Dynamics of two phosphorelays controlling cell cycle progression in *Caulobacter crescentus*

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Abstract

In *Caulobacter crescentus*, progression through the cell cycle is governed by the periodic activation and inactivation of the master regulator CtrA. Two phosphorelays, each initiating with the histidine kinase CckA, promote CtrA activation by driving its phosphorylation and by inactivating its proteolysis. Here, we examined whether the CckA phosphorelays also influence the down-regulation of CtrA. We demonstrate that CckA is bifunctional, capable of acting as either a kinase or phosphatase to drive the activation or inactivation, respectively, of CtrA. By identifying mutations that uncouple these two activities, we show that CckA's phosphatase activity is important for down-regulating CtrA prior to DNA replication initiation *in vivo*, but that other phosphatases may exist. Our results demonstrate that cell cycle transitions in *Caulobacter* require, and are likely driven by, the toggling of CckA between its kinase and phosphatase states. More generally, our results emphasize how the bifunctional nature of histidine kinases can help switch cells between mutually exclusive states.

Introduction

Caulobacter crescentus is a tractable model system for understanding the molecular mechanisms underlying cell cycle progression and the establishment of cellular asymmetry in bacteria. Each cell division for *Caulobacter* produces two morphologically different daughter cells, a swarmer cell and a stalked cell, which also differ in their ability to initiate DNA replication. A stalked cell can immediately initiate DNA replication following cell division, whereas a swarmer cell cannot initiate until after differentiating into a stalked cell. The swarmer-to-stalked cell transition thus coincides with a G1-S cell cycle transition. DNA replication occurs once-and-only-once per cell cycle, resulting in distinguishable G1, S, and G2 phases.

Progression through the *Caulobacter* cell cycle requires the precise temporal and spatial coordination of both morphological and cell cycle events. Previous genetic screens have uncovered numerous two-component signal transduction genes that help to regulate these events (10, 11, 17, 25, 29, 33, 34, 42). Two-component signaling pathways are typically comprised of a sensor histidine kinase that, upon activation, autophosphorylates and subsequently transfers its phosphoryl group to a cognate response regulator, which can then effect changes in cellular physiology (35). One common variation of this signaling paradigm is called a phosphorelay (3). Such pathways also initiate with the autophosphorylation of a histidine kinase and subsequent phosphotransfer to a response regulator, but these steps often occur intramolecularly within a hybrid histidine kinase. The phosphoryl group on the receiver domain of a hybrid kinase is then passed to a histidine phosphotransferase, which subsequently phosphorylates a soluble response

regulator to effect an output response. Relative to canonical two-component pathways, phosphorelays provide additional points of control and enable signal integration; they are often involved in regulating key cell fate decisions in processes such as sporulation, cell cycle transitions, and quorum sensing (1, 3, 8).

The master regulator of the *Caulobacter* cell cycle is CtrA, an essential response regulator that directly activates the expression of at least 70 genes (19, 29). CtrA also regulates DNA replication by binding to and silencing the origin of replication (30). Progression through the *Caulobacter* cell cycle thus requires the precise control of CtrA activity. CtrA must be abundant and active thoughout most of the cell cycle to drive gene expression and to silence the origin, but must be temporarily inactivated in stalked cells prior to S-phase to permit the initiation of DNA replication (also see Fig. 8).

CtrA is regulated on at least three levels: transcription, proteolysis, and phosphorylation (4, 5). During G1, CtrA is phosphorylated and proteolytically stable. At the G1-S transition, CtrA is dephosphorylated and degraded, thereby freeing the origin of replication to fire. After DNA replication initiates, *ctrA* is transcribed and the newly synthesized CtrA is again phosphorylated and protected from proteolysis. Following septation of the predivisional cell, CtrA remains phosphorylated and stable in the swarmer cell, but is dephosphorylated and degraded in the stalked cell to permit DNA replication initiation. Cells that constitutively transcribe *ctrA* are viable and display only a mild phenotype indicating that regulated phosphorylation and proteolysis alone can ensure the periodicity of CtrA activity (4). Cells producing nondegradable,

constitutively-active CtrA arrest in G1 because CtrA activity cannot be eliminated (4).

The regulation of CtrA activity involves two phosphorelays. Each initiates with CckA, a hybrid histidine kinase, and ChpT, a histidine phosphotransferase. After receiving a phosphoryl group from CckA, ChpT can act as the phosphodonor for either CtrA or the single-domain response regulator CpdR (1). Phosphorylation of CpdR prevents it from triggering CtrA proteolysis (1, 14). Unphosphorylated CpdR triggers CtrA degradation, by somehow influencing the polar localization of the protease ClpXP (14), although why the protease must be localized is unclear.

The down-regulation of CtrA prior to DNA replication involves the dephosphorylation of CtrA and CpdR, such that CtrA is both dephosphorylated and, ultimately, degraded. These events coincide with the time in the cell cycle when CckA's kinase activity is lowest (16). As the phosphoryl groups on CtrA~P and CpdR~P are relatively stable, at least *in vitro* (1), phosphatases are likely critical to eliminating CtrA activity prior to S-phase. For some phosphorelays, inactivation of the top-level kinase leads to a siphoning of phosphoryl groups from the terminal regulator back to the hybrid kinase's receiver domain. The bifunctional hybrid kinase then acts as a phosphatase, stimulating hydrolysis and loss of the phosphoryl group (7, 8). For other phosphorelays there are separate and dedicated phosphatases (23, 26, 27).

Here, we demonstrate that CckA is bifunctional and can act as both a kinase and a phosphatase such that inactivation of CckA as a kinase stimulates the dephosphorylation of CtrA~P and CpdR~P. We provide evidence that CckA's phosphatase activity contributes to the down-regulation of CtrA *in vivo*, but that other phosphatases may exist. Our results indicate that the

periodic toggling of CckA between kinase and phosphatase states is crucial to cell cycle progression in *Caulobacter*.

Results

CckA and ChpT are present throughout the cell cycle

CckA, unlike CtrA, is present throughout the cell cycle, but is only active at certain stages of the cell cycle (16, 17). To test whether the abundance of ChpT is cell cycle-regulated and hence a possible means of controlling the timing of CtrA activity, we generated polyclonal antibodies for ChpT. Immunoblotting with crude sera revealed a single major band in wild-type lysates that was absent in lysates from a *chpT* depletion strain and that was the correct approximate size (Fig. S1A). To examine the cell cycle abundance of ChpT, we synchronized a population of wild-type cells and isolated samples every 20 minutes. Immunoblotting of these samples demonstrated that ChpT was present throughout the cell cycle, in contrast to CtrA which showed a characteristic cell cycle-dependence (Fig. 1). These results suggest that phosphate flux from CckA to CtrA is probably not regulated by changes in ChpT abundance.

Our ChpT antiserum also recognized purified His_6-ChpT, although the molecular weight of this purified ChpT appeared slightly larger than that found in wild-type lysates (Fig. S1A). This difference could not be accounted for by the epitope tag, suggesting that the translational start-site for *chpT* might have been erroneous in the original annotation of the *C. crescentus* genome (22). The *chpT* open reading frame contains methionines at positions 19 and 29 (relative to the originally annotated protein), each of which could serve as the *bona fide* translational start site.

Alignment of *chpT* orthologs from several α-proteobacteria indicated that the first 28 amino acids of *C. crescentus* ChpT were not conserved (Fig. S2). We were able to complement the lethality of a chromosomal deletion of *chpT* with a plasmid expressing a version of *chpT* lacking the first 28 codons of the original annotation (Fig. S1B). This result strongly suggests that *C. crescentus chpT* encodes a protein of only 225 amino acids with a molecular weight of 23.4 kDa.

To verify that the smaller version of ChpT is capable of shuttling phosphate from CckA to CtrA and CpdR, we reconstituted the two cell cycle phosphorelays (CckA-ChpT-CtrA and CckA-ChpT-CpdR) using a purified version of the smaller ChpT, hereafter referred to simply as ChpT (Fig. S1C). Indeed, this shorter version of ChpT was able to efficiently shuttle phosphate from the receiver domain of CckA to either CtrA or CpdR.

Reconstitution of the CckA-based cell cycle phosphorelays

The reconstituted cell cycle phosphorelays shown in Figure S1 and those reported previously (1) involved a split version of CckA in which the histidine kinase (CckA-HK) and receiver domains (CckA-RD) were purified as separate polypeptides. Here, we wanted to examine the phosphotransfer behavior of a CckA construct containing both the kinase and receiver domains, as occurs *in vivo*. This construct, called CckA-HK-RD, lacking only the transmembrane domains, autophosphorylated and was an efficient phosphodonor for ChpT (Fig. 2A), which then transferred the phosphoryl group to either CtrA or CpdR, as with the split version of CckA (1). These data confirm that CckA initiates two phosphorelays, culminating in the phosphorylation of CtrA and CpdR.

Phosphorelays are often reversible, such that phosphoryl groups can flow either up or down the pathway according to the principles of mass-action equilibrium (7-9, 40). In some cases, the histidine kinase involved can be bifunctional, acting to stimulate dephosphorylation of its cognate response regulator or, in the case of a hybrid kinase, its receiver domain. These bifunctional kinases can thus drive the rapid dephosphorylation of the terminal response regulator when they are not stimulated to autophosphorylate. To test whether CckA is bifunctional, we isolated radiolabeled, phosphorylated CtrA (CtrA~P) and CpdR (CpdR~P) by phosphorylating each regulator for extended periods of time with the heterologous kinases PhoR (a histidine kinase from *C. crescentus*) and EnvZ(T247R) (a histidine kinase from *E. coli* that does not harbor significant phosphatase activity), respectively. The phosphorylated response regulators were then purified away from unreacted, radiolabeled ATP. This purification step was not 100% efficient and each preparation of phosphorylated CtrA or CpdR retains some radiolabeled ATP that runs at a similar position as inorganic phosphate at the bottom of each gel in Fig. 2B and 2C.

Incubation of each regulator in buffer alone demonstrated that their aspartyl-phosphates are both relatively stable against autodephosphorylation *in vitro*, showing only a minor production of radiolabeled inorganic phosphate after 60 minutes; the band at the bottom of lanes 2-4 in Fig. 2B-C increases in intensity only marginally relative to lane 1. By contrast, incubation of CtrA~P with ChpT and CckA-HK-RD led to a significant depletion of radiolabel from CtrA~P within 10 minutes with nearly complete depletion in 60 minutes (Fig. 2B, lanes 8-10). The loss of radiolabel from CtrA~P also coincided with the appearance of radiolabeled inorganic phosphate,

suggesting active dephosphorylation and not just partitioning of the phosphoryl groups among phosphorelay components. Incubation of CpdR~P with ChpT and CckA-HK-RD also led to a decrease in radiolabeled CpdR~P and an increase in inorganic phosphate (Fig. 2C, lanes 8-10), although not as much as with CtrA.

Notably, the dephosphorylation of CtrA and CpdR occurred at a much higher rate when the kinase and reciever domains of CckA were fused as a single polypeptide. Incubation of the radiolabeled response regulators with ChpT and the split version of CckA (CckA-HK and CckA-RD) did not lead to a significant production of inorganic phosphate (Fig. 2B-C, lanes 5-7). In these cases, phosphoryl groups did flow up the phoshorelay, as manifest by the appearance of radiolabeled ChpT and CckA-RD and the depletion of radiolabeled CtrA and CpdR. However, the levels of inorganic phosphate did not increase significantly indicating that CckA-RD must be tethered to CckA-HK for efficient dephosphorylation. Taken together, our data suggest that the cell cycle phosphorelays can run in reverse and that CckA is bifunctional such that it can stimulate the dephosphorylation of its own receiver domain. Together these two mechanisms, phosphorelay reversal and the phosphatase activity of CckA on its own receiver domain, can indirectly drive the dephosphorylation of CtrA~P and CpdR~P.

Mutations that genetically separate kinase and phosphatase activities of CckA

To assess whether CckA and phosphorelay reversal contribute to the dephosphorylation of CtrA or CpdR *in vivo*, we sought to identify mutations in *cckA* that uncouple its kinase and phosphatase activities to yield CckA with kinase-only (K^+P^-) or phosphatase-only (K^-P^+) activity.

To this end we generated ten mutant alleles of *cckA* based on mutations that render *E. coli* EnvZ either K^+P^- or K^-P^+ (Fig. 3A) (2, 6, 12, 21, 31, 39). We also made alanine mutations at the site of histidine autophosphorylation (H322) and at the site of aspartate phosphorylation in the receiver domain (D623), for a total of 12 mutations. We first introduced these mutations into our CckA-HK-RD construct and tested their abilities to autophosphorylate and phosphotransfer to ChpT *in vitro* (Fig. 3B). Four of the mutant kinases (harboring mutations G318T, G319E, and, V366P, and D623A) retained clear, detectable levels of autophosphorylation, and each construct could phosphotransfer to ChpT except for D623A. The mutations G318T and G319E each led to significantly higher levels of autophosphorylated CckA-HK-RD and higher levels of phosphorylated ChpT when compared to wild-type CckA-HK-RD. The V366P mutation, however, produced levels of CckA autophosphorylation and ChpT~P comparable to that seen with wild-type CckA-HK-RD.

Next, we tested whether any of the mutant kinase constructs that autophosphorylated could efficiently drive the dephosphorylation of CtrA~P via phosphorelay reversal and hydrolysis of phosphorylated CckA-RD (Fig. 3C). Each mutant construct that retained kinase activity was added to ChpT and CtrA~P and then incubated for 30 minutes at 30^{o}C. For the H322A, G318T, G319E, and V366P mutants, the radiolabeled phosphoryl groups flowed in reverse as seen by the appearance of radiolabeled bands corresponding to ChpT and CckA. For CckA(D623A) phosphoryl groups partitioned between CtrA and ChpT, but could not transfer back to CckA. CckA(D623A) lacks the aspartate phosphorylation site within the receiver domain and therefore cannot participate in phosphotransfer with ChpT. The dephosphorylation of CckA's receiver

domain by its kinase domain was assessed by examining the production of inorganic phosphate and the coincident depletion of radiolabel from all other bands. The only mutant with phosphatase activity comparable to wild-type CckA was that harboring the substitution H322A.

These *in vitro* data indicate that the V366P mutation produces a version of CckA that retains kinase activity but lacks significant phosphatase activity (K^+P^-) while the H322A mutation produces a version lacking kinase but not phosphatase activity (K^-P^+). To better characterize these two mutants, we analyzed time courses of CtrA~P dephosphorylation (Fig. 4A-C). CckA-HK-RD and CckA-HK-RD(H322A) each showed a depletion of radiolabel from the phosphorelay components along with an increase in inorganic phosphate. By contrast, the constructs harboring D623A and V366P showed little to no depletion of phosphorelay components and no significant production of inorganic phosphate. These data support the characterization of V366P as a K^+P^- mutant of CckA with kinase activity comparable to wild-type CckA.

Phosphatase activity of CckA is important, but not essential, for dephosphorylation of CtrA and CpdR

To test whether the phosphatase activity of CckA is important for cell cycle progression and viability, we tested whether the mutant alleles of *cckA* we created could complement a *cckA* chromosomal deletion. For these experiments, we placed a full-length copy of each mutant allele of *cckA*, driven by the native *cckA* promoter, on the low-copy plasmid pMR20. Each plasmid was transformed into wild type, followed by transduction of a gentamicin-marked *cckA* deletion

onto the chromosome. As expected, transduction of Δ*cckA* into a strain harboring the wild-type copy of *cckA* yielded thousands of colonies while transduction into a strain harboring an empty vector yielded none. Transduction of Δ*cckA* into a strain containing *cckA(D623A)* also produced no colonies, consistent with the notion that phosphorylation of the receiver domain is essential for viability. Unexpectedly, we recovered hundreds of colonies when transducing Δ*cckA* into a strain containing *cckA(H322A)*. However, sequencing of the plasmids in several of these colonies revealed that the mutation had reverted in each case, likely via recombination with the chromosomal copy of *cckA* prior to transduction. Reversion did not occur with the plasmid harboring *cckA(D623A)*, probably because the D623A mutation is toward the end of the *cckA* coding region and does not have sufficiently long regions of homology to efficiently drive recombination. Because we were unable to produce the CckA(H322A) + Δ*cckA* strain, we conclude that H322, like D623, is essential for CckA function.

As with H322A, transduction of Δ*cckA* into a strain expressing *cckA(G318T)* yielded abundant colonies, but plasmid sequencing from multiple colonies indicated reversion to wild-type CckA. *In vitro*, CckA(G318T) had shown significantly increased kinase activity relative to wild-type CckA-HK-RD and no detectable phosphatase activity (see Fig. 3). The inability of *cckA(G318T)* to complement a *cckA* deletion suggests that an imbalance in CckA activities is lethal. We cannot, however, say whether the lethality results from a lack of phosphatase activity or excessive kinase activity, or both.

For the G319E and V366P mutants we successfully constructed and sequence-verified strains in

which the chromosomal copy of *cckA* was deleted and the mutant allele of *cckA* was carried on a plasmid. The strain expressing *cckA(G319E)* grew more slowly than a strain expressing wild-type *cckA* and exhibited severe cellular filamentation (Fig. 5). These cells formed long, relatively straight filaments reminiscent of the morphology of a strain overproducing CtrA(D51E)Δ3Ω, a non-proteolyzable version of CtrA that mimics the phosphorylated state and induces a G1-arrest (4). Indeed, the *cckA(G319E)* + Δ*cckA* strain showed a significant increase in cells with one chromosome (Fig. 5). Our *in vitro* studies showed that CckA(G319E) exhibits a substantial increase in autophosphorylation relative to wild-type CckA. Taken together, these data suggest that the G319E mutation renders CckA hyperactive as a kinase, resulting in constitutive phosphorylation of CtrA and CpdR and hence, a G1-arrest.

The K^+P^- mutation V366P did not lead to a severe cell cycle phenotype (Fig. 5), suggesting that the phosphatase activity of CckA is either not strictly essential for viability or that V366P does not completely eliminate phosphatase activity *in vivo*. However, even if CckA phosphatase activity is not strictly essential, CckA could still be an important phosphatase *in vivo* for either CtrA or CpdR. To further test this possibility, we sought to examine whether the phenotype of a strain expressing *cckA(V366P)* as the only copy of *cckA* was exacerbated by the synthesis of CtrA(D51E) or CtrAΔ3Ω. For example, if CckA is a key phosphatase for CtrA, cells producing both CtrAΔ3Ω and a K^+P^- version of CckA may exhibit a G1-arrest phenotype, as with cells producing CtrA(D51E)Δ3Ω. For these experiments, we transformed the pMR20-*cckA(V366P)* + Δ*cckA* strain with medium-copy plasmids carrying *ctrA*, *ctrA(D51E)*, *ctrAΔ3Ω*, or *ctrA(D51E)Δ3Ω* under the control of a xylose-inducible promoter. For comparison, we

transformed the pMR20-*cckA* + Δ*cckA* strain with the same set of plasmids. Each strain was grown in the presence of xylose to mid-exponential phase and chromosome content measured by flow cytometry (Fig. 6). The strains synthesizing CtrA(D51E) or CtrAΔ3Ω each showed a small, but reproducible increase in G1-phased cells when combined with *cckA(V366P)* compared to *cckA*. No difference was seen between the strains synthesizing CtrA(D51E)Δ3Ω indicating that CckA(V366P) mediates its cell-cycle effect through the CckA-ChpT phosphorelays and not through other pathways. These data further suggest that CckA participates in the dephosphorylation of both CtrA~P and CpdR~P *in vivo*. However, the fact that CckA(V366P) does not yield a G1 arrest suggests that other phosphatases for CtrA and CpdR may exist. Or, as noted, the V366P mutation may be an imperfect K^+P^- allele that retains sufficient phosphatase activity *in vivo* to permit the dephosphorylation of CtrA~P and CpdR~P prior to DNA replication initiation.

Overproducing CckA drives the dephosphorylation of CtrA~P and CpdR~P

To further test whether phosphorelay reversal and CckA phosphatase activity can drive the dephosphorylation of CtrA~P and CpdR~P *in vivo*, we examined the effect of overexpressing *cckA*. We hypothesized that overproducing CckA should siphon phosphoryl groups back through the phosphorelay driving the dephosphorylation of CtrA and CpdR, leading to a decrease in CtrA activity. To test this prediction, we placed a full-length copy of *cckA* on the plasmid pJS14 under the control of a xylose-inducible promoter. After growth in xylose for 4 hours, this strain exhibited mild cellular filamentation and some accumulation of chromosomes, consistent

with a downregulation of CtrA (Fig. 7A). Overproducing a version of CckA lacking its transmembrane domains, CckAΔTM, produced more severe filamentation and led to excessive accumulation of chromosomal DNA (Fig. 7A), consistent with an even more significant downregulation of CtrA. This cellular filamentation and accumulation of chromosomes depended on backtransfer to the CckA receiver domain as overproducing CckAΔTM(D623A) did not severely disrupt the cell cycle (Fig. 7A). However, backtransfer alone was insufficient and CtrA downregulation also depended on the phosphatase activity of CckA as overproducing the CckA receiver domain alone (CckA-RD) or a version of CckA lacking phosphatase activity, CckAΔTM(V366P), did not lead to cellular filamentation or chromosome accumulation (Fig. 7A).

The more severe phenotype of overproducing CckAΔTM relative to full-length CckA may indicate that CckA in the membrane can adopt either a kinase or phosphatase state while a cytoplasmic fragment functions primarily as a phosphatase. Consistent with this hypothesis, we found that overproducing a full-length version of CckA(H322A), which can only function as a phosphatase, produced a more severe phenotype than overproducing wild type full-length CckA (Fig. 7A); CckA(H322A) may also have a dominant negative effect by forming inactive heterodimers with the chromosomally-expressed CckA.

Taken together, these data support a model in which the direction and flow of phosphoryl groups through the cell cycle phosphorelays *in vivo* is dictated by both mass-action equilibrium and the kinase/phosphatase balance of CckA. When CckA is stimulated to autophosphorylate, the net

result is an accumulation of phosphoryl groups on CtrA and CpdR. Conversely, when CckA is not activated as an autokinase, phosphoryl groups can flow back to the CckA receiver domain where the kinase domain stimulates their hydrolysis.

As noted, overproducing a full-length version of CckA did not yield a severe cell cycle phenotype, in contrast to the case of overproducing CckAΔTM, indicating that full-length CckA may retain a balance of kinase and phosphatase activities. If so, the overexpression of full-length CckA should, in principle, be exacerbated by mutations in other genes that regulate the activity of CckA. Our previous studies indicated that the response regulator DivK is a negative regulator of CckA (1). DivK phosphorylation is controlled by the reciprocal actions of a cognate histidine kinase, DivJ, and a cognate phosphatase, PleC (10, 41, 42). We therefore tested the effect of overproducing full-length CckA in either a *divJ* or a *pleC* mutant background. While CckA overproduction did not have a strong effect in the *divJ* mutant (data not shown), it appeared to be strongly synthetic with the *pleC* mutant (Fig. 7B). CckA overproduction and the *pleC* mutation each yield a relatively mild phenotype on their own; however, the combination produced cells that were extremely filamentous and that accumulated multiple chromosomes, consistent with a significant drop in CtrA~P (Fig.7B). This severe cell cycle phenotype was completely dependent on the phosphatase activity of CckA as overproducing full-length CckA(V366P) in a *pleC* mutant background had little to no effect on cells (Fig. 7B). These results indicate that *cckA* likely lies genetically downstream of *pleC* and further support a model in which phosphorylated DivK downregulates CtrA by influencing the kinase/phosphatase balance of

CckA.

Subcellular localization of CckA

In addition to changing from kinase to phosphatase during the cell cycle, CckA also dynamically changes its subcellular localization. CckA, which is present thoughout the cell cycle, was first reported to be polarly localized only in predivisional cells (17), with a second study indicating that CckA is also polarly localized in swarmer cells (1). Here, to further characterize CckA's polar localization and identify the source of this difference we fused full-length *cckA* to *gfp* and integrated this construct on the chromosome as the only copy of *cckA*. The fusion used here includes the last two amino acids, both alanines, of CckA that had been removed in fusing *cckA* to *gfp* in both of the previous studies. By following a synchronous population of swarmer cells isolated from an exponential phase culture (Fig. S3), we found that CckA-GFP was delocalized in nearly all swarmer cells and remained delocalized upon differentiation into stalked cells. CckA-GFP then localized to the nascent swarmer pole in late stalked and early predivisional cells before localizing bipolarly in late predivisional cells. CckA-GFP was delocalized in daughter swarmer cells following cell division. In the daughter stalked cells the pattern was variable with CckA-GFP delocalized in some cells but retained at the stalked pole in most (>75%) cells, in contrast to both of the previous studies showing, at least in the small number of cells examined, that CckA-GFP is delocalized following cell division. Finally, we found that CckA-GFP localization in the initial synchronized population of swarmer cells was strongly dependent on the density of the culture used for synchronization. As cells progressed through

early exponential phase and into late exponential phase, an increasing percentage of swarmer cells showed polarly localized CckA (Fig. S4) indicating that the localization of CckA-GFP in swarmer cells is dependent on culture conditions but is not typically localized in early exponential phase. The overall pattern of subcellular localization observed here for CckA-GFP is in accord with that described by the Jacobs-Wagner group (personal communication). Also, we note that a similar pattern of CckA-GFP localization during synchronous cell cycle progression was seen with a strain expressing CckA-GFP from the low-copy plasmid pMR20 (data not shown). Whether the subcellular localization of CckA affects its activity as a kinase or phosphatase, or *vice versa*, is not yet clear and will likely require the identification of polar factors that directly influence CckA.

Discussion

The *Caulobacter* cell cycle is ultimately driven by the periodic rise and fall in activity of the master regulator CtrA (Fig. 8A). Our previous work identified two phosphorelays that collaborate to activate CtrA, by promoting its phosphorylation and proteolytic stabilization, the latter via CpdR phosphorylation. Conversely, the down-regulation of CtrA depends critically on the dephosphorylation of CtrA and CpdR, but the mechanisms involved have been unknown previously. Here, we demonstrated that CckA, when not active as a kinase, can stimulate the dephosphorylation of CtrA and CpdR to help drive the initiation of DNA replication. We showed that phosphoryl groups can be transferred from CtrA~P and CpdR~P back to the CckA receiver domain, via ChpT, where the bifunctional CckA can stimulate hydrolysis (Fig. 8B).

Like phosphorelays in other organisms (7-9, 40), the direction of flow through the cell cycle phosphorelays in *C. crescentus* appears to be dictated by mass-action. Hence, when CckA is not active as a kinase to drive CtrA and CpdR phosphorylation, the flow of phosphate can reverse. Overexpressing full-length *cckA*, however, resulted in a relatively minor cell cycle phenotype, likely because the CckA produced retains a balance of kinase and phosphatase activities. By contrast, overproducing a version of CckA lacking the transmembrane domains, CckAΔTM, led to a severe disruption of the cell cycle and downregulation of CtrA activity as evidenced by chromosome accumulation. The more severe effect of overproducing CckAΔTM relative to full-length CckA may indicate that CckA must associate with other factors in the membrane to autophosphorylate. This downregulation requires both the reversed flow of phosphoryl groups and their active elimination by CckA phosphatase activity (Fig. 7A). The latter requirement is supported by the observation that overexpressing CckAΔTM(V366P) did not disrupt cell cycle progression. In wild type cells, CckA and ChpT are present at much lower levels (E.G.B, M.T.L, unpublished data) than CtrA, which is estimated to be present at ~20,000 molecules per cell (18). Such stochiometries imply that redistribution alone could only ever deplete a small fraction of the phosphate on CtrA without CckA participating as a phosphatase.

Phosphorelay reversal and CckA phosphatase activity together constitute one mechanism for inactivating CtrA prior to S-phase. Using a K^+P^- mutant of CckA, V366P, we demonstrated that the phosphatase activity of CckA contributes to the down-regulation of CtrA and CpdR *in vivo*. However, cells producing CckA(V366P) are still viable and able to initiate DNA replication indicating that other phosphatases likely exist. If other phosphatases do exist, they may be

difficult to identify owing to redundancy with CckA's phosphatase activity, either of which may be sufficient for survival. Moreover, aspartyl-phosphatases do not comprise a single, paralogous family and typically show little to no sequence homology with one another making their identification difficult (23, 26, 27, 36, 43). Alternatively, no other phosphatases may exist if the phosphoryl groups on CtrA~P and CpdR~P are intrinsically labile, as with CheY and other response regulators (28, 32, 38). However, our data suggest that the aspartyl-phosphates on CtrA and CpdR are relatively stable (Fig. 2B-C) indicating that active dephosphorylation is probably necessary and tightly regulated. Finally, as noted earlier, CckA could be the only phosphatase if the V366P mutation does not completely eliminate phosphatase activity. Our *in vitro* studies did not indicate any significant phosphatase activity for CckA(V366P), but the *in vitro* conditions may not perfectly reflect *in vivo* conditions.

How does the V366P mutation produce a kinase-positive and phosphatase-negative version of CckA? Notably, valine-366 in CckA is predicted, based on alignment to EnvZ, to lie at the C-terminal end of α-helix-2 in the DHp domain near the linker that connects the DHp and CA domains. It is thus tempting to speculate that a proline at this position (V366P in CckA, which was based on the previously reported L288P in EnvZ (12)) may interfere with kinase/phosphatase balance by affecting domain-domain interactions. Recent structural studies of a full-length histidine kinase provided evidence that modulating DHp-CA domain interactions significantly influences the kinase/phosphatase balance of bifunctional histidine kinases (20). It will be interesting to see whether mutations equivalent to V366P in CckA and L288P in EnvZ

can produce K^+P^- versions of other bifunctional histidine kinases.

In sum our results indicate that CckA switches between a kinase state and a phosphatase state to help drive the changes in CtrA activity crucial for proper cell cycle progression. *In vivo* measurements of CckA phosphorylation indicated that CckA kinase activity is detectable in swarmer cells, drops to its lowest levels in stalked cells, and then accumulates again to maximal levels in predivisional cells (16). CtrA and CpdR phosphorylation levels change in a similar fashion during the cell cycle (4, 14, 16), consistent with a model in which changes in CckA's kinase activity are translated into changes in CtrA activity (Fig. 8A).

What then regulates CckA activity? The essential single-domain response regulator DivK plays a key role. A *divK*^{cs} mutant is unable to down-regulate CtrA and consequently arrests with a single chromosome (13), as seen with cells overproducing CtrA(D51E)Δ3Ω (4) or as seen here with cells overproducing CckA(G319E), a version of CckA with high kinase activity. While DivK could control CtrA phosphorylation and degradation independently, a simpler model is that DivK regulates CckA, either directly or indirectly switching CckA from the kinase to phosphatase state. Consistent with this model, CckA phosphorylation levels per cell were found to increase in a *divK*^{cs} mutant (1). Although the increase was only four-fold, it should be noted that this measurement compared *divK*^{cs} to a mixed population of wild type which includes predivisional cells where CckA is most active. The *divK*^{cs} strain, however, is arrested at the G1-S transition when CckA kinase activity is normally at its lowest; in fact, the unabated activity of CckA as a kinase in the *divK*^{cs} strain may be responsible for its G1 arrest phenotype. We also

found here that *cckA* overexpression exhibits a strong synthetic interaction with *pleC*, which encodes a key phosphatase of DivK. This synthetic interaction was dependent on CckA's ability to act as a phosphatase as overexpressing the phosphatase-deficient CckA(V366P) in a *pleC* mutant did not cause cellular filamentation or chromosomal accumulation (Fig. 7B). In a *pleC* mutant, DivK~P levels are elevated (41) and our results suggest that this increase may bias CckA toward the phosphatase state when overproduced, leading to the down-regulation of CtrA and a severe cell cycle phenotype (Fig. 7B). If DivK functioned independently of CckA to regulate CtrA, the overexpression of *cckA* in a *pleC* background may have resulted in an additive, and consequently less severe, effect on the cell cycle.

DivK also affects CckA localization, with CckA-GFP present at the stalked pole but absent from the opposite pole in *divK*^{cs} mutants (1). However, this may be a secondary effect of DivK's effect on CckA activity and the consequent G1-arrest. Whether the localization of CckA influences its activity as a kinase or phosphatase, or *vice versa*, is not yet clear. CckA is most active as a kinase in predivisional cells when it is localized to the nascent swarmer pole and least active in stalked cells where it is either delocalized or only at the stalked pole. This may suggest that CckA receives an activation signal at the nascent swarmer pole or a repressing signal at the stalked pole. However, CckA also has moderate kinase activity in exponential phase swarmer cells when it is typically delocalized. A better understanding of the role of subcellular localization in modulating the kinase and phosphatase states of CckA will require the identification of factors that directly activate or repress CckA.

The model that DivK negatively regulates CckA is consistent with recent data suggesting that CpdR phosphorylation levels may increase after prolonged depletion of DivK (15). This observation could indicate that DivK functions in a second pathway to specifically stimulate CpdR dephosphorylation. Alternatively, or perhaps in addition, the depletion of DivK may simply lead CckA to remain in a kinase rather than phosphatase state; this would lead to increased phosphorylation of CpdR (and CtrA) and ultimately the G1-arrest phenotype characteristic of *divK* loss-of-function mutants. Further, *divK* mutants can be rescued if *cpdR* is replaced by a mutant allele that cannot be phosphorylated (15), and *divK* lethality was previously shown to be suppressed by other mutations that diminish CtrA activity (42). We thus favor a model in which DivK helps switch CckA, either directly or indirectly, from acting predominantly as a kinase to predominantly as a phosphatase, and that an inability to switch (in either direction) is lethal (Fig. 8). The switch in CckA from kinase to phosphatase likely depends on the phosphorylation of DivK by DivJ, its cognate kinase. DivJ is preferentially inherited by stalked cells and accumulates in stalked cells following the swarmer-to-stalked transition (41), presumably helping to temporally restrict the down-regulation of CckA kinase activity and the dephosphorylation of CtrA to stalked cells.

In sum, our results emphasize the critical role played by CckA in controlling cell cycle oscillations and cellular asymmetry in *Caulobacter*. Although CtrA is also regulated transcriptionally, constitutive expression of *ctrA* does not significantly disrupt or delay cell cycle progression, indicating that proteolysis and phosphorylation are likely the dominant modes of regulation. CckA controls both of these processes. In turn, a complex network of regulatory

molecules, including DivJ, PleC, and DivK, appear to regulate CckA activity, helping to toggle it between kinase and phosphatase states at the appropriate stages of the cell cycle.

Materials and Methods

Strain construction and growth conditions

E. coli and *C. crescentus* strains were grown as described previously (33). Strains, plasmids, and primers used in this study are listed in Table S1. All plasmids were introduced into *C. crescentus* by electroporation. PCR amplification of genes and promoters from CB15N genomic DNA was done with previously described conditions (33). For Gateway-based cloning, PCR amplicons of CB15N genes (primer sequences listed in Table S1) were first cloned into the pENTR/D-TOPO vector according to manufacturer's protocol and sequence-verified with M13F and M13R primers or primers within the gene. All site-directed mutagenesis was performed using the following PCR conditions: 75 ng pENTR clone, 50 μM each dNTP, 100 nM each primer, 1X Pfu Turbo buffer, 1.25 U Pfu Turbo polymerase (Strategene), 2% DMSO, and 60 mM Betaine. For each reaction, 17 cycles of the following sequence were run: 94^{o}C for 1 min, 55^{o}C for 1 min, and 68^{o}C for 15 minutes when using pENTR clones or 68^{o}C for 45 minutes when using other plasmids as templates. pENTR clones were then recombined into destination vectors following the manufacturer's protocols (Invitrogen, Carlsbad, CA).

To construct strain ML1054, *chpT* was amplified from the chromosome using primers alt_ChpT_fw and ChpT_rev to create pENTR:*chpT*. This pENTR clone was recombined into the destination vector pLXM-DEST and then transformed into a strain harboring a markerless deletion of *chpT* (1).

To construct strains ML1491-1499, a pENTR clone of the *cckA* gene (pENTR:P_{cckA}-*cckA*),

including 158 bp upstream of the translational start that presumably encompasses the *cckA* promoter, was amplified from CB15N genomic DNA using the primers PcckA-cckA-fw and PcckA-cckA-rev. This pENTR clone was recombined into the destination vector pMR20-DEST to produce a low-copy plasmid harboring a full-length copy of *cckA* under the control of its native promoter (pMR20-P_{cckA}-*cckA*). The plasmid pMR20-P_{cckA}-*cckA* was then transformed into CB15N followed by ΦCr30-based transduction of a gentamycin-marked *cckA* deletion from strain LS3382. To generate *cckA* point mutants, site-directed mutagenesis was performed on pENTR:P_{cckA}-*cckA* using primers listed in Table S1. These pENTR plasmids were sequence-verified and then recombined into the pMR20 destination vector prior to transformation and transduction of the marked *cckA* deletion.

Strains expressing mutant or wild-type *cckA* and overexpressing mutant *ctrA* (ML1567, ML1571, ML1572, ML1576, ML1578, ML1583, ML1585, ML1587) were made by transforming ML1491 and ML1497 with the following plasmids: pJS14, pJS14-P_{xyl}-*ctrA*, pJS14-P_{xyl}-*ctrA(D51E)*, pJS14-P_{xyl}-*ctrAΔ3Ω*, or pJS14-P_{xyl}-*ctrA(D51E)Δ3Ω* (Domian et al., 1997).

To construct strain ML1073, full-length *cckA* was amplified from CB15N genomic DNA with forward primer CckA_full_fw, which adds an NdeI site at the 5' end of the gene and reverse primer CckA_full_rev, which adds a SalI site at the 3' end. Both pML83 and the PCR product containing *cckA* were digested with NdeI and SalI and ligated to form plasmid pML83-P_{xyl}-*cckA*, which was then electroporated into a *pleC::Tn5* strain (41). ML1709 was constructed similarly, but pML83-P_{xyl}-*cckA* was modified by site-directed mutagenesis PCR with primers V366P_fw

and V366P_rev before being electroporated into the *pleC::Tn5* strain.

To construct a strain overexpressing full-length *cckA* (ML1688), we first made pENTR:P_{xyl}-*cckA* by using primers Pxyl_fw and CckA_full_rev to amplify a fragment containing a xylose-inducible promoter and *cckA* from the plasmid pML83:P_{xyl}-cckA . This pENTR clone was then recombined into the destination vector pJS14-DEST. To construct a strain overexpressing full-length *cckA(H322A)* (ML1738), we used primers H322A_fw and H322A_rev for site-directed mutagenesis on the plasmid pJS14:P_{xyl}-*cckA* from ML1688.

To construct strains overexpressing pieces of *cckA* containing no transmembrane domain (ML1689) or only the receiver domain (ML1692), we generated PCR products from CB15N genomic DNA using the following primers: HK7_fw and RR53_rev (ML1689) or RR53_fw and RR53_rev (ML1692). pENTR clones containing these PCR fragments were then recombined into pHXM2-DEST using the Gateway cloning method. To construct ML1690 and ML1691, we performed site-directed mutagenesis on pENTR:*cckA-HK-RD* with primers D623A_fw and D623A_rev (ML1690) or V366P_fw and V366P_rev (ML1691) before recombining into pHXM2 -DEST.

To construct strain ML1681, the last 519 codons (without the stop codon) of *cckA* were amplified by PCR with primers CckA_GFP_fw and CckA_GFP_rev. The reverse primer removed the stop codon, added two nucleotides to keep it in frame with the downstream GFP fusion, and contains an EcoRI site. The forward primer contains a KpnI site. The *cckA* PCR product was cloned in-frame with the *egfp* gene in pGFP-c4 (37) using KpnI and EcoRI

restriction sites. The coding region was sequence verified and the plasmid was recombined into CB15N by electroporation to generate chromosomally encoded CckA-GFP.

To create pJS14-DEST and pMR20-DEST for Gateway cloning, the RfA Gateway cassette was blunt cloned into an EcoRV site in pJS14 and pMR20. To create pHXM2-DEST, the SacI-KpnI fragment containing a xylose-inducible promoter and M2 tag (P_{xyl}-M2) was digested out of pHXM-DEST and then cloned into pJS14.

Differential interference contrast microscopy was performed on mid-exponential phase cells after fixing in PBS with 0.5% paraformaldehyde.

Immunoblotting and synchronization

Mixed populations of wild-type cells grown in M2G were synchronized using Percoll density centrifugation as previously described (24). Cell samples were taken every 20 minutes for 140 minutes, resolved on a 12% SDS polyacrylamide gel, transferred to PVDF transfer membrane (Pierce), and probed with anti-ChpT serum at a 1:10,000 dilution. Polyclonal rabbit antisera (Covance) was generated using His_6-ChpT.

Flow cytometry

Single colonies were inoculated into 5-10 mL liquid cultures from plates and grown overnight at 30^{o}C under appropriate antibiotic selection, but were always maintained at an OD_{600} less than 0.7. Cultures were then diluted to an OD_{600} of 0.005-0.01 and grown to OD_{600} ~ 0.2-0.4 before processing. All strains were grown in PYE except for strains overexpressing *ctrA* alleles (Fig.

6), which were grown in M2G. Strains overexpressing *cckA* or *ctrA* were induced by the addition of 0.3% xylose to culture media, or maintained in 0.2% glucose and processed after 4 or 8 hours. After 8 hours of induction, rifampicin (20 μg/ml) was added to strains overexpressing *ctrA*, which were grown for 3 more hours to allow for completion of DNA replication. Cells were fixed in 70% EtOH overnight at 4°C and stored at 4°C for up to a week. They were spun at 6000 rpm for 4 minutes, resuspended in 1 ml 50 mM sodium citrate, and incubated for 4 hours at 50°C with 2 ug/ml RNAse to allow complete RNA digestion. After digestion, cells were incubated in 2.5 μM SYTOX Green nucleic acid stain (Invitrogen) for 15 minutes at room temperature before analyzing by flow cytometry using an Epics C analyzer (Beckman-Coulter). For quantification of flow cytometry data in Figure 6, we gated 1N DNA content peaks, using the same gate for all samples. The percentages shown in the bar graph were obtained by dividing the gated number cells with 1N DNA content by the total number of cells, which were gated to exclude cellular debris on the far left of the flow cytometry profiles.

In vitro analysis of kinase, phosphatase, and phosphotransfer reactions

All protein purifications were done as reported previously (33). Primers CC3470_HPT_for and ChpT_Hbox_rev were used to amplify the H-box-containing N-terminus of ChpT for constructing the plasmid pENTR:*chpTΔC*. Primers phoR_fw and phoR_rev were used to amplify the last 415 amino acids of PhoR (CC0289) for constructing the plasmid pENTR:*phoR*. Primers EnvZ_T247R_fw and EnvZ_T247R_rev were used for site-directed mutagenesis on the plasmid pENTR:*envZ* to create the plasmid pENTR:*envZ(T247R)*. Creation of other pENTR

clones for protein purification has been described previously (33).

Phosphatase reactions: First, 10 μM TRX-His_6-CtrA was incubated with 0.2 μM PhoR with 5 μC_i [γ^{32}P]ATP (~6000 Ci/mmol, Amersham Biosciences) in storage buffer supplemented with 2 mM DTT and 5 mM $MgCl_2$. Reactions were incubated at 30°C for 60 minutes and then depleted of any remaining ATP by the addition of 1.5 U hexokinase (Roche) and 5 mM D-glucose for 5 minutes at room temperature. Reactions were then washed in 10 kD Nanosep columns four times with 10X reaction volumes of HKEDG buffer (10 mM HEPES-KOH pH 8.0, 50 mM KCL, 10% glycerol, 0.1 mM EDTA, 1 mM DTT (added fresh)) with a final resuspension in the original reaction volume of HKEDG buffer. A similar procedure was used to prepare CpdR~P except that EnvZ(T247R) was used instead of PhoR. These preparations of CtrA~P or CpdR~P were then incubated with upstream components, as indicated in figure panels, each at a final concentration of 5 μM. Phosphatase reactions were supplemented with 5 mM $MgCl_2$ and incubated at 30°C before being stopped at indicated timepoints by the addition of 3.5 μL 4X sample buffer (500 mM Tris [pH 6.8], 8% SDS, 40% glycerol, 400 mM beta-mercaptoethanol). Samples were heated at 30°C for 2 minutes before loading onto 10% Tris-HCl gels (Bio-Rad) with electrophoresis at room temperature for 40 minutes at 150 V. Gels were exposed to phosphor screens overnight at -80°C and then scanned using a Storm 86 imaging system (Amersham Biosciences).

Autophosphorylation reactions: Histidine kinase constructs at 5 μM were incubated with 0.5 μM ATP and 5 μC_i [γ^{32}P]ATP in HKEDG buffer supplemented with 5 mM $MgCl_2$ at 30°C for 60

minutes. Reactions were stopped at indicated timepoints by the addition of 4X sample buffer and analyzed as with phosphatase reactions, described above.

Phosphotransfer reactions: To autophosphorylation reactions, His_6-ChpT or TRX-His_6-ChpT was added to a final concentration of 12.5 μM and reactions incubated at 30°C before being stopped at indicated timepoints and processed as above.

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Figure Legends

Figure 1. Cell cycle abundance of ChpT. Wild-type cells were synchronized and allowed to proceed through a single cell cycle with lysates collected every 20 minutes and used for immunoblotting with anti-CtrA or anti-ChpT serum. The cell cycle diagram above indicates the approximate cell cycle stage at each time point.

Figure 2. Full-length CckA can drive the phosphorylation and dephosphorylation of CtrA and CpdR. (A) Phosphorelay reconstitutions using the purified components indicated by pluses and minuses. The components indicated were mixed together without ATP. Phosphotransfer reactions were started with the addition of ATP and incubated for 30 minutes at 30^{o}C. The position of each phosphorylated component is marked with an arrowhead on the right. **(B-C)** Dephosphorylation of CtrA and CpdR. Purified CtrA~P **(B)** or CpdR~P **(C)** was incubated with the components indicated, but without ATP, to test for backtransfer and dephosphorylation to yield inorganic phosphate (P_i). Phosphatase reactions were started with the addition of CckA and, if indicated, ChpT. Each reaction was allowed to proceed for 0, 10, 30, or 60 minutes before being stopped. Note that the faint bands appearing in the first lane of panels **(B)** and **(C)** correspond to the components used to phosphorylate CtrA and CpdR (see Methods). In each panel, CckA-HK-RD and CckA-HK contain His_6-MBP tags, CckA-RD, Trx-ChpT, CpdR, and CtrA contain thioredoxin-His_6 tags, and ChpT contains a His_6 tag. Different tags for ChpT were used to optimize band separation.

Figure 3. Identification of mutants that uncouple kinase and phosphatase activity in CckA.

(A) Summary of CckA mutations tested. For mutations reported previously to produce K^+P^- (green) or K^-P^+ (blue) EnvZ, the same amino acid substitutions were introduced at the corresponding sites of CckA, based on an alignment of EnvZ and CckA sequences. Mutations are listed below the domain of CckA in which they were constructed. Domains include transmembrane (TM), dimerization and histidine phosphotransfer (DHp), catalytic and ATPase (CA), and receiver domain (RD). Phosphorylation sites (H322 and D623) are indicated above their respective locations. **(B)** Each mutant version of CckA-HK-RD was tested for autophosphorylation (top) and phosphotransfer to ChpT (bottom panel). Autophosphorylation reactions were started with the addition of ATP to preincubated mixtures of the indicated mutant CckA and reaction buffer. Reactions were incubated for 30 minutes at 30°C. Phosphotransfer reactions were performed using autophosphorylated CckA. These reactions were started with the addition of ChpT and allowed to proceed for 30 minutes before being stopped. **(C)** Each mutant version of CckA-HK-RD was tested for dephosphorylation of CtrA~P. Purified CtrA~P was isolated and reactions were started with the addition of ChpT and mutant versions of CckA, and then allowed to proceed for 30 minutes at 30°C. The position of phosphorylated components in each panel are marked with arrowheads on the left.

Figure 4. Biochemical characterization of CckA(V366P). (A) Time-course of CtrA dephosphorylation by CckA-HK-RD constructs. Point mutations are indicated above each time course. **(B)** Quantification of CckA-HK-RD band intensities in time-courses from panel A. The intensities for each construct were normalized to the percent maximum. (C) Quantification of

inorganic phosphate band intensities in time-courses from panel A.

Figure 5. Complementation analysis of mutant alleles of *cckA*. Cellular morphology (left) and flow cytometry (right) analysis of strains in which the chromosomal copy of *cckA* was deleted and the *cckA* allele indicated on the far left was driven by the native *cckA* promoter on a low-copy plasmid. Scale bar, 4 μm.

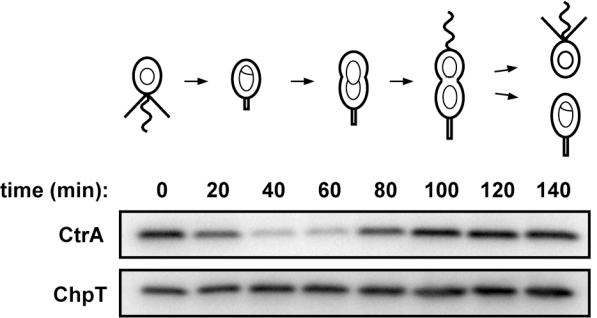
Figure 6. CckA contributes to, but is not essential for, the inactivation of CtrA and CpdR. Cells expressing various combinations of *cckA* and *ctrA* alleles were analyzed by flow cytometry to assess chromosomal content as a readout of CtrA activity. Each strain harbored a *cckA* chromosomal deletion and expressed either *cckA* (light grey) or *cckA(V366P)* (dark grey) from a low-copy plasmid using the native *cckA* promoter. Strains expressed the *ctrA* allele indicated above each panel from a medium-copy plasmid using the xylose-inducible promoter P_{xyl}. High levels of CtrA(D51E) partially mimics phosphorylated CtrA. CtrAΔ3Ω is a non-proteolyzable version of CtrA. All strains were grown in M2G to mid-exponential phase (OD_{600} ~ 0.2-0.4) in the presence of xylose for 8 hours, followed by the addition of rifampicin for 3 additional hours, and then analyzed by flow cytometry. **(A)** Representative flow cytometry profiles. **(B)** Quantification of the percentage of cells with one chromosome in the flow cytometry profiles. Error bars represent the standard error of the mean (n=3).

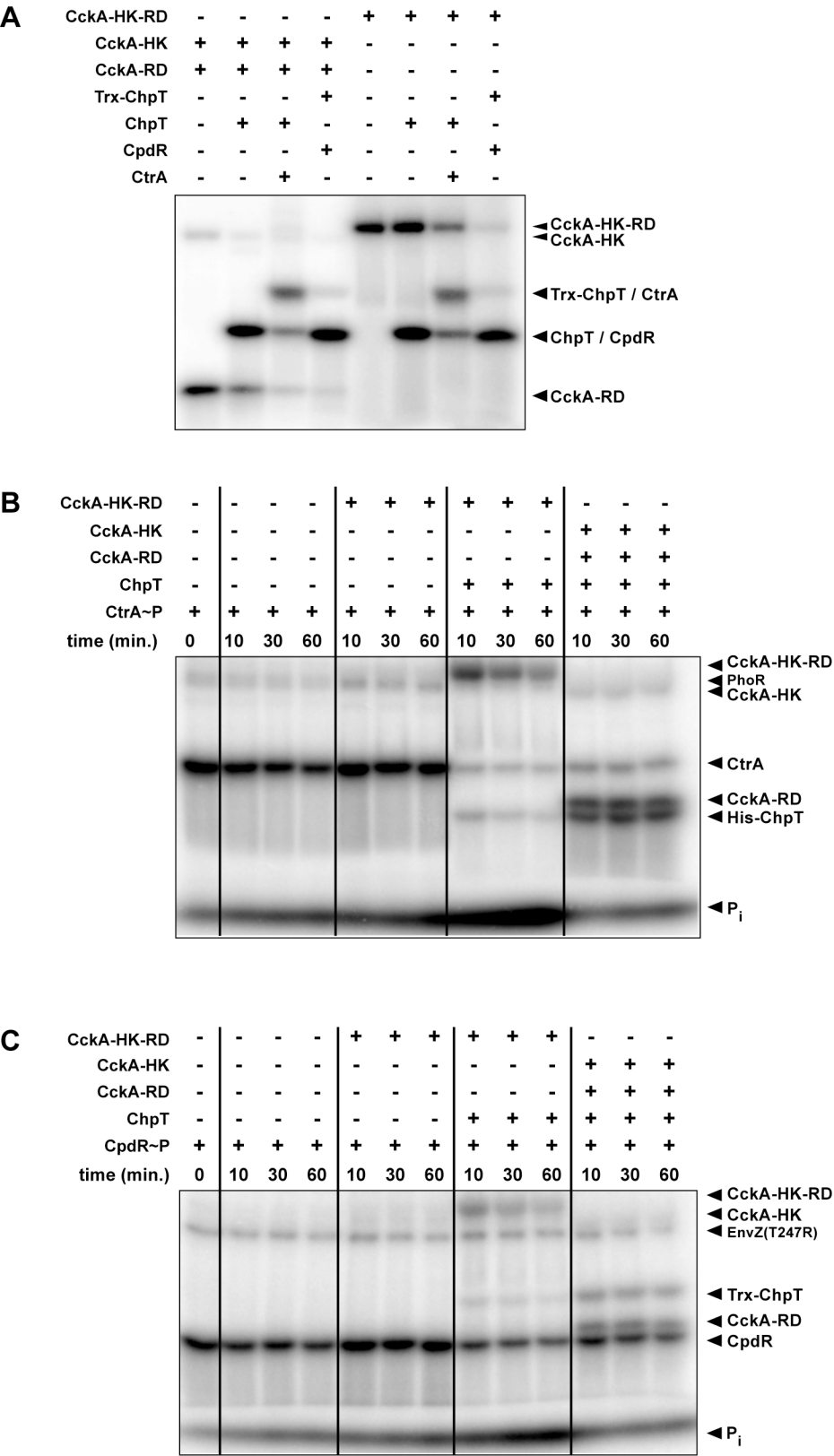
Figure 7. Overproducing CckA inactivates CtrA. (A) The effect of overproducing various CckA constructs was examined by light microscopy and flow cytometry. A diagram of the overexpression constructs used is shown at the top with abbreviations as in Fig. 3. Cells

harbored the construct indicated above each pair of micrograph and flow cytometry profile. Each construct was expressed from a xylose-inducible promoter on a medium-copy plasmid. Cultures were grown to mid-exponential phase (OD_{600} ~ 0.2-0.4) in the presence of xylose for four hours and then fixed for microscopy and flow cytometry analysis. **(B)** Genetic interactions between *pleC* and *cckA*. Cells harbored a transposon insertion in *pleC* and carried a full-length copy of *cckA* or *cckA(V366P)* under the control of a xylose-inducible promoter. Note that in the flow cytometry profiles, the far right edge of the profile includes an integration of all cells that have chromosome accumulation beyond the range shown, if any. Scale bar, 4 μm.

Figure 8. Regulation of the balance between CckA kinase and phosphatase activities controls cell cycle. **(A)** Summary of regulatory pathway controlling CtrA activity (left). Schematic of *Caulobacter* cell cycle indicating temporal pattern of CtrA activity (right). **(B)** Summary of cell cycle phosphorelays. Net phosphate flow depends on the activity of CckA. As a kinase, CckA drives the phosphorylation of CtrA and CpdR. As a phosphatase, CckA drives the dephosphorylation of CtrA and CpdR. Cell cycle transitions and changes in CtrA activity are thus driven by changes in the kinase/phosphatase balance of CckA. DivK influences CckA's switching between kinase and phosphatase states.

Figure 1




A
CckA-HK-RD - - - - + + + +
CckA-HK + + + + - - - -
CckA-RD + + + + - - - -
Trx-ChpT - - - + - - - +
ChpT - + + - - + + -
CpdR - - - + - - - +
CtrA - - + - - - + -
CckA-HK-RD
CckA-HK
Trx-ChpT / CtrA
ChpT / CpdR
CckA-RD
B
CckA-HK-RD - - - - + + + + + + - - -
CckA-HK - - - - - - - - - - + + +
CckA-RD - - - - - - - - - - + + +
ChpT - - - - - - - + + + + + +
CtrA~P + + + + + + + + + + + + +
time (min.) 0 10 30 60 10 30 60 10 30 60 10 30 60
CckA-HK-RD
PhoR
CckA-HK
CtrA
CckA-RD
His-ChpT
P i
C
CckA-HK-RD - - - - + + + + + + - - -
CckA-HK - - - - - - - - - - + + +
CckA-RD - - - - - - - - - - + + +
ChpT - - - - - - - + + + + + +
CpdR~P + + + + + + + + + + + + +
time (min.) 0 10 30 60 10 30 60 10 30 60 10 30 60
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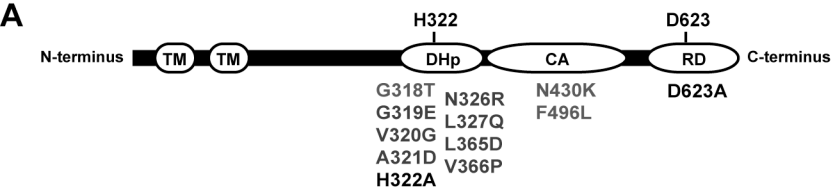
CckA-HK
EnvZ(T247R)
Trx-ChpT
CckA-RD
CpdR
P i


A
H322
D623
N-terminus
TM
TM
DHp
CA
RD
C-terminus
G318T
G319E
V320G
A321D
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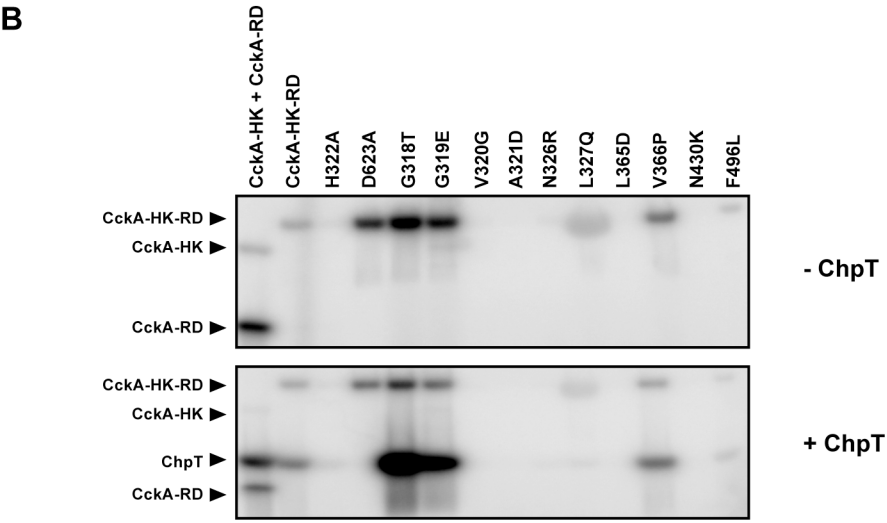
L365D
V366P
N430K
F496L
D623A


B
CckA-HK + CckA-RD
CckA-HK-RD
H322A
D623A
G318T
G319E
V320G
A321D
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L327Q
L365D
V366P
N430K
F496L
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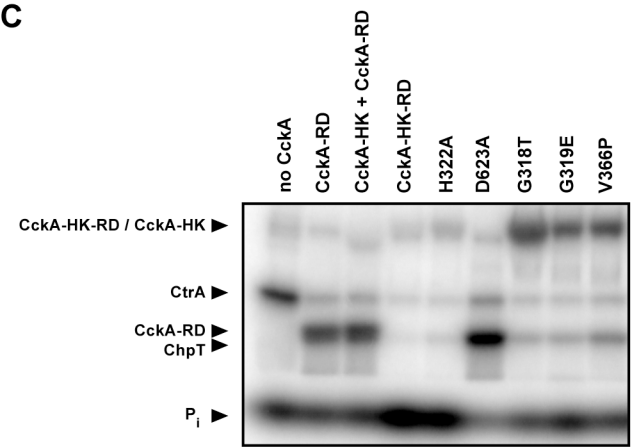
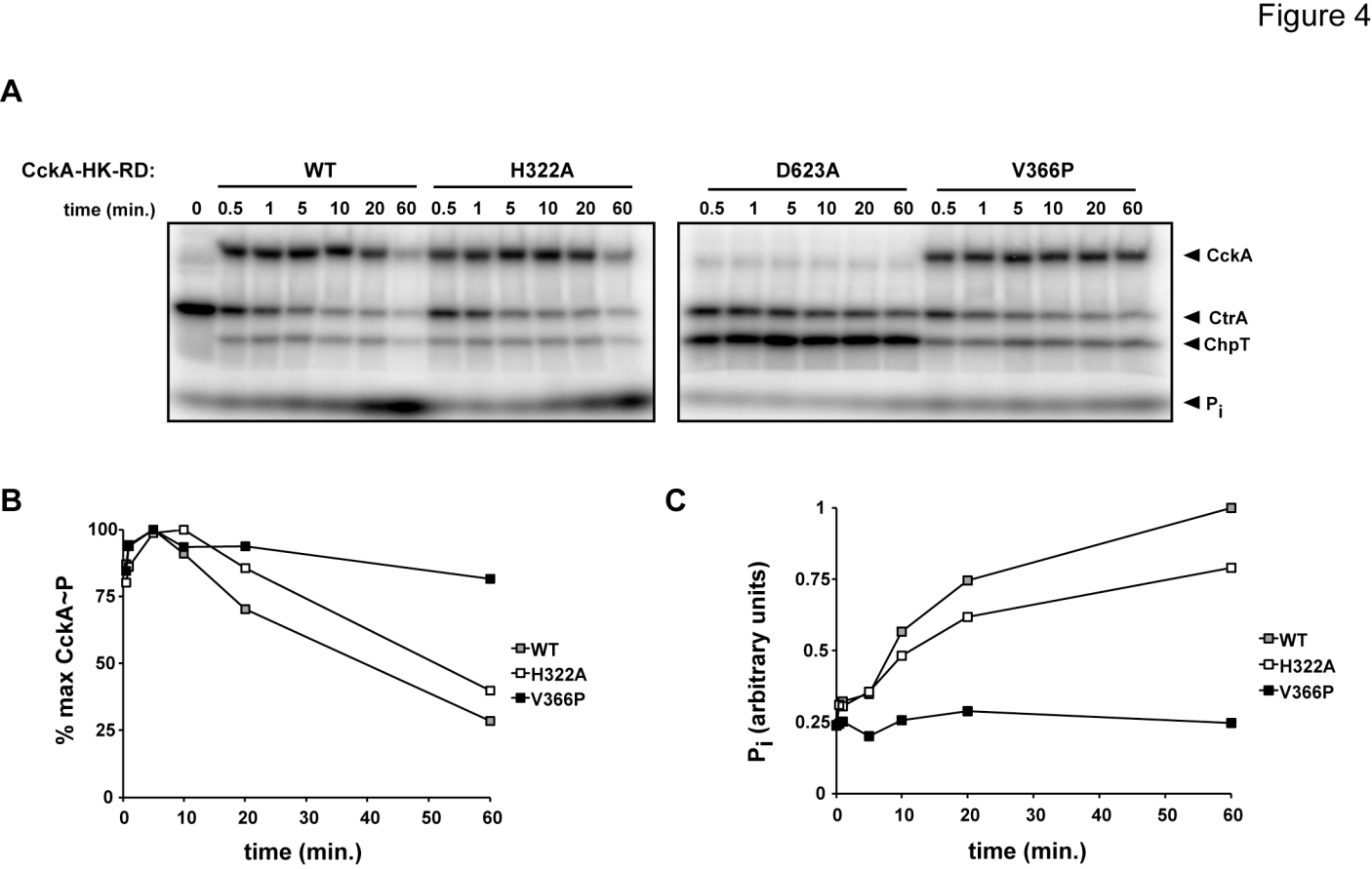
CckA-HK
ChpT
CckA-RD
+ ChpT


C
no CckA
CckA-RD
CckA-HK + CckA-RD
CckA-HK-RD
H322A
D623A
G318T
G319E
V366P
CckA-HK-RD / CckA-HK
CtrA
CckA-RD
ChpT
Pi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CckA-HK-RD:
WT
H322A
D623A
V366P
time (min.)
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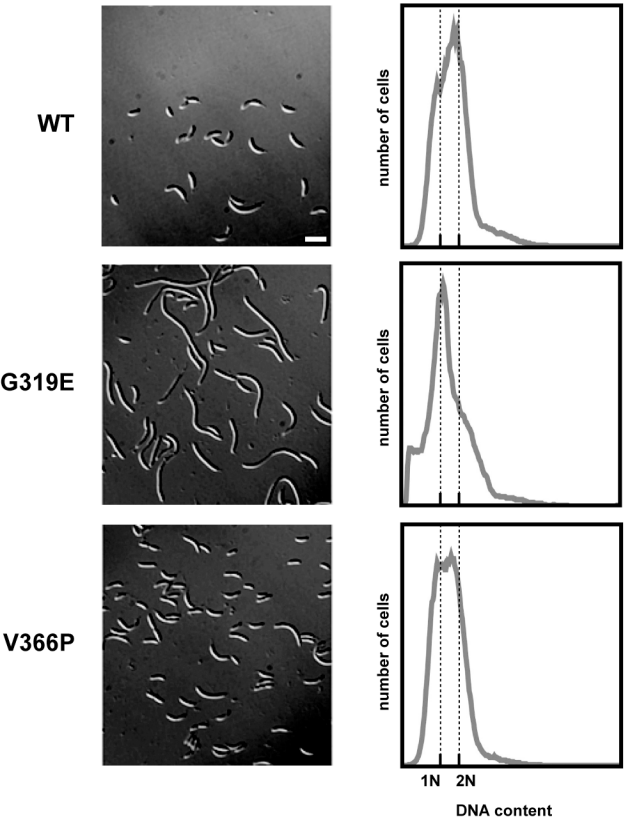
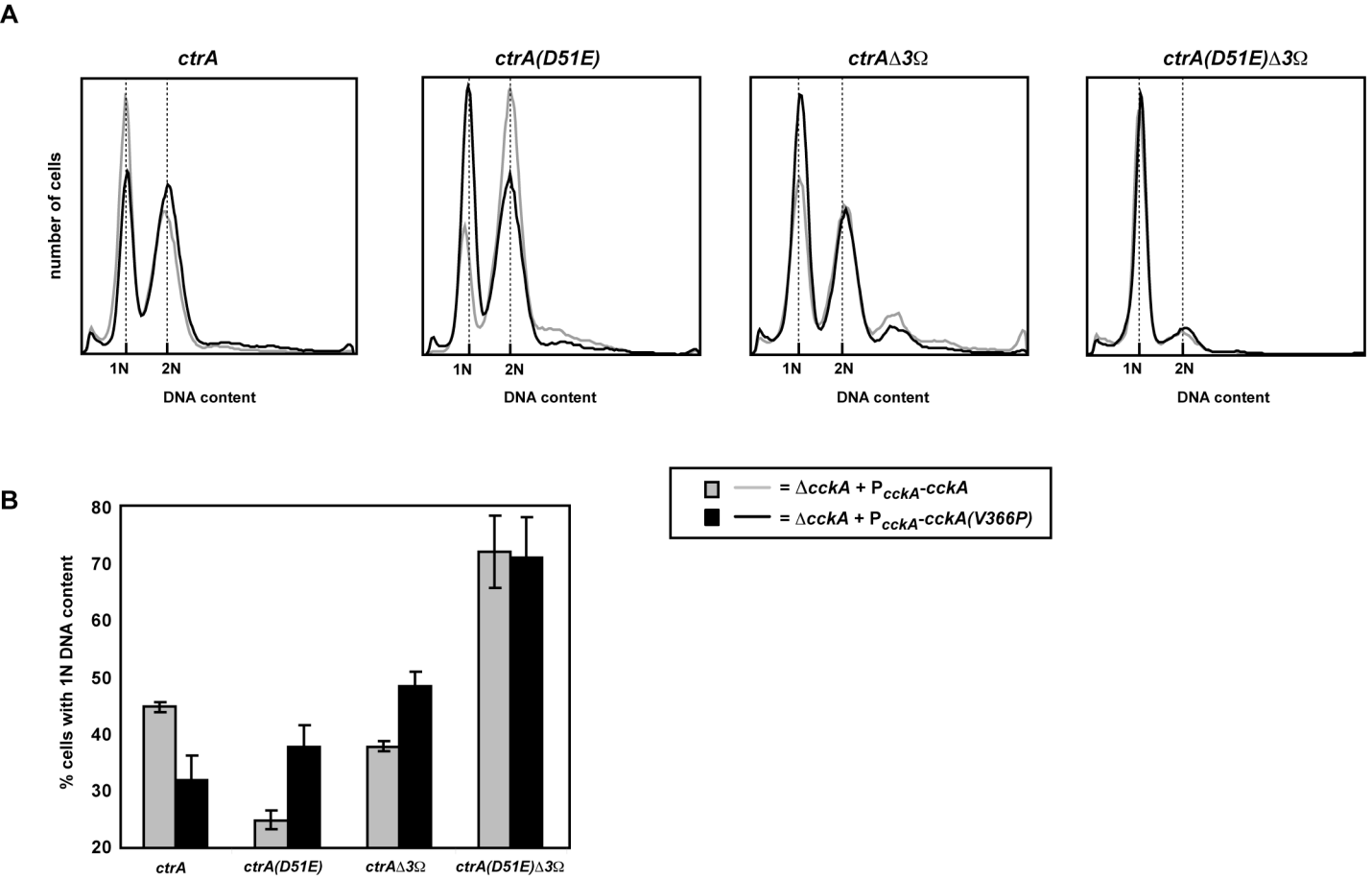
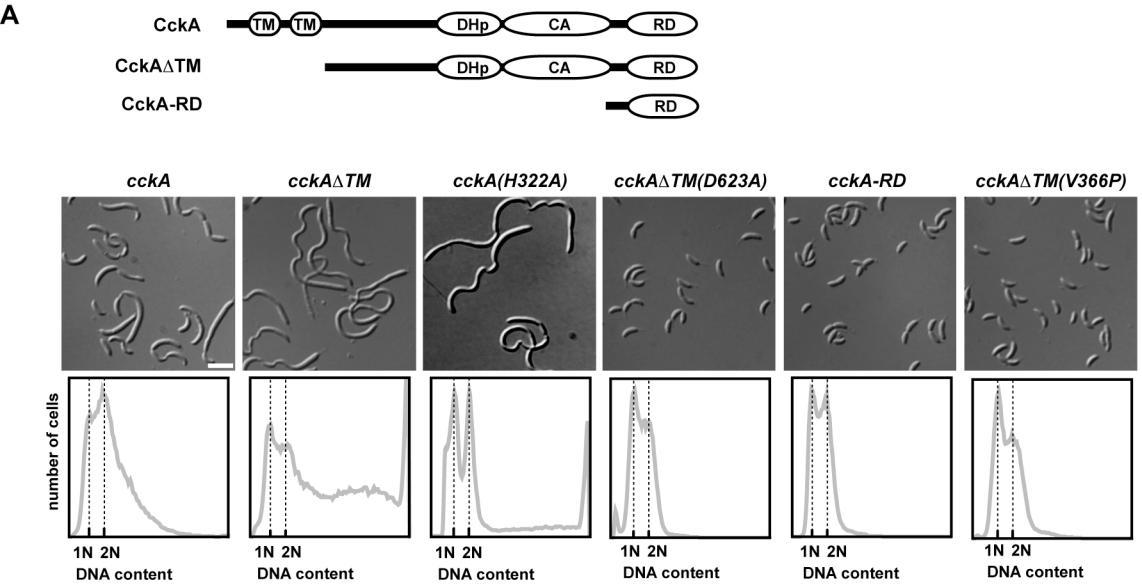
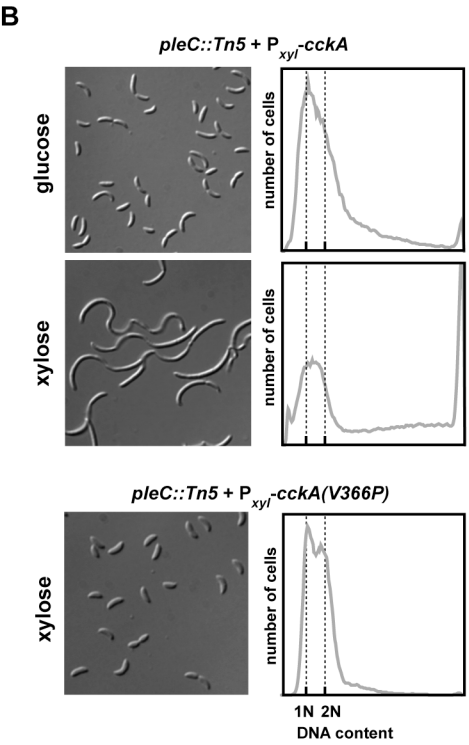
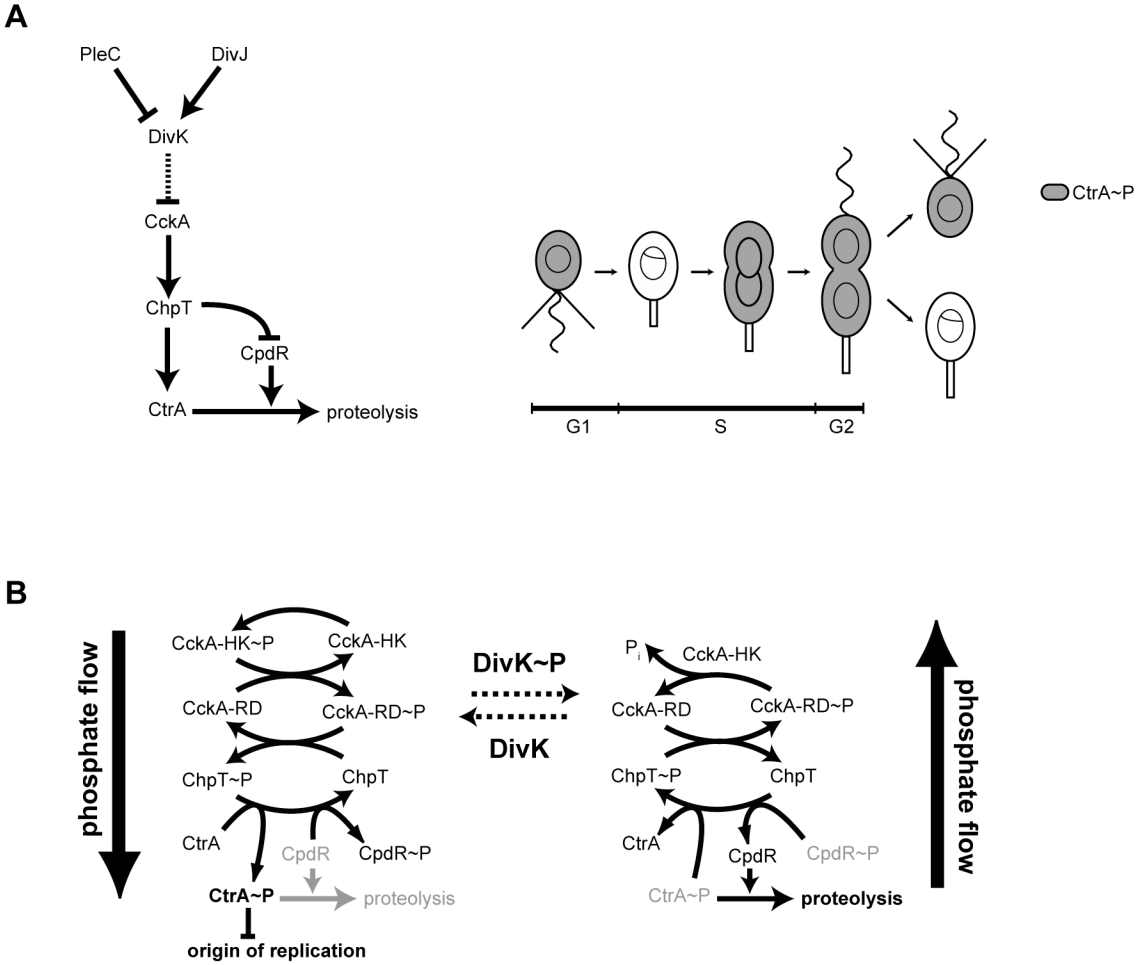
0.5 1 5 10 20 60 0.5 1 5 10 20 60
CckA
CtrA
ChpT
Pi
B
% max CckA~P
100
75
50
25
0
0 10 20 30 40 50 60
time (min.)
WT
H322A
V366P
C
Pi (arbitrary units)
1
0.75
0.5
0.25
0
0 10 20 30 40 50 60
time (min.)
WT
H322A
V366P


WT
G319E
V366P
number of cells
number of cells
number of cells
1N
2N
DNA content


A
ctrA
ctrA(D51E)
ctrAΔ3Ω
ctrA(D51E)Δ3Ω
number of cells
1N
2N
DNA content
B
= ΔcckA + P cckA -cckA
= ΔcckA + P cckA -cckA(V366P)
% cells with 1N DNA content
80
70
60
50
40
30
20
ctrA
ctrA(D51E)
ctrAΔ3Ω
ctrA(D51E)Δ3Ω


A
CckA
CckAΔTM
CckA-RD
TM
TM
DHp
CA
RD
cckA
cckAΔTM
cckA(H322A)
cckAΔTM(D623A)
cckA-RD
cckAΔTM(V366P)
number of cells
1N 2N
DNA content


B
pleC::Tn5 + P xyl -cckA
glucose
xylose
number of cells
pleC::Tn5 + P xyl -cckA(V366P)
xylose
number of cells
1N 2N
DNA content


A
PleC
DivJ
DivK
CckA
ChpT
CpdR
CtrA
proteolysis
CtrA~P
G1
S
G2
B
phosphate flow
CckA-HK~P
CckA-HK
CckA-RD
CckA-RD~P
ChpT~P
ChpT
CtrA
CpdR
CpdR~P
CtrA~P
proteolysis
origin of replication
DivK~P
DivK
P i
CckA-HK
CckA-RD
CckA-RD~P
ChpT~P
ChpT
CtrA
CpdR
CpdR~P
CtrA~P
proteolysis
phosphate flow