Developmental Cell, Vol. 5, 149–159, July, 2003, Copyright ©2003 by Cell Press

The Asymmetric Spatial Distribution of Bacterial Signal Transduction Proteins Coordinates Cell Cycle Events

Hubert Lam, Jean-Yves Matroule, and Christine Jacobs-Wagner*
Department of Molecular, Cellular, and Developmental Biology
Yale University
New Haven, Connecticut 06520

Summary

The polar localization of signaling proteins that are essential for *Caulobacter* cell cycle control is temporally regulated. Here we provide evidence that phosphorylation of the essential response regulator, DivK, is required for both its function and its cell cycle-regulated localization. The asymmetric location of the DivJ and PleC histidine kinases and their antagonistic activities on the cellular concentration of phosphorylated DivK provide positional and temporal information for the ordered sequence of DivK localization during the cell cycle. DivJ activity on DivK affects its correct localization, which, in turn, is required for PleC function. Since DivJ and PleC regulate different cell cycle events, the interconnected function of these two histidine kinases through localization of a common response regulator provides a mechanism for coordinating cell cycle progression. Study of a DivK homolog in the morphologically symmetric bacterium *Sinorhizobium meliloti* suggests that this type of cell cycle mechanism is widespread in prokaryotes.

Introduction

Protein localization is a frequently encountered form of regulation in eukaryotic signal transduction pathways and plays a crucial role in many cellular and developmental processes. Localization of signal transduction proteins at discrete cellular locations also occurs in primitive organisms such as the intrinsically polarized bacterium *Caulobacter crescentus*, whose cell cycle depends on obligate cell differentiation and asymmetric cell division. In this organism, most components of the signal transduction network that regulates polar differentiation and cell cycle progression exhibit a dynamic cell cycle-dependent behavior of localization (Jacobs et al., 1999, 2001; Wheeler and Shapiro, 1999). In this case, however, the biological relevance and the mechanisms responsible for protein localization remain largely unknown.

Here we investigate the function and mechanisms for localization of an important component of the *C. crescentus* cell cycle signaling network, DivK. DivK is a single-domain response regulator that is essential for viability (Hecht et al., 1995). A temperature-sensitive mutant of *divK* displays a polar morphogenesis defect, a block in initiation of chromosome replication, and a cell filamentation phenotype at the restrictive temperature (Hecht et al., 1995; Hung and Shapiro, 2002). Genetic evidence suggests that DivK's function lies, at least in part, in the regulation of the master transcriptional regulator, CtrA, which, in turn, controls multiple morphogenetic and essential cell cycle events (Hung and Shapiro, 2002; Wu et al., 1998). How is DivK activity regulated during the cell cycle? Experiments have shown that the level of phosphorylated DivK (DivK∼P) is significantly lower in swarmer cells than in later cell cycle stages, where it remains fairly constant (Jacobs et al., 2001). However, the most distinctive cell cycle regulation of DivK is its dynamic spatial distribution within the cell (Jacobs et al., 2001). As illustrated in Figure 1A, DivK is evenly distributed in the cytoplasm of swarmer cells (G1 phase), but, during the swarmer to stalked (G1-S) cell transition, DivK migrates to the old cell pole, where a stalk develops. Soon after, it accumulates at both poles in the early predivisional cell. Localization of DivK remains bipolar for most of the predivisional stage. Prior to cell separation, however, DivK is released specifically from the new swarmer pole while it remains anchored at the old stalked pole (Figure 1A). Thus, the completion of cell division results in a differential localization of DivK in the two daughter cells, with DivK being evenly distributed in the cytoplasm of the swarmer daughter cell, but polarly localized in the stalked sibling.

The histidine kinases DivJ and PleC are involved in the regulation of DivK localization, although the mechanism whereby this occurs is unknown. DivJ is required for DivK localization at the poles; in a Δ*divJ* mutant, DivK is uniformly dispersed in the cytoplasm in all cell types (Jacobs et al., 2001). Conversely, DivK is unable to delocalize from either pole at cell division in a *pleC*::Tn*5* mutant, indicating that PleC is involved in the pole-specific release of DivK at the end of the cell cycle (Jacobs et al., 2001). DivJ and PleC also display a discrete cell cycle-dependent pattern of localization (Wheeler and Shapiro, 1999). The two histidine kinases are asymmetrically localized at opposite poles (PleC at the new swarmer pole and DivJ at the old stalked pole) in the predivisional stage, suggesting that their asymmetric location may be important in governing DivK spatial distribution. Genetic data and biochemical experiments indicate that DivK activity is also regulated by PleC and DivJ (Hecht et al., 1995; Sommer and Newton, 1991). The purified kinase domains of PleC and DivJ can serve as efficient phosphodonors to DivK and as phosphatases to DivK∼P in vitro (Hecht et al., 1995). Consistent with DivJ serving as a kinase for DivK in vivo, the level of DivK∼P is considerably reduced in a Δ*divJ* mutant (Wheeler and Shapiro, 1999). Conversely, the DivK∼P level is significantly increased in a *pleC*::Tn*5* mutant (Wheeler and Shapiro, 1999), suggesting that PleC may primarily act as a phosphatase for DivK∼P in vivo; alternatively, PleC may reduce the level of DivK∼P by an indirect means. The fact that DivK is essential for viability, while DivJ and PleC are not, suggests that there must be at least one other kinase for DivK. Consistent with this conclusion, the DivK∼P level is reduced, but not abolished, in Δ*divJ* single or Δ*divJ pleC*::Tn*5* double

*Correspondence: christine.jacobs-wagner@yale.edu

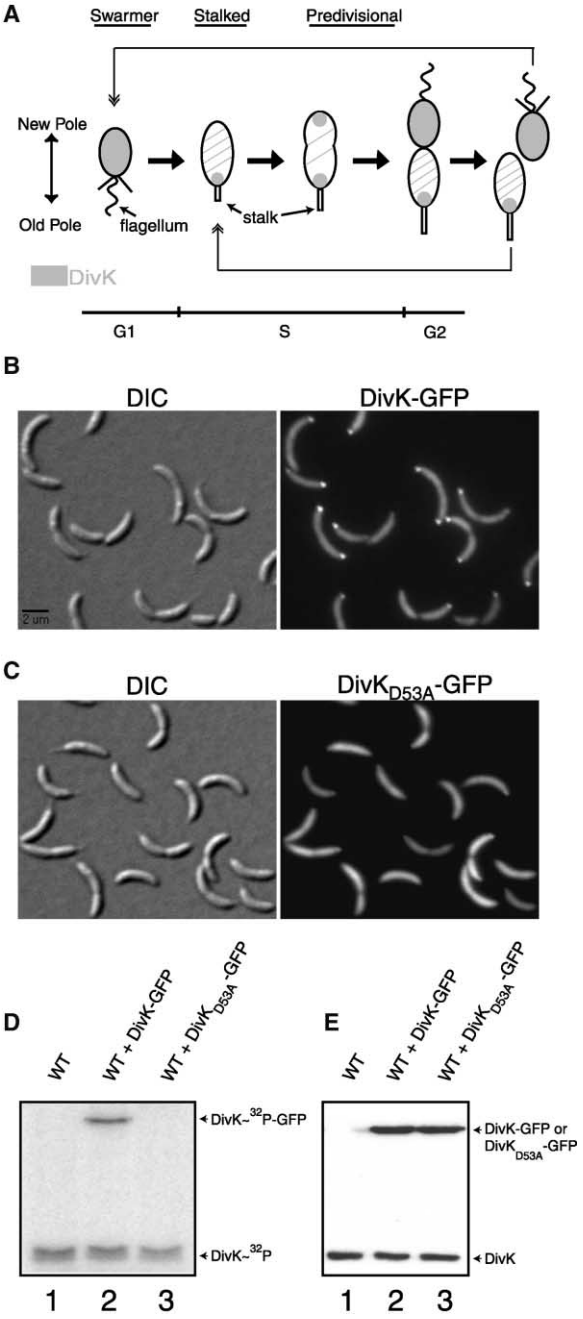

Figure 1. Phosphorylation of DivK Is Essential for Polar Localization of DivK

(A) Dynamic localization of DivK (gray) during the *C. crescentus* cell cycle.

(B–E) A nonphosphorylatable DivK mutant is impaired in polar localization. DIC and corresponding fluorescent images of wild-type CB15N strains producing either (B) wild-type DivK-GFP or (C) mutant DivK$_{D53A}$-GFP from a low-copy plasmid.

(D) In vivo phosphorylation of DivK, DivK-GFP, and DivK$_{D53A}$-GFP. CB15N cells (lane 1; WT) and CB15N cells producing either wild-type DivK-GFP (lane 2) or DivK$_{D53A}$-GFP (lane 3) from a low-copy plasmid were labeled with [γ-^{32}P]ATP for 3 min. GFP-tagged and untagged DivK$\sim^{32}$P proteins were immunoprecipitated with anti-DivK antibodies and resolved on a 12% SDS polyacrylamide gel.

(E) Samples of cells used in (D) were evaluated for the presence of total DivK proteins by immunoblot.

mutants (Wheeler and Shapiro, 1999). Interestingly, PleC and DivJ control different aspects of polar morphogenesis and the cell division cycle. *pleC* mutants are defective in chemotaxis, stalk formation, pili synthesis, and flagellar rotation (Sommer and Newton, 1989, 1991; Wang et al., 1993). On the other hand, *divJ* mutants have long stalks (sometimes more than one per cell and/or placed at the wrong location) and exhibit varying degrees of cell filamentation (Ohta et al., 1992; Sommer and Newton, 1991; Wheeler and Shapiro, 1999).

Here we demonstrate that the phosphorylation of DivK is essential for both the function and the dynamic cell cycle-dependent localization of DivK. We also provide evidence that DivJ has two dissociable functions that serve to regulate the temporal and spatial targeting of DivK$\sim$P to the distinct cell poles. The asymmetric location of DivJ, irrespective of its kinase activity, provides positional information that aids in recruiting a low level of DivK$\sim$P to the stalked pole, while DivJ's kinase activity subsequently targets DivK$\sim$P to the new swarmer pole of the predivisional cell by increasing the cellular concentration of DivK$\sim$P. The activity of asymmetrically localized PleC is responsible for the next step in the cell cycle pattern of DivK localization: it mediates the release of DivK$\sim$P specifically from the new swarmer pole by decreasing the DivK$\sim$P concentration in the swarmer compartment of the late predivisional cell. Since DivJ and PleC regulate different morphogenetic and cell cycle events, their interconnected function through the polar localization of a shared response regulator, DivK, provides a mechanism for coordinating polar morphogenesis and cellular events with cell cycle progression. Furthermore, asymmetric spatial distribution of DivK is not limited to intrinsically polarized bacteria but is also employed by a rod-shaped bacterium with no polar identifier organelles. Taken together, our data suggest that asymmetric spatial distribution of signal transduction proteins may constitute a widespread regulatory mechanism to control cell cycle progression in prokaryotes.

Results

Phosphorylation of DivK Is Essential for Both Its Activity and Its Dynamic Cell Cycle-Dependent Localization

Because the analysis of the phosphorylation and localization of DivK in wild-type, Δ*divJ*, and *pleC*::Tn*5* cells suggested that there was a positive correlation between polar localization of DivK and a high level of DivK$\sim$P and between nonpolar distribution of DivK and a low level of DivK$\sim$P in the cell (Jacobs et al., 2001; Wheeler and Shapiro, 1999), we tested the possibility that phosphorylation of DivK directly controls its localization. A DivK mutant carrying an alanine substitution in place of Asp53, the predicted phosphorylation site of DivK, was fused to GFP (DivK$_{D53A}$-GFP) and expressed from a low-copy plasmid in wild-type CB15N. The D53A mutation dramatically disrupted the ability of DivK to localize at the poles (Figure 1C) when compared with plasmid-encoded wild-type DivK-GFP (Figure 1B). In vivo phosphorylation experiments with wild-type CB15N and CB15N carrying a plasmid with either *divK-gfp* or

divK$_{D53A}$*-gfp* confirmed that the D53A alteration disrupted the phosphorylation site of DivK. Unlike wild-type DivK and DivK-GFP, DivK$_{D53A}$-GFP had no detectable phosphorylation (Figure 1D). Corresponding immunoblot analysis with anti-DivK antibodies showed that full-length GFP fusions to DivK and DivK$_{D53A}$ were produced at similar levels (Figure 1E). Furthermore, no degradation products were observed in immunoblots with anti-GFP antibodies, indicating that no soluble GFP was released from the GFP fusions (data not shown). We also examined the spatial distribution of DivK$_{D53A}$-GFP in Δ*divJ* and *pleC*::Tn*5* mutants and found that the mutant protein remained homogeneously dispersed in the cytoplasm, irrespective of the genetic background (data not shown).

These data strongly suggested that phosphorylation of DivK has a critical role in polar localization of DivK. Since it remained possible that the Asp53 residue itself, rather than its phosphorylation, was involved in the localization of DivK at the poles, we examined the subcellular distribution of two other plasmid-encoded DivK-GFP mutants, DivK$_{E9A}$-GFP and DivK$_{D10A}$-GFP. These mutants carry an alanine substitution in place of the active site residue Glu9 or Asp10, known to be essential in the phosphorylation process of response regulators by coordinating an Mg^{2+} ion (Stock et al., 1995). Similar to DivK$_{D53A}$-GFP, nonphosphorylatable DivK$_{E9A}$-GFP and DivK$_{D10A}$-GFP were impaired in their ability to localize at either pole in wild-type CB15N (data not shown). Taken together, our data indicate that it is the phosphorylated form of DivK that preferentially binds to the poles.

Typically, the activity of a response regulator is controlled by its phosphorylation state. Thus, it was likely that the phosphorylation of DivK was required for its function and, therefore, for cell viability, since DivK is known to be essential (Hecht et al., 1995). To determine whether this was the case, we generated a phage lysate carrying a *divK* null mutation, Δ*divK*::Spec^R. Using this phage lysate and selecting for spectinomycin resistance, we attempted to transduce Δ*divK*::Spec^R into wild-type CB15N or CB15N producing either wild-type DivK-GFP or nonphosphorylatable DivK$_{D53A}$-GFP from a low-copy plasmid. The expectation was that the Δ*divK*::Spec^R mutation would be transduced only if a second functional copy of *divK* were present. Consistently, over 200 Spec^R colonies were recovered in the strain with plasmid-encoded DivK-GFP, whereas none were recovered in CB15N alone or in cells with plasmid-encoded DivK$_{D53A}$-GFP. From these results, we conclude that phosphorylation of DivK is essential, not only for its polar localization, but also for cell viability.

DivJ Has Two Dissociable Functions in the Control of DivK∼P Targeting to the Distinct Poles

How can phosphorylation of DivK account for the protein's complex temporal and pole-specific localization pattern during the cell cycle (Figure 1A)? Previous studies suggested that the activities of DivJ and PleC and, possibly, their asymmetric localization at opposite poles provide spatial and temporal cues for the dynamic localization of DivK during the course of the cell cycle (Jacobs et al., 2001; Wheeler and Shapiro, 1999). To investigate the molecular mechanisms whereby DivJ contributes to the polar localization of DivK, we generated a catalytically inactive mutant of DivJ carrying an alanine substitution in place of the conserved phosphorylatable histidine residue His338 (DivJ$_{H338A}$). As expected, cells in which chromosomal *divJ* was replaced by *divJ*$_{H338A}$ fused to the *yfp* gene (*divJ*$_{H338A}$*-yfp*) exhibited all phenotypes characteristic of a Δ*divJ* mutant, such as long stalks and a mild cell division defect, indicating that the conserved His338 residue of DivJ is essential for its activity. Nevertheless, DivJ$_{H338A}$-YFP was able to localize at the stalked pole (Figure 2A). This is consistent with a recent report indicating that the localization determinants of DivJ lie within its sensor domain and not within its transmitter domain, where His338 is located (Sciochetti et al., 2002).

As shown in Figure 2B (strain CJ846), the presence of DivJ$_{H338A}$ was able to promote DivK-GFP localization at the stalked pole of stalked and predivisional cells, but not at the new swarmer pole of predivisional cells. For comparison, Figure 2B also shows the localization pattern of DivK-GFP in a strain expressing wild-type *divJ* where the polar localization of DivK-GFP is normal (strain CJ509) and in a Δ*divJ* strain where DivK-GFP remains dispersed in the cytoplasm in all cell types (strain CJ513). Since catalytically inactive DivJ$_{H338A}$ localizes at the stalked pole (Figure 2A), the observation that a fluorescent focus of DivK-GFP forms at the stalked pole in the presence of DivJ$_{H338A}$ (Figure 2B, CJ846) suggests that the mere accumulation of DivJ at the stalked pole, and not the kinase activity of DivJ, recruits DivK-GFP to the stalked pole. The fact that DivK-GFP localizes at the stalked pole, but not at the new swarmer pole, of predivisional cells when catalytically inactive DivJ$_{H338A}$ is present (Figure 2B, CJ846) implies that DivJ's kinase activity is required for promoting DivK-GFP localization at the incipient swarmer pole.

How can these findings be reconciled with our previous conclusion that phosphorylation of DivK is required for localization at both poles, including the stalked pole? As mentioned above, the DivK∼P level is reduced, but not abolished, in a Δ*divJ* mutant, indicating that an unidentified DivJ-independent kinase pathway contributes to DivK phosphorylation (Wheeler and Shapiro, 1999). We performed in vivo phosphorylation experiments on the strains used for the localization study (Figure 2B) to determine the level of phosphorylated DivK-GFP in the different *divJ* backgrounds. Consistent with the existence of another DivK kinase besides DivJ, a residual level of phosphorylated DivK-GFP was detected in Δ*divJ* and *divJ*$_{H338A}$ mutants (Figure 2C, CJ513 and CJ846, respectively). For comparison, a wild-type level of phosphorylated DivK-GFP is also shown (Figure 2C, CJ509). Thus, it may be the residual amount of phosphorylated DivK-GFP in the *divJ*$_{H338A}$ mutant that is recruited to the stalked pole by DivJ$_{H338A}$ (Figure 2B, CJ846). If correct, this hypothesis predicts that the nonphosphorylatable DivK$_{D53A}$ mutant will be unable to localize at the stalked pole, even in the presence of DivJ$_{H338A}$. Consistent with this prediction, we found that, unlike wild-type DivK-GFP (Figure 2B, CJ846), nonphosphorylatable DivK$_{D53A}$-GFP failed to localize at the stalked pole in the presence of DivJ$_{H338A}$ and instead remained evenly dispersed in

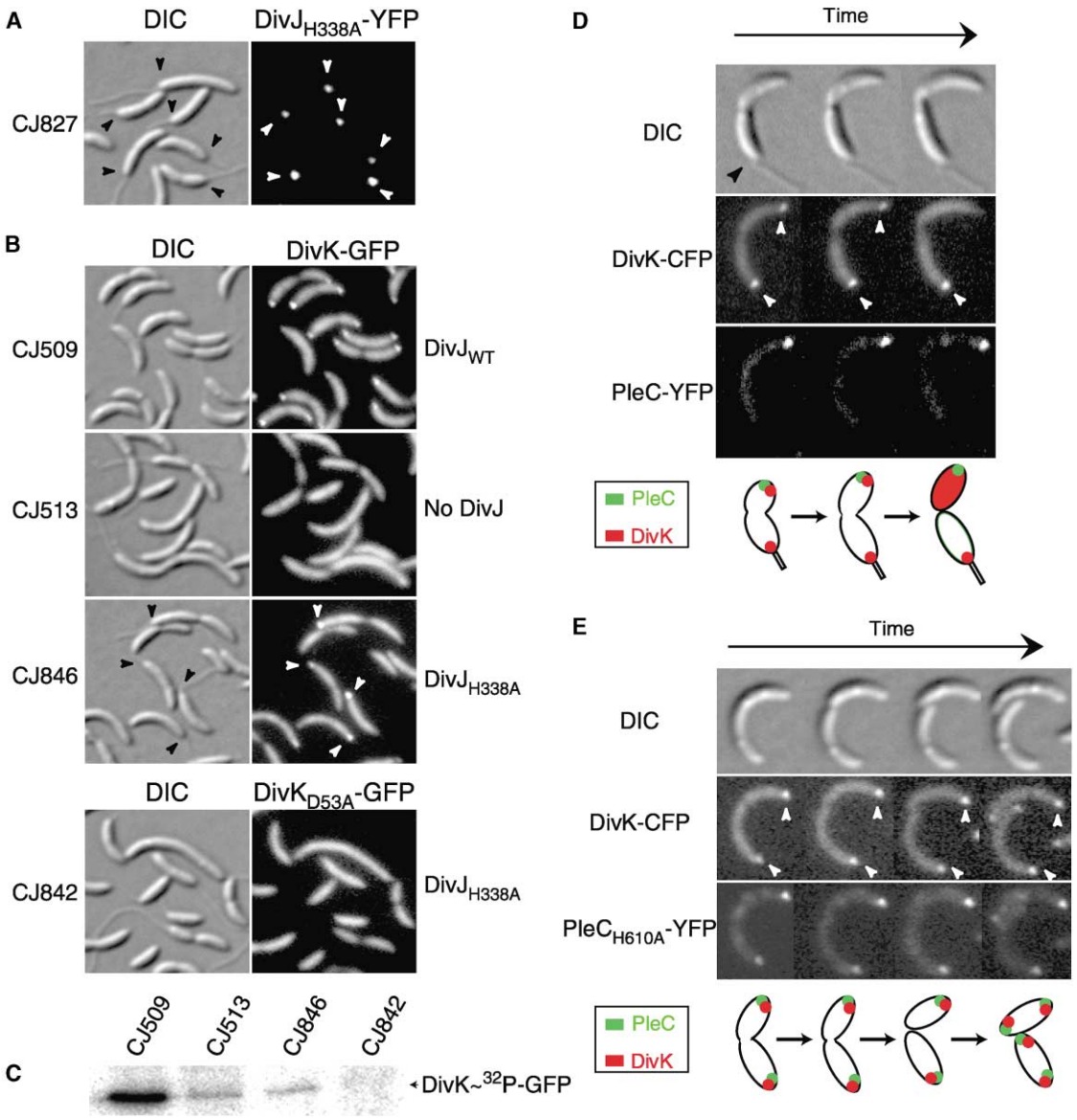

Figure 2. The Cellular Locations and Activities of DivJ and PleC Control the Cell Cycle-Dependent Localization of DivK

(A) $DivJ_{H338A}$-YFP localizes to the stalked pole. DIC and fluorescent images of cells producing chromosomally encoded $DivJ_{H338A}$-YFP in place of wild-type DivJ. White and black arrowheads show polar fluorescent foci and stalk locations, respectively.

(B) The interplay between the polar localization of DivJ and its kinase activity govern DivK localization at the distinct poles. The first three horizontal panels show the localization of chromosomally encoded DivK-GFP in the presence of wild-type DivJ (strain CJ509), in the absence of DivJ (strain CJ513), and in the presence of catalytically inactive $DivJ_{H338A}$ as the only DivJ protein (strain CJ846). The fourth panel shows the localization of chromosomally encoded $DivK_{D53A}$-GFP in the presence of $DivJ_{H338A}$ as the only DivJ protein (strain CJ842).

(C) The level of phosphorylated DivK-GFP or $DivK_{D53A}$-GFP was evaluated for the strains shown in (B) after [γ-^{32}P]ATP cell labeling and immunoprecipitation with anti-DivK antibodies.

(D) Both PleC and DivK$\sim$P colocalize at the new swarmer pole throughout most of the predivisional stage. Time-lapse fluorescence microscopy of *pleC*::Tn*5* cells producing DivK-CFP and PleC-YFP from a low- and medium-copy plasmid, respectively. The white and black arrowheads indicate polar foci of DivK-CFP and the location of the stalk, respectively. Below is a schematic representation of DivK-CFP and PleC-YFP localization.

(E) The activity of PleC is required for the release of DivK$\sim$P from the swarmer pole at cell division. Time-lapse fluorescence microscopy of Δ*pleC* cells producing DivK-CFP and $PleC_{H610A}$-YFP from a low- and medium-copy plasmid, respectively. The white arrowheads indicate that DivK-CFP remains anchored at both poles, even after cell division is completed. Below is a schematic representation of DivK-CFP and $PleC_{H610A}$-YFP localization.

the cytoplasm (Figure 2B, CJ842). In the four strains shown in Figures 2B and 2C, the *divJ* alleles and *divK-gfp* fusions were chromosomally encoded (see Table 1); these four strains also contain the endogenous *divK* gene. To eliminate the possibility that the $DivJ_{H338A}$-mediated localization of wild-type DivK-GFP at the stalked

Table 1. Strains and Plasmids

Strain	Relevant Genotype or Description	Reference or Source
C. crescentus		
CB15N (also NA1000)	Synchronizable variant strain of CB15	Evinger and Agabian, 1977
LS3196	CB15N Δ*divJ*	Wheeler and Shapiro, 1999
LS2732	CB15N *pleC*::Tn5	Wang et al., 1993
CB15N Δ*pleC*	CB15N Δ*pleC*	Urs Jenal
CJ403	CB15N *divK*::Δ*divK*Ω\\pdivK-EGFP	This study
CJ509	CB15N *divK-gfp*	This study
CJ513	CB15N Δ*divJ divK-gfp*	This study
CJ826	CB15N *divJ*::*divJ-yfp*	This study
CJ827	CB15N *divJ*::$divJ_{H338A}$-*yfp*	This study
CJ828	CB15N xylX::*divK-cfp*	This study
CJ833	CB15N *divK*::*divK-cfp*	This study
CJ842	CB15N *divJ*::$divJ_{H338A}$ $divK_{D53A}$-*gfp*	This study
CJ845	CB15N *divJ*::$divJ_{H338A\ divK}$::*divK-cfp*	This study
CJ846	CB15N *divJ*::$divJ_{H338A}$ *divK-gfp*	This study
CJ850	CB15N *divJ*::$divJ_{H338A}$	This study
S. meliloti		
MB501	Electroporable derivative of *S. meliloti* 1021	William Margolin
E. coli		
DH5α	Cloning strain	Invitrogen
S17-1	RP4-2, Tc::Mu,Km-Tn7, for plasmid mobilization	Simon et al., 1983
Plasmid	**Relevant Genotype or Description**	**Reference or Source**
pBluescriptKS+	Amp^R cloning vector	Stratagene
pMR20	Tet^R low copy number broad host range vector	Roberts et al., 1996
pJS14	Chl^R pBBR1-derived medium copy number broad host range vector	Jeffrey Skerker
pNPTS138	Kan^R pLitmus38-derived vector with *oriT* and *sacB*	Alley, 2001
pBGST18	A derivative of pBGS18 (Spratt et al., 1986)	Alley, 2001
pEGFP, pEYFP, pECFP	Variants of the *Aequorea victoria* green fluorescent protein (GFP)	Clonetech
pdivK-EGFP	pMR20 carrying *divK-gfp*	Jacobs et al., 2001
pMR20divK-cfp	pMR20 carrying *divK-cfp*	This study
pMR20$divK_{D53A}$-gfp	pMR20 carrying $divK_{D53A}$-*gfp*	This study
pMR20$divK_{D10A}$-gfp	pMR20 containing $divK_{D10A}$-*gfp*	This study
pMR20$divK_{E9A}$-gfp	pMR20 containing $divK_{E9A}$-*gfp*	This study
pJS14P_{xylX}divK-cfp	pJS14 carrying *divK-cfp* under the control of xylose-inducible promoter	This study
pBGST18P_{xylX}divK-cfp	pBGST18 carrying *divK-cfp* under the control of xylose-inducible promoter	This study
pBGST18TdivJ_{H338A}	pBGST18 carrying 5′-truncated $divJ_{H338A}$	This study
pBGST18TdivJ-yfp	pBGST18 carrying 5′-truncated *divJ-yfp*	This study
pBGST18TdivJ_{H338A}-yfp	pBGST18 carrying 5′-truncated $divJ_{H338A}$-*yfp*	This study
pBGST18ΩdivK-gfp	pBGST18 carrying $spec^R$/str^R linked to *divK-gfp*	This study
pBGST18ΩdivK$_{D53A}$-gfp	pBGST18 carrying $spec^R$/str^R linked to $divK_{D53A}$-*gfp*	This study
pBGENTTdivK-cfp	pBGST18 derivative ($gent^R$ replacing kan^R) carrying 5′-truncated *divK-cfp*	This study
pJS14pleC-yfp	pJS14 carrying *pleC-yfp*	This study
pJS14pleC$_{H610A}$-yfp	pJS14 carrying $pleC_{H610A}$-*yfp*	This study
pMR20$_{sm}$divK-gfp	pMR20 carrying a *S. meliloti divK* homolog fused to *gfp*	This study
pMR20$_{sm}$divK$_{D53A}$-gfp	pMR20 carrying $_{sm}divK_{D53A}$-*gfp*	This study
pJS14$_{sm}$divK-gfp	pJS14 carrying $_{sm}$*divK-gfp*	This study
pNPTS138ΔdivKΩ	pNTPS138 carrying *divK* with an internal deletion linked to $spec^R$/str^R	This study

pole is affected by the presence of an extra chromosomal copy of *divK*, we generated a strain (CJ845) in which both $divJ_{H338A}$ and *divK-cfp* were expressed from the chromosome as the only *divJ* and *divK* copies. In this strain, similar to what was observed with strain CJ846 (Figure 2B), DivK-CFP was recruited at the stalked pole by catalytically inactive $DivJ_{H338A}$ (data not shown).

From these experiments, we conclude that DivJ has two dissociable functions that participate in the temporal and spatial control of DivK∼P localization at the distinct poles. The asymmetric location of DivJ, irrespective of its kinase activity, helps recruit a low level of DivK∼P at the stalked pole, while DivJ's kinase activity subsequently promotes the additional localization of DivK∼P at the new swarmer pole by increasing the cellular concentration of DivK∼P.

PleC Activity Is Required for the Pole-Specific Release of DivK∼P before Cell Separation

While DivJ participates in DivK∼P localization at the poles, PleC is involved in the pole-specific release of DivK∼P (Jacobs et al., 2001), and it causes a decrease in the cellular concentration of DivK∼P (Wheeler and Shapiro, 1999). Since PleC is specifically localized at the new swarmer pole in predivisional cells (Wheeler and Shapiro, 1999), its asymmetrically localized activity on the concentration of DivK∼P may be involved in the release of DivK∼P from the new swarmer pole in late predivisional cells; alternatively, PleC may displace DivK∼P from the new swarmer pole by simply competing for the same binding sites. If the latter were correct, one would expect PleC and DivK localization at the new swarmer pole to be mutually exclusive. To conclusively

test this prediction, we examined the simultaneous localization of PleC and DivK using protein fusions to YFP and CFP. As clearly shown in time-lapse experiments (Figure 2D), DivK-CFP and PleC-YFP colocalize at the new swarmer pole throughout most of the predivisional stage, which rules out a simple competitive mechanism for PleC-dependent delocalization of DivK∼P. Instead, delocalization of DivK∼P from the new swarmer pole requires PleC activity. This was demonstrated with a Δ*pleC* mutant that expressed plasmid-encoded DivK-CFP and $PleC_{H610A}$-YFP, which carried an alanine in place of the conserved phosphorylatable His610. As recently reported for a chromosomal GFP fusion to $PleC_{H610A}$ (Viollier et al., 2002), $PleC_{H610A}$-YFP was unable to suppress the characteristic *pleC* phenotypes and failed to delocalize from the old pole at the swarmer to stalked transition, resulting in a bipolar localization in predivisional cells (Figure 2E). Importantly, in the presence of catalytically inactive $PleC_{H610A}$-YFP only, the level of DivK∼P was significantly elevated relative to that in the wild-type (data not shown), and the DivK-CFP fusion was unable to delocalize from the new swarmer pole at cell division, producing two daughter cells with identical polar localization of DivK-CFP (Figure 2E). Thus, the site of PleC phosphorylation, His610, is required for both the negative control of DivK phosphorylation and the pole-specific release of DivK∼P at cell division, which sets up the differential distribution of DivK in the two daughter cells.

Collectively, our results suggest that the mechanism for the complex temporal and spatial pattern of DivK localization during the cell cycle involves the asymmetric location and opposing activities of DivJ and PleC on the cellular concentration of DivK∼P.

A High Level of Mislocalized DivK Results in a Cell Division Defect

We reasoned that overproduction of DivK may saturate the DivJ-mediated DivK binding to the poles and thereby induce mislocalization of DivK. To test this hypothesis, we placed a *divK-cfp* fusion under the control of the inducible xylose promoter (P*xylX*) (Meisenzahl et al., 1997) on a medium-copy plasmid ($pJS14P_{xylX}$divK-cfp), which was introduced into wild-type CB15N cells. In the absence of the xylose inducer, a basal level of *divK-cfp* expression created a low level of DivK-CFP that had a normal localization pattern (Figure 3, 0 min). Addition of the xylose inducer resulted quickly in overproduction and mislocalization of DivK-CFP throughout the cytoplasm (Figure 3, 20 min), which, in turn, caused cell filamentation (Figure 3, 7 hr). The fact that mislocalization of DivK occurs before cell filamentation argues against the possibility that inhibition of cell division causes mislocalization of DivK. Instead, it suggests that a high level of DivK dispersed throughout the cell inhibits cell division. Induction of *divK-cfp* expression from the chromosomal *xylX* locus for 13 hr gave rise to a lower level of overproduction of DivK-CFP, causing only a partial mislocalization of DivK-CFP and a cell filamentation of three to four times the normal cell length on average (data not shown; strain CJ828). Thus, the severity of the cell division defect correlates with the amount of DivK delocalized throughout the cytoplasm.

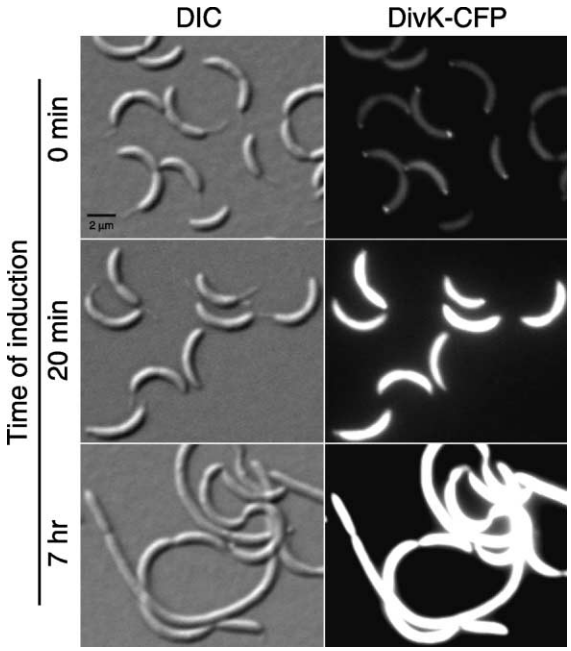

Figure 3. Overproduction of DivK Results in Protein Mislocalization and Cell Filamentation

DIC and fluorescent images were taken of CB15N cells expressing *divK-cfp* under the xylose promoter from a medium-copy plasmid ($pJS14P_{xylX}$divK-cfp) grown in the presence of the xylose inducer for 0 min, 20 min, or 7 hr (the culture was diluted so that it remained in the exponential phase of growth).

$_{sm}$DivK-GFP Localizes Polarly in *Sinorhizobium meliloti* Using a Phosphorylation-Dependent Mechanism

Given the role of DivK in the coordination of polar morphogenesis with the cell cycle in *C. crescentus*, it was intriguing that *Sinorhizobium meliloti* had a DivK homolog (SMc01371) that shared 74% protein sequence identity with *C. crescentus* DivK. Unlike *C. crescentus*, the rod-shaped *S. meliloti* is thought to divide symmetrically to produce two identical daughter cells. There is no evidence of polar organelles. Instead, it typically has five to ten peritrichously inserted flagella (Pleier and Schmitt, 1989). The conservation of DivK protein identity prompted the question of whether bacterial species that are not conspicuously asymmetric also utilize spatial regulation of signaling proteins such as DivK.

To investigate the function and the cellular distribution of the *S. meliloti* DivK homolog ($_{sm}$DivK), we amplified the sequence of $_{sm}$*divK* from the genome of *S. meliloti* 1021 and fused it to *gfp* ($_{sm}$*divK-gfp*). Using a Δ*divK*:: $Spec^R$-transducing lysate, we showed that $_{sm}$*divK-gfp* harbored on a low-copy plasmid was able to support viability in *C. crescentus* as the only copy of *divK*, arguing that $_{sm}$DivK is a functional homolog of *C. crescentus* DivK. When expressed from a low-copy plasmid in *S. meliloti* MB501 (electroporable derivative of *S. meliloti* 1021), $_{sm}$DivK-GFP accumulated at one pole in 50% of the cells (Figure 4A), indicating that $_{sm}$DivK-GFP was able to localize polarly in *S. meliloti*. An alanine substitution disrupting the conserved phosphorylatable Asp53

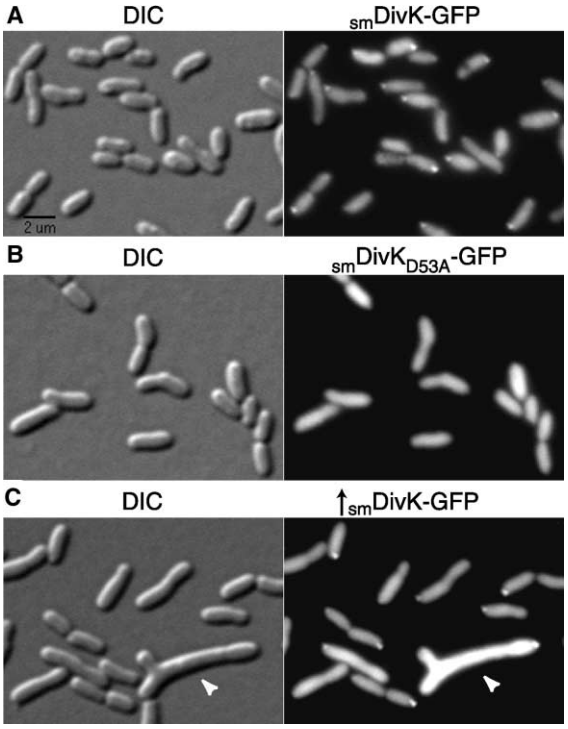

Figure 4. The *S. meliloti* Homolog of DivK Fused to GFP ($_{sm}$DivK-GFP) Polarly Localizes in *S. meliloti* Using a Phosphorylation-Dependent Mechanism

DIC and corresponding fluorescent images were taken of *S. meliloti* cells producing either wild-type $_{sm}$DivK-GFP from a low-copy plasmid (A), mutant $_{sm}$DivK$_{D53A}$-GFP from a low-copy plasmid (B), or wild-type $_{sm}$DivK-GFP from a medium-copy plasmid (C). The arrowhead indicates a cell with a cell division defect.

of $_{sm}$DivK-GFP ($_{sm}$DivK$_{D53A}$-GFP) prevented the protein from forming a polar fluorescent focus in *S. meliloti* cells (Figure 4B), implying that, as for its *C. crescentus* homolog, phosphorylation of $_{sm}$DivK-GFP is required for its polar localization.

Expression of *$_{sm}$divK-gfp* from a medium-copy plasmid (pJS14$_{sm}$divK-GFP) in *S. meliloti* cells resulted in a heterogeneous cell population in terms of cell morphology (Figure 4C). Approximately 80% of cells had a wild-type morphology, divided normally, and had normal unipolar localization of $_{sm}$DivK-GFP. Conversely, the remaining 20% of the cells exhibited a cell division defect (Figure 4C, arrowhead), which, in *S. meliloti*, is characterized by a cell filamentation and branching phenotype (Latch and Margolin, 1997). Fluorescence microscopy showed that the cell division phenotype correlated with high levels of mislocalized $_{sm}$DivK-GFP throughout the cytoplasm, as evidenced by the fluorescent signal intensity (Figure 4C). The variations in wild-type *$_{sm}$divK-gfp* expression responsible for the cell population heterogeneity (normal versus filamentous and branched) likely results from an unequal segregation of plasmid copy number between cells. In any event, a high level of mislocalized $_{sm}$DivK-GFP correlated with a cell division defect, suggesting that, as for *C. crescentus*, an increased level of DivK functioning in an inappropriate cellular location leads to a loss in cell division control.

Dynamic Subcellular Distribution of $_{sm}$DivK during the *S. meliloti* Cell Cycle Culminates in an Asymmetric Cell Division

The fact that $_{sm}$DivK-GFP is polarly localized in only 50% of the cell population (Figure 4A) suggested that, similar to what happens in *C. crescentus*, $_{sm}$DivK-GFP may dynamically change its spatial distribution during the *S. meliloti* cell cycle. To resolve the spatial and temporal coordinates of $_{sm}$DivK-GFP during the division cycle, we performed time-lapse fluorescence microscopy on free-living *S. meliloti* cells carrying plasmid-encoded $_{sm}$DivK-GFP. The doubling time of *S. meliloti* in a slide on the microscope stage was approximately 270 min. The cell cycle was visualized by DIC microscopy, and a cell pole was denoted "new pole" if it originated from the last cell division or "old pole" otherwise. Figure 5A illustrates a representative example of a time-lapse experiment. The $_{sm}$DivK-GFP fluorescent signal was evenly distributed within a young *S. meliloti* cell. As the cell grew, $_{sm}$DivK-GFP accumulated at one pole. From the analysis of $_{sm}$DivK-GFP localization during two cell cycle lengths, we determined that $_{sm}$DivK-GFP accumulated at the old pole. After polar localization, the cell went through a transient period where $_{sm}$DivK-GFP was released from the old pole and uniformly distributed throughout the cell to only later reaccumulate at the same pole prior to cell separation. The differential distribution of $_{sm}$DivK-GFP culminated in an asymmetric division producing two different daughter cells: one with a uniform distribution of $_{sm}$DivK-GFP and the other with an accumulation of $_{sm}$DivK-GFP at the old pole. In the former, $_{sm}$DivK-GFP soon accumulated at the old pole in a new round of cell cycle-dependent localization, whereas, in the latter (where $_{sm}$DivK-GFP was already polarly localized after cell division), the first cell transition of polar localization was bypassed, as schematically illustrated in Figure 5B. The difference in the DivK localization pattern between the two daughter cells can be viewed in Supplemental Movie S1 at http://www.developmentalcell.com/cgi/content/full/5/1/149/DC1, which shows a sequence of time-lapse images of a period close to two cell cycle lengths.

In *C. crescentus*, cell division is not only morphologically asymmetric, but also unequal, with the swarmer cell being shorter than the stalked cell (Terrana and Newton, 1975). The calculated ratio between the cell length of the stalked daughter cell and that of its swarmer sibling is 1.14 (Terrana and Newton, 1975). Interestingly, the *S. meliloti* daughter cell with a nonpolar distribution of $_{sm}$DivK-GFP, which in that respect resembled the *C. crescentus* swarmer cell, appeared to be shorter than its sibling with polar $_{sm}$DivK-GFP (as shown Figure 5, 280 min time point). To determine whether the noted difference in cell size was significant, we measured the cell length of 140 daughter cells with a polar $_{sm}$DivK-GFP focus and that of their siblings without a polar $_{sm}$DivK-GFP focus and calculated their ratio as described in Experimental Procedures. The calculated mean of 140 cell length ratios (daughter cells with a polar $_{sm}$DivK-GFP focus versus siblings without a polar $_{sm}$DivK-GFP focus) was 1.12, with a 95% confidence interval from 1.10 to 1.14. A paired t test of the data set gave a two-tailed p value less than 0.0001, which, by

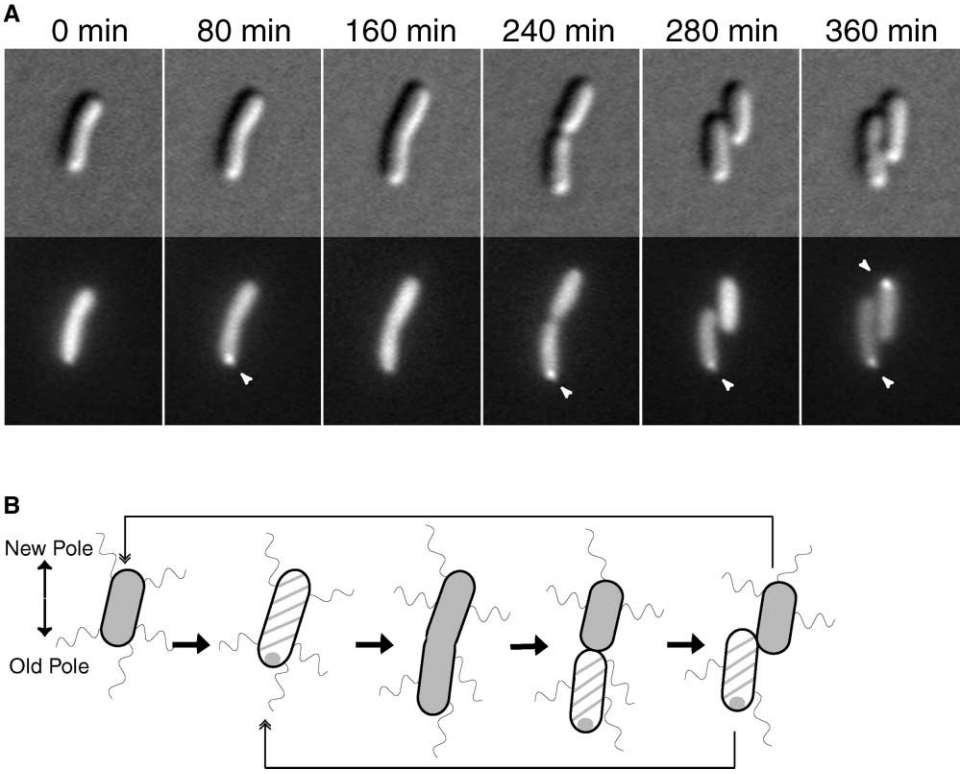

Figure 5. Dynamic Cell Cycle-Dependent Localization of $_{sm}$DivK-GFP in *S. meliloti* Culminates in an Asymmetric Cell Division

(A) Representative time-lapse fluorescence microscopy experiment on *S. meliloti* cells containing plasmid-encoded $_{sm}$DivK-GFP. In a young cell (0 min), $_{sm}$DivK-GFP is uniformly distributed in the cytoplasm. As the cell progresses through the cell cycle (80 min), $_{sm}$DivK-GFP localizes at the old pole. In the early predivisional stage (160 min), $_{sm}$DivK-GFP is released from the old pole and distributed evenly throughout the cytoplasm. Just prior to cell separation (240 min), $_{sm}$DivK-GFP reaccumulates at the old pole, ending in an asymmetric cell division, with $_{sm}$DivK-GFP being diffusely distributed in one of the daughter cells, but polarly localized in the other daughter cell (280 min). Later, the cell with delocalized $_{sm}$DivK-GFP regains a polar focus of $_{sm}$DivK-GFP at the old pole (360 min), whereas the cell with polarly localized $_{sm}$DivK-GFP bypasses this step. Arrowheads indicate the polar fluorescent foci.

(B) Schematic representation of the cell cycle-dependent localization of $_{sm}$DivK in *S. meliloti*. Wavy lines represent peritrichous flagella.

conventional criteria, is considered to be extremely statistically significant. Thus, the *S. meliloti* daughter cell that inherits a polar localization of $_{sm}$DivK-GFP is statistically longer than its sibling where the subcellular distribution of $_{sm}$DivK-GFP is not polarized, indicating an unequal cell division. Therefore, as in *C. crescentus*, the $_{sm}$DivK protein has a dynamic cell cycle-dependent localization in *S. meliloti* that ends in an asymmetric and unequal cell division, giving rise to two daughter cells that differ in their size and in their temporal and spatial pattern of $_{sm}$DivK localization.

Discussion

To address the role of localization of signaling proteins and the mechanisms whereby these proteins accumulate at discrete locations in bacteria, we investigated the control mechanisms of DivK localization in *C. crescentus* and the respective roles of DivJ and PleC in the temporal and spatial regulation of this process. Using nonphosphorylatable DivK mutants, we first demonstrated that phosphorylation of DivK is critical for both cell viability and the polar localization of DivK in *C. crescentus*. This indicates that it is the phosphorylated, active form of DivK (DivK∼P) that preferentially binds at the cell poles.

DivJ has two separable functions that provide spatial and temporal cues for the localization of DivK∼P at the distinct poles. First, the asymmetric location of DivJ contributes to the polar localization of DivK∼P at the stalked pole. Second, the kinase activity of DivJ mediates the transition from unipolar to bipolar localization of DivK∼P by increasing the overall cellular level of DivK∼P. The first function was suggested by the following results (Figures 2A–2C). (1) In the absence of DivJ protein, the level of phosphorylated DivK-GFP is decreased (but not abolished), and the fluorescent signal of DivK-GFP is evenly distributed in the cytoplasm of all cell types. This is consistent with published data (Jacobs et al., 2001; Wheeler and Shapiro, 1999). (2) In the presence of kinase-inactive DivJ$_{H338A}$, which localizes at the stalked pole, the level of phosphorylated DivK-GFP is similarly reduced, yet DivK-GFP forms a fluorescent focus at the stalked pole. (3) It is the residual amount of phosphorylated DivK-GFP that localizes at the stalked pole in the *divJ$_{H338A}$* mutant because a nonphosphorylatable DivK-GFP mutant remains homogeneously distributed in the cytoplasm throughout the cell cycle, even when DivJ$_{H338A}$ is present. Taken together, these findings suggest that it is the mere presence of DivJ at the stalked pole, irrespective of its kinase activity, that recruits DivK∼P at that location. The fact that

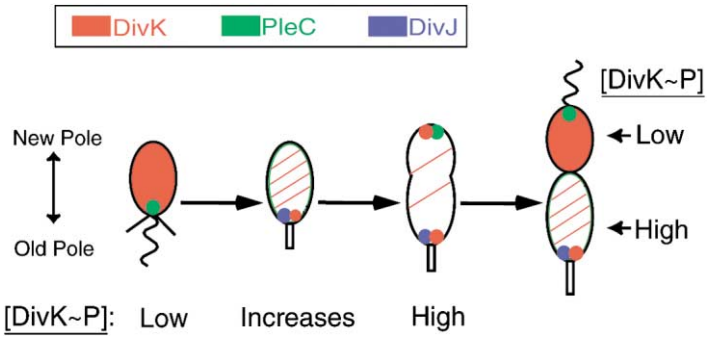

Figure 6. Model for the Control of DivK Dynamic Localization during the *C. crescentus* Cell Cycle

Temporal and spatial control of DivK localization is mediated by the asymmetric locations of DivJ and PleC and their opposing activities on DivK∼P levels. In the swarmer cell, DivK∼P is prevented from binding at the poles by PleC activity that maintains a low concentration of DivK∼P and by the absence of DivJ protein at the old pole. During the swarmer to stalked cell transition, PleC delocalizes and DivJ is synthesized and localized at the pole previously occupied by PleC, where a nascent stalk is being formed. By providing positional information, DivJ recruits DivK∼P at the old stalked pole. Through its kinase activity, DivJ increases the cellular concentration of DivK∼P, thereby increasing DivK∼P binding at the stalked pole as well as promoting DivK∼P targeting to the new swarmer pole, resulting in a bipolar localization of DivK∼P. In the predivisional cell, PleC relocalizes at the new swarmer pole and remains there together with DivK∼P until compartmentalization of the late predivisional cell, at which time PleC activity releases DivK∼P from the new swarmer pole by decreasing the DivK∼P concentration in the swarmer compartment. In contrast, DivK∼P polar localization is maintained at the stalked pole by DivJ, setting up the differential distribution of DivK between the two daughter cells.

no fluorescent focus of DivK-GFP is observed at the new swarmer pole of predivisional cells in the $divJ_{H338A}$ mutant (Figure 2B) implies that DivJ's kinase activity is essential for targeting DivK to the new swarmer pole by increasing the cellular concentration of DivK∼P.

Two observations suggest that DivJ's kinase activity also increases DivK∼P binding at the stalked pole. First, on the basis of the intensity of the fluorescent signal, the accumulation of DivK-GFP at the stalked pole tends to increase when DivK-GFP becomes bipolarly localized (Jacobs et al., 2001). This suggests that the increased level of DivK∼P generated by DivJ's activity favors binding of DivK∼P, not only at the new swarmer pole, but also at the stalked pole. Second, in the stalkless *pleC*::Tn*5* mutant, in which the level of DivK∼P is increased relative to wild-type, DivJ is no longer polarly localized, yet DivK∼P is able to localize at both poles (Jacobs et al., 2001; Wheeler and Shapiro, 1999), suggesting that an increased cellular level of DivK∼P may circumvent the need for DivJ asymmetric location. Taking these observations together, we propose that, in the wild-type situation, the presence of DivJ at the stalked pole provides positional information to recruit DivK∼P to the stalked pole when the DivK∼P level is low. Subsequently, DivJ's kinase activity increases DivK∼P binding stability at the stalked pole in addition to promoting DivK∼P targeting to the new swarmer pole.

While DivJ participates in the regulation of DivK∼P targeting to the distinct poles, it is the activity of PleC that triggers the subsequent release of DivK∼P specifically from the new swarmer pole prior to cell separation. In the presence of catalytically inactive $PleC_{H610A}$-YFP in place of wild-type PleC, regulation of DivK-CFP is affected at two levels: the cellular level of phosphorylated DivK-CFP is increased, and DivK-CFP is prohibited from delocalizing from the new swarmer pole at cell division. The fact that DivK-CFP and $PleC_{H610A}$-YFP (or wild-type PleC-YFP) colocalize at the new swarmer pole precludes a simple mechanism of competition between PleC and DivK∼P for binding sites. Instead, our data argue that PleC activity is responsible for reducing the concentration of DivK∼P in the swarmer compartment of the late predivisional cell, which, in turn, causes the dissociation of DivK∼P from the new swarmer pole.

Figure 6 illustrates a tentative model in which the asymmetric cellular location of DivJ and PleC and their opposite activities on the cellular concentration of DivK∼P contribute in a concerted way to regulate the localization of DivK during the *C. crescentus* cell cycle. In a swarmer cell where the DivK∼P level is the lowest relative to later cell cycle stages (Jacobs et al., 2001), the activity of the polarly localized PleC prevents DivK∼P targeting to the poles by maintaining a low concentration of DivK∼P. During the swarmer to stalked cell transition, PleC delocalizes and the newly synthesized DivJ accumulates at the old pole where a stalk develops (Wheeler and Shapiro, 1999). By providing positional information, DivJ contributes to the recruitment of DivK∼P at the old stalked pole. Through its kinase activity, DivJ increases the cellular level of DivK∼P, thereby promoting DivK∼P targeting to the new swarmer pole in addition to stabilizing DivK∼P binding at the stalked pole. In the predivisional cell, PleC relocalizes at the new swarmer pole (Wheeler and Shapiro, 1999). Just before cell separation, the activity of PleC at the new swarmer pole mediates the pole-specific release of DivK∼P by decreasing the DivK∼P level in the swarmer cell compartment of the late predivisonal cell. At the opposite pole, the compartmentalized activity of DivJ maintains DivK∼P localization, setting up the asymmetric cell division.

The direct coupling between activity and polar localization of DivK through phosphorylation suggests that localization of DivK has a regulatory function. Furthermore, overexpression of *divK*, creating a high level of mislocalized DivK throughout the cytoplasm, results in inhibition of cell division (Figure 3). The severity of the cell filamentation phenotype increases with the amount of DivK and its resulting mislocalization as shown by placing *divK* under the inducible *xylX* promoter from either a chromosomal or a plasmidic location. It is unclear how this cell division defect occurs, but it is consistent with the observation that *divJ* mutants exhibit varying degrees of filamentation (Ohta et al., 1992; Sommer and Newton, 1991; Wheeler and Shapiro, 1999). These data suggest that a loss of spatial control of DivK localization results in a cell division defect whose severity is dependent on the amount of mislocalized DivK. On the other hand, *pleC* mutants in which DivK remains poised at both poles at cell division (Jacobs et al., 2001) are

defective in temporally regulated morphogenetic events, including stalk and pili synthesis, flagellar rotation, and ejection of the flagellum (Burton et al., 1997; Wang et al., 1993). If the pleiotropic defects of *pleC* mutants result from the inability of DivK∼P to delocalize from the new swarmer pole at cell division, it predicts that a *divJ* mutation will be epistatic to a *pleC* mutation, since, as mentioned above, localization of DivK∼P at the new swarmer pole is dependent on DivJ kinase activity. Accordingly, *divJ* mutations suppress *pleC* phenotypes (Sommer and Newton, 1991; Wheeler and Shapiro, 1999). A Δ*divJ pleC*::Tn*5* double mutant has all the characteristic phenotypes of a single Δ*divJ* mutant, including a low DivK∼P level and a dispersed DivK distribution (Jacobs et al., 2001; Wheeler and Shapiro, 1999). The mechanisms by which DivJ and PleC govern the temporal and spatial regulation of DivK localization provide an explanation as to why. Since DivJ and PleC regulate distinct morphogenetic and cell cycle events, the dependence of PleC function on DivJ activity through localization of a shared response regulator provides an efficient way for the cell to coordinate distinct morphogenetic and cellular events with cell cycle progression.

Several lines of evidence suggest that this cell cycle mechanism observed in the intrinsically polarized bacterium, *C. crescentus,* also operates in the rod-shaped, seemingly symmetric bacterium, *S. meliloti*. (1) In *C. crescentus*, the *divK* gene locus is organized in an operon with a downstream gene encoding a nonessential response regulator, *pleD* (Hecht et al., 1995). The genetic locus is conserved in *S. meliloti*. Furthermore, $_{sm}$DivK supports viability in a *C. crescentus* Δ*divK* strain, arguing that $_{sm}$DivK is a functional homolog of *C. crescentus* DivK. (2) We have shown that phosphorylation and polar localization are coupled for DivK in *C. crescentus*. This is also true in *S. meliloti* (Figure 4). (3) In *S. meliloti*, $_{sm}$DivK exhibits a dynamic cyclical localization with two consecutive rounds of dispersed distribution and accumulation at the old pole per cell cycle (Figure 5). This cell cycle-controlled localization profile is different from that in *C. crescentus*, but the end result is the same in both organisms: the predivisional cell divides unequally to produce two daughter cells with a different size and a differential distribution of DivK. Similar to the *C. crescentus* progenies, the shorter daughter cell has a uniform cytoplasmic distribution of DivK in *S. meliloti*, while its longer sibling has $_{sm}$DivK polarly localized. The shorter daughter cell reiterates the cell cycle-controlled localization of $_{sm}$DivK described above, whereas the longer daughter cell skips the first cell transition of polar localization (Figure 5B; see also Supplemental Movie S1 at http://www.developmentalcell.com/cgi/content/full/5/1/149/DC1/). Thus, despite their lack of conspicuous differences, *S. meliloti* daughter cells differ in size and in the temporal and spatial regulation of DivK localization in a fashion that parallels that of the *C. crescentus* swarmer and stalked daughter cells, suggesting that asymmetric divisions may be more common in the prokaryotic world than originally thought. The apparent lack of polar organelles in *S. meliloti* also suggests that they are not a prerequisite for the asymmetric localization of DivK and, therefore, are unlikely to participate to the process. (4) Finally, we observed a correlation between high levels of mislocalized $_{sm}$DivK and a cell division defect in *S. meliloti*, arguing that, as in *C. crescentus*, DivK and its spatial regulation may play a critical role in regulating the *S. meliloti* division cycle.

DivK and its *divK-pleD* genetic locus are conserved among most α-proteobacteria for which the genome sequence is available (data not shown; a DivK homolog, CelR1, has been previously identified in *Rhizobium leguminosarum* [Ausmees et al., 1999]). It was difficult to assess with confidence the conservation of DivK across subdivisions of proteobacteria because of the already existing high sequence similarity between single-domain response regulators. In light of our results with *S. meliloti*, other DivK homologs may also exhibit a dynamic polar localization during the cell cycle in other bacteria, culminating in an asymmetric division. Therefore, asymmetric localization of signaling proteins may provide a general mechanism of prokaryotic regulation to control complex signal transduction networks such as those involved in cell cycle control.

Experimental Procedures

Strains, Plasmids, and Media

C. crescentus strains were grown in peptone-yeast extract (PYE complex media), M2G (minimal medium), or M5GG (low-phosphate medium supplemented with 1 mM glutamate) (Ely, 1991; Jacobs et al., 2001). Plasmids were mobilized from *Escherichia coli* strain S17-1 into *C. crescentus* by bacterial conjugation (Ely, 1991). *S. meliloti* strains were grown in LB or in the minimal medium M2Gsm (M2G plus 250 μM $FeCl_2{\cdot}4H_2O$, 20 μM $CaCl_2{\cdot}2H_2O$, 10 μM H_3BO_3, 1 μM $CoCl_2{\cdot}6H_20$, 0.3 μM $CuCl_2{\cdot}2H_2O$, 25 μM $ZnCl_2$, 25 μM $Na_2MoO_4{\cdot}2H_2O$, 2 μM $MnCl_2{\cdot}4H_2O$, 3 μM biotin, and 2.5 μM thiamine). Plasmids were introduced into *S. meliloti* by electroporation. Oxytetracycline and chloramphenicol concentrations for *S. meliloti* were used at 5 μg/ml.

The mode of construction of strains and plasmids (Table 1) as well as the sequences of all primers is available upon request. To determine whether phosphorylation of DivK was essential for viability, we made a φCR30 bacteriophage lysate (Ely, 1991) from strain CJ403 and used it to transduce the Δ*divK*::Spec^R mutation into the strains CB15N, CB15N/pdivK-EGFP, and CB15N/pMR20divK$_{D53A}$-GFP. Δ*divK*::Spec^R transductants were only recovered from CB15N/pdivK-EGFP.

Immunobloting and In Vivo Phosphorylation Experiments

Immunoblot analysis was carried out as described with anti-DivK serum at a dilution of 1/5000 (Jacobs et al., 2001), anti-DivJ serum at 1/15,000 (Wheeler and Shapiro, 1999), and anti-GFP serum at 1/1000 (Covance, NJ). In vivo phosphorylation experiments were performed as described (Jacobs et al., 2001).

Microscopy and Photography

For fluorescence imaging, cell populations of *C. crescentus* or *S. meliloti* strains were placed on a microscope slide that was layered with a pad of 1% agarose containing M2G or M2Gsm (Jacobs et al., 1999). This slide was placed on a microscope stage at room temperature (∼22°C). Samples were observed on a Nikon E1000 microscope through a differential interference contrast (DIC) 100× objective with a Hamamatsu Orca-ER LCD camera. Images were taken and processed with Metamorph software (Universal Imaging, PA). The cell size measurements were done as follows: MB501 cells containing plasmid-encoded $_{sm}$DivK-GFP were grown to log phase in M2Gsm and then placed on an M2Gsm-agarose slide for 1 hr. Only cells in the cell cycle stages illustrated in Figure 5, time points 240 min and 280 min, were considered for cell length measurements. The cell boundaries in the fluorescence images were delimited in an unbiased manner with a masking option in Metamorph. Cell length values for each sibling were obtained from the length of a line drawn parallel to the lateral cell boundaries and along the midline of the cell with Metamorph. Cell length ratios between 140 daughter

cells with polar $_{sm}$DivK-GFP focus and their siblings with even $_{sm}$DivK-GFP distribution were determined by this procedure. The calculated mean of these ratios was 1.12, with a 95% confidence interval from 1.10 to 1.14. A paired t test gave a two-tailed p value inferior to 0.0001.

Acknowledgments

We would like to thank Bill Margolin and Urs Jenal for the gift of the MB501 and CB15N Δ*pleC* strains, respectively. We also thank Adam Arkin, Lucy Shapiro, and the members of the Jacobs-Wagner laboratory for critical reading of the manuscript. This work was supported by the National Institutes of Health.

Received: April 23, 2003
Accepted: April 30, 2003
Published: July 7, 2003

References

Alley, M.R. (2001). The highly conserved domain of the *Caulobacter* McpA chemoreceptor is required for its polar localization. Mol. Microbiol. *40*, 1335–1343.

Ausmees, N., Jonsson, H., Hoglund, S., Ljunggren, H., and Lindberg, M. (1999). Structural and putative regulatory genes involved in cellulose synthesis in *Rhizobium leguminosarum bv. trifolii*. Microbiology *145*, 1253–1262.

Burton, G.J., Hecht, G.B., and Newton, A. (1997). Roles of the histidine protein kinase pleC in *Caulobacter crescentus* motility and chemotaxis. J. Bacteriol. *179*, 5849–5853.

Ely, B. (1991). Genetics of *Caulobacter crescentus*. Methods Enzymol. *204*, 372–384.

Evinger, M., and Agabian, N. (1977). Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. J. Bacteriol. *132*, 294–301.

Hecht, G.B., Lane, T., Ohta, N., Sommer, J.M., and Newton, A. (1995). An essential single domain response regulator required for normal cell division and differentiation in *Caulobacter crescentus*. EMBO J. *14*, 3915–3924.

Hung, D.Y., and Shapiro, L. (2002). A signal transduction protein cues proteolytic events critical to *Caulobacter* cell cycle progression. Proc. Natl. Acad. Sci. USA *99*, 13160–13165.

Jacobs, C., Domian, I.J., Maddock, J.R., and Shapiro, L. (1999). Cell cycle-dependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division. Cell *97*, 111–120.

Jacobs, C., Hung, D., and Shapiro, L. (2001). Dynamic localization of a cytoplasmic signal transduction response regulator controls morphogenesis during the *Caulobacter* cell cycle. Proc. Natl. Acad. Sci. USA *98*, 4095–4100.

Latch, J.N., and Margolin, W. (1997). Generation of buds, swellings, and branches instead of filaments after blocking the cell cycle of *Rhizobium meliloti*. J. Bacteriol. *179*, 2373–2381.

Meisenzahl, A.C., Shapiro, L., and Jenal, U. (1997). Isolation and characterization of a xylose-dependent promoter from *Caulobacter crescentus*. J. Bacteriol. *179*, 592–600.

Ohta, N., Lane, T., Ninfa, E.G., Sommer, J.M., and Newton, A. (1992). A histidine protein kinase homologue required for regulation of bacterial cell division and differentiation. Proc. Natl. Acad. Sci. USA *89*, 10297–10301.

Pleier, E., and Schmitt, R. (1989). Identification and sequence analysis of two related flagellin genes in *Rhizobium meliloti*. J. Bacteriol. *171*, 1467–1475.

Roberts, R.C., Toochinda, C., Avedissian, M., Baldini, R.L., Gomes, S.L., and Shapiro, L. (1996). Identification of a *Caulobacter crescentus* operon encoding *hrcA*, involved in negatively regulating heat-inducible transcription, and the chaperone gene *grpE*. J. Bacteriol. *178*, 1829–1841.

Sciochetti, S.A., Lane, T., Ohta, N., and Newton, A. (2002). Protein sequences and cellular factors required for polar localization of a histidine kinase in *Caulobacter crescentus*. J. Bacteriol. *184*, 6037–6049.

Sommer, J.M., and Newton, A. (1989). Turning off flagellum rotation requires the pleiotropic gene *pleD*: *pleA*, *pleC*, and *pleD* define two morphogenic pathways in *Caulobacter crescentus*. J. Bacteriol. *171*, 392–401.

Sommer, J.M., and Newton, A. (1991). Pseudoreversion analysis indicates a direct role of cell division genes in polar morphogenesis and differentiation in *Caulobacter crescentus*. Genetics *129*, 623–630.

Stock, J.B., Surette, M.G., Levit, M., and Park, P. (1995). Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis. In Two-Component Signal Transduction, J.A. Hoch and T.J. Silhavy, eds. (Washington, D.C.: ASM Press), pp. 5–51.

Terrana, B., and Newton, A. (1975). Pattern of unequal cell division and development in *Caulobacter crescentus*. Dev. Biol. *44*, 380–385.

Viollier, P.H., Sternheim, N., and Shapiro, L. (2002). A dynamically localized histidine kinase controls the asymmetric distribution of polar pili proteins. EMBO J. *21*, 4420–4428.

Wang, S.P., Sharma, P.L., Schoenlein, P.V., and Ely, B. (1993). A histidine protein kinase is involved in polar organelle development in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA *90*, 630–634.

Wheeler, R.T., and Shapiro, L. (1999). Differential localization of two histidine kinases controlling bacterial cell differentiation. Mol. Cell *4*, 683–694.

Wu, J., Ohta, N., and Newton, A. (1998). An essential, multicomponent signal transduction pathway required for cell cycle regulation in *Caulobacter*. Proc. Natl. Acad. Sci. USA *95*, 1443–1448.