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Functions of the CckA histidine kinase in *Caulobacter* cell cycle control

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Summary

The CtrA master transcriptional regulator is a central control element in *Caulobacter* cell cycle progression and polar morphogenesis. Because of its critical role, CtrA activity is temporally regulated by multiple mechanisms including phosphorylation and ClpXP-dependent degradation of CtrA. The CckA histidine kinase is known to contribute to CtrA phosphorylation. We show here that genes differentially expressed in a *ctrA* temperature-sensitive (ts) mutant are similarly affected in a *cckA* ts mutant, that the phosphorylation of CckA coincides temporally with CtrA phosphorylation during the cell cycle, and that CckA is essential for viability because it is required for CtrA phosphorylation. Thus, it is the signal transduction pathway mediated by CckA that culminates in CtrA activation, which is temporally regulated and essential for cell cycle progression. CckA also positively regulates CtrA activity by a mechanism that is independent of CtrA phosphorylation. CtrA is more stable in the presence of CckA than it is absence, suggesting that CckA may also be involved, directly or indirectly, in the regulation of CtrA proteolysis.

Introduction

Two-component signal transduction proteins play a critical role in the control of cell cycle progression and polar morphogenesis in the bacterium *Caulobacter crescentus*. A small number of these regulatory proteins control the expression of a large portion of cell cycle-regulated genes. The CtrA master transcriptional regulator controls multiple cell cycle events and couples polar morphogenesis with cell cycle progression (Quon *et al*., 1996; Kelly *et al*., 1998; Quon *et al*., 1998; Laub *et al*., 2000; Skerker and Shapiro, 2000; Laub *et al*., 2002). In addition, CtrA controls the initiation of chromosome replication by directly binding to the origin of replication, preventing replisome formation in G1 cells (Quon *et al*., 1998).

Tight control of CtrA activity is critical to the coordinated progression of cell cycle events and to cellular morphogenesis (Fig. 1). In the swarmer cell, the chromosome replication origin is silenced by the binding of the phosphorylated form of CtrA (CtrA~P), but as the swarmer cell differentiates into a stalked cell, CtrA~P is degraded by the ClpXP protease complex (Jenal and Fuchs, 1998), allowing initiation of chromosome replication (Domian *et al*., 1997; Quon *et al*., 1998). After the initiation of replication in the stalked cell, CtrA is synthesized *de novo* and is immediately phosphorylated, presumably to prevent further rounds of replication initiation (Quon *et al*., 1996; Domian *et al*., 1997). In the predivisional cell, CtrA~P activates the cascade of flagellar gene transcription and the expression of the *ccrM* gene encoding an essential DNA methyltransferase (Quon *et al*., 1996). The CtrA protein is preferentially cleared from the stalked compartment of the late predivisional cell, allowing the initiation of replication in the progeny stalked cell, while maintaining CtrA in the progeny swarmer cell where it functions to prevent the initiation of DNA replication (Domian *et al*., 1997).

Whereas much is known about when CtrA is present and active during the cell cycle, very little is understood about how its regulation is accomplished. As expected for the response regulator family of proteins, the activity of CtrA is controlled by phosphorylation on the conserved aspartate residue (Asp51) (Quon *et al*., 1996). Phosphorylation of CtrA is essential for cell viability and greatly enhances its DNA binding and the consequent transcriptional activity of its target genes *in vitro* (Quon *et al*., 1998; Wu *et al*., 1998; Domian *et al*., 1999). Three signal transduction pathways have been proposed to control CtrA phosphorylation levels (Wu *et al*., 1998; Jacobs *et al*., 1999; Wu *et al*., 1999; Osteras and Jenal, 2000). One of the putative pathways involves the histidine kinase, CckA

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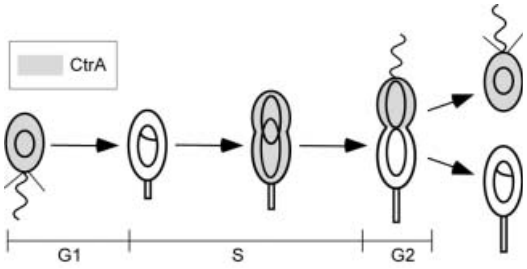

Fig. 1. CtrA activity during the *Caulobacter crescentus* cell cycle. The *C. crescentus* cell cycle is shown schematically; oval and theta structures represent quiescent and replicating chromosomes, respectively. The presence of the CtrA protein in specific cell types is shown in grey.

(Jacobs *et al*., 1999). We showed previously that a *cckA* temperature-sensitive (ts) mutant (*cckATS1*) is essential for viability and exhibits a pleiotropic phenotype, including defects in flagella and pili biogenesis, and initiation of DNA replication and cell division, that are characteristic of a *ctrA* loss-of-function mutant (Jacobs *et al*., 1999). CtrA phosphorylation was shown to be dramatically reduced in *cckATS1* cells. The *cckA* gene encodes a hybrid histidine kinase that contains a receiver domain at its C-terminus with the conserved aspartate phosphorylation site usually found in response regulators. This hybrid structure is not uncommon (Grebe and Stock, 1999), and suggests that CckA may be part of a multicomponent phosphorelay.

We report here that CckA is essential for CtrA phosphorylation during the cell cycle and that, in the absence of CckA, CtrA is inactive. Phosphorylation of both CckA and CtrA is temporally controlled and coincident, reflecting the pivotal role of CckA-mediated signal transduction in *Caulobacter* cell cycle progression. CckA has an additional function that is independent of CtrA phosphorylation. We present data suggesting that this additional function may be the protection of CtrA from proteolysis.

Results

The CckA histidine kinase and the CtrA response regulator control the same genetic pathways

The essential CckA histidine kinase has been proposed to play a role in the phosphorylation of CtrA. The most compelling evidence is that CtrA phosphorylation is dramatically reduced in the *cckATS1* mutant (Jacobs *et al*., 1999). If CckA function mainly resides in the regulation of CtrA activity, the expectation is that CckA controls CtrA-dependent genes or a subset of them. Therefore, we used whole-genome microarrays and two-dimensional gel electrophoresis to compare the respective RNA and protein levels of ts strains mutant for CtrA or CckA function. Three different strains were used: wild-type CB15N, *ctrAV148F* and *cckATS1*. The *ctrA* and *cckA* ts mutant strains were isolated in the same genetic screen (Jacobs *et al*., 1999). All three strains were grown in rich media at 28°C (permissive temperature for viability) to early log phase and then shifted to 37°C (restrictive temperature) for 4 h. The cell morphology and DNA content of each strain after 4 h at 37°C are shown in Fig. 2A and B. Strains bearing ts alleles of *ctrA* and *cckA* exhibit similar phenotypes after a 4 h shift to 37°C (Jacobs *et al*., 1999). They show excessive filamentation, accumulation of multiple chromosomes per cell, and an eventual loss in viability with follows similar kinetics (data not shown). RNA from each of the ts strain cultures, grown at 28°C in rich media and then shifted to 37°C for 0, 2 and 4 h, was obtained and compared to RNA from the wild-type strain, grown under the same conditions, using whole-genome microarrays. The entire experiment was repeated twice and results were averaged (see *Supplementary material*, 'Microarray ctrA cckA ts series'). We then selected the cell cycle-regulated genes (Laub *et al*., 2000) whose expression level changed greater than twofold in either of mutant strains. The expression profiles for 212 genes at the 4 h time-point is shown in Fig. 2C. They are nearly identical for *ctrAV148F* and *cckATS1*, with a correlation coefficient of 0.91 for the genes shown. This level of correlation is on par with that seen when comparing two RNA samples from the same strain, and so the differences between the *ctrAV148F* and *cckATS1* profiles is probably due to experimental noise. As an additional test of profile similarity, we averaged data from the first replicate of *ctrAV148F* and the second replicate of *cckATS1* and compared that to an average of the second replicate of *ctrAV148F* and the first replicate of *cckATS1*. The correlation coefficient for such a comparison is 0.8, indicating that the profiles amongst all replicates, and hence, between each strain, are very similar. These results support the notion that CckA and CtrA are in the same signalling pathway. Finally, for the genes that were previously identified as direct targets of CtrA (Laub *et al*., 2002), we note here that all of these genes were found to be significantly changed in the *ctrAV148F* strain and ~95% were also found to be strongly affected in the *cckATS1* strain (data not shown).

To determine whether loss of CckA function also causes CtrA-dependent effects at the translational or post-translational levels, the global protein profiles of *ctrAV148F* and *cckATS1* cell cultures after 4 h at 37°C were examined in comparison to wild-type CB15N cells grown under the same conditions (see *Supplementary material*, Fig. S1). Total cell extracts were separated using two-dimensional gel electrophoresis and the resulting images were compared to detect proteins with different levels. In total, 30 differentially abundant proteins, defined by a minimum twofold change in spot abundance and replicated across three independent gel series, were detected (see *Supple-*

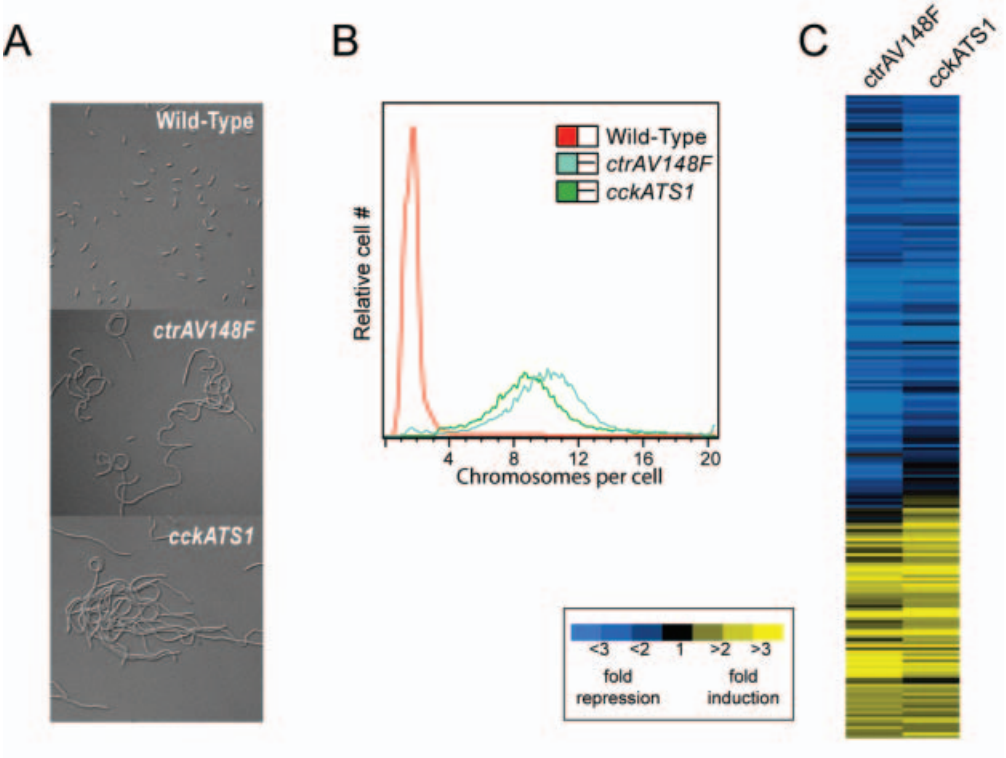

Fig. 2. The CckA histidine kinase and the CtrA response regulator control the same genetic pathways
A. DIC images of CB15N, *ctrAV148F* and *cckATS1* logarithmic cultures grown at 37°C for 4 h.
B. The DNA content profiles of samples from cultures used in (A) were compared by flow cytometry of chromomycin A3-stained cells. Chromosome equivalents are indicated on the *x*-axis and relative number of cells on the *y*-axis.
C. Expression profiles for *ctrAV148F*, and *cckATS1* 4 h after a shift to restrictive temperature (37°C). Expression data is shown for 212 cell cycle-regulated genes (Laub *et al*., 2000) whose expression level changes greater than twofold in either of the mutant strains. Changes in mRNA levels relative to a common reference derived from wild-type cells are represented using the colour scale shown at the bottom.

mentary material, Fig. S1 for gels, and Table S1 for protein spot identity). Out of this set, only two spots (identified as CC2258, heat-shock protein Hsp20 family and CC3494, OmpA family protein) appeared to be down- or upregulated in one of the mutants but not in the other (see *Supplementary material*, Table S1). Thus, the protein level profiles, like the gene transcript profiles, are very similar between *ctrA* and *cckA* mutants supporting the argument that CckA function is mediated through CtrA.

CckA phosphorylation during the cell cycle correlates with that of CtrA

CtrA is phosphorylated in swarmer and predivisional cells, but not in stalked cells (Domian *et al*., 1997). This was demonstrated using a proteolysis-resistant form of CtrA. As the phosphorylated form of CtrA (CtrA~P) has a short half-life (under 5 min) relative to the length of the cell cycle (Domian *et al*., 1997), an active signal transduction pathway must continuously phosphorylate CtrA in swarmer and predivisional cells, but not in stalked cells. If CckA contributes to the regulation of CtrA phosphorylation throughout the cell cycle, one would expect that the times of its activity would correlate with the times when CtrA phosphate is present. Alternatively, CckA may be involved in CtrA phosphorylation only at specific times during the cell cycle, but not at others. Thus, to determine when CckA acts upon CtrA phosphorylation during the cell cycle, we sought to determine when CckA is active. Histidine kinases form dimers that act as autokinases with each subunit catalysing the transfer of a phosphoryl group from ATP to the conserved histidine of the other subunit upon signal activation (Grebe and Stock, 1999; Hoch, 2000). The phosphoryl group is subsequently transferred onto the conserved aspartate phosphorylation site of the cognate response regulator. Thus, the phosphorylation state of histidine kinases is usually considered a valid measure of their activity.

To determine when the CckA histidine kinase is phosphorylated during the cell cycle and how its phosphorylation pattern correlates with that of CtrA phosphorylation, we performed *in vivo* phosphorylation experiments on synchronized populations of wild-type CB15N cells. In this assay, both CtrA and CckA were immunoprecipitated after radiolabelling with ^{32}Pi to assess their phosphorylation

levels at specific times during the cell cycle (Fig. 3A). CckA has two predicted phosphorylation sites (His322 in the transmitter domain and the Asp623 in the C-terminal receiver domain) that cannot be distinguished by this technique. We performed the *in vivo* phosphorylation assay three times and, in each case, the phosphorylation level of the CckA histidine kinase was found to be highest in predivisional cells (around 110 min) and lowest in stalked cells (around 50 min) (Fig. 3B), following a temporal pattern parallel to that of CtrA phosphorylation (Fig. 3C). The coincidence of CckA and CtrA phosphorylation levels during the cell cycle suggests that the CckA histidine kinase controls CtrA phosphorylation throughout the cell cycle.

Besides its essential role in CtrA phosphorylation, CckA has another function that is important for normal cell cycle progression

Mutation of the phosphorylation site of CtrA from an aspartate residue to a glutamate residue (CtrAD51E) has been shown to result in constitutive, although partial, phosphorylation-independent activity (Domian *et al*., 1997). This *ctrAD51E* mutant allele, when expressed in high copy number (pCtrAD51E), supports viability in a Δ*ctrA* strain in the absence of phosphorylation (Domian *et al*., 1997). We reasoned that if the CckA histidine kinase was essential for viability because of its requirement for CtrA phosphorylation, the *cckA* gene may become dispensable in the presence of the functional phosphorylation-independent CtrAD51E. Using a transducing phage lysate carrying *C. crescentus* chromosomal sequences in which most of the *cckA* sequence had been replaced by the gentamycin resistance gene cassette (Δ*cckA*::Gent^R), we confirmed our previous observation (Jacobs *et al*., 1999) that chromosomal disruptions of *cckA* do not occur in wild-type CB15N unless a second functional copy of *cckA* is present (Table 1). We used the same lysate to transduce wild-type CB15N or Δ*ctrA* strains expressing either wild-type *ctrA* or *ctrAD51E* from a high copy number plasmid (pCtrA and pCtrAD51E respectively). Consistent with our hypothesis, Gent^R transductants were only recovered in strains overexpressing the phosphorylation-independent CtrAD51E mutant creating CJ395 (Δ*cckA*/pCtrAD51E) and CJ396 (Δ*ctrA*Δ*cckA*/pCtrAD51E) but none were recovered in strains expressing wild-type *ctrA* from the same high copy number plasmid (Table 1). Immunoblot blot analysis confirmed the chromosomal disruption of *cckA* in CJ396 and CJ395 as no CckA protein was detected (Fig. 4A, lanes 4 and 5). Strains like wild-type CB15N, CB15N/pCtrAD51E, and LS2716 (Δ*ctrA*/pCtrAD51E), which all carry *cckA* on the chromosome, were used as controls (Fig. 4A).

In vivo phosphorylation experiments with the same strains showed that CtrAD51E was not phosphorylated in either Δ*ctrA* or Δ*cckA* strains (Fig. 4B, lanes 3 and 4). This is consistent with previous *in vivo* and *in vitro* studies showing that CtrAD51E mutant protein cannot be phosphorylated (Domian *et al*., 1997; Reisenauer *et al*., 1999).

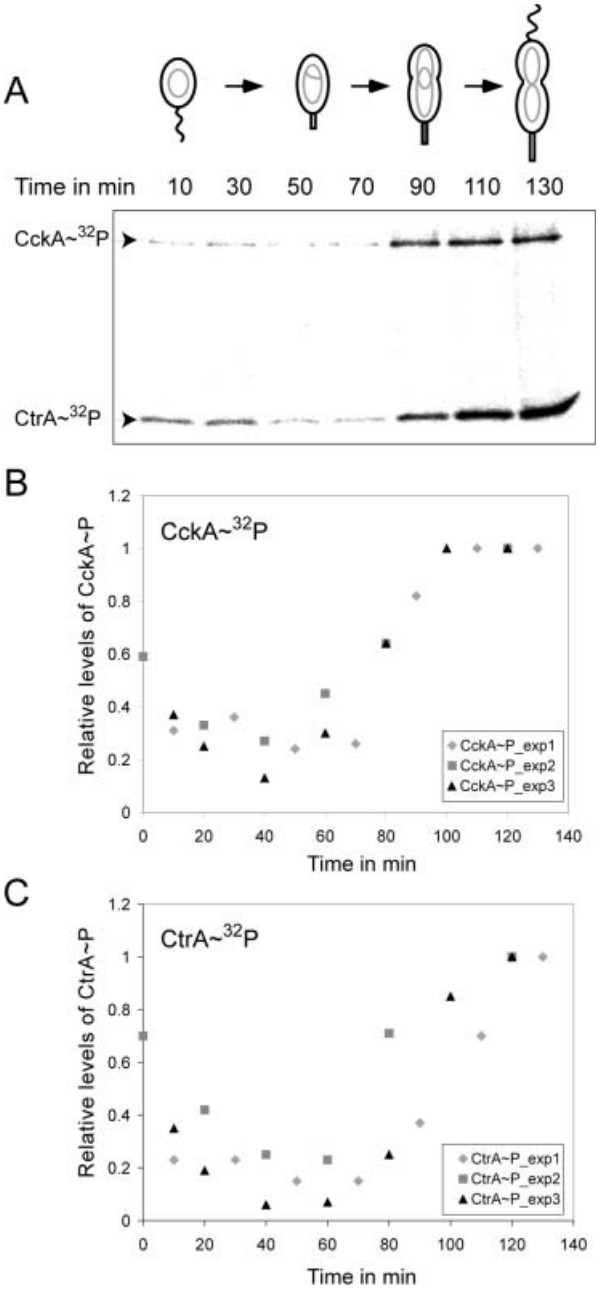

Fig. 3. CckA phosphorylation follows the pattern of CtrA phosphorylation during the cell cycle.
A. Cells from a synchronous populations of wild-type CB15N were labelled with [^{32}P]-H_3PO_4 for 10 min every 20 min. CtrA~^{32}P and CckA~^{32}P were immunoprecipitated together with anti-CtrA and anti-CckA antibodies. Relative CckA~^{32}P (B) and CtrA~^{32}P (C) levels of three independent *in vivo* phosphorylation experiments in synchronized CB15N were plotted over time. Both CckA~^{32}P and CtrA~^{32}P levels were the highest in predivisional cells (≈110 min) and the lowest in stalked cells (≈50 min).

Table 1. CtrAD51E supports viability in a *cckA* null background.

Strains	Gene expressed from a plasmid	*cckA*::Gent colonies	Phenotype of *cckA*::Gent^R cells
CB15N (wild type)	–	0	–
CB15N/pMR10cckA	*cckA*	≈480	wild-type
CB15N/pCtrA	*ctrA* (wild type)	0	–
CB15N/pCtrAD51E	*ctrAD51E*	≈50	Filamentous; extra chromosomes; poor viability at high cell density
Δ*ctrA*/pCtrA	*ctrA* (wild type)	0	–
Δ*ctrA*/pCtrAD51E	*ctrAD51E*	≈50	Filamentous; extra chromosomes; poor viability at high cell density

The phosphorylation level of wild-type CtrA was reduced in CB15N/pCtrAD51E relative to wild-type CB15N (Fig. 4B, lane 2 and 1 respectively). This effect is probably due to the known autoregulation of *ctrA* expression

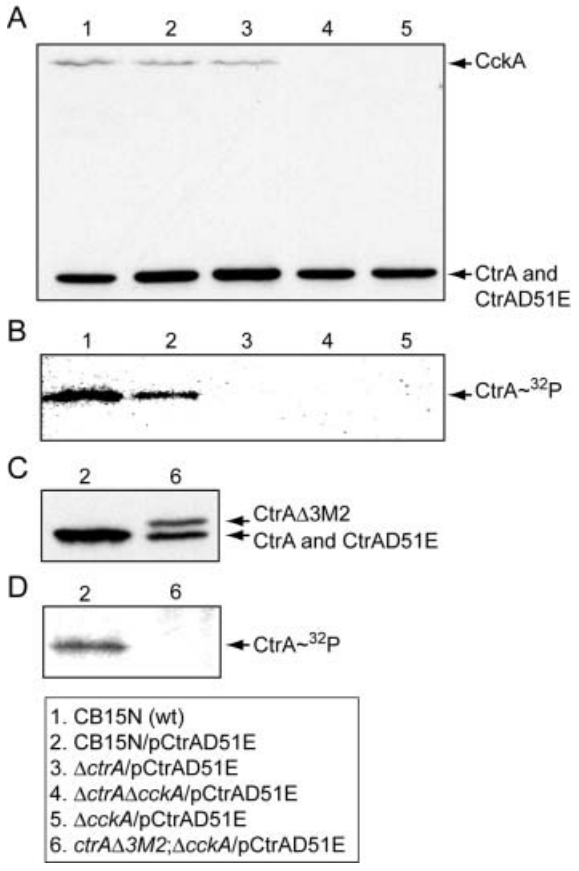

Fig. 4. Analysis of the levels of CtrA and CtrAD51E proteins and of their phosphorylation status in the presence or absence of CckA.
A and C. The abundance of CtrA, CtrAD51E, CtrAΔ3M2, and CckA proteins in wild-type CB15N (lane 1), CB15N/pCtrAD51E (lane 2), in LS2716 (Δ*ctrA*/pCtrAD51E, lane 3), CJ396 (Δ*ctrA*Δ*cckA*/pCtrAD51E, lane 4), CJ395 (Δ*cckA*/pCtrAD51E, lane 5) and *ctrAΔ3M2*;Δ*cckA*/pCtrAD51E (lane 6) was analysed by immunoblot using anti-CtrA and anti-CckA antibodies.
B and D. CtrA phosphorylation levels in cells from cultures used in (A) and (C), respectively, were compared in *in vivo* phosphorylation assays. Cells from cultures with the same optical densities at 660 nm were labelled with ^{32}P-ATP for 3 min, lysed, and immunoprecipitated with anti-CtrA antibodies. CtrA~^{32}P were resolved on 12% SDS polyacrylamide gel and visualized on a Molecular Dynamics Phosphoimager.

(Domian *et al.*, 1999). In CB15N/pCtrAD51E, the multicopy number of pCtrAD51E results in overexpression of *ctrAD51E*, which probably inhibits to some extent the synthesis of wild-type CtrA from the chromosome, and therefore the level of CtrA~^{32}P. This presumably also occurs for the CJ395 strain (Δ*cckA*/pCtrAD51E). It was, however, interesting that no CtrA~^{32}P could be detected in the latter strain even though it still carries an intact copy of wild-type *ctrA* on the chromosome (Fig. 4B, lane 5 to be compared with lane 2). To ascertain that CtrA protein was indeed present in this background, the chromosomal *ctrA* gene was replaced by a tagged copy of *ctrA*, *ctrAΔ3M2* whose product is known to be under a normal phosphorylation control when CckA is present but, unlike wild-type CtrA, can be distinguish electrophoretically from CtrAD51E because of its size difference (Domian *et al.*, 1999). In this Δ*cckA* strain (*ctrAΔ3M2*;Δ*cckA*/pCtrAD51E), CtrAΔ3M2 protein was clearly present as shown by immunoblot (Fig. 4C; lane 6), yet no phosphorylation could be detected in an *in vivo* phosphorylation assay (Fig. 4D, lane 6). This observation is of interest since other histidine kinases besides CckA have been proposed to contribute to CtrA phosphorylation (Wu *et al.*, 1998; 1999). One might have expected that in the absence of CckA, the activity of these other histidine kinases would have contributed to some residual phosphorylation of CtrA. It is possible that the phosphorylation of CtrA by CckA-independent pathways resides below the detection level of our technique. Alternatively, the other proposed CtrA phosphorylation pathways may be dependent on CckA function. In any case, the fact that the phosphorylation-independent CtrAD51E mutant protein can support viability in the absence of CckA when the wild-type CtrA cannot (Table 1) provides strong genetic evidence that the CckA histidine kinase is essential for viability because it is required for CtrA phosphorylation.

Interestingly, the Δ*cckA* strains (CJ395 and CJ396) exhibited parallel phenotypes, including severe filamentation, accumulation of chromosomes (Fig. 5A) and lysis at high cell density. These phenotypes were absent in LS2716 cells (Δ*ctrA*/pCtrAD51E). As CtrAD51E does not require phosphorylation for its activity (Domian *et al.*, 1997), we reasoned that if the sole function of CckA was to regulate CtrA phosphorylation, LS2716 and CJ396 (Δ*ctrA*/pCtrAD51E and Δ*ctrA*Δ*cckA*/pCtrAD51E

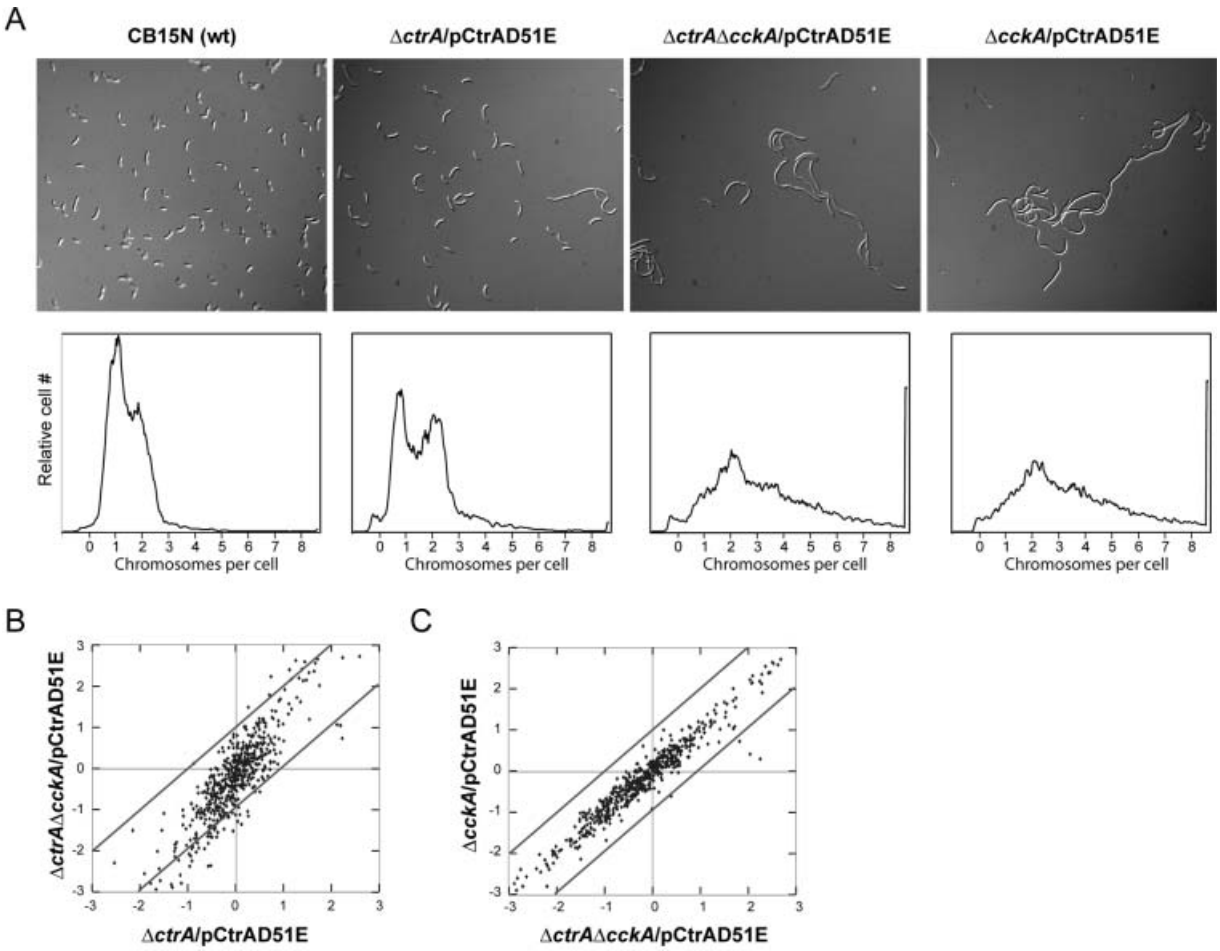

Fig. 5. CckA also has a CtrA phosphorylation-independent function that is critical for normal cell cycle progression.
A. Cell morphologies of wild-type CB15N, LS2716 (Δ*ctrA*/pCtrAD51E), CJ396 (Δ*ctrA*Δ*cckA*/pCtrAD51E) and CJ395 (Δ*cckA*/pCtrAD51E) were visualized by DIC light microscopy. The corresponding DNA content profile for each strain was analysed by flow cytometry of chromomycin A3-stained cells. Chromosome equivalents are indicated on the *x*-axis and relative number of cells on the *y*-axis.
B. The RNA levels of LS2716 (Δ*ctrA*/pCtrAD51E) and CJ396 (Δ*ctrA*Δ*cckA*/pCtrAD51E) were compared, on whole-genome microarrays, to a common reference RNA derived from wild-type cells (see *Experimental procedures*). The ratios for all cell cycle-regulated genes (544 in total) were then plotted against each other as a scatterplot. Genes falling above or below the parallel lines had expression levels at least twofold higher in one strain compared with the other.
C. Comparison of RNA levels, performed as in (B), between CJ396 (Δ*ctrA*Δ*cckA*/pCtrAD51E) and CJ395 (Δ*cckA*/pCtrAD51E).

respectively) should have the same phenotypes. Their phenotypic differences suggest that in addition to mediating the phosphorylation of CtrA, the CckA histidine kinase has another function that is critical for cell cycle progression.

The additional role of CckA appears to be a phosphorylation-independent activation of CtrA

To directly examine if the additional CckA function that is independent of CtrA phosphorylation affects gene expression, we compared the RNA levels of cell cycle-regulated genes between LS2716 (Δ*ctrA*/pCtrAD51E) and CJ396 (Δ*ctrA*Δ*cckA*/pCtrAD51E). Total RNA from each strain was analysed on whole-genome microarrays and compared with RNA from wild-type CB15N cells grown under the same conditions. These comparisons were done in triplicate and results averaged (see *Supplementary material*, 'Microarray CtrAD51E series'). Out of the 3536 genes for which reliable data were available, 226 (6%) were induced or repressed greater than twofold in CJ396 relative to LS2716. Many of the observed expression changes may be a reflection of the organism compensating for its perturbed cell cycle. To filter out these expression differences and focus on the additional role CckA plays in controlling cell cycle progression, only the genes that are known to be cell cycle-regulated (Laub *et al.*, 2000) were selected for further examination. The relative RNA levels for each of 544 cell cycle-regulated genes were compared for the

two strains (shown as scatterplots in Fig. 5B). Consistent with the dramatic phenotypic differences between the two strains (Fig. 5A), the gene expression profiles were also significantly different. Out of these, 47 genes were repressed and 10 genes were induced in CJ396 relative to LS2716 (a list of them is provided as *Supplementary material*). These two strains are isogenic except for the presence or absence of a functional copy of *cckA*. We anticipated that the functions and identities of these genes would help unravel the additional role CckA plays in governing cell cycle progression. Surprisingly, inspection of this list revealed that of these 57 genes whose expression was significantly altered between the two strains, 55 of them (96%) were similarly induced or repressed in the *ctrA* ts mutants relative to wild-type (see *Supplementary material*, Gene list). The fact that nearly all of these genes are CtrA-dependent suggests that CckA's additional role is a phosphorylation-independent activation of CtrA. However, we cannot rule out the possibility that CckA participates in an additional signal transduction pathway that regulates, independently of CtrA, this particular subset of CtrA-dependent genes.

In the absence of CckA, CtrA is inactive

The fact that the presence of wild-type *ctrA* in CJ395 (Δ*cckA*/pCtrAD51E) strain does not rescue or suppress the severity of the cell division and DNA replication phenotypes (Table 1; Fig. 5A) seen in CJ396 (Δ*ctrA*Δ*cckA*/pCtrAD51E) suggests that in the absence of CckA, CtrA is inactive. To obtain a sensitive readout of any putative CtrA activity in CJ395, we performed a comparative gene expression analysis between CJ395 and CJ396 (which only differ by the presence or absence of a functional copy of *ctrA* on the chromosome). Each strain was grown to mid-log phase and total RNA was harvested for comparison, on whole-genome microarrays, to RNA from wild-type CB15N cells grown under the same conditions. These comparisons were carried out in triplicate and the results were averaged (see *Supplementary material*, 'Microarray CtrAD51E series'). As with other comparisons, we analysed only those genes shown previously to be cell cycle regulated. The relative RNA levels for each cell cycle-regulated gene on the microarrays were then compared for the two strains (shown as a scatterplot, Fig. 5C), resulting in a correlation coefficient of 0.97. Only three out of 544 cell cycle-regulated genes (CC0782 transcriptional regulator of LuxR family, CC0707 conserved hypothetical protein and CC0559 hypothetical protein) showed greater than twofold induction or repression, and they probably represent noise or false positives. Thus, in agreement with the observed cell cycle defects, the gene expression profiles of the Δ*cckA* strains, CJ395 and CJ396, are nearly indistinguishable despite the fact that one strain carries an intact copy of *ctrA* on the chromosome. These data, together with the fact that no CtrA phosphorylation can be detected in *cckA* deletion strains (Fig. 4B, lane 5 relative to lane 2 and Fig. 4D, lane 6 relative to lane 2), suggest that in the absence of CckA, CtrA is unphosphorylated and inactive.

CckA appears to be also involved in the control of CtrA stability

The DNA microarray analysis of CJ396 transcripts relative to LS2716 (see above) suggest that CckA's additional role in cell cycle control is a positive regulation of CtrA. If correct, what could be the molecular basis for this phosphorylation-independent regulation of a response regulator by a histidine kinase? An important aspect of CtrA regulation is its cell cycle-regulated proteolysis (Domian *et al*., 1997). One possibility is that CckA may affect the stability of CtrA and thereby alter CtrA activity, regardless of the phosphorylation state. This possibility was suggested by the immunoblots shown in Fig. 4A. CtrAD51E protein levels were lower in Δ*cckA* strains (lanes 4 and 5 relative to lanes 3 and 2 respectively). As only high levels of CtrAD51E proteins, but not low levels, can support viability in a Δ*ctrA* strain (Domian *et al*., 1997), a decrease in CtrAD51E protein levels could be sufficient to explain the phenotypic defects observed in Δ*cckA* strains.

In order to test if CckA affects CtrAD51E stability, we determined the half-life of CtrAD51E and the proteolysis resistant mutant protein CtrAΔ3M2 in two genetic backgrounds that only differ by the presence or absence of CckA. The first strain is CJ507 in which the wild-type *ctrA* is replaced by *ctrAΔ3M2* (an allele whose product is resistant to proteolysis at the G1-S transition) on the chromosome (*ctrAΔ3M2*/pCtrAD51E). The second strain, CJ508, is genetically identical except for the chromosomal deletion of the *cckA* gene (*ctrAΔ3M2*;Δ*cckA*/pCtrAD51E). Both strains harboured the multicopy pCtrAD51E plasmid. The CJ507 strain had wild-type cell morphology whereas the CJ508 strain had the characteristic filamentation phenotype of Δ*cckA* strains (data not shown). Because of the cell cycle defects seen in Δ*cckA* strains, we were concerned that an increased instability of CtrA in such strains may be due to induction of overall protein degradation. If true, we speculated that the degradation would not be specific and would similarly affect the G1-S proteolysis resistant CtrAΔ3M2 protein. Cultures of both strains were pulse-labelled with ^{35}S-methionine in early log phase, and samples were withdrawn at several times during the following 60 min. The CtrA derivatives were immunoprecipitated using anti-CtrA antibodies. As shown in Fig. 6A, the proteolysis resistant CtrAΔ3M2 was equally stable in the

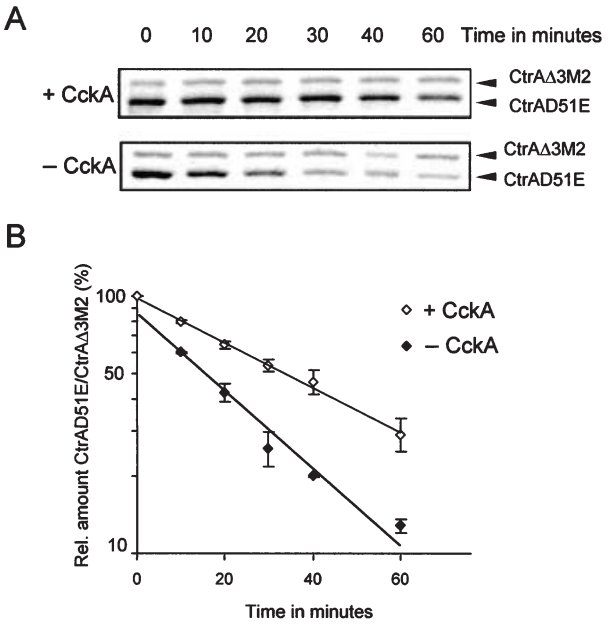

Fig. 6. The stability of CtrAD51E is reduced in the absence of CckA.
A. Phosphoimages of SDS-PAGE gels containing ^{35}S-labelled CtrA-derivatives from two strains, CJ507 (+CckA) and CJ508 (–CckA). Samples were removed from log-phase cultures at indicated time-points after pulse-labelling with ^{35}S-methionine (= time 0) and immunoprecipitated using anti-CtrA antibodies.
B. Three independent pulse-chase experiments were performed for each strain and the amounts of the labelled CtrA proteins were quantified using IMAGEQUANT software.
Mean values of the amounts of ^{35}S-labelled CtrAD51E and CtrAΔ3M2 for each time-point were calculated and expressed as percentages of the corresponding amount labelled at time 0. These values were semi-logarithmically plotted as CtrAD51E/CtrAΔ3M2 ratios for each sample as a function of time. Bars indicate standard deviations for each time-point.

presence or absence of CckA, arguing that there is little or no induction of general protein degradation in the absence of CckA.

We observed that the ^{35}S-labelled CtrAD51E levels decreased at a faster rate in the Δ*cckA* deletion strain (Fig. 6A). As CtrAΔ3M2 stability was not significantly affected by the presence or absence of CckA, the ^{35}S-labelled CtrAD51E levels were analysed over time as an expression of the ratio of CtrAD51E and CtrAΔ3M2 levels to reduce handling and loading-induced errors. As shown in Fig. 6B, the relative CtrAD51E degradation rate was significantly higher in the Δ*cckA* strain (CJ508) when compared with the CJ507 strain, with half of the CtrAD51E protein level being degraded in approximately 15 min when CckA was absent and in 35 min when CckA was present. As *ctrAD51E* is only a partial functional allele of *ctrA*, which has to be overexpressed to support viability (Domian *et al*., 1997), a higher turnover of its gene product in the absence of CckA provides an explanation for the phenotypes seen in the Δ*cckA* strains. It has been demonstrated that CtrAD51E is normally degraded during the G1-S transition, even when overexpressed from a high copy number plasmid (Domian *et al*., 1997), suggesting that CckA histidine kinase may contribute, directly or indirectly, to the stability of the master response regulator CtrA during the cell cycle.

Discussion

Because of its central role, CtrA activity is tightly regulated by several mechanisms including temporally controlled synthesis, ClpXP-dependent degradation, and phosphorylation of CtrA (Quon *et al*., 1996; Domian *et al*., 1997; Jenal and Fuchs, 1998). We present evidence here that the CckA histidine kinase plays a central role in the regulation of CtrA activity. This conclusion is based on several observations: (i) CtrA phosphorylation is dramatically reduced in a *cckA* loss-of-function mutant, suggesting that CckA controls CtrA phosphorylation (Jacobs *et al*., 1999); (ii) an analysis of the RNA and protein levels in *ctrA* and *cckA* ts mutants relative to wild-type shows that most genes that are differentially expressed in a *ctrA* ts mutant were similarly affected in the *cckA* ts mutant. The strong correlation in global gene expression profiles and protein levels between *ctrA* and *cckA* ts mutants indicates that a loss of CckA function corresponds in a loss of CtrA activity; (iii) the fact that CtrA phosphorylation itself, and not only the mere presence of the protein, is cell cycle regulated (Domian *et al*., 1997) predicts that the signal transduction pathway that activates CtrA follows the same cell cycle pattern as that of CtrA phosphorylation. In support of this, phosphorylation of CckA, and therefore presumably its activity, coincides temporally with CtrA phosphorylation during the course of the cell cycle. It suggests that CckA is involved in the regulation of CtrA phosphorylation at each stage of the cell cycle; (iv) only the phosphorylation-independent CtrAD51E mutant protein supports viability in the absence of CckA protein, providing strong genetic evidence that CckA is essential for viability because it is required for CtrA phosphorylation; and (v) finally, it appears that CckA has an additional role in controlling CtrA activity, that of stabilizing the CtrA protein.

The expression of phosphorylation-independent CtrAD51E mutant protein from a multicopy plasmid allows the cells to live without a functional copy of *cckA*. However, in the absence of CckA, the cells have several phenotypes including severe filamentation and accumulation of chromosomes. The CtrAD51E protein cannot be phosphorylated *in vitro* or *in vivo* (Domian *et al*., 1997; Reisenauer *et al*., 1999), and comparison of gene expression profiles using two Δ*ctrA* strains with CtrAD51E that only differ by

the presence or absence of CckA showed that most genes that were differentially expressed are known to be CtrA dependent. These results argue that CckA may affect CtrA activity by a means other than phosphorylation. In the absence of CckA, the stability of the CtrAD51E protein, which, like wild-type CtrA, is normally degraded during the swarmer-to stalked (G1-S) cell transition (Domian *et al*., 1997), is significantly reduced. A reduced level in CtrAD51E proteins can easily explain the phenotypes observed in Δ*cckA* strains as elevated levels of CtrAD51E protein are necessary for its functionality (Domian *et al*., 1997). When CckA interacts with its CtrA substrate, it may protect it from proteolysis by ClpXP. In *Bacillus subtilis*, it has been shown that the anti-sigma factor SpoIIAB not only phosphorylates the anti-anti sigma factor, SpoIIAA, but also form a complex with it that protects SpoIIAB from ClpCP-mediated proteolysis (Pan *et al*., 2001). However, it is not known if CckA directly interacts with CtrA. Given its hybrid histidine kinase structure with a response regulator-like domain at its C-terminus, it is likely that CckA is involved in a phosphorelay with an unidentified histidine phosphotransferase as an intermediate between CckA and CtrA. Another possibility is that CckA may affect the phosphorylation state of another response regulator that directly controls the proteolysis of CtrA by ClpXP. There is precedent for regulated proteolysis by a signal transduction pathway. The RssB response regulator (also termed SprE or MviA) directly targets σ^S, the master regulator of the general stress response, to the ClpXP protease (Bearson *et al*., 1996; Muffler *et al*., 1996; Pratt and Silhavy, 1996). RssB promotes σ^S degradation while having no effect on ClpXP activity with other substrates (Zhou and Gottesman, 1998). RssB activity is modulated by phosphorylation suggesting that a histidine kinase, yet-to-be identified, controls σ^S proteolysis (Zhou *et al*., 2001). Finally, CckA may negatively regulate ClpXP protease activity irrespective of CtrA. However, we think that this possibility is unlikely because both *clpX* and *clpP* RNA levels were found to be twofold reduced in Δ*cckA* strains relative to wild-type CB15N (see *Supplementary material* 'Microarray CtrAD51E series') although we cannot rule out a post-transcriptional effect of CckA on the global ClpXP protease activity.

Thus, we have presented evidence that CckA plays a critical role in the signal transduction pathway culminating in CtrA phosphorylation and it, at least partly, contributes to CtrA stability.

Besides CckA, two other signal transduction pathways have been proposed to control CtrA phosphorylation levels (Wu *et al*., 1998; 1999). Genetic and biochemical evidence clearly showed that PleC and DivJ histidine kinases control the activity of the essential single domain response regulator, DivK (Hecht *et al*., 1995; Burton *et al*., 1997; Wu *et al*., 1998; Wheeler and Shapiro, 1999). A mutant *ctrA* allele (*sokA*) suppresses the lethality phenotype of a *divK* null mutant and the cell division defect of a *divJ* point mutant, providing a genetic link between the DivK signal transduction pathway and CtrA (Wu *et al*., 1998). The *sokA* allele can also suppress the cell division phenotype of cold-sensitive point mutations in a gene encoding the essential DivL kinase suggesting that DivL also contributes to CtrA phosphorylation (Wu *et al*., 1999). If several signal transduction pathways independently participate in increasing CtrA~P levels, one would expect that in the absence of CckA, the other pathways would contribute to a residual activation of CtrA. To address this question, we compared two Δ*cckA* strains, both containing CtrAD51E but differing by the presence or absence of wild-type *ctrA* on the chromosome. The two strains were undistinguishable; they were filamentous and they had multiple chromosomes and an identical altered profile of global gene expression. Thus, the presence of *ctrA* does not rescue any of the phenotypes even though the other putative CtrA signal transduction pathways are present. These results, together with the observation that no CtrA~P was detected in the Δ*cckA* strain with *ctrA* on the chromosome, argue that, in the absence of CckA, CtrA is unphosphorylated and inactive. To affect CtrA~P levels, the DivK and DivL signalling pathways may be genetically upstream of CckA or dependent on CckA function to be active. Another possibility is that these other signalling pathways negatively control CtrA activity by, for example, contributing to its dephosphorylation.

Considering the pivotal role of CckA in differentiation and cell cycle control, the next critical question is: what mechanisms control CckA activity? The level of the CckA protein is constant during the cell cycle, suggesting that mechanisms using controlled proteolysis and/or synthesis are unlikely (Jacobs *et al*., 1999). On the other hand, CckA phosphorylation is cell cycle controlled (Fig. 3). Identifying the mechanisms regulating CckA phosphorylation, and therefore its activity, will be key. The striking observation that CckA changes its cellular location during the cell cycle suggests that this cell cycle-controlled localization may play an important role in CckA regulation (Jacobs *et al*., 1999). We have shown that CckA is found around the entire cell membrane in swarmer and stalked cells. It then moves from this dispersed distribution to the cell pole in early predivisional cells, and disperses at cell division. The polar localization of CckA in predivisional cells coincides with the time of CckA peak phosphorylation. The integration of multiple temporal and spatial levels of CtrA activation mediated by the CckA histidine kinase yields a finely tuned regulatory pathway. The challenge is now to understand how a unicellular organism that lives in a dilute aquatic environment sends cues to signal transduction proteins to effect multiple cell cycle changes.

Experimental procedures

Bacterial strains, plasmids and growth

Strains and plasmids are listed in Table 2. *Caulobacter crescentus* strains were routinely grown in peptone-yeast extract (PYE, complex media), M2G (minimal media) or M5GG (low-phosphate media) at 28°C unless otherwise stated (Ely, 1991; Jacobs *et al*., 2001). Plasmids were mobilized from *Escherichia coli* strain S17-1 into *C. crescentus* by bacterial conjugation (Ely, 1991). Chromosomal disruptions of *cckA* were generated by ΦCR30 phage transduction (Ely, 1991) of Δ*cckA*::Gent^R into different *C. crescentus* strains carrying the *ctrAD51E* allele on a multicopy plasmid, pCtrAD51E (Jacobs *et al*., 1999).

Flow cytometry

Samples for flow cytometry were prepared as described previously (Winzeler and Shapiro, 1995) and analysed in a Becton Dickinson FACStar Plus machine. Data were collected and analysed using FACS&SOL;DESK software (Stanford University, Stanford, CA).

Synchronization, immunoblots and immunoprecipitations

Homogenous populations of swarmer cells were isolated as described (Quon *et al*., 1996), resuspended to A_{660} of approximately 0.15, and allowed to proceed synchronously throughout the cell cycle. Samples were taken at different time intervals. Immunoblot and immunoprecipitations were performed as described (Jacobs *et al*., 1999). CtrA stability was determined by performing a 5 min pulse with [^{35}S]-methionine and a chase with unlabelled methionine and casamino acid. At different time intervals after pulse, aliquots were lysed and immunoprecipitated with anti-CtrA serum as described previously (Domian *et al*., 1997). [^{35}S]-CtrAD3M2 and [^{35}S]-CtrAD51E were separated on 12% SDS-PAGE and quantified with a Molecular Dynamics Phosphoimager.

In vivo *phosphorylation*

In vivo experiments were performed as described (Jacobs *et al*., 2001) with the following modifications: each sample was immunoprecipitated with 2 μl of anti-CtrA and 0.5 μl of anti-CckA antibodies.

Microscopy and photography

Cells were immobilized using a thin layer of agarose as described (Webb *et al*., 1998). Normaski differential interference contrast (DIC) images were taken with a Princeton Micromax 5600 camera or a Hamamatsu ORCA-ER and processed by Metamorph (Universal Imaging).

DNA microarray, two-dimensional gel electrophoresis and data analysis

Peptone-yeast extract cultures were grown at 28°C until they reached an A_{660} of approximately 0.3. For the temperature-sensitive series of microarray experiments, PYE cultures of CB15N, *ctrAV148F* and *cckATS1* were grown at 28°C until they reached an A_{660} of 0.1 ($t = 0$ h). The cultures were then shifted for 2 h at 37°C ($t = 2$ h); at which time their A_{660} was approximately 0.35. The cultures were then diluted four times with prewarmed PYE media and incubated at 37°C for an

Table 2. Strains and plasmids.

Strains or plasmids	Description	Source or reference
Strains		
Caulobacter crescentus		
CB15N	Wild type; all the other *C. crescentus* strains derived from it	Evinger and Agabian, (1977)
LS2722	CB15N *ctrA*:: *ctrAΔ3M2*; carrying wild-type *ctrA* and *ctrAΔ3M2* alleles on the *ctrA* locus	Domian *et al*. (1997)
cckATS1	*cckA* ts allele	Jacobs *et al*. (1999)
ctrAV148F	*ctrA* ts allele	Jacobs *et al*. (1999)
LS2515	CB15N *ctrAΔ3M2*; chromosomal *ctrA* gene replaced by *ctrAΔ3M2* allele	Domian *et al*. (1997)
LS2713	CB15N/pCtrA	Domian *et al*. (1997)
LS2715	CB15N/pCtrAD51E	Domian *et al*. (1997)
LS2714	Δ*ctrA*/pCtrA	Domian *et al*. (1997)
LS2716	Δ*ctrA*/pCtrAD51E	Domian *et al*. (1997)
CJ395	Δ*cckA*/pCtrAD51E; *cckA* gene replaced by gentamycin-resistant cassette	This study
CJ396	Δ*ctrA*Δ*cckA*/pCtrAD51E; chromosomal *ctrA* and *cckA* genes, respectively, replaced by spectomycin and gentamycin resistant cassettes	This study
CJ507	CB15N *ctrAΔ3M2*/pCtrAD51E; derived from LS2515	This study
CJ508	CJ507 *cckA*::Gent^{R}; *cckA* gene replaced by gentamycin resistant cassette	This study
Escherichia coli		
DH10B	Cloning strain	Gibco BRL
S17-1	RP4-2, Tc::Mu Km::Tn*7*, for plasmid mobilization	Simon *et al*. (1983)
Plasmids		
pJS14	Broad range multi-copy cloning vector, Chl^R pBBR1MCS derivative with unique *Eco*RI site	Skerker and Shapiro, 2000)
pMR10	Broad range low copy cloning vector	Roberts *et al*. (1996)
pMR10cckA	pMR10 carrying wild-type *cckA*	Jacobs *et al*. (1999)
pCtrA	pJS14 carrying wild-type *ctrA*, also named pSAL14	Domian *et al*. (1997)
pCtrAD51E	pJS14 carrying *ctrAD51E* allele, also named pCTD14	Domian *et al*. (1997)

additional 2 h ($t = 4$ h). RNA extraction was harvested for each sample and prepared for hybridization as described previously (Laub *et al*., 2002).

For the proteomic studies, 10 mg of freeze-dried *C. crescentus* cells were suspended in 1 ml two-dimensional buffer (5 M urea, 2 M thiourea, 0.1% carrier ampholytes pH 3-10 (Bio-Rad), 2% (w/v) CHAPS, 2% (w/v) sulphobetaine 3–10, 2 mM tributyl phosphine) and subjected to three rounds of tip-probe sonication for 30 s with 2 min on ice between each round. The lysate was centrifuged at 6000 *g* for 12 min to remove insoluble material. Then, 250 μg protein was used to perform each two-dimensional gel. Two-dimensional electrophoresis was performed as described (Cullen *et al*., 2002). Duplicate gel sets for each of two independent sample preparations were used to perform image analysis as described (Cordwell *et al*., 2002). Protein spots showing a minimum twofold change in abundance were selected for characterization by mass spectrometry. MALDI-TOF MS peptide mass mapping was performed as described (Cullen *et al*., 2002). Peptide masses were used to search the translated *C. crescentus* genome (http://www.tigr.org) using the programs PROTEINPROBE and MASSLYNX (Micromass).

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mole/mole3379/mmi3379sm.htm

Fig. S1. Two-dimensional gels.

Table S1. Proteomic data for CB15N (wt), ctrAV148F and cckATS1 mutants 4 h post temperature shift.

Microarray ctrA cckA ts series.

Microarray CtrAD51E series.

Gene expression profiles.

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