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An essential single domain response regulator required for normal cell division and differentiation in *Caulobacter crescentus*

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Signal transduction pathways mediated by sensor histidine kinases and cognate response regulators control a variety of physiological processes in response to environmental conditions. Here we show that in *Caulobacter crescentus* these systems also play essential roles in the regulation of polar morphogenesis and cell division. Previous studies have implicated histidine kinase genes *pleC* and *divJ* in the regulation of these developmental events. We now report that *divK* encodes an essential, cell cycle-regulated homolog of the CheY/SpoOF subfamily and present evidence that this protein is a cognate response regulator of the histidine kinase PleC. The purified kinase domain of PleC, like that of DivJ, can serve as an efficient phosphodonor to DivK and as a phospho-DivK phosphatase. Based on these and earlier genetic results we propose that PleC and DivK are members of a signal transduction pathway that couples motility and stalk formation to completion of a late cell division cycle event. Gene disruption experiments and the filamentous phenotype of the conditional *divK341* mutant reveal that DivK also functions in an essential signal transduction pathway required for cell division, apparently in response to another histidine kinase. We suggest that phosphotransfer mediated by these two-component signal transduction systems may represent a general mechanism regulating cell differentiation and cell division in response to successive cell cycle checkpoints.

Keywords: *Caulobacter*/cell division/differentiation/signal transduction

Introduction

Receptor-mediated signal transduction pathways are a central mechanism controlling cellular responses to stimuli. One of the best understood of these systems is the so-called two-component system, which typically contains a histidine kinase, or sensor protein, that initiates signal transduction by autophosphorylation at a conserved histidine residue. Autophosphorylation is followed by phosphotransfer to a conserved aspartate of the cognate response regulator, which serves to regulate the activity of a target protein or the expression of one or more target genes. Two-component systems control a wide array of physiological processes in bacteria in response to a variety of environmental conditions (reviewed in Stock *et al.*, 1989; Parkinson and Kofoid, 1992; Volz, 1993) and recently they have been implicated in the control of mitogen-activated protein (MAP) kinases in both *Saccharomyces cerevisiae* and *Arabidopsis thaliana* (Chang *et al.*, 1993; Ota and Varshavsky, 1993; Maeda *et al.*, 1994). Two histidine kinase genes described in the aquatic bacterium *Caulobacter crescentus*, *divJ* and *pleC*, have been shown to regulate morphogenic events that occur independently of the environment (Ohta *et al.*, 1992; Wang *et al.*, 1993) and *divJ* also appears to function in cell division (Sommer and Newton, 1991; Ohta *et al.*, 1992). The work described here, which was undertaken to further define the roles of these histidine kinases, has now identified an essential response regulator that functions with PleC in signal transduction pathways to regulate both cell differentiation and division.

Differentiation in *C.crescentus* involves a series of discrete morphogenic events and results in an asymmetric cell division that produces an old stalked cell and a new, motile swarmer cell. These events, which occur at the one pole of the stalked cell during the course of the cell division cycle, include flagellum biosynthesis, assembly of polar bacteriophage receptor sites and the start of flagellum rotation (Figure 1A). Development continues after cell division at the flagellated pole of the new swarmer cell with pili formation, loss of motility and stalk formation (see description in Figure 1A; reviewed in Newton and Ohta, 1990; Lane *et al.*, 1995; Brun *et al.*, 1994).

Analysis of conditional cell division cycle mutants has suggested that successive steps in the cell division and DNA synthetic pathways are checkpoints for specific developmental events and in this way the cell cycle effectively acts as a developmental 'clock' (see Figure 1 for details; Osley and Newton, 1980). Thus DNA synthesis is required for flagellum formation (Sheffery and Newton, 1981; Stephens and Shapiro, 1993), completion of the late cell division step division progression (DIVp; Figure 1C) is necessary for the gain of motility (Huguenel and Newton, 1982) and completion of cell separation (CS) is necessary for pili formation (Sommer and Newton, 1988). Insight into how development is regulated by these cell cycle events has come from studies of mutations in the pleiotropic gene *pleC* that display a phenotype similar to that of division mutants blocked early in the cell cycle. The observation that both *pleC* and DIVp mutants are arrested in the developmental program after flagellum biosynthesis, but prior to motility and stalk formation (Figure 1B and C), originally suggested that the *pleC* gene product might act in response to a late cell division checkpoint for motility and subsequent developmental events (Sommer and Newton, 1991).

Pseudoreversion analysis of a temperature sensitive (Ts)

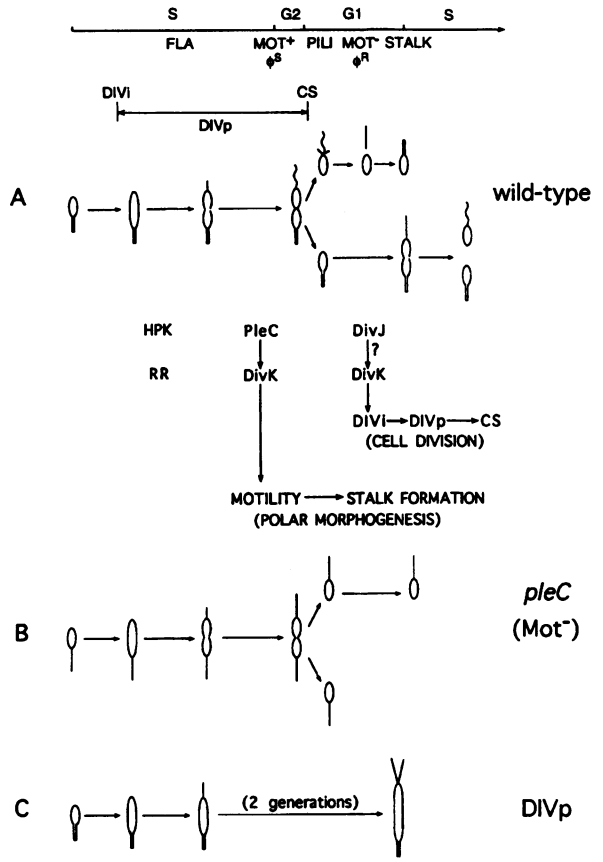

Fig. 1. *Caulobacter crescentus* cell cycle. The sequence of developmental events includes flagellum formation (FLA), appearance of polar bacteriophage receptors (ϕ^S), motility or flagellum rotation (MOT^+), pili formation (PILI), loss of motility (MOT^-), loss of bacteriophage receptors (ϕ^R) and stalk formation (STALK). Cell cycle events correspond to DNA synthesis (S), post-synthetic gap (G_2), pre-synthetic gap (G_1), division initiation (DIVi), division progression (DIVp) and cell separation (CS). (**A**) Cell cycle for wild-type strain CB15 is shown above the working model for PleC, DivK and DivJ in cell division and polar morphogenesis. The response regulator (RR) DivK acts at two different times in the cell cycle: late in the cell cycle DivK responds to the histidine kinase (HPK) PleC to initiate motility and stalk formation and early in the next cell cycle it responds to another sensor HPK, shown here as DivJ, to regulate cell division (for details see text). (**B**) Non-motile *pleC* mutants assemble inactive flagella (designated as straight lines), are bacteriophage φCbK resistant and fail to shed the flagellum and form stalks, but they divide normally. (**C**) Cells blocked before the completion of DIVp form long unpinched filaments and, like *pleC* mutants, are arrested in development before gain of motility and stalk formation (Huguenel and Newton, 1982).

pleC mutation identified cold sensitive (Cs) suppressors that restore motility and normal development at 37°C and also confer a filamentous cell division defect at 25°C. These suppressors map to the three new cell division (*div*) genes *divJ*, *divK* and *divL* (Sommer and Newton, 1991). The *divJ* (Ohta *et al.*, 1992) and *pleC* (Wang *et al.*, 1993) genes encode predicted histidine kinases and we now describe experiments demonstrating that *divK* encodes a 125 residue protein homologous to a subfamily of small response regulators. Both genetic and biochemical experiments presented below show that the DivK protein behaves as a cognate response regulator of the PleC kinase and we propose that PleC and DivK are members of a signal transduction pathway required for motility and stalk

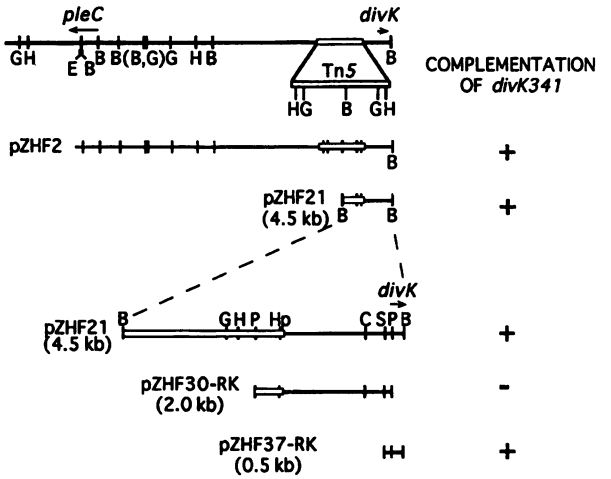

Fig. 2. Location of the *divK* gene by complementation. Restriction map of the *pleC–divK* region of the chromosome is at the top, showing the direction of *pleC* and *divK* transcription (arrows) and the linked *zhf372*::Tn*5* transposon. DNA fragments from plasmid pZHF2 were subcloned into pRK2L1 (Ohta *et al.*, 1990) and examined for complementation of the Div^{Cs} defect in strain PC4175 (*divK341 recA526 zbe406*::Tn*5*) in liquid culture after growth at the non-permissive temperature of 25°C for at least 12 h. +, complementation; – no complementation. Open boxes indicate Tn*5* sequences. B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; P, *Pst*I; S, *Sst*I.

formation, developmental events controlled by a late cell division cycle checkpoint. We also describe experiments demonstrating that *divK* is an essential gene required for an early step in cell division. Thus the *divK* gene product may function in two different signal transduction pathways to control both polar morphogenesis and cell division (Figure 1A). The essential role of DivK in regulating the cell cycle sets it apart from previously described response regulators that control cellular responses to environmental conditions.

Results

Cloning of the divK gene

To clone the *divK* gene we isolated cosmid clones containing Tn*5* insertion element *zhf372*::Tn*5* (Materials and methods), which is 83% linked by transduction to *divK*, and screened 12 of them for complementation of the Div^{Cs} phenotype of the *divK341* allele. Cosmid pZHF2 restored normal division to *divK341* strains at 25°C (see Figure 2) and it was used in all subsequent experiments.

We located the *zhf372*::Tn*5* insertion on the pZHF2 insert by DNA restriction site analysis (Figure 2; Jorgenson *et al.*, 1979) and partial nucleotide sequencing (data not shown). Subcloning experiments showed that plasmid pZHF37-RK, which carries a 0.5 kb *Sst*I–*Bam*HI fragment, is sufficient to complement *divK341* in a *rec* (O'Neill *et al.*, 1985) background (Figure 2), suggesting that this sequence contains the intact *divK* gene and all transcriptional regulatory sequences necessary for its expression. We confirmed that the cloned insert is not a multicopy suppressor by subcloning the 0.5 kb *Sst*I–*Bam*HI fragment into the integrative vector pGH500 and showing that it fully complemented the Div^{Cs} phenotype of *divK341* when maintained as a stable co-integrant (data not shown).

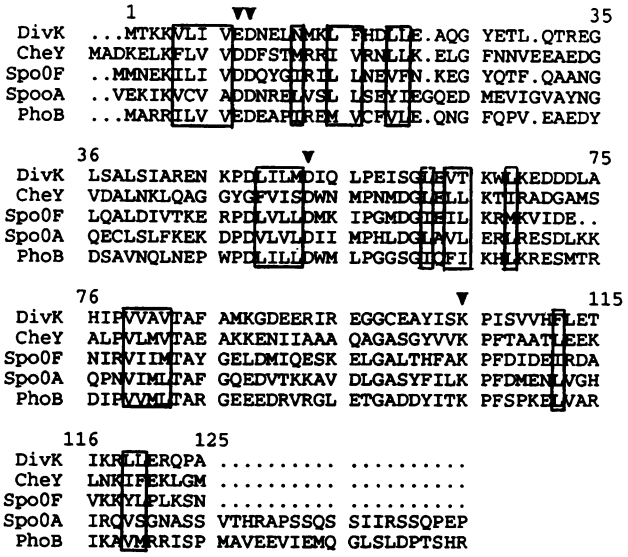

Fig. 3. Alignment of the translated *divK* sequence with CheY (Stock *et al.*, 1985) and Spo0F (Trach *et al.*, 1988) and the response regulator domains of Spo0A (Ferrari *et al.*, 1985) and PhoB (Makino *et al.*, 1986). Arrowheads designate the invariant residues of the response regulator family (Volz, 1993); conserved hydrophobic regions are boxed (Stock *et al.*, 1989). Numbering refers to amino acid residues in DivK.

divK encodes a single domain response regulator

The nucleotide sequence of the insert in pZHF37-RK (Figure 2B) was determined on both strands. A single long open reading frame (ORF) was identified which encodes a predicted protein of 125 amino acids and displays a codon usage typical of CB15 genes characterized previously (T.Lane and A.Newton, unpublished results). We have located the *divK341* mutation within this ORF (G.B.Hecht and A.Newton, in preparation), which confirms that it corresponds to *divK*.

Analysis by the method of Lipman and Pearson (1985) revealed extensive homology between the translated *divK* sequence and the N-terminal regulatory domains of the large family of bacterial response regulators that are phosphorylated by histidine protein kinases (Figure 3; Stock *et al.*, 1989; Parkinson and Kofoid, 1992; Volz, 1993). Phosphorylation by cognate histidine kinases typically controls the activity of C-terminal domains present in most response regulators. DivK, however, belongs to a small subfamily of response regulators that contain only the conserved regulatory domain (Figure 3), a group of proteins that includes the 125 residue CheY chemotaxis protein (Stock *et al.*, 1985) and the 123 residue *Bacillus subtilis* Spo0F protein (Trach *et al.*, 1988), to which DivK is 25 and 31% identical respectively. In particular, the Glu9, Asp10, Asp53 and Lys105 residues of DivK (arrowheads in Figure 3) correspond to the four typically invariant residues found in response regulators, aligning with Asp12, Asp13, Asp57 (the site of phosphorylation; Sanders *et al.*, 1989) and Lys109 of CheY. DivK contains other sequence features characteristic of the response regulators, including the hydrophobic sequence motifs (boxed regions in Figure 3) necessary for proper folding of response regulators (reviewed in Stock *et al.*, 1989; Parkinson and Kofoid, 1992; Volz, 1993). Genetic data (Sommer and Newton, 1991), biochemical experiments and the regulation of *divK* expression (see below) support the conclusion that DivK functions as a cognate response regulator of the histidine kinase PleC and we discuss below implications of the single domain structure of DivK for its mechanism of action (see Discussion).

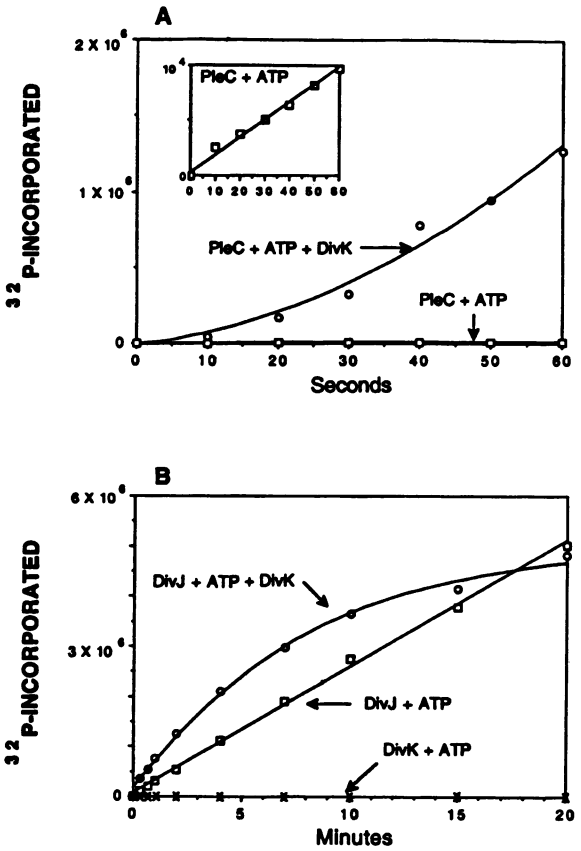

Fig. 4. Autophosphorylation of PleC′ and DivJ′ and phosphotransfer to DivK. (**A**) Autophosphorylation of PleC′ (□) was measured in the presence of PleC′ (100 pmol) and [γ-^{32}P]ATP (20 pmol); phosphotransfer to DivK (○) was measured in an identical reaction mixture to which DivK (100 pmol) protein was added. (**B**) Autophosphorylation of DivJ′ (□) was measured in the presence of DivJ′ (100 pmol) and [γ-^{32}P]ATP (20 pmol) and phosphotransfer to DivK (○) was measured in an identical reaction to which DivK (100 pmol) was added. No phosphorylation of DivK by ATP was detected when PleC′ (data not shown) or DivJ′ (×, panel B) was omitted from the reaction. Samples were withdrawn from reaction mixtures at the times indicated and the levels of phospho-PleC′, phospho-DivJ′ and phospho-DivK were determined as described in Materials and methods.

Catalytic activities of purified PleC′, DivJ′ and DivK proteins

Response regulators function in response to the activities of histidine kinases which regulate their levels of phosphorylation. The pseudoreversion analysis of *pleC* (Sommer and Newton, 1991) has implicated the histidine kinases PleC (Wang *et al.*, 1993) and DivJ (Ohta *et al.*, 1992) in the regulation of DivK and we examined this possibility directly using purified proteins to assay the catalytic activities of PleC and DivJ in the phosphorylation of DivK. The full-length 125 amino acid *divK* gene product was purified as a soluble protein after overexpression in *Escherichia coli* cells, while the catalytic domains of the 842 residue PleC protein (PleC′, a 313 residue C-terminal

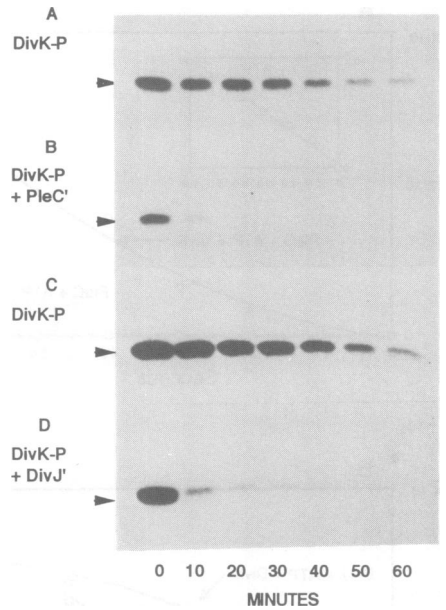

Fig. 5. Stability of phospho-DivK in the presence and absence of PleC′ and DivJ′. Purified phospho-DivK (400 pmol) was incubated alone (**A** and **C**) or in the presence of 40 pmol PleC′ (**B**) or DivJ′ (**D**) as described in Materials and methods. Samples were withdrawn at the times indicated and the amount of remaining phospho-DivK was visualized after electrophoresis by autoradiography. The position of the band corresponding to phospho-DivK is indicated by the arrowhead.

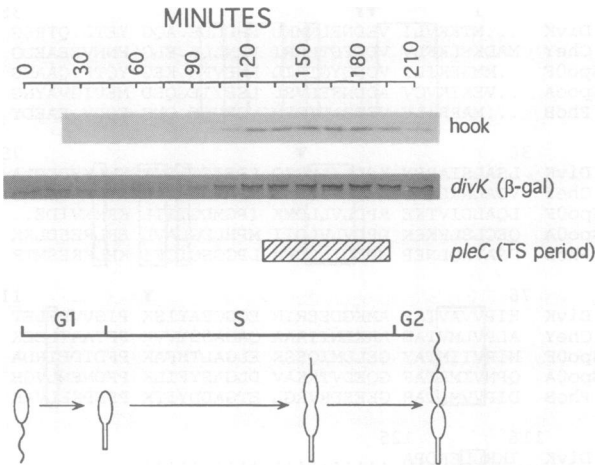

Fig. 6. Cell cycle regulation of the *divK* promoter. Synchronous cells of strain CB15F/pANZ443 were pulse-labeled with [^{35}S]methionine for 9 min at the times indicated. The rates of *lacZ* expression driven by the *divK* promoter and flagellar hook protein synthesis were determined by immunoprecipitation (Materials and methods; Sheffery and Newton, 1981; Minnich and Newton, 1987). The Ts period of the *pleC319* mutation for the gain of motility and stalk formation is indicated by the hatched box (Sommer and Newton, 1989).

polypeptide) and the 596 residue DivJ protein (DivJ′, a 286 residue C-terminal polypeptide) were purified as poly(His)-tagged proteins, as described in Materials and methods. Examination of the reaction kinetics shows that PleC′ (Figure 4A) and DivJ′ (Figure 4B) are autophosphorylated in the presence of ATP and Mg^{2+} and that the phosphate is rapidly transferred to DivK when the response regulator is added to the reaction. Ely and coworkers have reported previously the *in vitro* phosphorylation of a LacZ–PleC fusion protein (Wang *et al.*, 1993). In other experiments we have shown that purified phospho-PleC′ or phospho-DivJ′ will also serve as a phosphodonor to DivK (data not shown), but that the purified DivK protein is not phosphorylated in the presence of ATP alone (Figure 4B).

The kinetics of phosphotransfer to DivK by the two kinases are strikingly different. The initial rate of DivK phosphorylation was ~100-fold higher than can be accounted for by the rate of PleC′ autophosphorylation when DivK was not present in the reaction mixture (Figure 4A). Thus there appears to be a strong synergistic effect between PleC′ and DivK on PleC′ autophosphorylation. A similar, but much weaker, effect was observed between DivJ′ and DivK (Figure 4B).

We also examined the stability of phospho-DivK (Figure 5). Purified PleC′ (Figure 5B) and DivJ′ (Figure 5D) each decreased the stability of phospho-DivK, which shows that both kinases have phosphatase activity that recognizes phospho-DivK as a substrate. These results and those in Figure 4 demonstrate that PleC and DivJ display the autokinase, transphosphorylase and protein phosphatase activities required to regulate levels of phospho-DivK. They are also consistent with our conclusion from the pseudoreversion analysis of *pleC* that DivK is the cognate response regulator of the histidine kinase PleC (Sommer and Newton, 1991; see Discussion).

Cell cycle regulation of divK expression

The patterns of periodically expressed mammalian cell cycle genes frequently mirror the times at which the individual gene products are required (reviewed in Müller, 1995). We have also observed that the *C.crescentus dnaC* (Ohta *et al.*, 1990) and *divJ* (Ohta *et al.*, 1992) genes, which are required for DNA synthesis and an early cell division step respectively, are also under cell cycle control and expressed maximally at the G_1–S phase boundary. Consequently we have examined the regulation of *divK* expression using plasmid pANZ443, which carries a transcriptional fusion of the *divK* promoter to the *lacZ* reporter gene, and measured activity of the *divK* promoter during the cell cycle by determining the rate of β-galactosidase synthesis in a synchronous culture of strain CB15F/pANZ443 (Materials and methods). Transcription of *divK*, which was periodic and peaked at about the same time as hook protein synthesis, began at ~105 min and was restricted to a period from 0.5 to ~0.95 of the swarmer cell cycle (Figure 6). This period of synthesis includes the time that the *pleC* gene product is required for flagellum rotation and stalk formation (0.6–0.95 of the cell cycle, as shown in Figure 6; Sommer and Newton, 1989). The coincidence of *divK* expression with the temperature-sensitive period for PleC activity is consistent with the functional relationship proposed above for PleC and DivK.

Cell division and developmental phenotypes of divK341

The *divK341* allele suppresses the non-motile phenotype of *pleC* mutations at 37°C, but at 25°C it causes extensive filamentation. DNA synthesis is not affected at the non-permissive temperature, however, and thus the primary effect of this mutation appears to be on cell division

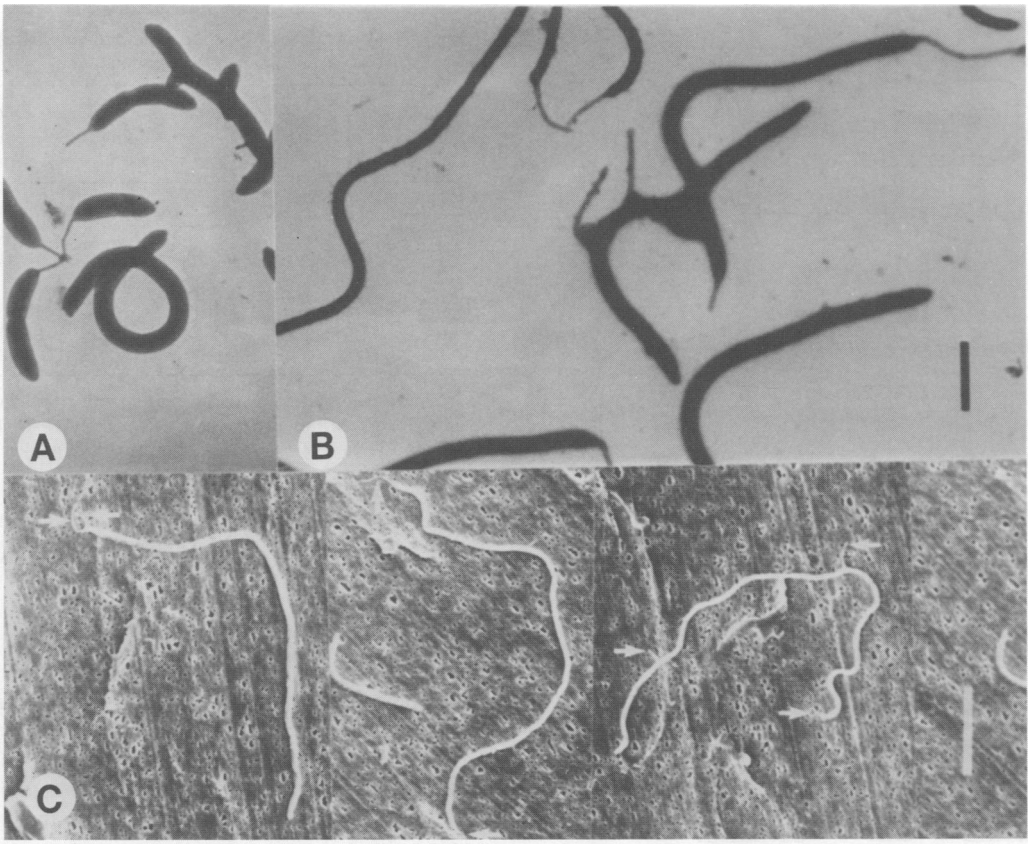

Fig. 7. Cell division phenotype of strain PC4160 (*divK341*). (**A**) Transmission electron micrograph of cells grown at the permissive temperature of 37°C. (**B**) Transmission electron micrograph after growth at the non-permissive temperature of 25°C for ~12 h. Bar, 2 mm for (A) and (B). (**C**) Scanning electron micrograph after growth at 25°C for ~12 h. Arrows indicate unusual stalk phenotypes. Bar, 5 mm.

(Sommer and Newton, 1991), a function not previously associated with prokaryotic response regulators. To define the terminal cell division phenotype we examined strain PC4160 (*pleC*$^+$ *divK341*) by scanning and transmission electron microscopy. In cultures grown at 25°C (Figure 7B and C) >95% of the cells were straight, unpinched, unflagellated filaments that ranged in length from 10 to 56 μm. At the permissive temperature of 37°C, however, stalked cells were ~2 μm long and the patterns of division, flagellum biosynthesis and stalk formation were normal (Figure 7A).

The unpinched phenotype of the *divK* mutant is a characteristic previously shown to be typical of cells blocked very early in cell division, at DIVi (Huguenel and Newton, 1982), but the polar morphology of this strain sets it apart from previously described cell division mutants in two respects. First, a large fraction of the filaments (22%) had more than one stalk and many of these were stalked at both poles (Figure 7B and C), indicating that the cells had completed the last stage of polar morphogenesis. This phenotype is normally associated with highly pinched filamentous cells blocked at CS, the last stage of cell division (Huguenel and Newton, 1982). Thus, as considered in the Discussion, mutations in *divK* appear to disrupt the tight coupling normally observed between cell division and the formation of new stalks. We also observed that the second stalk was frequently not polar, but protruded instead from an envelope bleb at the side of the cell (arrows, Figure 7C), a developmental pattern not reported in other cell division mutants (Osley and Newton, 1980; Huguenel and Newton, 1982).

The second unusual feature of the filamentous *divK* mutants was the absence of flagella at either of the cell poles or in the culture medium, as determined by transmission electron microscopic studies (Figure 7B) and Ryu staining (Kodaka *et al.*, 1982; data not shown). Failure to synthesize the polar flagellum has been associated with mutants blocked in DNA synthesis (Sheffery and Newton, 1981; Stephens and Shapiro, 1993), but not with mutants blocked in cell division (Huguenel and Newton, 1982). Although flagellin proteins were not detected by Western analysis of cell cultures (G.B.Hecht and A.Newton, unpublished), we know that unassembled flagellar proteins are unstable *in vivo* (Kornacker and Newton, 1994) and additional experiments will be required to determine whether flagellum assembly or gene expression is affected by the *divK* mutation.

Disruption of divK requires a second copy of the gene

The cell division phenotype of *divK* mutants suggests an essential role for the DivK protein. To test this possibility we attempted to construct a disruption of *divK*. A plasmid-borne copy of a *divK* null allele containing the gentamicin-resistant (Gm^R) *aacC1* cassette (Schweizer, 1993) was first integrated into the chromosome by a single crossover within the *divK* sequence or flanking DNA to generate a disrupted copy and an intact copy of the gene (crossover i or ii, Figure 8A). Replacements were constructed in a

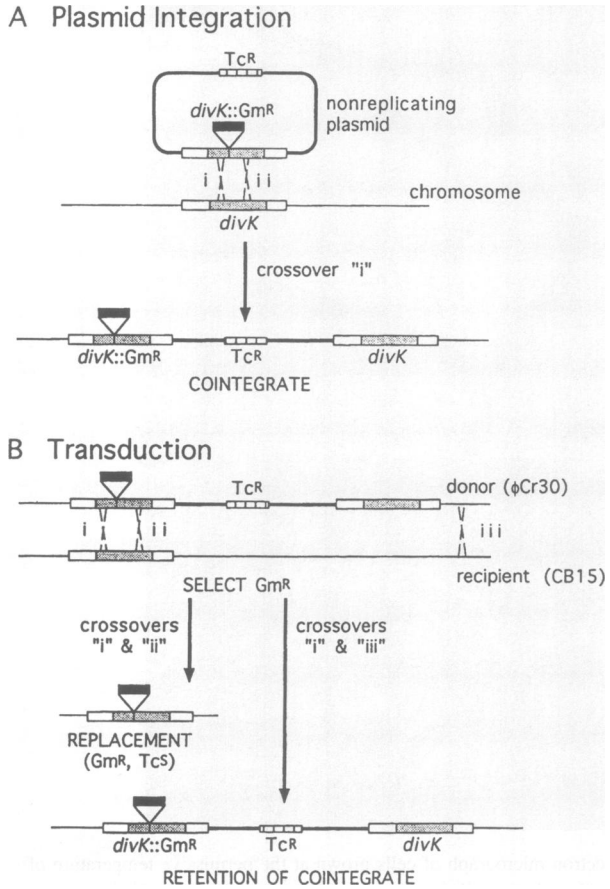

Fig. 8. Strategy for gene replacement of *divK* in *C.crescentus*. (**A**) Co-integrate formation. A non-replicative plasmid carrying a Tc^R marker and the *divK*::*aacC1* allele was introduced into wild-type *C.crescentus* strain CB15. A single crossover (i or ii) yields co-integrates carrying one intact and one disrupted copy of *divK*. Shaded box indicates *divK* ORF. (**B**) Replacement by transduction. A lysate of the co-integrate was prepared and used to transduce either strain CB15 (wild-type) or strain CB15/pZHF37-RK (see text). Selection for the disruptive Gm^R marker should give only two recombinant classes: replacements (crossover events i and ii to excise the integrated plasmid; Gm^R Tc^S) and maintenance of the co-integrate (crossover events i and iii; Gm^R Tc^R).

second step (Figure 8B) by transducing the gene duplication into wild-type strain CB15 (Minnich *et al.*, 1988), selecting for the disrupting Gm^R marker and screening for loss of the linked plasmid marker (crossovers i and ii). In one set of experiments we screened a total of 2100 transductants isolated at 25, 30 and 37°C for loss of the plasmid tetracycline resistant (Tc^R) marker. None of the recombinants yielded potential gene replacements (Gm^R Tc^S), which is consistent with an essential function for the *divK* gene (data not shown).

To eliminate the possibility that failure to recover a *divK* replacement resulted from the inability of DNA in the *divK* region to undergo the requisite recombination events, we carried out a second set of replacement experiments in the presence of an additional, functional copy of *divK* on a replicating plasmid. A lysate was prepared on a co-integrant strain made using a kanamycin-resistant (Km^R) integrative plasmid (see Materials and methods) and used to transduce strains containing either the Tc^R pZHF37-RK (*divK*$^+$) plasmid or the pRK2L1 vector. Gm^R recombinants were selected and screened for loss of the

Table I. Construction of *divK* null allele in the chromosome in the presence of a *divK* complementing plasmid

Transduction recipient	Temperature (°C)	Gm^R Tc^R transductants (total)[a]	Frequency of gene replacement (Km^S/Gm^R)[b]
CB15/pRK2L1 (vector)	37	191	0/191 (0%)
	30	540	0/540 (0%)
	24	351	0/351 (0%)
CB15/pZHF37-RK (*divK*$^+$)	37	68	27/68 (40%)
	30	469	249/469 (53%)
	24	545	171/545 (31%)

[a]φCr30 transduction was carried out using a donor strain carrying an integrated copy of the *divK*::*aacC1* allele (Figure 8A). Transductants were selected on PYE + gentamicin (disruptive marker) + Tc (replicating vector).
[b]Because the integrated vector confers Km^R, the Km^R Gm^R transductants carry the co-integrate construct from the donor strain. Replacement of the wild-type chromosomal copy of *divK* was indicated by the appearance of Km^S Gm^R transductants. For more details see text and Figure 8.

Km^R vector marker. Table I shows that disruptions (Gm^R Km^S transductants) were obtained at a frequency of 31–53% in the CB15/pZHF37-RK (*divK*$^+$) strain, depending on the temperature examined. In contrast, all 1082 Gm^R transductants in the CB15/pRK2L1 (vector) strain were Km^R. We conclude from these results that replacements occur only in the presence of a second functional copy of *divK*. None of the pZHF37-RK plasmids in these Gm^R Km^S strains conferred Gm^R when they were transferred into *E.coli* (data not shown), which indicates that the *divK*::*aacC1* gene replacement had occurred on the chromosome. We confirmed this conclusion and the location of the *aacC1* insertion within the *divK* gene by genomic Southern analysis (data not shown).

Discussion

Two-component systems play a central role in receptor-mediated signal transduction in bacteria, where they regulate responses to environmental changes (Stock *et al.*, 1989; Parkinson and Kofoid, 1992). Our results now suggest that these systems also respond to internal cell cycle cues required for polar morphogenesis and play an essential role in the control of cell division. We have identified the predicted *C.crescentus divK* gene product as a single domain response regulator. Genetic results and biochemical experiments presented here provide evidence that the DivK protein and the histidine kinase PleC are members of a signal transduction pathway that couples motility and stalk formation to the postulated cell division cycle clock (Osley and Newton, 1980; Newton and Ohta, 1990). We also show that *divK* is an essential gene and present results suggesting a second function of the DivK protein in regulation of an early cell division step. The indispensable role of the *divK* gene product in a signal transduction pathway required for cell division make it unusual, if not unique, among the reported bacterial response regulators. The essential nature of *divK* also underscores the potential of the two-component systems as targets for new classes of antibiotics (Roychoudhury *et al.*, 1993).

The PleC–DivK signal transduction pathway

The isolation of bypass suppressors of mutations in histidine kinase genes has been a useful approach to the identification of corresponding cognate response regulator genes (Parkinson, 1995). The kinase PleC is required for motility in *C.crescentus* cells and the strongest evidence that PleC and DivK are cognate kinase–response regulator proteins is the original isolation of *divK341* as a bypass suppressor of the non-motile phenotype of the Ts *pleC319* mutation (Sommer and Newton, 1991) and the identification of four additional Cs alleles of *divK* as suppressors of a *pleC*::Tn*5* null mutation (G.B.Hecht and A.Newton, in preparation). Supporting this conclusion is the demonstration that PleC′ mediates the transphosphorylation of purified DivK in the presence of ATP (Figure 4A). Although *in vitro* phosphorylation of response regulators by non-cognate kinases is known to occur (Ninfa *et al.*, 1988), our results also show that PleC′ exhibits phospho-DivK phosphatase activity (Figure 5B). In addition, we have demonstrated that *divK* is under cell cycle regulation and is maximally expressed at the time PleC is required for motility and stalk formation (Figure 6). Based on the completely non-motile phenotype displayed by *pleC* null mutations (Sommer and Newton, 1989; G.J.Burton *et al.*, in preparation), there is also no genetic evidence for a role of another histidine kinase activity in motility regulation during the critical late S–G_2 phase, when the cells gain motility (Figure 6). None of these results individually proves that PleC is responsible for regulating the level of phospho-DivK *in vivo*, but taken together they do strongly support this conclusion.

How does DivK function to regulate motility and stalk formation? Analysis of Spo0F and CheY, the two single domain response regulators that have been extensively characterized, suggests that DivK could function either as a phospho-relay protein or by direct interaction with a target protein. Spo0F is phosphorylated by the KinA and KinB kinases early in *B.subtilis* sporulation and then acts as a phospho-relay protein to phosphorylate Spo0B (Burbulys *et al.*, 1991). This phosphotransferase in turn phosphorylates Spo0A, a canonical response regulator that controls transcription of several target genes (Burbulys *et al.*, 1991). In contrast, phosphorylation of CheY induces a conformational change which allows the protein to physically interact with flagellar switch proteins and change the direction of flagellar rotation (reviewed in Lukat and Stock, 1993). If DivK functions in a phospho-relay, then it is likely that the *divK341* mutant allele encodes a protein with altered specificity that allows it to be phosphorylated by a histidine kinase not normally involved in the regulation of motility, a mechanism reported recently in the *B.subtilis* phospho-relay system (LeDeaux and Grossman, 1995). If DivK acts by direct interaction with an effector protein, then the same mechanism of suppression could apply or, alternatively, the DivK341 protein could mimic the conformation of the active, presumably phosphorylated, protein. To elucidate the mechanism of DivK function we are currently using genetic approaches to identify the downstream target(s) of the PleC–DivK pathway.

Cell cycle checkpoints and regulation of development

Although sensor proteins in two-component systems typically respond to extracellular or environmental signals, all of our observations are consistent with the idea that PleC controls motility and stalk formation in response to autogenous signals generated by progress through the cell cycle. Internal cues regulating the sequence of developmental events have been described in a number of organisms (reviewed in Horvitz and Herskowitz, 1992) and in *C.crescentus* we have previously proposed that these cues are provided by the completion of steps in the cell division and DNA synthetic pathways (Huguenel and Newton, 1982; Newton and Ohta, 1990). In particular, we have shown that activation of motility and stalk formation requires completion of DIVp (Huguenel and Newton, 1982; Figure 1C), a cell cycle checkpoint coincident with maximal *divK* expression and the time in the cell cycle that PleC function is required for development (Figure 6). Thus, as suggested below (see also Figure 1A), it seems likely that the function of the PleC–DivK signal transduction pathway in the regulation of motility and stalk formation is to couple these developmental events to completion of a cell division checkpoint, in this case DIVp.

Signal transduction by two component systems, as described for PleC–DivK, may provide a general mechanism for coupling morphogenesis to progression through the cell cycle. The reported cell cycle-dependent phosphorylation of FlbD, a response regulator required for transcriptional activation of flagellar genes at the bottom of the *C.crescentus* flagellar hierarchy (Ramakrishnan and Newton, 1990; Benson *et al.*, 1994), may be another example (Wingrove *et al.*, 1993), although a cognate kinase for FlbD has not been identified. Regulation by cell cycle checkpoints is, however, widespread in eukaryotic cells. For example, DNA damage in budding yeast induces a MEC1- and MEC2-mediated block in the cell cycle that prevents entry into mitosis (Weinert *et al.*, 1994) and the MAD protein represents a checkpoint that allows progression from metaphase to anaphase only in the presence of correct spindle and microtubule structures (Li and Murray, 1991).

DivK and the regulation of cell division

In addition to its requirement for motility and stalk formation, the filamentous phenotype of the *divK341* mutant suggests that DivK is also required for an early cell division step (Figure 7). Consistent with two functions of the DivK protein are the results of genetic complementation showing that *divK* mutations are gain of function alleles for suppression of the *pleC* motility phenotype (G.B.Hecht and A.Newton, in preparation) and loss of function alleles for the division phenotype (Figure 2). Also supporting a dual function of the DivK protein is the observation that some *divK* filaments have multiple stalks (Figure 7B and C), which is not expected in mutants blocked at an early stage of cell division (Figure 1C). We suggest that at 25°C, where the regulation of cell division by *divK* is lost, the mutant response regulator is still able to bypass the DIVp checkpoint and activate polar differentiation, as it does in *pleC* mutants. Thus under these conditions the DivK341 protein appears to effectively 'uncouple' morphogenesis from normal checkpoint control and allow stalk formation. This explanation might also account for lateral stalk formation at what are presumably incipient division sites (Figure 7B and C).

How is the function of DivK in cell division regulated?

The observation that *divK* is an essential gene (Table I), while *pleC* is not (G.J.Burton *et al.*, in preparation), argues that DivK must respond to a second histidine kinase. One candidate is DivJ (Ohta *et al.*, 1992). The *divJ* gene, like *divK*, was identified in the pseudoreversion analysis of *pleC* (Sommer and Newton, 1991) and conditional mutations in *divJ* display a largely unpinched, filamentous phenotype at the non-permissive temperature that is similar to *divK* mutants (Ohta *et al.*, 1992). The observations that the C-terminal domain of DivJ catalyzes phosphotransfer to DivK (Figure 4B) and dephosphorylation of purified phospho-DivK (Figure 5D) and that *divJ* expression is restricted to the late G_1–early S phase period of the cell cycle, when DivK is presumed to function in division (Ohta *et al.*, 1992), are also consistent with the involvement of DivJ in the regulation of DivK. Multiple histidine kinases have been described previously in the regulation of *B.subtilis* sporulation (Burbulys *et al.*, 1991) and in nitrate- and nitrite-regulated gene expression in *E.coli* (reviewed in Stewart, 1993), but not in the regulation of essential cell cycle genes.

In Figure 1A we speculate that PleC phosphorylates DivK to turn on motility late in the cell cycle and that another histidine kinase, shown here as DivJ, functions early in the subsequent cell cycle to alter the level of phospho-DivK to permit cell division to occur. The timing of histidine kinase activities by transit through successive cell cycle checkpoints could in this way account for the temporal regulation of the two different activities ascribed here to the DivK protein. A detailed genetic analysis is needed to elucidate more fully the role of DivJ in DivK regulation, given that our working model is necessarily an oversimplification. In addition to the genes considered in Figure 1A, other genes identified in the pseudoreversion analysis of *pleC* (Sommer and Newton, 1989, 1991) have not been fully characterized and their gene products are also likely to be involved in the regulation of this complex signal transduction network. One of these, *pleD*, which is also known to be involved in the regulation of motility and stalk formation (Sommer and Newton, 1989), has recently been shown to encode a compound response regulator with a C-terminal domain belonging to new family of 'GGDEF' proteins (G.B.Hecht and A.Newton, submitted; reviewed in Lane *et al.*, 1995).

Conclusions

The predicted structures and observed biochemical activities of histidine kinases PleC and DivJ and response regulator DivK discussed above are typical of those described for homologous proteins in other bacterial two-component systems. What sets the *C.crescentus* proteins apart is, first, their requirement for developmental events and an essential cell division function and, second, the regulation of their activities by internal cell cycle cues, rather than any identified environmental signal. The roles played by these proteins in *C.crescentus* may not be unique and it is possible that similar two-component switches are a general feature of cell cycle or developmental regulation in prokaryotes and in other organisms, as already observed in the ETR1 and SLN1/SSK1 two-component systems described in plants and yeast (Chang *et al.*, 1993; Maeda *et al.*, 1994). Study of the dual roles of *divK* in the regulation of cell differentiation and division promises to provide insights into how the emerging family of histidine kinase–response regulator networks controls these processes.

Materials and methods

Bacterial strains, plasmids and growth

All *C.crescentus* strains described in the text were derived from CB15 (ATCC 19089). Replicative and integrative plasmids were transferred to *C.crescentus* strains by triparental crosses (Ohta *et al.*, 1984). *Escherichia coli* strains MC1061 and HB101 were used as hosts for cloning and mating and XL1-Blue (Stratagene) for some cloning and for rescuing phage from pBluescript (Stratagene) clones. *Caulobacter crescentus* strains were grown in PYE rich medium or in M2 minimal medium supplemented with 2 μg/ml gentamicin or other antibiotics, as described (Ohta *et al.*, 1984). *Escherichia coli* strains were grown in ML medium (Ohta *et al.*, 1984) supplemented with antibiotics where indicated.

Cloning, complementation and sequencing of divK

The pLAFR1-7 cosmid library containing random inserts of DNA from *C.crescentus* strain PC5468, which contains Tn*5* 83% linked to the wild-type *divK* gene, was constructed in *E.coli* (Ohta *et al.*, 1990). Cosmids containing DNA with the linked Tn*5* insertion were obtained by plating cells from the library on ML+Tc plates and screening for Km^R clones. Cosmid pZHF2 (Figure 2) was identified as a Tc^R Km^R clone that complemented the cell division defect of a *divK341 rec* strain. The ability of pZHF2 subclones to complement the *divK* phenotype was assayed using either the broad host range replicative plasmid pRK2L1 (Ohta *et al.*, 1990) or integrative plasmid pGH500, a non-replicative vector in *C.crescentus* for which stable Tc^R exconjugants must arise by recombination between inserts subcloned into the vector and the chromosome. Plasmid pGH500 was constructed by replacing the 1348 bp *Sca*I fragment of pSUP202 (Simon *et al.*, 1983) with the 448 bp *Pvu*II fragment from pBluescript KS (+) (Stratagene) containing the polylinker region. The resulting plasmid no longer carries the ampicillin or chloramphenicol resistance markers, but confers Tc^R. The nucleotide sequence of the minimal *divK* complementing region was determined as described (Ohta *et al.*, 1992). The nucleotide sequence reported in this paper has been deposited in the GenBank database (accession no. U13765).

Purification of DivJ', PleC' and DivK

The entire *divK* sequence was fused to the initiation codon of the T7 gene 10 bp downstream from the T7 promoter of the T7-7 vector (Tabor and Richardson, 1985) and overproduced as discussed above. Debris and membranes were removed from the sonicated cell lysate by ultra-centrifugation at 65 000 *g* and the high speed supernatant was precipitated with 60% ammonium sulfate. The precipitate was resusupended in TEDG (20 mM Tris–HCl, pH 7.0, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol), dialyzed in the same buffer and chromatographed on DEAE Sephacel (Bio-Rad) with a gradient of 50–500 mM NaCl in TEDG. Final purification of DivK was achieved by sieving through a Sephadex G50 column (Pharmacia). Purification steps were monitored by electrophoresis of chromatographic fractions on SDS–PAGE gels and staining with Coomassie brilliant blue (Figure 9).

The *Nru*I–*Xba*I restriction fragment of the cloned *divJ* gene (Ohta *et al.*, 1992) and the *Bam*HI–*Pst*I restriction fragment of *pleC* (Wang *et al.*, 1993) were each cloned in the pRSET vector downstream from a T-7 promoter (Kroll *et al.*, 1993). The resulting constructs allow the expression of the 286 amino acid C-terminal DivJ fragment (DivJ') and the 313 amino acid C-terminal PleC fragment (PleC'), which contain all of the conserved residues of typical histidine kinases (Stock *et al.*, 1989; Parkinson and Kofoid, 1992) fused to a poly(His), nickel binding domain. Inclusion bodies were formed by PleC' upon IPTG induction in *E.coli* BL21(DE3) containing the pLysE plasmid (Tabor and Richardson, 1985). The protein was then solubilized in guanidine hydrochloride, purified on a nickel chelate column (Hochuli *et al.*, 1987) and renatured as described (McCleary and Zusman, 1990). DivJ' was overexpressed in the same manner, but remained soluble and was purified in native form on a nickel chelate column. Samples of purified PleC' and DivJ' preparations were examined for purity as described in Figure 9.

Enzyme assays

For autophosphorylation assays 100 pmol of each kinase were incubated at 25°C in 100 μl kinase buffer (50 mM Tris–HCl, pH 7.8, 25 mM KCl,

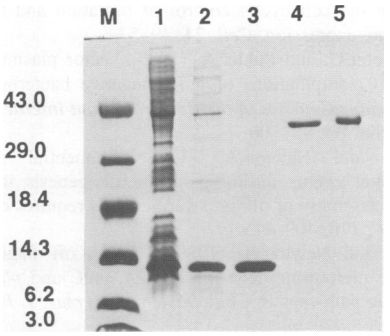

Fig. 9. Purified DivK, DivJ′ and PleC′ proteins. Lanes 1–3 show steps in the purification of DivK: high speed supernatant of cell extract (lane 1), pooled fractions from DEAE column (lane 2) and pooled fractions of Sephadex G50 column (lane 3). Preparations of PleC′ (lane 4) and DivJ′ (lane 5) were purified as described in Materials and methods. Lane M contains protein size standards.

5 mM $MgCl_2$) containing 100 μM ATP with 20 pmol [γ-^{32}P]ATP. Samples (10 μl) were withdrawn at the time intervals indicated and the reaction was stopped by the addition of 1/5 vol. SDS–PAGE sample buffer (0.4 M Tris–HCl, pH 6.8, 10% SDS, 50% glycerol, 3.5 M β-mercaptoethanol, 0.1% bromophenol blue) containing 50 mM EDTA. ^{32}P incorporation was assayed by electrophoresis on 15% SDS–PAGE gels and subsequent phosphoimaging of labeled DivJ′ and PleC′ bands. Phosphotransfer to DivK was assayed by the same procedure in reactions that also contained 1000 pmol DivK.

Phospho-DivK was produced by incubating 2000 pmol DivK in kinase buffer containing 100 μM ATP with 20 pmol [γ-^{32}P]ATP and 40 pmol PleC′ for 10 min at 25°C. The phospho-DivK was then purified by passing the reaction mixture over a 12 ml Sephadex G50 column and collecting 200 μl fractions. Phospho-DivK-containing fractions were pooled and then divided into five aliquots each containing ~400 pmol DivK. Either PleC′ or DivJ′ (40 pmol) was added to the samples as indicated. Each reaction mixture was then incubated at 25°C for 60 min, samples withdrawn and the reaction stopped by addition of 1/5 vol. SDS–PAGE sample buffer containing 50 mM EDTA. Storage of the samples was on dry ice until completion of the experiment. The fraction of remaining phospho-DivK was visualized after electrophoresis on 15% SDS–PAGE by autoradiography.

Construction of divK promoter–lacZ fusions and radioimmunassays

The *divK*p–*lacZ* transcriptional fusion plasmid pANZ443 was constructed by directionally cloning the 2.0 kb *Pst*I–*Pst*I fragment from plasmid pZHF30-RK (Figure 2), first in pBluescript KS+ and then moving it into pANZ5 (Ohta *et al.*, 1991), using the flanking *Kpn*I and *Xba*I sites in the pBluescript vector. The cloned 2.0 kb fragment contained ~1.9 kb of DNA upstream of the *divK* gene plus the first 93 bp of the *divK* ORF. The *divK*p–*lacZ* fusion in pANZ443 expressed 260 U β-galactosidase activity (Miller, 1972), compared with a basal level of 38 U expressed by the pANZ5 vector. The rate of β-galactosidase synthesis in a synchronous culture of strain CB15F/pANZ443 was determined by radioimmunoassay, as described in detail previously (Ohta *et al.*, 1990). The boundaries of the G_1, S and G_2 periods were determined by incorporation of base-stable [^{3}H]guanosine (Ohta *et al.*, 1990).

Microscopy

For transmission electron microscopy (JEOL JEM-100C) mid-log cell cultures were fixed with 1% glutaraldehyde, washed and negatively stained with 0.5% uranyl acetate. For scanning electron microscopy (JEOL JSM-840) mid-log cells were fixed in 0.5% glutaraldehyde followed by 2% osmium tetroxide, then deposited on a 0.25 μm pore size Nucleopore filter and rotary shadowed with carbon and sputter coated with palladium (T.Lane, J.Goodhouse and A.Newton, in preparation). Length measurements were performed using the public domain NIH Image program written by Wayne Rasband at the US National Institutes of Health.

divK disruption experiments

A plasmid-borne copy of the *divK* null allele was constructed by inserting an *aacC1* Gm^R cassette (Schweizer, 1993) into the unique *Pst*I site of *divK* (Figure 2) in plasmid pGH527, a pUC18 derivative containing ~6 kb of contiguous *C.crescentus* DNA extending from the right edge of the cloned *zhf372*::Tn*5* to an *Eco*RI site 4 kb downstream of *divK* (G.B.Hecht and A.Newton, unpublished). The *aacC1* cassette was inserted in both orientations to generate polar and non-polar disruptions of *divK* and these constructs were then shuttled into pGH500 (see above) or pGH551, a Tc^R Km^R derivative of pGH500 constructed using a Km^R cassette (a gift of S.Inouye). Large segments of flanking DNA in the *divK*::*aacC1* constructs were used to increase the crossover frequency on both sides of the disruptive marker, thus maximizing the recovery of replacements.

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