

## The Core Dimerization Domains of Histidine Kinases Contain Recognition Specificity for the Cognate Response Regulator

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**Histidine kinases DivJ and PleC initiate signal transduction pathways that regulate an early cell division cycle step and the gain of motility later in the *Caulobacter crescentus* cell cycle, respectively. The essential single-domain response regulator DivK functions downstream of these kinases to catalyze phosphotransfer from DivJ and PleC. We have used a yeast two-hybrid screen to investigate the molecular basis of DivJ and PleC interaction with DivK and to identify other His-Asp signal transduction proteins that interact with DivK. The only His-Asp proteins identified in the two-hybrid screen were five members of the histidine kinase superfamily. The finding that most of the kinase clones isolated correspond to either DivJ or PleC supports the previous conclusion that DivJ and PleC are cognate DivK kinases. A 66-amino-acid sequence common to all cloned DivJ and PleC fragments contains the conserved helix 1, helix 2 sequence that forms a four-helix bundle in histidine kinases required for dimerization, autophosphorylation and phosphotransfer. We present results that indicate that the four-helix bundle subdomain is not only necessary for binding of the response regulator but also sufficient for *in vivo* recognition specificity between DivK and its cognate histidine kinases. The other three kinases identified in this study correspond to DivL, an essential tyrosine kinase belonging to the same kinase subfamily as DivJ and PleC, and the two previously uncharacterized, soluble histidine kinases CckN and CckO. We discuss the significance of these results as they relate to kinase response regulator recognition specificity and the fidelity of phosphotransfer in signal transduction pathways.**

Asymmetric cell division in the alpha proteobacterium *Caulobacter crescentus* produces a mother stalked cell and a new motile swarmer cell. The mother cell initiates chromosome replication immediately after cell separation. During the ensuing S phase, the cell elaborates the flagellum and other polar surface structures required to construct the incipient swarmer cell. The new progeny swarmer cell produced at division enters a presynthetic gap ( $G_1$  phase) during which the cell loses motility and elaborates a polar stalk at the point of flagellum attachment before initiating chromosome replication and entering the cell division cycle. The sequence of developmental events is closely coordinated with cell cycle progression (2, 10, 24). Genetic analysis of conditional cell division cycle mutants has provided evidence that successive steps in the cell division cycle provide checkpoints for the execution of developmental events, including flagellum biosynthesis, gain of motility, and stalk formation (9; reviewed in reference 22).

Differentiation in *C. crescentus* is controlled and coordinated by multiple signal transduction pathways mediated by members of the His-Asp family of proteins (10, 22, 24). The *C. crescentus* genome encodes 61 histidine protein kinases (23), and genetic analysis has shown that at least 4 of them initiate signal transduction pathways that regulate cell cycle progression and differentiation. These include the transmembrane kinases DivJ (38), PleC (35), and DivL (38), which belong to kinase subfamily 1. This subfamily contains the majority of the *C. crescentus* histidine kinases (24). The fourth kinase involved in cell cycle regulation, CckA, is a hybrid histidine kinase (12) in subfamily 4. There is only limited sequence similarity in the

conserved catalytic domains of the subfamily 1 and 4 kinases (24).

Pseudoreversion analyses of mutants blocked in polar morphogenesis or cell division identified two signal transduction pathways initiated by DivJ and PleC, both of which are mediated by the essential single-domain response regulator DivK (32, 35). The pathway initiated by DivJ (25) controls an early step in the cell division cycle. The second signal transduction pathway, which is initiated by the PleC kinase, regulates cell motility late in the cell division cycle and the swarmer cell to stalked cell transition after cell separation (32, 35). A target of the DivJ  $\rightarrow$  DivK cell division pathway and the PleC  $\rightarrow$  DivK motility pathway was identified as the essential response regulator CtrA (29, 37) on the basis of suppression of the DivJ and PleC phenotypes by a conditional *sokA* allele of *ctrA* (31, 37). Recent work suggests that a target of the DivK-mediated signal transduction pathway regulating the early cell division cycle step is the ClpX/P-dependent turnover of CtrA (11).

The genetic analyses described above suggested that DivJ and PleC are cognate DivK kinases and that DivK participates in at least two different signal transduction pathways that are initiated in response to different cell cycle checkpoints (24). Biochemical experiments support this conclusion. The purified catalytic domains of the two kinases are autophosphorylated in the presence of ATP and transfer the phosphoryl group to DivK (8). The catalytic fragments of DivJ and PleC also catalyze dephosphorylation of phospho-DivK in a purified system (8). Experiments measuring DivK phosphorylation in intact cells (36) and by isolated membranes (T. Hofmeister and A. N., unpublished observation) indicate that DivK phosphorylation depends primarily on the DivJ kinase.

The function of DivK in programming polar morphogenesis and cell cycle progression in response to DivJ and PleC raises

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or construction
<b>Caulobacter strains</b>		
CB15	Wild type	ATCC 19089
PC1131	CB15/pRK2L1	This study
PC3017	<i>divJ356::Ω</i>	31
PC3026	<i>cckO302::Gm<sup>r</sup> divJ356::Ω (Spc<sup>r</sup>)</i>	φCr30(PC3332) × PC3017
PC3327	<i>cckN<sup>+</sup> cckN301::Kan<sup>r</sup> Gm<sup>r</sup></i>	CB15::pNOR702 (Materials and Methods)
PC3331	<i>cckO<sup>+</sup> cckO302::Gm<sup>r</sup> Kan<sup>r</sup> sacB</i>	CB15::pNOR1302 (Materials and Methods)
PC3332	<i>cckO303::Gm<sup>r</sup></i>	Sucrose selection of PC3331
PC3335	<i>cckO303::Gm<sup>r</sup></i>	φCr30(PC3332) × CB15
PC3356	<i>cckN301::Kan<sup>r</sup></i>	φCr30(PC3327) × CB15
<b>Yeast strains</b>		
YE001	<i>MATa trp1-901 leu2-3 leu2-112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met3::GAL7-lacZ</i>	Same as PJ69-4A; 14
YE002	YE001/pNOR001	pGBDU- <i>divK</i> <sup>+</sup> ; this study
<b>Plasmids</b>		
pAROGm <sup>r</sup>	Derivative of pARO181 (ATCC 77125; 27); Kan <sup>r</sup> was replaced with a Gm resistance-encoding <i>aacC1</i> cassette (30)	This study
pNPTS128	Kan <sup>r</sup> derivative of pLITMUS28 with <i>sacB</i> and <i>oriT</i>	1
pGBDU-C3	Bait vector	14
pGAD-C1-, 2-, 3	Prey vector	14

the questions of how the specificity of DivK interaction with these kinases is determined and the identity of other His-Asp signal transduction proteins that might interact with DivK. To address these issues, we have used DivK as the bait protein in a yeast two-hybrid screen of random DNA fragments of the *C. crescentus* genome. Although candidates for interaction include the 61 histidine kinases encoded by the *C. crescentus* genome (23), only 5 histidine kinases were identified in the screen and many of the isolated clones encoded fragments of either DivJ or PleC. This result supports our previous conclusion that DivJ and PleC are the cognate kinases regulating DivK activity (7). Of particular interest is the identification of a conserved 66-amino-acid sequence shared by all of the interacting fragments of the DivJ and PleC kinases. We present results indicating that this conserved  $\alpha$ -helical sequence, which is expected to form a four-helix bundle in the kinase structure necessary for dimerization and phosphotransfer (33), is sufficient in vivo for recognition of the cognate response regulator. We discuss this conclusion and three additional histidine kinases identified in the two-hybrid screen.

#### MATERIALS AND METHODS

**Media and growth conditions.** The *Caulobacter* strains, yeast strains, and plasmids used in this study are described in Table 1. Yeast cells were grown in yeast peptone dextrose or synthetic complete (SC) medium as described by Miller and Rose (19). The *C. crescentus* strains were derived from strain CB15 (ATCC 19089), and cultures were grown in PYE medium at 30°C (28). Spectinomycin (50  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), gentamicin (2  $\mu$ g/ml), and tetracycline (2  $\mu$ g/ml) were added when appropriate.

**Construction of *C. crescentus* yeast two-hybrid libraries.** Plasmid libraries containing the wild-type DNA were constructed as described by James et al. (14), with minor modifications. The purified DNA from *C. crescentus* strain CB15 was partially digested with three restriction enzymes, *AclI*, *HinPII*, and *MspI* (New England Biolabs), separately. Each of these three enzymes cuts the GC-rich *Caulobacter* chromosome very frequently (on average, once in less than 100 bp), rendering the resulting partial digests extremely random. Aliquots of digested DNA samples were examined by Southern hybridization analysis to ascertain that partial and complete digestions were evenly distributed among restriction fragments, by using the <sup>32</sup>P-labeled 501-bp *SstI*-*Bam*HI fragment containing *divK* as a probe. DNA fragments in the range of 0.5 to 2 kb from each of the three

enzyme digests were separately purified from agarose gel and then combined and fused to the *GAL4*-activating domain at the *ClaI* site in the pGAD vectors in three frames (14), generating libraries, CB-C1, CB-C2, and CB-C3. Electrophoretic examination of 16 random transformants from each library has shown that 90% of the clones contained insert DNA (data not shown).

**Two-hybrid screen with DivK as the bait protein.** The bait plasmid containing the entire *divK* gene was constructed by creating the *ClaI* site at the 5' end of the *divK* open reading frame, taking advantage of a preexisting *ClaI* half site, GAT. DivK, encoded by the 387-bp *ClaI*-*Bam*HI fragment (see the *divK* DNA sequence [GenBank accession no. U13765]), was fused in frame to the *GAL4* DNA binding domain in the vector pGBDU-C3 (14) to generate plasmid pNOR1001. pNOR1001 was transformed into yeast strain PJ69-4A (renamed YE001 for this study) (Table 1), which contains three reporter genes, *GAL1-HIS3*, *GAL2-ADE2*, and *GAL7-lacZ* (14), generating strain YE002. The library DNAs in three different frames (CB-C1, CB-C2, and CB-C3) were then independently transformed into YE002. Approximately  $4 \times 10^6$  transformants were screened as described by Miller and Rose (18), except that the retesting of Ade<sup>+</sup> prey plasmids was omitted. The omission may have resulted in the further examination of some of the false-positive clones.

**Analysis of interacting clones.** Prey plasmid DNAs isolated from the yeast cells were transformed into *Escherichia coli* strain DH5 $\alpha$  (Bethesda Research Laboratories) for further analysis. Identities of interacting clones were determined either by DNA sequencing or by Southern hybridization analyses. The 5' nucleotide sequences of 127 clones were sequenced from the upstream end of the 5' *GAL4*-insert fusion junction. Among 76 clones that encode either DivJ or PleC, 28 clones were also sequenced from the 3' end to determine the end points of the respective polypeptide fragments. Among the sequenced clones, 32 represented unique hits but 28 of them were not in-frame fusions to open reading frames in the *Caulobacter* database (23) and were presumably false positives. The four single-hit in-frame fusions, which are not discussed in Results, are in the CC1471, CC0013, CC3043, and CC0766 genes. Several of these clones encode very short peptides. The probes used for Southern hybridization analyses were <sup>32</sup>P-labeled DNA fragments containing the core catalytic (H-box) domain of either *divJ* (1,067-bp *NotI*-*XhoI* fragment; see the DNA sequence with GenBank accession no. M98873) or *pleC* (1,030-bp *Bam*HI-*ClaI* fragment; see the DNA sequence with GenBank accession no. M91449).

**Disruption of *cckN* and *cckO*.** Since *cckN* is located adjacent to *divJ* on the chromosome (23), the 1.5-kb *Bam*HI-*Eco*RI fragment containing *cckN* (CC1062 [23]) was subcloned from a clone containing *divJ* and the upstream sequence (25). *cckN* was disrupted by deleting the 718-bp *NcoI*-*NcoI* fragment, which removed most of the gene, and replacing it with the 1.3-kb Kan<sup>r</sup>-encoding fragment (from pKM18-5; obtained from S. Inouye). The disrupted construct was cloned into the suicide vector pAROGm (Table 1; a Gm<sup>r</sup> derivative of pARO181 [27]) to give pNOR702 and integrated into the chromosome, resulting in a strain containing both wild-type *cckN* and *cckN::Kan<sup>r</sup>* (strain PC3327; Table 1). Strain

PC3356 containing only *ckkN::Kan<sup>r</sup>* was obtained by phage Cr30-mediated transduction (20).

The 1,866-bp *BglII-BamHI* fragment covering the C-terminal two-thirds of *ckkO* (CC0026 [23]) was amplified from CB15 genomic DNA and cloned as an *EcoRI-BamHI* fragment in the suicide vector pNPTS128 (Table 1) (1) to give plasmid pNOR1301. The internal 681-bp *EcoRV-NotI* fragment of *ckkO* was then deleted from pNOR1301 and replaced with the *Gm<sup>r</sup>* cassette (30), generating pNOR1302. The deletion removed the linker region connecting the kinase catalytic domain and the response regulator domain. Plasmid pNOR1302 was integrated into the CB15 genome to give PC3331, followed by sucrose selection (15) to obtain a *ckkO::Gm<sup>r</sup>* replacement strain (PC3332) that had lost the plasmid sequence. Isogenic *ckkO::Gm<sup>r</sup>* strain PC3335 was constructed by transduction of the *ckkO::Gm<sup>r</sup>*-encoding allele of PC3332 into wild-type strain CB15 (Table 1). A ca. 3-kb-long DNA fragment presumed to contain the full-length *ckkO* (CC0026) gene and the promoter region was also amplified from genomic DNA and cloned into pRK2L1 (21) to give pNOR1306. The *ckkN::Kan<sup>r</sup>* and *ckkO::Gm<sup>r</sup>* disruption mutations were confirmed by PCR amplification of the chromosome region containing the deletion-insertion and comparing them to those obtained from the wild-type strain CB15 DNA.

**Flow cytometry (fluorescence-activated cell sorter [FACS] analysis).** Early exponential cultures (optical density at 650 nm of ca. 0.1 to 0.3) were treated with rifampin (Sigma) at a final concentration of 10 µg/ml for 3 h at 30°C to allow completion of chromosome replication but to prevent reinitiation of DNA synthesis. Cells were then harvested, stained with Sytox green nucleic acid stain (Molecular Probes, Eugene, Oreg.), and analyzed with a FACSsort flow cytometer as previously described (31).

## RESULTS

### Yeast two-hybrid screen for proteins interacting with DivK.

Previous genetic and biochemical results (7, 32) have provided evidence that DivJ and PleC are cognate histidine kinases of response regulator DivK. To examine these interactions and to screen for other His-Asp proteins that interact with DivK by using the yeast two-hybrid assay, we constructed prey libraries of small, random fragments of *C. crescentus* DNA in the three reading frames in the pGAD shuttle vectors (see Materials and Methods and reference 14). As the bait clone, the *divK* gene was fused in frame to the DNA binding domain of GAL4 in plasmid pGBDU-C3 (14). Approximately  $4 \times 10^6$  yeast transformants harboring both the bait and library DNAs were screened first for activation of *ADE2* and then for activation of *HIS3* in a secondary screen (18). A total of 182 interacting clones were identified. DNA sequence analysis of 88 clones from the region upstream of the 5' fusion junction demonstrated that 23 clones contained inserts that encode PleC polypeptides and 38 contained inserts that encode DivJ polypeptides. The remaining 94 clones were examined by Southern blot analysis with <sup>32</sup>P-labeled DNA probes containing either *divJ* or *pleC* restriction fragments (data not shown; see Materials and Methods). This analysis identified an additional 26 PleC clones and 35 DivJ clones. Clones that were not unambiguously identified as *divJ* or *pleC* by the Southern hybridization analysis were further analyzed by DNA sequencing.

The majority of the isolates examined by the two procedures just outlined correspond to either PleC (49 clones) or DivJ (73 clones) clones. An additional eight clones encoded polypeptide fragments of the tyrosine kinase DivL (38). We also identified 11 clones encoding fragments of either the histidine kinase CckN (3 clones) or CckO (8 clones). CckN (CC1062) and CckO (CC0026) are in the *C. crescentus* database (23), but they have not been previously studied (see below). None of the four remaining in-frame clones (see Materials and Methods) identified a predicted His-Asp signal transduction protein, including a pos-

sible phosphotransferase predicted to be downstream of DivK (37). It is also noteworthy that no DivK clones were recovered in this screen, although DivK forms dimers in vitro (6).

A second, smaller-scale screen with the same *divK* bait clone that was carried out as a control in another screen identified an additional 39 interacting clones. The majority of these clones again corresponded to either PleC (19 clones) or DivJ (16 clones). One DivL and one CckN clone were also isolated. These results confirmed our findings from the larger screen described above. They are consistent with the conclusion that only a small subset of the histidine kinases in *C. crescentus* recognize the DivK bait protein in the yeast two-hybrid screen.

**DivJ, PleC, and DivL clones that interact with DivK contain the conserved helix 1, helix 2 sequence.** The DivJ and PleC fragments, a subset of which are shown in Fig. 1, form an overlapping array of sequences. The 76 PleC and DivJ clones examined by DNA sequencing have in common the translated H-box sequence, which contains the His residue that is the site of autophosphorylation and phosphotransfer in these histidine kinases (data not shown; reviewed in reference 4). Alignment of these common sequences showed that they are similar to the helix 1, helix 2 sequence of the homodimeric core domain of the *E. coli* EnvZ protein and other histidine kinases (Fig. 2) (33). The helix 1, helix 2 sequence of EnvZ forms a four-helix bundle that is responsible for dimerization of the histidine kinase and contains the site of autophosphorylation and phosphotransfer (33). Computer analysis of the PleC and DivJ fragments identified in the yeast two-hybrid screen supports the idea that the conserved sequence containing the H box also forms a four-helix bundle, although the exact location of helix 2 relative to helix 1 differs slightly from that of EnvZ (Fig. 2; analysis not shown).

Experiments with the purified catalytic domains of PleC, DivJ, and DivL have shown that DivL is relatively inefficient at catalyzing phosphotransfer to DivK (38). Given this observation, the interaction between DivL and DivK in the two-hybrid assay was unexpected. As described above, however, we isolated a total of nine *divL* clones in the two screens. All of the cloned fragments contain the conserved helix 1, helix 2 sequence (Fig. 1 and 2) and the Tyr-550 residue, which is the site of phosphorylation in the DivL kinase (38). The N-terminal end of the shortest cloned DivL fragment, the 228-amino-acid insert encoded by pNOR1243, lies just upstream of the conserved helix 1 sequence and extends to the end of the protein (Fig. 2). We conclude from these results that DivK also interacts with the four-helix bundle of the DivL kinase, as proposed for DivJ and PleC.

We have used spot tests and β-galactosidase assays of representative PleC, DivJ, and DivL clones to assess their interaction with the DivK bait protein. There is generally a good correlation between the growth of these hybrid clones on the spot tests (Fig. 3) and the enzyme activities (Fig. 1; see also Fig. 4). Although all of the clones interacted with DivK in the His<sup>-</sup> plate assay, two of the DivL clones, pNOR1216 and pNOR1284, displayed the interaction only at one of the lower dilutions (Fig. 3). The more stringent assay on the Ade<sup>-</sup> plate (after 2 days) confirmed this result and indicates that the interaction of the four DivL clones with DivK is weaker than that of many of the PleC and DivJ clones (Fig. 3). The results of the β-galactosidase assays suggest a similar

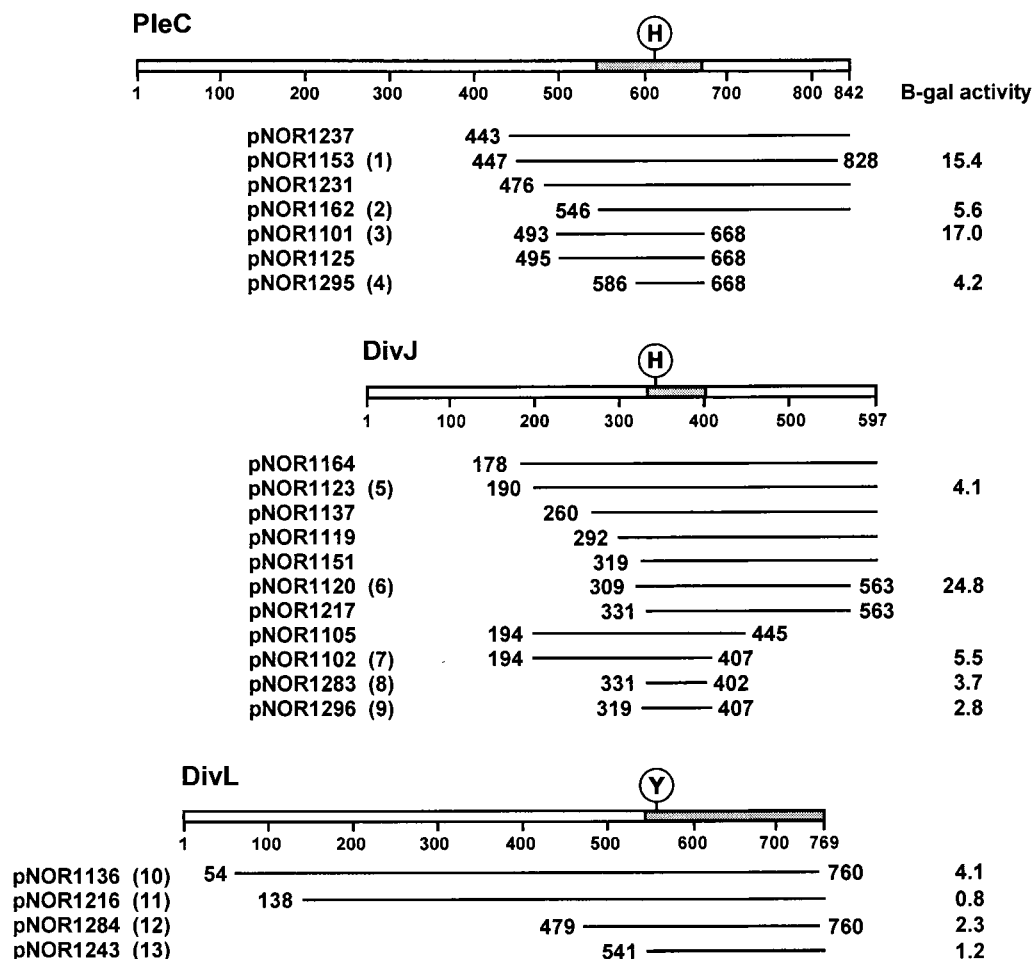


FIG. 1. Polypeptide fragments of kinases DivJ, PleC, and DivL that interact with DivK in the yeast two-hybrid screen. An alignment of translated inserts in representative DivJ, PleC, and DivL clones is shown. The positions of the N- and C-terminal residues are indicated for each fragment, except those that extended to the C-terminal end of the proteins. Locations of the conserved sites of phosphorylation, histidine (H) in DivJ and PleC and tyrosine (Y) in DivL, are shown. Shaded areas of each kinase represent overlapping sequences common to the respective DivJ, PleC, and DivL clones. The numbers in parentheses following plasmid names identify the clones tested in the spot test shown in Fig. 3.  $\beta$ -Galactosidase (B-gal) assays (17) were performed on the cultures of the same clones that were grown in SC medium lacking leucine and uracil to mid-exponential phase. At least three independent cultures of each strain were assayed. Including the clones shown here, the fusion joints of 76 clones were sequenced. Among 34 *pleC* clones sequenced, the most frequent fusion points were either at amino acid 456 (7 clones) or between amino acids 492 and 495 (21 clones). Of the 42 DivJ clones sequenced, a total of 21 DivJ fragments started between residues 190 and 196 and 6 started between residues 328 and 331. The C-terminal ends of most clones were not sequenced.

conclusion (Fig. 1). A weaker interaction of the DivL kinase with DivK could account for the smaller number of DivL clones isolated in our screen.

**The four-helix bundle domains of the DivJ and PleC kinases are sufficient for recognition of DivK.** Among the 17 *divJ* and 11 *pleC* clones that were sequenced from both the 5' and 3' ends, the sizes of the translated inserts ranged from 72 to 420 amino acids for DivJ and from 174 to 400 amino acids for PleC. The isolation of a DivJ fragment of only 72 amino acids (pNOR1283) and a PleC fragment of 174 amino acids (pNOR1125; Fig. 1) indicates that the conserved 66-residue helix 1, helix 2 sequence may be sufficient, as well as necessary, for recognition of DivK. To examine this possibility in more detail, we constructed two additional prey clones with inserts encoding PleC (amino acid residues 586 to 668; plasmid pNOR1295) or DivJ (residues 319 to 407; plasmid pNOR1296)

that contain primarily the conserved helix 1, helix 2 sequence (Fig. 1). The pNOR1295 and pNOR1296 clones, like the 72-amino-acid DivJ fragment encoded by the pNOR1283 clone, interacted with DivK, as determined by spot tests (Fig. 3) and  $\beta$ -galactosidase activity assays (Fig. 1). These short PleC and DivJ fragments displayed interactions with DivK that are similar to or stronger than those observed between DivK and the DivL kinase fragments. Although the interactions of these PleC and DivJ clones are weaker than those observed for the longer PleC clones pNOR1153 and pNOR1101 and the DivJ clone pNOR1120, they are comparable to those observed for other, longer clones, including the PleC clone pNOR1162 and the DivJ clone pNOR1123. Thus, the strength of interaction between the various kinase fragments and DivK is not strictly related to the length of the sequences flanking the helix 1, helix 2 motif. These findings support the conclusion that the recog-

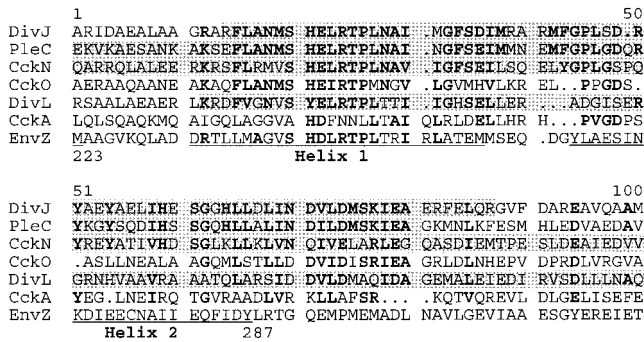


FIG. 2. Amino acid sequence alignment of the helix 1, helix 2 sequences in histidine kinases. The amino acid sequences of conserved DivJ, PleC, CckN, and DivL fragments identified in the yeast two-hybrid screen with DivK as the bait protein (shaded) are aligned with the core helix 1, helix 2 domain of the *E. coli* EnvZ protein. CckA and CckO are included for comparison, although *cckA* clones were not isolated in the screen and *cckO* clones did not encode the helix 1, helix 2 sequence (see text). Locations of helices 1 and 2 in EnvZ were determined as described previously (33). Residues conserved in both PleC and DivJ are in bold.

nition specificity of the kinases for DivK is confined to the four-helix bundle domain.

**Genetic characterization of *cckN* and *cckO*.** CckN is a cytoplasmic kinase that is closely related in sequence to DivJ (Fig. 2). The four CckN (CC1062) clones identified by their interaction with DivK encode all or most of the kinase sequence, including the core dimerization domain. The *cckN* gene is divergently transcribed from *divJ* on the *C. crescentus* genome. This gene organization and the sequence similarity of DivJ, PleC, and CckN (Fig. 2) suggested that CckN might belong to a network of kinases controlling DivK activity. To determine if a *cckN* mutant displayed a defective cell cycle or a developmental phenotype similar to those caused by mutations in either *divJ* or *pleC*, we constructed a Kan<sup>r</sup> disruption allele of *cckN* and replaced the genomic *cckN*<sup>+</sup> allele to give

*cckN*::Kan<sup>r</sup> knockout strain PC3356 (see Materials and Methods) (Table 1).

Our ability to construct the disruption strain indicates that *cckN* is not essential. Moreover, we were unable to detect mutant phenotypes, e.g., no obvious defects in cell division, motility, or stalk formation, as determined by light microscopy (data not shown). We also examined cell cycle regulation in the *cckN* disruption mutant by FACS analysis and observed a significant and reproducible increase in the number of cells with one chromosome, suggesting a possible G<sub>1</sub> defect in DNA replication (see Fig. 5).

The second novel kinase identified in this screen was CckO (CC0026), a cytoplasmic, hybrid kinase of 900 amino acids with a C-terminal receiver domain. Surprisingly, none of the eight CckO clones identified in the two-hybrid screen contain the conserved helix 1, helix 2 sequence (Fig. 4). Instead, all of the isolated clones contain the same 87-amino-acid sequence that includes the kinase response regulator linker region along with the N-terminal half of the CckO receiver domain, including the presumptive site of phosphorylation (Asp-821; Fig. 4). The isolation of eight independent *cckO* clones argues that the observed CckO-DivK interaction is not fortuitous. To confirm this interaction, we have reconstituted an isogenic set of interacting strains by retransforming the parent yeast strain containing the DivK bait plasmid (YE002; Table 1) with the eight *cckO* prey plasmids isolated from *E. coli*. The levels of two-hybrid interaction in these strains tested in a plate spot test (data not shown) and β-galactosidase assays (Fig. 4) were comparable to those observed for many of the *divJ*, *pleC*, and *divL* clones shown in Fig. 1 and 3.

To examine the possible function of *cckO*, we constructed a *cckO*::Gm<sup>r</sup> knockout strain. We were able to obtain the disruption construct in the absence of a complementing wild-type *cckO* sequence (see Materials and Methods). This indicates that *cckO* is not essential for colony formation on plates under the conditions used in this selection. Surprisingly, however, *cckO*::Gm<sup>r</sup> knockout strain PC3335 (see Materials and Meth-

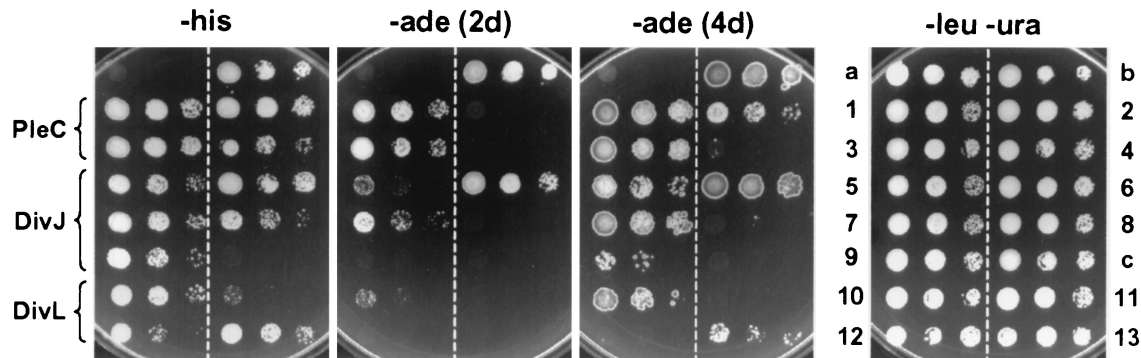


FIG. 3. Interactions of DivK with DivJ, PleC, and DivL in the yeast two-hybrid assay. Yeast strains containing both bait and prey plasmids were grown overnight at 30°C in SC medium lacking uracil and leucine. Cultures were serially diluted 10-fold and spotted onto SC plates lacking leucine and uracil (-leu -ura), histidine (-his), or adenine (-ade). All of the strains tested contained the *divK* bait plasmid and interacting clones, except a, b, and c. a, *divK* bait plus empty prey vector; b, pGDB-KAR9 plus pGAD-BIMI (18); c, pNOR1196 plus empty bait vector. The rationale behind the method is that stronger interactions between bait and prey polypeptides result in higher enzymatic activities for the synthesis of histidine and adenine and thus permit more rapid growth on the selective His<sup>-</sup> and Ade<sup>-</sup> plates. The numbered clones correspond to those indicated in parentheses in Fig. 1. Before being photographed, the Leu<sup>-</sup> Ura<sup>-</sup>, His<sup>-</sup>, and Ade<sup>-</sup> plates were incubated for 2 days (2d), and the Ade<sup>-</sup> plate was incubated for 4 days (4d), at 30°C.

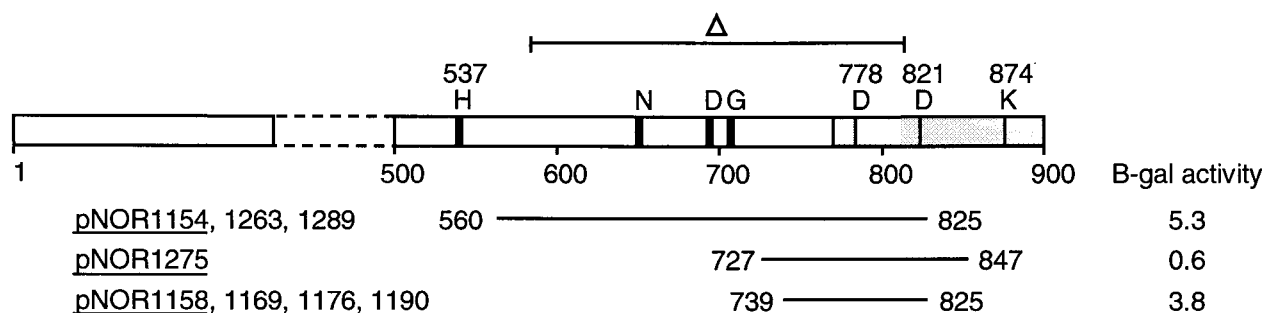


FIG. 4. Alignment of CckO clones. Alignment of translated inserts in *cckO* clones with the translated CckO sequence (CC0026). The unshaded area represents the kinase domain, and the shaded area represents the receiver domain, of the hybrid kinase. The predicted sites of phosphorylation at H537, D821, other conserved motifs in the kinase domain (N, D, and G boxes [reviewed in reference 5]), and conserved residues in the receiver domain are shown.  $\beta$ -Galactosidase (B-gal) assays on each of the CckO clones underlined were performed as described in the legend to Fig. 1.

ods) (Table 1) grew very poorly in liquid medium and exhibited a significant increase in the number of predivisional cells and short filaments (data not shown). FACS analysis of strain PC3335 revealed that a large number of cells contain greater than 1n but smaller than 2n chromosomes both before and after incubation for 3 h in the presence of rifampin (Fig. 5). This suggests that a defect in DNA synthesis in the mutant prevents or slows the completion of DNA replication. Double-knockout mutant strain PC3026, containing both *cckO::Gm<sup>r</sup>* and *divJ:: $\Omega$* , exhibited the same profile as PC3335, while in *divJ* knockout strain PC3017 (31), DNA replication was completed in the presence of rifampin (Fig. 5), as reported previously (36). This suggests that the *cckO* mutant block is epistatic to the *divJ* mutant block.

## DISCUSSION

The yeast two-hybrid screen reported here identified five members of the histidine kinase superfamily in *C. crescentus* that interact with the essential response regulator DivK. These kinases include PleC, DivJ, and DivL, which have been previously characterized both genetically and biochemically (22), and the two novel kinases CckN and CckO. DivK plays a role in at least two signal transduction pathways regulating cell differentiation. The first of these pathways is initiated by the histidine kinase DivJ and controls a cell division step early in the cell cycle (10, 25), while the second pathway is initiated by the kinase PleC and controls motility in late predivisional cells (31, 32). The observation that two-thirds of the independently isolated clones in our screen correspond to either DivJ or PleC (Fig. 1) supports the previous conclusion (7, 32) that these kinases are cognates of DivK. In addition, all of the interacting DivJ and PleC clones, as well as the DivL and CckN clones, share a conserved  $\alpha$ -helical sequence of 66 residues. We suggest below that this kinase sequence motif, which is expected to form a four-helix bundle that is the site of autophosphorylation, phosphotransfer, and kinase dimerization (26, 33, 34), is sufficient in vivo for recognition specificity between kinases and their cognate response regulators.

The failure to isolate clones identifying certain His-Asp proteins in our screen may be significant. These include proteins with sequence homology to a histidine phosphotransferase that would function downstream of DivK in a signal transduction pathway regulating CtrA activity (37). Clones encoding DivK

and the essential kinase CckA were also not recovered. DivK clones may not have been isolated either because the DivK-DivK interaction does not occur in vivo or because the interaction could not be detected under our assay conditions. The failure to identify CckA, which is required for CtrA phosphorylation (10), is not surprising. The core dimerization domain sequence of this subfamily IV kinase (24) diverges from that of the histidine kinases shown in this study to interact with DivK (Fig. 2).

**Response regulator interactions with the four-helix bundle subdomain of kinases and phosphotransferases.** The four-helix bundle appears to be a general structural feature of histidine kinases and phosphotransferases. As shown in studies of the *E. coli* histidine kinase EnvZ (33) and the *Bacillus subtilis* phosphotransferase Spo0B (34, 39), it plays an essential role in interaction with the respective cognate response regulators. Structural studies have shown that Spo0F of *B. subtilis* interacts with  $\alpha 1$  and  $\alpha 2'$  of the two Spo0B protomers, as well as two  $\alpha$ - $\beta$  loops in the C-terminal domain of the phosphotransferase (39). Nuclear magnetic resonance titration experiments indicate that OmpR interacts with multiple residues on both helices 1 and 2 of the four-helix bundle of EnvZ (33). These structural studies suggest that an interaction between the response regulator DivK and its cognate histidine kinases in the two-hybrid assay described here would also occur with the four-helix bundle dimerization domain.

A genetic approach has not previously been used to address the question of whether the four-helix bundle of histidine kinases alone is sufficient to confer recognition specificity for cognate response regulators. The screen for *C. crescentus* proteins that interact with the response regulator DivK described here suggests that kinase response regulator interactions can be studied with the yeast two-hybrid assay. Our results indicate that the four-helix bundle sequence is both necessary and sufficient to confer specificity of recognition between this response regulator and its cognate histidine kinases in vivo. This screen of a genomic library of prey clone inserts also suggests that the specificity of the interaction of the core kinase dimerization domain with the cognate response regulator may be sufficient to exclude unacceptable levels of cross talk between noncognate kinase response regulator pairs, an issue explored previously in studies of His-Asp signal transduction in *B. subtilis* (39). An indication of the specificity of kinase response

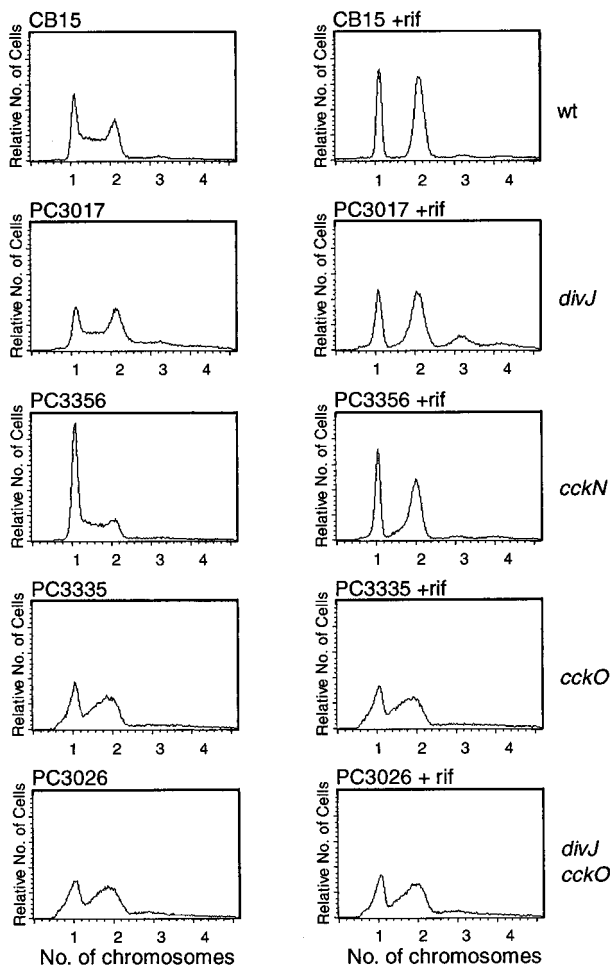


FIG. 5. FACS analysis of *divJ*, *cckN*, and *cckO* null mutants. Cultures were grown in PYE medium supplemented with appropriate antibiotics to optical densities at 650 nm of ca. 0.1 to 0.3, and a portion of each culture was further incubated with rifampin (rif) for 3 h at 30°C before harvesting. Samples were prepared for flow cytometry as described in Materials and Methods, and DNA content (chromosome number) was measured. Normally, swarmer cells ( $G_1$  phase) contain one chromosome per cell and predivisional cells ( $G_2$  phase) contain two chromosomes per cell (3). wt, wild type.

regulator interactions was the identification of only five protein kinases in the yeast two-hybrid screen, despite the fact that *C. crescentus* is predicted to encode 61 histidine kinases that could potentially interact with DivK (23).

On the basis of the finding that all of the interacting DivJ and PleC clones contain the helix 1, helix 2 sequence (as shown in the alignment in Fig. 2), we concluded that the specificity of the DivK-kinase interaction is confined to this conserved 66-amino-acid sequence. The ability of the newly constructed DivJ and PleC prey clones that encode only the helix 1, helix 2 sequence to interact with DivK in the two-hybrid assay supports this conclusion (Fig. 1). However, the extents of PleC-DivK and DivJ-DivK interactions for individual clones measured by growth on spot test plates and assays of  $\beta$ -galactosidase activity differed (Fig. 1 and 3). Although the strength of the interactions seemed to be influenced by the presence of sequences flanking the helix 1, helix 2 sequence, it did not depend on the length of these sequences.

Relatively few DivL and CckN clones were isolated, but all of them contain the conserved helix 1, helix 2 sequence. These  $\alpha$ -helical sequences in DivJ, PleC, and CckN are very similar to one another. The cytoplasmic domains of the three kinases cluster as a group in a PILEUP analysis of the 29 subfamily 1 kinases, while DivL and CckO, also members of subfamily 1, are not adjacent to these kinases in the analysis (data not shown) (24).

**Interactions of DivK with DivL, CckN, and CckO.** Several clones encoding the polypeptides of DivL kinase (38) were recovered in the screen (Fig. 1). Because phosphotransfer experiments have previously shown that DivL is much more efficient in phosphotransfer to the response regulator CtrA than to DivK (38), the interaction between DivK and DivL was unexpected. The nine DivL clones isolated contain a presumptive four-helix bundle sequence, however, and we propose that this structure subdomain also functions in DivL as the site of dimerization and interaction with DivK. The affinity of DivK-DivL interactions estimated for DivL clones is somewhat less than that for most of the PleC and DivJ clones. It should also be noted that the amino acid sequence in this subdomain in DivL diverges to some extent from those in PleC and DivJ, particularly in presumptive helix 2 (Fig. 2).

Genes encoding the two remaining kinases identified in this screen, CckN and CckO, were reported in the *C. crescentus* genome sequencing project (23). As discussed above, the soluble CckN kinase is similar in sequence to DivJ and PleC around the phosphotransfer domain (Fig. 2). The *cckN* gene, like *divJ* and *pleC*, is not essential, but unlike *divJ* and *pleC*, the *cckN* disruption mutant displays no detectable morphological phenotype. The only indication that this kinase may play a role in the cell division cycle was the accumulation of *cckN* mutant cells arrested in  $G_1$  (Fig. 5).

The *cckO* gene is also not essential, as judged by the successful isolation of a disruption in this gene. The *cckO::Gm<sup>r</sup>* mutant strain did, however, display a severe growth phenotype in liquid medium, and it also showed an increase in the number of predivisional cells, as well as an increase in the number of short filaments (data not shown). Consistent with the latter observation were the results of FACS analysis of *cckO* mutant cells, which displayed a distribution profile suggesting that the mutant is defective in the completion of DNA replication (Fig. 5). The interaction of the hybrid kinase CckO with DivK in the clones isolated is unusual. None of the clones contained the four-helix bundle sequences; instead, all contained the kinase response regulator linker region and the N-terminal portion of the receiver module. This unexpected interaction is under investigation.

**Stability of kinase response regulator interactions.** One question raised by the present studies is whether the yeast two-hybrid system, which is not typically used for detection of catalytic interactions, might be of general use in the identification of cognate kinases or phosphotransferases for orphan response regulators. Our failure to identify any kinases in a yeast two-hybrid screen with the response regulator CtrA (29) or PleD (8) as the bait protein (unpublished results) suggests that many kinase response regulator interactions may not be stable enough for detection in a genomewide screen. The transmitter (catalytic) domain of the NtrB kinase and its cognate response regulator NtrC have been shown to interact in a

pairwise combination in the yeast two-hybrid system (16), but neither of these proteins was used as bait to screen a prey library in that study.

The stability of DivK interactions with its cognate kinases may explain the spatial colocalization of DivK with the DivJ and PleC kinases at the *C. crescentus* cell poles: DivK colocalizes to the stalked pole with the DivJ kinase and to the flagellated pole with the PleC kinase (13). We propose that the localization of DivK may depend directly on the subcellular targeting of this soluble response regulator to the cell poles via its interaction with the four-helix bundles of the localized DivJ and PleC kinases (31, 36). Structural studies of DivK have shown that the metal ion coordination required for activity of the response regulator is unorthodox (6). These results have suggested that the required active-site geometry of DivK is achieved by its interaction with the cognate kinase. It is thus tempting to speculate that the observed colocalization of DivK with PleC or DivJ represents enzymatically active DivK-kinase complexes at the cell poles.

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