate that its C-terminal GGDEF domain has adopted a novel function as c-di-GMP effector domain. This discovery that a GGDEF domain can act as molecular switch in response to c-di-GMP might explain the existence of abundant GGDEF domain proteins suggesting a more global role for GGDEF domains with a degenerate A-site, but conserved I-site, as c-di-GMP effector proteins in bacteria.

## 4.3 Polar sequestration mechanism of PopA

This study demonstrates that PopA dynamically localizes to the cell poles during the cell cycle. PopA sequestration to the incipient stalked pole is dependent on an intact I-site and the absence of PopA at the incipient stalked pole correlates with the failure of CtrA degradation at the pole. Based on this observation, one can speculate that upon c-di-GMP binding to the conserved I-site of PopA, CtrA sequesters to the incipient stalked pole and gets degraded by the ClpXP protease complex.

What is the molecular mechanism for the I-site dependent sequestration to the incipient stalked pole? Is the molecular mechanism, which triggers PopA localization, comparable to PleD sequestration to the stalked pole? Extensive studies on PleD elaborated a molecular mechanism for PleD sequestration, in which PleD dimerizes upon phosphorylation by the cognate histidine kinases, and is sequestered to the stalked pole as a catalytic active PleD dimer (Figure 4.3, (Paul et al., 2007)). The assumption that PopA polar sequestration to the incipient stalked might involve similar mechanisms is based on the following observations. PopA is a paralog of PleD, shows identical domain organization, and the putative phosphorylation site of the first receiver domain, $Asp_{55}$, is conserved. Further, the modeled three-dimensional structure of PopA is very similar to the crystal structure of PleD except the missing A-site (Figure 4.2) and finally, the key residues, which form the dimerization interface in activated PleD, are all conserved except one residue.

The first model for PopA localization is mainly based on the mechanism of PleD sequestration to the cell pole. Similarly, to PleD, PopA localization to the incipient stalked pole would be triggered by dimerization, which in contrast to PleD is mediated by c-di-GMP binding to the I-site. PopA in its c-di-GMP-bound dimeraize and because of its

dimerization localize to the incipient stalked pole (Figure 4.3B). Importantly, in contrast to PleD, the *popA*$_{D55N}$ mutant (P-site mutant) shows wild-type like localization to the incipient stalked pole. One possible explanation might be that phosphorylation plays no regulatory role in PopA, dimerization is exclusively triggered by c-di-GMP binding, or alternatively the non-phosphorylated form of PopA might be active, similar to the regulation of CpdR sequestration. The speculation that non-phosphorylated PopA might be active is not supported by the observation, that a *popA*$_{D51E}$ mutant, which often mimics a constitutive active allele in response regulators, shows no effect *in vivo*. This finding still does not completely exclude this possibility, but favors the idea that phosphorylation is not important for PopA function. This is consistent with the finding, that CtrA degradation is independent of an intact P-site.

Furthermore, one could think of a slightly different model, in which dimerization of PopA, in contrast to PleD, prevents polar localization to the incipient stalked pole. C-di-GMP binding would facilitate PopA sequestration by converting an inactive PopA dimer into the localization-competent monomeric form. This model is supported by preliminary biochemical studies, which reveal that although PopA I-site mutants forms dimers *in vitro*, PopA wild-type is unable to dimerize (A. Moser, unpublished; Figure 4.3C)

A completely different possibility for PopA localization to the incipient stalked pole is shown in Figure 4.3D. This model suggests that PopA sequestration is not regulated by monomerization/dimerization, but the by a simple conformational change of the GGDEF domain. PopA, always present in a monomeric form, binds c-di-GMP at the I-site of the GGDEF domain, which then triggers a conformational change of the GGDEF domain, and makes PopA accessible to a receptor structure at the pole (Figure 4-3 D).

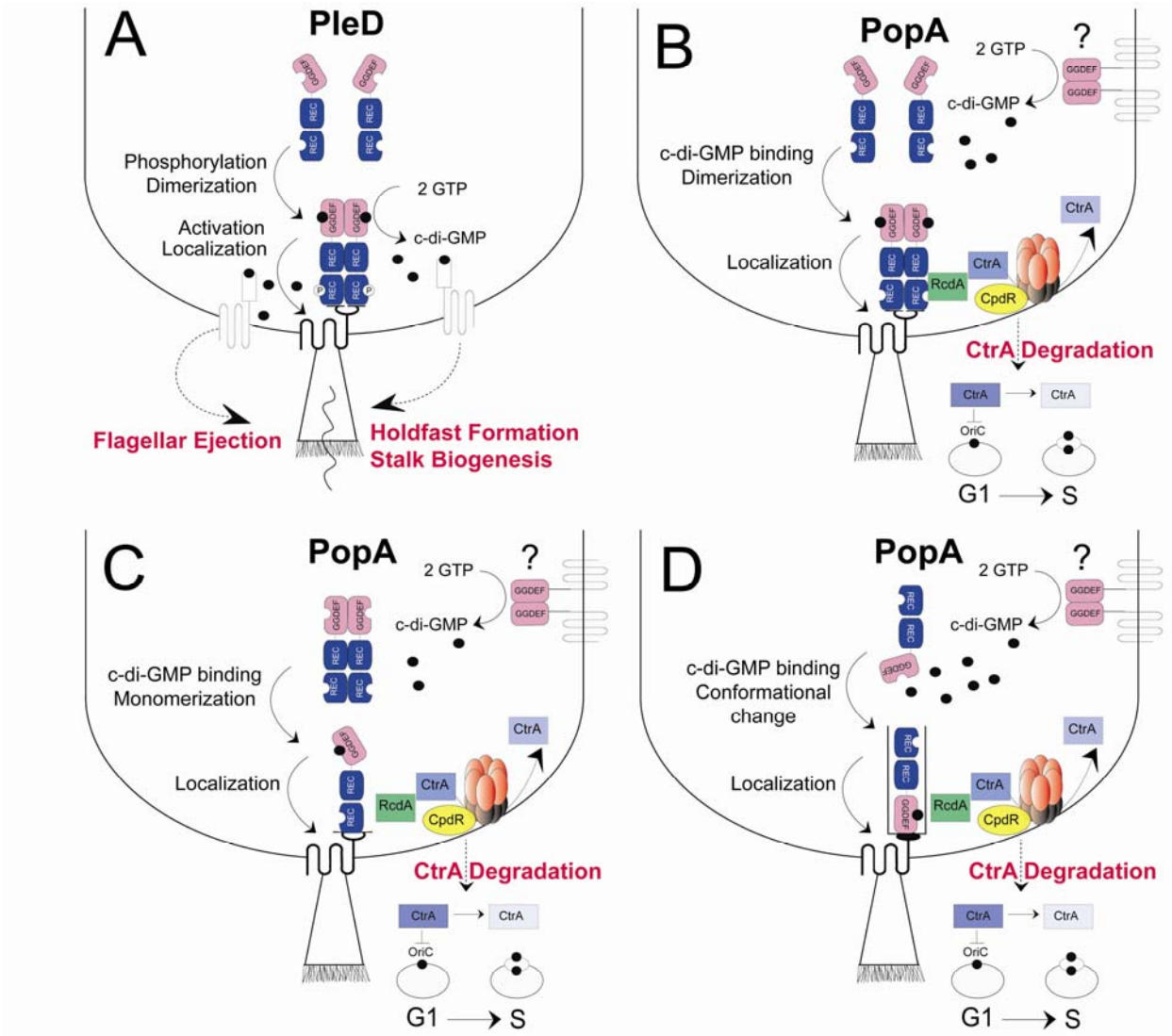



**Figure 4.3**

Schematic representation of PleD (A) and PopA (B,C,D) localization to the cell pole. A) Schematic representation of PleD localization to the stalked cell pole. Phosphorylation-mediated dimerization sequesters PleD to the stalked pole where PleD orchestrates the remodeling of the pole B) Schematic representation of possible PopA sequestration to the incipient stalked pole. C-di-GMP binding triggers PopA dimerization and sequesters PopA to the incipient stalked pole. At the incipient stalked pole localized PopA acts as recruitment factor for CtrA degradation by the localized ClpXP protease complex, which leads to DNA replication initiation. C) c-di-GMP binding to the I-site facilitates monomerization of PopA. The monomeric active form is sequestered to the incipient stalked pole. D) Alternative model for PopA sequestration to the pole. C-di-GMP binds to the I-site of the GGDEF domain and triggers a conformational change, which makes PopA accessible to the receptor at the pole.

Importantly, all obvious mechanisms for PopA sequestration to the cell pole imply the presence of a receptor structure for PopA at the cell pole, which retains PopA from diffusing away. One can speculate, if another, general localization factor, similar to a landmark protein, is required or if a specific characteristic of the membrane at the pole, including special lipid composition, is responsible for PopA sequestration to the right subcellular site. Beyond that, it is still unclear, if the c-di-GMP binding to PopA itself triggers the localization to the incipient stalked pole, or if PopA independently of c-di-GMP binding sequesters to the cell pole, where it binds c-di-GMP, which ensures that PopA is retained at the cell pole.

## 4.4 PopA controls polar development

PopA, in addition to its prominent role in cell cycle progression during the *C. crescentus* cell cycle (see Section 3.2.8), also controls polar development. PopA is an additional player and coordinates the complex c-di-GMP based regulatory network controlling the switch between motility and sessility. Based on the current knowledge, it suggests that PopA might act as a bifunctional enzyme, controlling cell cycle progression and cell development.

*PopA controls flagellar motor function*

PopA was shown to be partially impaired in swimming on semi-solid agar plates despite the fact that the mutant is still able to assemble flagellar structures. One possible explanation for the partially impaired motility is that fewer cells are flagellated compared to wild-type. To rule out this possibility statistical analysis of the electron micrographs counting flagellated cells should be performed. Another way to test this hypothesis would be to measure the overall protein concentration of flagellar proteins. An alternative explanation for reduced motility of *popA*, the two possibilities are not mutually exclusive, is that the flagellar function is reduced in *popA* mutants. This possibility is supported by the results collected by the analysis of the Δ*popA* mutant with the automated Hobson BacTracking system. These experiments not only revealed a reduced number of motile cells compared to wild-type, but also indicated a reduction in swimming speed.

Apparently, the flagellar motors of the cells recorded in this assay are only partially functional. The reason for the reduced number of motile cells remains unclear. One possible explanation for the reduced number of motile cells could be a shortened swarmer phase of the Δ*popA* mutant. Since the overall growth rate of Δ*popA* is not affected, this possibility seems unlikely. Alternatively, fewer cells might be flagellated (see above). A careful qualitative and quantitative analysis of motor assembly, function, and cell cycle timing should shed more light on this particular role of the multifunctional PopA protein.

Interestingly, the function of PopA in cell motility is not dependent on the phosphorylation (P-site) or the active site motif “GGDEF” (A-site), but requires an intact I-site. Apparently, despite the fact that the I-site is not required to sequester PopA to the flagellated pole, it is critical to maintain proper functioning of the flagellar motor.

*Cell motility and attachment are inversely regulated by a complex network of different c-di-GMP signaling proteins*

It is long known that the PleD diguanylate cyclase has a prominent role in controlling the switch between the motile and sessile cell types during the *C. crescentus* life cycle (Aldridge and Jenal, 1999; Aldridge et al., 2003; Paul et al., 2004). Here we have identified two additional c-di-GMP signaling proteins, DgcB and PopA, as members of the regulatory network controlling *C. crescentus* cell motility and attachment (see Section 3.2.8).

We could show that DgcB, an active DGC *in vitro*, contributes to cell motility and surface attachment in *C. crescentus*. Analysis of the Δ*dgcB* mutant on semi-solid agar plates and by Hobson BacTraking showed more motile and faster swimming cells for the Δ*dgcB* mutant compared to wild-type. Based on these findings one can suggest, that DgcB negatively regulates cell motility. However, the molecular mechanism remains unclear. One can think of several possibilities, including changed timing of flagellar ejection or more efficient motor function. In addition to the increased motility, the Δ*dgcB* mutant shows reduced surface attachment, in contrast to PleD, only a minor reduction of 20%. In agreement with the only slight reduced attachment, no obvious phenotype for holdfast formation was observed in exponentially growing cells.

However, deleting *dgcB* in a *pleD* mutant background, results in the Δ*pleD*Δ*dgcB* mutant to the complete loss of attachment and holdfast, and impaired stalk biogenesis. This strongly argues that DgcB and PleD, two active DGCs, regulate together the same downstream pathway, which controls stalk biogenesis, surface attachment and holdfast formation. The effect of *dgcB* on attachment and holdfast is minor compared to PleD arguing for a modulating or enhancing function for DgcB.

Consistent with the finding, that PopA has no DGC activity *in vitro*, the Δ*popA* mutant has no effect on stalk biogenesis, surface attachment or holdfast formation. Nevertheless, PopA is involved in the regulation of cell motility (see above). Unexpected, combining the *popA* with the *pleD* mutant results in mutant cells with microscopically non-visible stalks, the complete loss of surface attachment as well as holdfast. In contrast, introducing the *popA* mutant into the Δ*dgcB* background had no effect. This suggests an additional function for PopA regulating together with PleD pole development, including stalk biogenesis, surface attachment and holdfast formation, but only and exclusively in the Δ*pleD* background.

Based on the presented motility and attachment data for Δ*pleD*, Δ*popA*, Δ*dgcB* and the multiple deletion strains we propose three different models in which all three GGDEF domain proteins together control the switch between motility and sessility in *C. crescentus* (Figure 4.4). Model A and B suggest two redundant pathways controlling together the same downstream regulating pathway, including motility, attachment and holdfast formation. The first pathway consists of PleD and the other of PopA together with DgcB, whereas the hierarchy of PopA and DgcB is unclear. In model A, PopA is located upstream of DgcB. This would suggest that PopA might act as localization factor for DgcB and therefore regulate the DGC activity of DgcB at the cell pole. Nevertheless, one could also think about an opposite arrangement in which DgcB is positioned upstream of PopA. This would suggest that the DGC DgcB produces and delivers the c-di-GMP for PopA. A completely different possibility is shown in model C. Motility, attachment and holdfast formation are regulated by three independent pathways, which converge and contribute to the regulation of the same downstream target. Importantly, under normal conditions the contribution of PopA would be masked by the presence of PleD. PopA only contributes for correct polar morphogenesis in absence of PleD. A possible influence of additional players controlling polar development can not be excluded.

However, one main focus is to elucidate the role of PopA in controlling cell development in more detail, including the swarmer pole specific localization of PopA. One could speculate that the swarmer pole specific localization of PopA, which is dependent on the general localization factor PodJ, is directly linked to its function in controlling motility, stalk biogenesis and holdfast formation? This would be consistent with the observation that PodJ acts as a general localization factor for proteins, including PleD and CpaE, which are involved in pole morphogenesis in *C. crescentus*.

Finally, it will be interesting to test if an intact I-site, in addition to its control in CtrA degradation, is also required for optimal holdfast formation and stalk biogenesis.

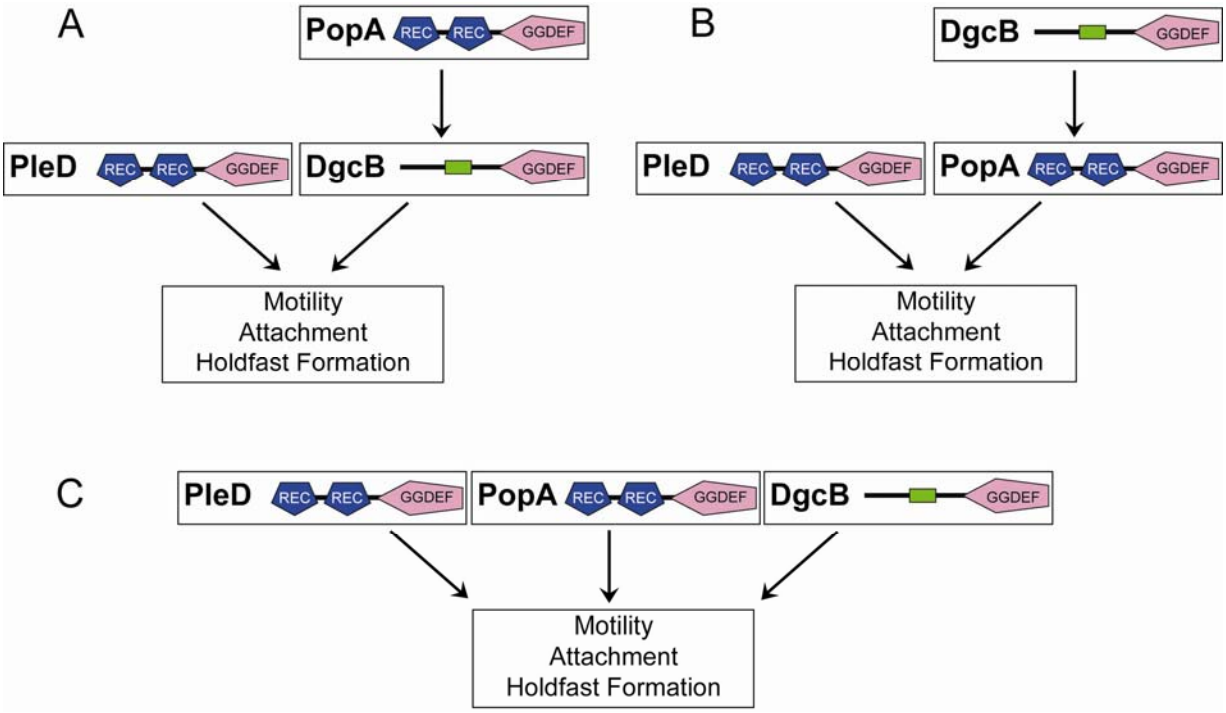



**Figure 4.4**

Three different models for the regulation of cell development in *C. crescentus*.

# 5 Outlook

We have shown for the first time that PopA, a GGDEF domain protein, acts as a c-di-GMP effector protein and regulates cell cycle control in *Caulobacter crescentus*. PopA is required for CtrA sequestration to the incipient stalked pole and for CtrA degradation during the G1-to-S phase transition. However, there remain many unanswered questions about the function of PopA. Is PopA specific for CtrA or does it control the degradation of additional proteins. Is PopA a more global recruitment factor regulating polar sequestration of other proteins?

Time laps fluorescence microscopy showed that PopA itself dynamically localizes to the cell poles during the cell cycle. So far, we could demonstrate that an intact PopA c-di-GMP binding site is required for PopA localization to the incipient stalked pole. However, the exact molecular sequestration mechanism to the incipient stalked pole is still not identified. What are upstream the components, delivers the c-di-GMP for PopA sequestration to the incipient stalked pole? Moreover, once localized at the cell pole, how is PopA retained at the pole? Which kind of receptor structures are required to keep PopA at the pole?

In addition to its prominent role in cell cycle control, PopA regulates together with additional GGDEF domain proteins cell motility, holdfast formation and surface attachment. How does PopA contribute to cell development and what is the exact role of PopA in this complex network controlling development? Is the function of PopA regulating cell pole morphogenesis directly coupled to its localization to the new swarmer pole? How is PodJ involved in PopA sequestration to the new swarmer pole?

From a more general point of view, it would be interesting to identify possible external signals, which activate the c-di-GMP mediated PopA pathway leading to polar CtrA sequestration and degradation. Is the c-di-GMP signaling cascade cross connected to additional regulatory pathways?

Finally, it would be challenging to postulate a general role for c-di-GMP controlling cell cycle looking in different bacterial species.

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# 7 Appendices

## 7.1 Acknowledgements

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## 7.4 Curiculum Vitae

# Curriculum Vitae

### Personal Details


| | |
|---|---|
| Name | **Duerig Anna** Elisabeth |
| Address | Davidsbodenstrasse 72<br>CH-4056 Basel<br>Switzerland |
| Telephone | +41 61 311 45 15<br>+41 61 267 21 29 |
| Mobile | +41 79 305 61 15 |
| E-mail | anna.duerig@unibas.ch |


| | |
|---|---|
| Nationality | Swiss |
| Date of birth | 24.10.1979 |

### Education

| | |
|---|---|
| Postgraduate Degree | **PhD in Molecular Microbiology**<br>" c-di-GMP signalling in *Caulobacter crescentus* " |
| Institution | Department of Molecular Microbiology<br>Biozentrum, University of Basel<br>Switzerland |
| Supervisor | Prof. Dr. Urs Jenal |
| Date | March 2004 – April 2008 |
| Main activities / responsibilities | Basic research on c-di-GMP signalling in *Caulobacter crescentus*<br>- Planning and organization of the research project<br>- Main work techniques: genetics, molecular biology, 4D fluorescence microscopy<br>- Protocol and evaluation of the acquired data<br>Teaching assistance<br>- Preparation of lab courses and mentoring of students (2004-2007)<br>- Supervision of master students |
| Graduate Degree | **Master of Science in Biochemistry**<br>" Gensynthese, Klonierung, Expression und Charakterisierung von $PD_1$ and $DHP_1$ " |
| Main courses | Genetics, Molecular Microbiology, Biochemistry, Physical Chemistry and Organic Chemistry |

| | |
|---|---|
| Institution | Department of Chemistry and Biochemistry<br>University of Berne<br>Switzerland |
| Supervisor | Prof. Dr. Ulrich Baumann |
| Date | October 1999 – October 2003 |
| Secundary Education | **Matura Typ B** |
| | Gymnasium Kirchenfeld, Bern, Switzerland |
| Main courses | German, English, French, Mathematics |
| Date | 1994 –1999 |

## Working Experience

| | |
|---|---|
| Occupation | **Scientific assistance** for Index Nominum –<br>The International Drug Directory |
| Employer | Swiss Pharmaceutical Society SAV<br>Stationsstrasse 12,<br>CH-3097 Liebefeld |
| Date / Pensum | Part-time, during the studies |
| Main activities / responsibilities | - Scientific and administrative assistance for the project "Index Nominum" |
| | - Data research and data acquisition (active pharmaceutical ingredient, international brand name, company) |
| | - Instruction and supervision of students for data acquisition |
| | *Index Nominum is an international drug directory that provides access to approximately 5'300 substances and derivates, 12'800 synonyms, and 41'800 trade names from 45 countries* |

## Publication Record

| | |
|---|---|
| 04/2008 | The following manuscript is ready for submission |
| | "Second messenger mediated spatiotemporal control of protein degradation during the cell cycle" |
| | A. Duerig, M. Folcher, S. Abel, T. Schwede and U. Jenal |

## Scientific Conferences

| | |
|---|---|
| 10/2004 | Poster, Biozentrum Symposium, St. Chrischona (CH) |
| 03/2005 | Oral presentation, SWIMM-Meeting, Zuerich (CH) |
| 10/2005 | Poster, Biozentrum Symposium, Basel (CH) |
| 03/2006 | Oral presentation, SSM-Meeting, Lausanne (CH) |
| 05/2006 | Poster, General ASM Conference, Orlando (USA) |
| 04/2007 | Poster, Microbial-Adaption-Meeting, Marburg (D) |

| | |
|---|---|
| **Personal Skills** | |
| Mother language | **German** |
| Other languages | |
| **English** | Fluent (PhD studies in English) |
| **French** | Good command of written and spoken French |
| **Spanish** | Basic knowledge, Spanish course in Nerja, 1999 |
| **References** | Prof. Dr. Urs Jenal<br>Klingelbergstrasse 50/70<br>CH-4056 Basel<br>Tel: +41 61 267 21 35<br>urs.jenal@unibas.ch<br><br>Dr. Alexander Boehm<br>Klingelbergstrasse 50/70<br>CH-4056 Basel<br>Tel: +41 61 267 21 29<br>alexander.boehm@unibas.ch |