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Identification of a Novel Response Regulator Required for the Swarmer-to-Stalked-Cell Transition in *Caulobacter crescentus*

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The onset of motility late in the *Caulobacter crescentus* cell cycle depends on a signal transduction pathway mediated by the histidine kinase PleC and response regulator DivK. We now show that *pleD*, whose function is required for the subsequent loss of motility and stalk formation by the motile swarmer cell, encodes a 454-residue protein with tandem N-terminal response regulator domains D1 and D2 and a novel C-terminal GGDEF domain. The identification of *pleD301*, a semidominant suppressor of the *pleC* Mot phenotype, as a mutation predicted to result in a D-53→G change in the D1 domain supports a role for phosphorylation in the PleD regulator. Disruptions constructed in the *pleD* open reading frame demonstrated that the gene is not essential and that the *pleC* phenotype can also be suppressed by a recessive, loss-of-function mutation. These results suggest that PleD is part of a signal transduction pathway controlling stalked-cell differentiation early in the *C. crescentus* cell cycle.

Caulobacter crescentus is an asymmetrically dividing bacterium, which produces a nonmotile stalked mother cell and a motile, flagellated swarmer cell at the end of each cell cycle. During cell division, the stalked cell undergoes a series of discrete developmental events at one pole, including flagellum biosynthesis, the appearance of receptors for polar bacteriophage φCbK, and the start of flagellum rotation when the cell gains motility. Upon completion of cell division, the stalked cell immediately reenters the DNA synthetic period (S phase) and the new swarmer cell enters a G_1 period, during which polar morphogenesis continues with pilus formation, loss of motility, and other events required for stalked-cell formation (Fig. 1A) (reviewed in references 3 and 20).

Genetic experiments with *C. crescentus* indicate that cues for the temporal and spatial control of polar differentiation are provided by the completion of successive cell cycle events (11, 29, 36), and more recent work on the regulation of motility has suggested that development may be coupled to the underlying cell cycle by the so-called two-component signal transduction systems (8, 21, 39) (reviewed in references 12 and 19). Histidine kinases initiate signal transduction pathways by autophosphorylation of the conserved histidine residue, normally in response to a signal received by the sensory domain of the kinase. Autophosphorylation is followed by phosphotransfer to an aspartyl residue of a member of the second family of conserved proteins, the response regulators. Depending on the response regulator, phosphorylation may change either the enzymatic activity or the regulatory function of the protein (25, 26, 35, 37).

Gain of motility in the *C. crescentus* cell cycle is controlled by histidine kinase PleC and the single domain response regulator DivK (8). Although *pleC* mutants divide and assemble flagella normally, they fail to turn on motility, shed the flagellum, or make stalks (5, 31) (Fig. 1B). Mutations in *divK* were identified as suppressors of the nonmotile (Mot) phenotype of *pleC* alleles (10, 32). These and other results have suggested that the PleC-DivK signal transduction pathway regulates the start of flagellum rotation and gain of motility, presumably in response to a late cell cycle checkpoint (8, 32, 39) (reviewed in references 12 and 19). Genetic analysis has also indicated that *divK* plays an essential role in the regulation of an early cell division step (8). Thus, DivK may act at two different times in the cell cycle, during late S phase in response to PleC to effect the gain of motility and then early in the subsequent cell cycle to regulate cell division, possibly in response to histidine kinase DivJ (8, 12, 19).

Another locus involved in *C. crescentus* development is *pleD*. Strains carrying the *pleD301* mutations bypass the motility defect of *pleC* mutations, but they are blocked at subsequent stages of morphogenesis, including loss of motility, shedding the flagellum, and stalk formation. Consequently, *pleD* mutants are motile at all stages of the cell cycle, a phenotype designated "supermotile" (Mot*) (Fig. 1C). Genetic analysis of *pleD* has been complicated, however, because the close linkage of the *pleD* and *divK* alleles has prevented their separation by generalized transduction (32). This result, along with the observation that mutations in both *divK* and *pleD* behave as bypass suppressors of the nonmotile *pleC* phenotype (31, 32), raised the possibility that the *divK* and *pleD* suppressors might be allelic.

We present here a genetic and molecular analysis demonstrating that *pleD* is an open reading frame (ORF) adjacent to, but separate from, *divK*. The *pleD* gene encodes a translated product of 454 residues containing two tandem, N-terminal domains with homology to the response regulator family of proteins and a novel GGDEF domain at the C terminus. The GGDEF sequence is highly similar to translated sequences of unknown function reported for six other bacterial species. The inferred D-53→G change in the semidominant *pleD301* mutant protein suggests that phosphorylation of PleD may be important in the regulation of polar morphogenesis. Gene disruptions demonstrate that *pleD* is not an essential gene and that loss-of-function mutations also suppress the Mot defect of *pleC* mutations. We discuss the implication of these findings for the regulation of the swarmer-cell-to-stalked-cell transition.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth, and complementation. All *C. crescentus* strains were derived from CB15 (ATCC 19089). *Escherichia coli* MC1061 and

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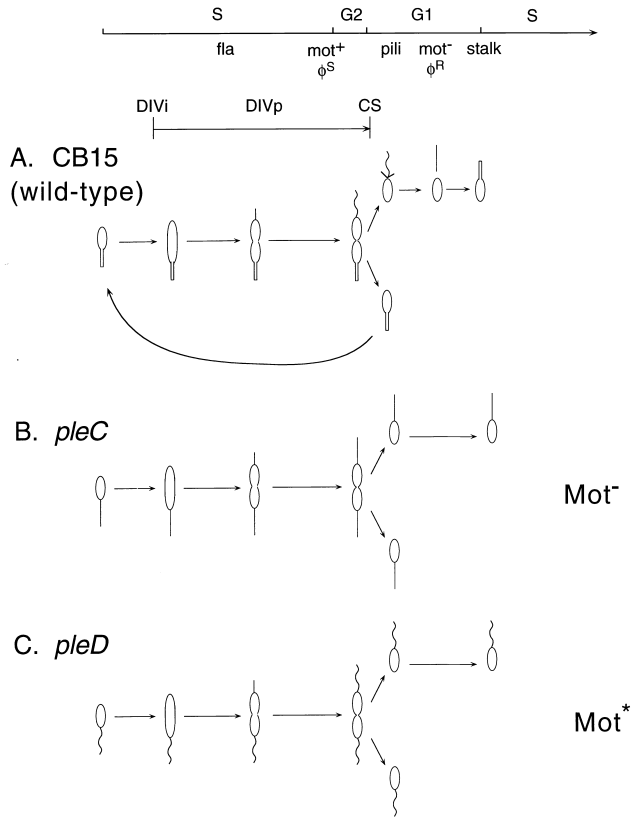

FIG. 1. *C. crescentus* cell cycle. (A) The sequence of developmental events in the wild-type strain CB15 includes flagellum formation (fla), appearance of polar bacteriophage receptors (ϕ^s), activation of flagellum rotation (Mot^+), pilus formation (pili), loss of motility (Mot^-), loss of polar bacteriophage receptors (ϕ^r), and stalk formation. The periods corresponding to DNA synthesis (S), postsynthetic gap (G_2), and presynthetic gap (G_1), as well as division initiation (DIVi), division progression (DIVp), and cell separation (CS), are indicated. (B) Nonmotile *pleC* mutants assemble inactive flagella (straight lines), are resistant to polar bacteriophage, and fail to form stalks, but they divide normally. (C) The *pleD301* mutant assembles active flagella but is unable to undergo the developmental transformation into a stalked cell. As a result, the flagellum is retained, motility is never turned off, and no stalk is formed. Thus, each cell division produces two motile swarmer cells. Because all of the cells in a *pleD* culture are motile, this phenotype is referred to as "supermotility" (Mot^*).

HB101 were used as hosts for cloning and mating. Growth of bacterial strains in PYE (peptone-yeast extract) medium (1, 8) and generalized transductions (6) were performed as described previously. Replicative and integrative plasmids were transferred to *C. crescentus* strains by triparental crosses as described previously (24). Motility and supermotility were assayed by using motility agar (24) and phase-contrast microscopy of exponential cultures. Sensitivity to the polar bacteriophage φCbK was determined by streaking *C. crescentus* strains across fresh streaks of φCbK on solid medium. Bacterial strains and plasmids are listed in Tables 1 and 2, respectively.

Cloning and complementation of *pleD*. The *pleD* gene was cloned as contiguous DNA 3′ of the *divK* complementing region by a strategy similar to that of Binnie et al. (2). Integration of plasmid pZHF37-GH, which carries the 511-bp *Sst*I-*Bam*HI minimal *divK* complementing fragment, into *C. crescentus* CB15 results in cointegrate formation. Chromosomal DNA from the cointegrate was isolated, digested to completion with *Eco*RI, and ligated, and tetracycline-resistant transformants were selected in *E. coli*. Given the locations of the *Eco*RI sites within the pZHF37-GH plasmid and the genomic DNA 3′ of *divK* (9), this procedure allowed the isolation of pGH501, which carried *divK* plus a defined 4.3-kb segment of wild-type DNA 3′ of the gene as a continuous insert. Plasmid pGH503 was isolated from an integrant of plasmid pZH37-GH in the *pleD301* strain PC4166 by the same procedure. Plasmids pGH509 and pGH510, which are truncated versions of pGH501 and pGH503, respectively, were isolated by digesting chromosomal DNAs from the same cointegrates with *Pvu*I to generate clones carrying *divK* and only 323 bp of downstream genomic DNA.

Complementation of *pleD* was carried out with the broad-host-range replicative plasmids pRK2L1 and pRK2L10 (Table 2), and the motility phenotype was assayed as described above. Cointegrate strains were constructed by using derivatives of the pGH500 vector (Table 2). Restriction digestions, ligation reactions, and other molecular manipulations were carried out by standard techniques (17).

TABLE 1. Strains[a]

Strain	Genotype	Description or reference
CB15	Wild type	ATCC 19089
PC4160	*divK341*	8
PC4166	*pleC319 pleD301 str301*	φ(PC5344) × PC8940→Aux^+ (Tc^s Mot^*, $\phi CbK^{r,37°}$)
PC4695	*pleD302*::*kan*	*pleD* gene replacement strain generated from pooled φCr30 lysate grown on CB15/pGH516 cointegrates[b]
PC4707	*pleC319 pleD302*::*kan*	φ(PC4695) × PC5262→Kan^r ($\phi CbK^{r,37°}$)
PC5344	*pleC319 pleD301 zbg354*::Tn*5*	32
PC5349	*pleD301 zhf341*::Tn*5*	31
PC5225	*pleC301*::Tn*5*	31
PC5262	*pleC319*	31
PC8940	*aux*::Tn*5-132* (Tc^r) *str301*	Tc^r Tn*5* derivative linked to *pleC*[c]
PC8981	*pleD301 str301*	φ(PC5349) × PC8940→Aux^+ (Tc^s Mot^* Kan^s)
PC8992	*pleC319 divK341*	8
PC8993	*pleC301*::Tn*5 pleD301 str301*	φ(PC5225) × PC8981→Kan^r (Mot^*)
PC8997	*pleC301*::Tn*5 divK341*	8

[a] Aux^+, prototrophic; Tc^r and Tc^s, resistance and sensitivity to tetracycline, respectively; Mot^*, supermotility; $\phi CbK^{r,37°}$, resistance to φCbK at 37°C; Kan^r and Kan^s, resistance and sensitivity to kanamycin, respectively.
[b] See references 8 and 18.
[c] See references 8 and 10.

Construction and physical mapping of the *pleD* gene replacement. The previous sequence analysis of *divK* demonstrated that the *Bam*HI_2 restriction site (Fig. 2) lies 3′ of the stop codon of the *divK* ORF (8), and a *pleD* disruption allele

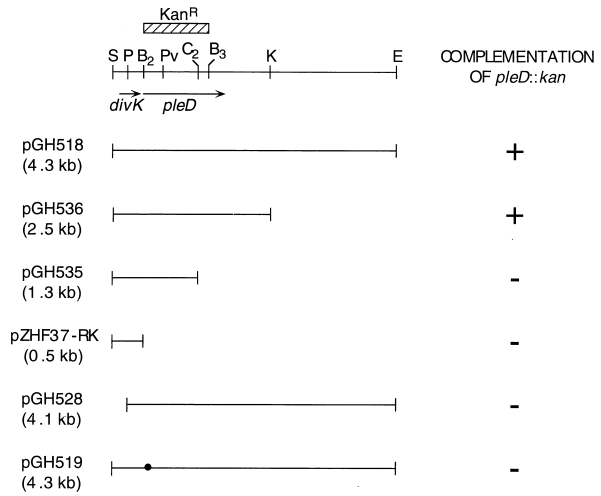

FIG. 2. Location of the *pleD* gene by complementation analysis. A restriction map of the *divK-pleD* region of the chromosome is shown at the top, with the direction of *divK* and *pleD* transcription and the region replaced in the construction of the *pleD*::*kan* strain indicated. DNA fragments from the *Sst*I-*Eco*RI fragment were subcloned into the pRK2L1 or pRK2L10 replicating vector and examined for complementation of the Mot^* phenotype conferred by the *pleD*::*kan* allele in the PC4707 strain (*pleC319 pleD*::*kan*) in liquid culture and in motility agar. +, complementation of the *pleD*::*kan* allele to give the Mot(Ts) phenotype; −, no complementation. ●, *pleD301* mutation. B, *Bam*HI; C, *Cla*I (not all sites are shown); E, *Eco*RI; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I (not all sites are shown); S, *Sst*I.

TABLE 2. Plasmids

Plasmid[a]	Description[b]	Source or reference
pBluescript II KS+	Amp^r	Stratagene
pDIVK-DGH	Tc^r; pRK2L10 derivative carrying the *divK* allele from strain PC8981 as a 511-bp *Sst*I-*Bam*HI fragment obtained by PCR	This study
pGH500	Tc^r; *C. crescentus* integrating vector; replicates in *E. coli*	8
pGH501	Tc^r; pGH500 derivative carrying the 511-bp *divK* complementing region plus 3.8 kb of contiguous downstream DNA; isolated from the CB15/pZHF37-GH cointegrate strain	This study[c]
pGH503	Tc^r; pGH500 derivative carrying the 511-bp *divK* complementing region plus 3.8 kb of contiguous downstream DNA; isolated from the PC8981/pZHF37-GH cointegrate strain	This study[c]
pGH509	Tc^r; pGH500 derivative carrying the 511-bp *divK* complementing region plus 300 bp of contiguous downstream DNA; isolated from the CB15/pZHF37-GH cointegrate strain	This study[c]
pGH510	Tc^r; pGH500 derivative carrying the 511-bp *divK* complementing region plus 300 bp of contiguous downstream DNA; isolated from the PC8981/pZHF37-GH cointegrate strain	This study[c]
pGH511	Amp^r; 4.3-kb *Sst*I-*Eco*RI insert cloned from pGH501 into pBluescript II KS+	This study
pGH512	Amp^r; 4.3-kb *Sst*I-*Eco*RI insert cloned from pGH503 into pBluescript II KS+	This study
pGH513	Amp^r Kan^r; derivative of pGH511 with the 1.1-kb *Bam*HI fragment replaced by a Kan^r cassette from pUCKm5Pst$^-$, thus generating a *pleD* null allele	This study
pGH516	Tc^r Kan^r; pGH500 derivative carrying the entire insert from pGH513 cloned as an *Sst*I-*Eco*RI fragment	This study
pGH518	Tc^r; pRK2L10 derivative carrying the entire insert from the *pleD*$^+$ clone pGH511 as an *Sst*I-*Eco*RI fragment	This study
pGH529	Amp^r; pRSETA derivative carrying the same insert as pGH535	This study[c]
pRK2L1	Tc^r; *C. crescentus* replicating vector used in complementation experiments	22
pRK2L10	Tc^r; *C. crescentus* replicating vector used in complementation experiments, similar to pRK2L1 but carrying the polylinker from M13tg131[d]	23
pRSETA	Amp^r	Invitrogen
pUCKm5Pst$^-$	Amp^r Kan^r; pUC derivative carrying a Kan^r cassette	S. Inouye
pZHF37-GH	Tc^r; pGH500 derivative carrying the 511-bp *Sst*I-*Bam*HI *divK* complementing region	8
pZHF37-RK	Tc^r; pRK2L1 derivative carrying the 511-bp *Sst*I-*Bam*HI *divK* complementing region	8

[a] Plasmids appearing only in Fig. 2 are not listed.
[b] Tc^r, resistance to tetracycline; Amp^r, resistance to ampicillin; Kan^r, resistance to kanamycin.
[c] See the text and Fig. 2.
[d] Obtained from Amersham.

was constructed in vitro by replacing the 1.15-kb *Bam*HI$_2$-*Bam*HI$_3$ fragment (Fig. 2) in plasmid pGH511 with the Kan^r cassette from plasmid pUCKm5Pst$^-$ (Table 2). The resulting plasmid, pGH513, was used to replace the wild-type *pleD* allele of strain CB15 with the *pleD*::*kan* allele to generate strain PC4695 (*pleD302*::*kan*), as described previously (8, 18). Southern blot analysis confirmed that the Kan^r cassette in PC4695 is at the predicted location and that the *Bam*HI$_2$-*Bam*HI$_3$ fragment is not present (data not shown). Isolation of chromosomal DNA, Southern transfer, nick translation, and hybridization procedures followed standard protocols (17).

DNA sequencing. The nucleotide sequence of the coding strand of the wild-type *pleD* gene was determined by using a series of nested deletions in pGH511 generated by exonuclease III (Stratagene), incubation with S1 nuclease for 30 min at 30°C, Klenow digestion (5 min at 37°C), and fill-in (15 min at 24°C) reactions. Oligonucleotide primers designed to anneal to the *pleD* coding strand were used to determine the nucleotide sequence of the anticoding strand of *pleD*. The sequence of 300 bases at the 5′ end of the *pleD301* gene was determined by using plasmid pGH512 and a primer designed to hybridize to the coding strand of *divK*. The dideoxy chain termination reaction was carried out with the Sequenase sequencing kit (United States Biochemical). DNA sequences were analyzed with the Genetics Computer Group software package (University of Wisconsin). Potential DNA binding motifs were analyzed with the PROSITE Dictionary of Protein Sites and Patterns or the ProfileScan library of structural motifs.

Nucleotide sequence accession numbers. The *pleD* sequence has been deposited in the GenBank database under accession number L42554. GenBank numbers for sequences in Fig. 4B are as follows: *Vibrio anguillarum*, VAU17054; *E. coli*, D12597; *Mycobacterium leprae*, U00015; *Streptomyces ambofaciens*, Z19594; *Streptomyces griseus*, M77841; *Rhodobacter capsulatus*, Z15088.

RESULTS

***pleD* and *divK* are different genes.** Previous studies failed to detect recombination between the *pleD301* and *divK341* mutations, both of which were isolated as bypass suppressors in a pseudoreversion analysis of *pleC* (32). The recent cloning and sequencing of *divK* (8) allowed a direct test of whether the *pleD301* mutation lies within the *divK* ORF. Replicative plasmid pZHF37-RK, which contains the intact *divK* ORF (8) (Fig. 2), did not complement the *pleD301* mutation either in the wild-type or mutant *pleC* genetic background (data not shown), indicating that *pleD301* is not a recessive allele of *divK*.

We examined the possibility that *pleD301* is a dominant allele of *divK* by amplifying the *divK* gene from *pleD301* PC8981 by PCR (10). Four independent *divK* clones in integrative vector pGH500 were examined, and none conferred a Mot* phenotype to the wild-type strain CB15 or a *pleC* mutant (data

TABLE 3. Integration experiments with plasmids containing intact and truncated copies of wild-type *pleD* and *pleD301*

Recipient strain and plasmid insert (source)[a]	Phenotype of cointegrates[b] [%]
PC5262 *pleC319*	
No plasmid	Mot(Ts)
pGH503 (PC8981 *pleD301*)	Mot$^{+/-}$
pGH510 (PC8981 *pleD301′*)	Mot(Ts) [72], Mot* [28]
PC4166 *pleC319 pleD301*	
No plasmid	Mot*
pGH501 (CB15 *pleD*$^+$)	Mot$^{+/-}$
pGH509 (CB15 *pleD*$^+$′)	Mot* [76], Mot(Ts) [24]

[a] See Materials and Methods for construction; in addition to *divK*, inserts contained the complete *pleD* ORF from the wild-type (*pleD*$^+$) or mutant (*pleD301*) strain or only the first 324 bp of the *pleD* ORF from the wild-type (*pleD*$^+$′) or mutant (*pleD301′*) strain.
[b] Motility and cell division phenotypes were determined by a phase-contrast microscopy examination of mid-log-phase cultures growing in PYE liquid at 37°C. Mot$^+$, wild-type motility; Mot*, supermotility; Mot(Ts), temperature-sensitive wild-type motility; Mot$^{+/-}$, intermediate percentage of motile cells (between those observed for CB15 [wild type] and the nonmotile PC5225 strain [*pleC301*::Tn*5*]).

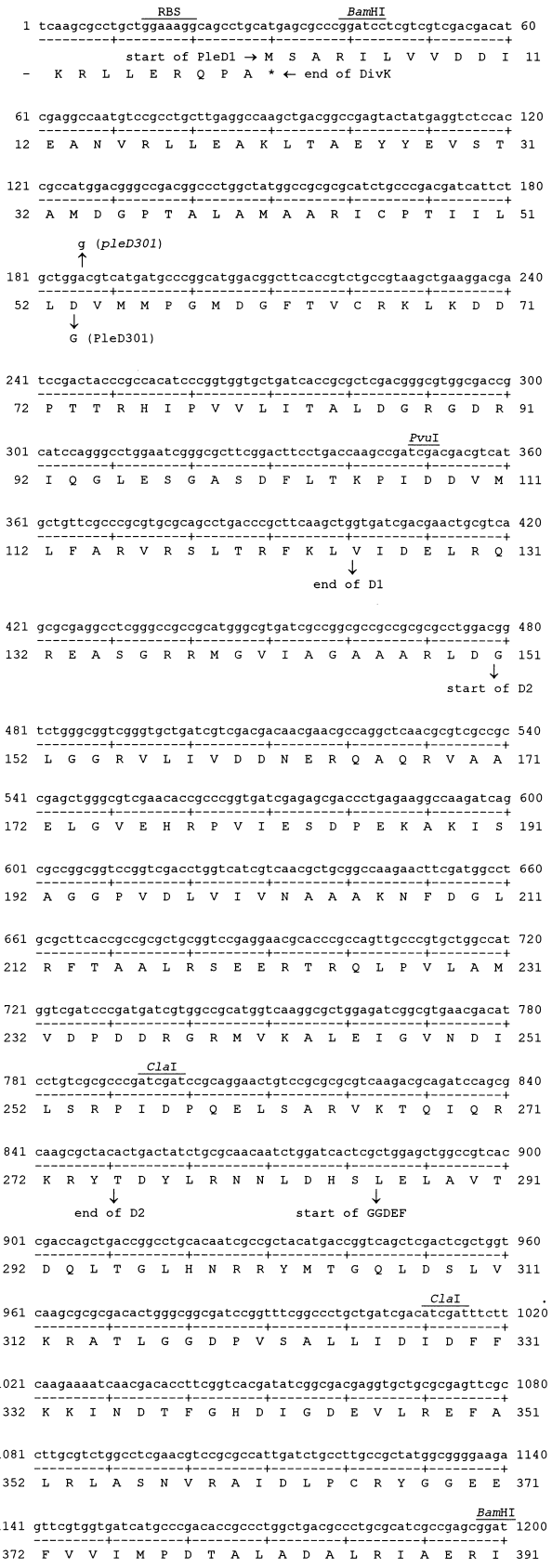

FIG. 3. Nucleotide and translated amino acid sequences of *pleD*. The putative ribosome binding sequence (RBS) and the nucleotide and amino acid changes conferred by the *pleD301* mutation are indicated. The 3′ region of the *divK* ORF is also translated.

not shown). Failure of these clones to display a dominant supermotility phenotype suggested that they carry the wild-type *divK* gene and that the *pleD301* allele is not located within the *divK* ORF, a conclusion confirmed by the DNA sequence analysis reported below.

Cloning of the *pleD* gene. Because DNA sequencing did not identify an ORF upstream of *divK*, we cloned chromosomal DNA 3′ of the gene from both wild-type and *pleD301* strains (see Materials and Methods). Integrative plasmid pGH503, which carries *divK* and 4.3 kb of DNA from a *pleD301* strain, was mated into the *pleC319* tester strain PC5262, which is Mot(Ts). The resulting cointegrate strain, which is heterozygous for the *pleD* allele (*pleD*$^+$ *pleD301*), was motile at 37°C (Table 3), as expected if the *pleD301* mutation suppresses the *pleC319* mutation and confers a semidominant motility phenotype. Reciprocal crosses were carried out by integrating the *pleD*$^+$ plasmid pGH501 into the Mot* *pleC319 pleD301* tester strain PC4166 to confirm the semidominant phenotype of *pleD301* (Table 3).

Construction and phenotype of a *pleD*-null allele. Complementation assays to define the limits of the *pleD* gene were difficult when the semidominant *pleD301* allele was used, and, consequently, we constructed the *pleD*-null strain PC4695 (*pleD302*::*kan*; see Materials and Methods) with the expectation that it might confer a tighter phenotype. Strain PC4695 divides normally, indicating that *pleD* is not required for viability or normal cell division. Microscopic analysis also revealed that the *pleD*::*kan* allele, like *pleD301*, confers a supermotile phenotype and suppresses the Mot(Ts) phenotype of *pleC319* (data not shown) (31), but unlike the semidominant *pleD301* allele, it is recessive to the wild-type gene in complementation experiments (Fig. 2) (see below). Neither the null allele nor the *pleD301* mutation suppressed the resistance to bacteriophage ϕCbK (ϕCbK^r) conferred by *pleC* (data not shown), however, demonstrating that *pleD* is not required for the appearance of polar phage receptors.

Defining the minimum *pleD* complementation region. The limits of the *pleD* gene were determined by complementation of the supermotile *C. crescentus pleC319 pleD*::*kan* tester strain with replicating plasmids carrying subclones of the DNA insert in plasmid pGH518, which contains the 511-bp *divK* region and 4.3 kb of contiguous 3′ flanking DNA. Complementation of the *pleD302*::*kan* allele in the *pleC319* genetic background results in a Mot(Ts) phenotype (Table 3), and the smallest fragment sufficient for complementation was the 2.5-kb *Sst*I-*Kpn*I fragment carried by plasmid pGH536. As expected, neither the minimal *divK* complementing region in plasmid pZHF37-RK

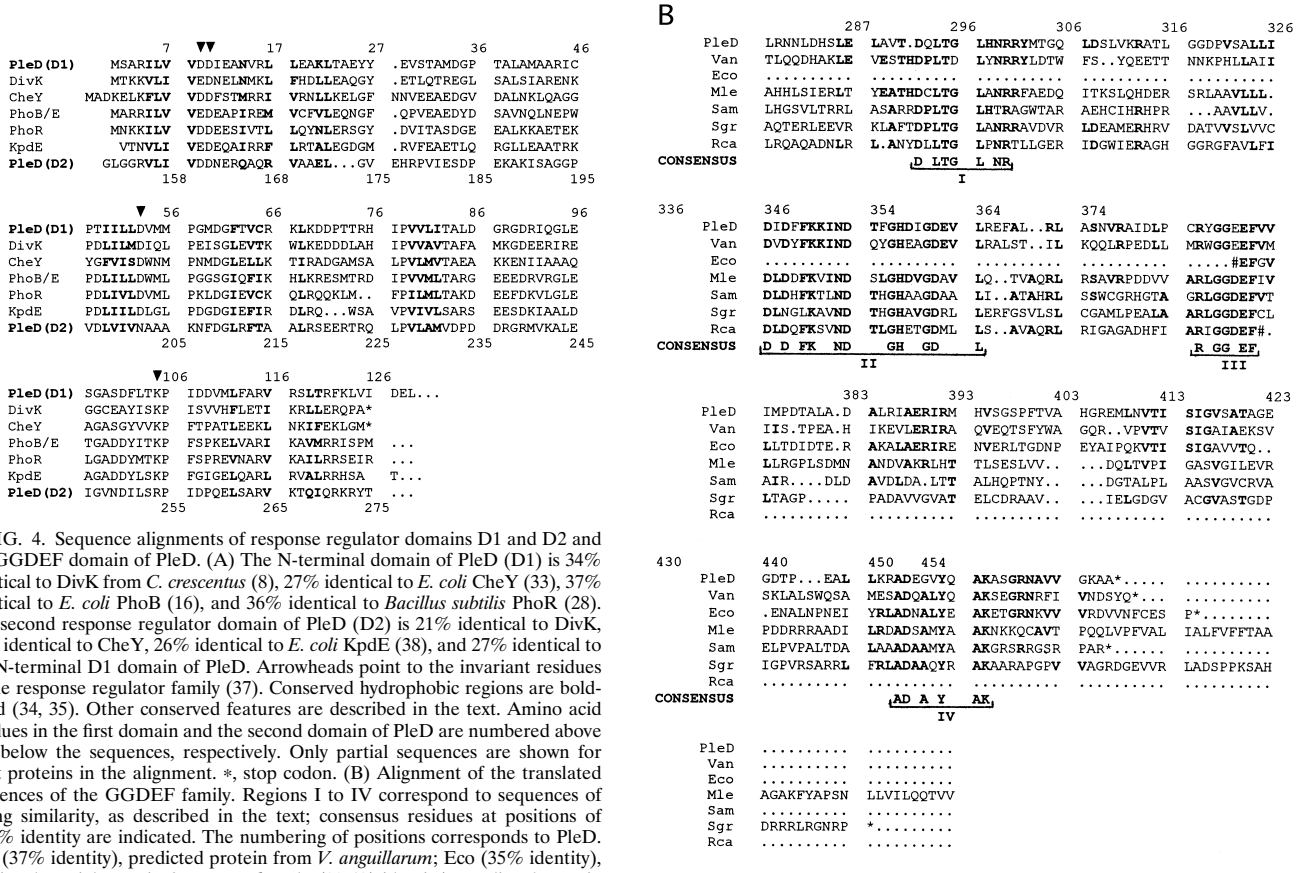

FIG. 4. Sequence alignments of response regulator domains D1 and D2 and the GGDEF domain of PleD. (A) The N-terminal domain of PleD (D1) is 34% identical to DivK from *C. crescentus* (8), 27% identical to *E. coli* CheY (33), 37% identical to *E. coli* PhoB (16), and 36% identical to *Bacillus subtilis* PhoR (28). The second response regulator domain of PleD (D2) is 21% identical to DivK, 22% identical to CheY, 26% identical to *E. coli* KpdE (38), and 27% identical to the N-terminal D1 domain of PleD. Arrowheads point to the invariant residues of the response regulator family (37). Conserved hydrophobic regions are boldfaced (34, 35). Other conserved features are described in the text. Amino acid residues in the first domain and the second domain of PleD are numbered above and below the sequences, respectively. Only partial sequences are shown for most proteins in the alignment. *, stop codon. (B) Alignment of the translated sequences of the GGDEF family. Regions I to IV correspond to sequences of strong similarity, as described in the text; consensus residues at positions of >80% identity are indicated. The numbering of positions corresponds to PleD. Van (37% identity), predicted protein from *V. anguillarum*; Eco (35% identity), predicted partial protein from *E. coli*; Mle (32.5% identity), predicted protein from *M. leprae*; Sam (29% identity), *S. ambofaciens* predicted ORF 183 protein (7); Sgr (29% identity), predicted partial protein from *S. griseus*; Rca (42% identity), predicted partial protein from *R. capsulatus*. #, gap or break.

nor the plasmid clone containing *pleD301* complemented the allele (Fig. 2). Our results also show that deletion of a 200-bp fragment containing the *divK* promoter (8) results in loss of complementation (cf. plasmid pGH528 [Fig. 2]), which suggests that *pleD* is in the *divK* transcription unit.

Sequences of *pleD* and the *pleD301* allele. We determined the sequence of ca. 2.0 kb of DNA located immediately downstream of *divK* on the 2.5-kb *Sst*I-*Kpn*I insert of plasmid pGH536 (Fig. 2). The longest continuous ORF contains 1,365 nucleotides and encodes a predicted polypeptide of 454 amino acids (Fig. 3) with a molecular mass of 49.6 kDa. The ORF is predicted to begin at the ATG codon at nucleotide 29, which has a possible ribosome binding site 9 bp upstream, and to terminate at the TGA stop codon at nucleotide 1393. This sequence displays a codon usage typical of *C. crescentus* (13). The *pleD* start codon overlaps the stop codon of *divK* (Fig. 3), suggesting that translation of the two genes could be coupled.

Campbell recombination experiments with plasmids pGH509 and pGH510, which contain 5′ fragments of the *pleD* gene isolated from the wild-type strain and a *pleD301* strain, respectively (see Materials and Methods), demonstrated that the *pleD301* mutation lies outside *divK* and within the first 324 bp of the *pleD* ORF (Table 3). Nucleotide sequence analysis of this region of the cloned *pleD301* allele showed that the *pleD301* mutation is a single A-to-G base pair change at nucleotide 186, a nonsilent change that is predicted to result in a D-53→G substitution (Fig. 3). This result confirmed that the ORF identified is *pleD*.

***pleD* encodes a predicted compound response regulator.** Analysis of the translated *pleD* sequence by the method of Lipman and Pearson (14) revealed two tandem sequences with extensive homology to the N-terminal regulatory domains of bacterial response regulators that are typically phosphorylated by histidine kinases (26, 35, 37). These sequences, designated domains D1 and D2, are aligned with the response regulators to which they are most similar, including DivK and CheY (Fig. 4A). Domain D1, corresponding to the N-terminal 125 amino acids (Fig. 3), is 25 to 37% identical to members of the response regulator superfamily. In particular, Asp-9, Asp-10, Asp-53, and Lys-105 (arrowheads in Fig. 4A) correspond to the four typically invariant residues found in these proteins. As pointed out above, Asp-53, the predicted site of phosphorylation (27), is changed to Gly by the *pleD301* mutation (Fig. 3). Domain D1 also contains hydrophobic sequence motifs (boldfaced in Fig. 4A) that are conserved in most of the response regulator proteins and believed to be necessary for correct protein folding (26, 34, 35, 37). In addition, the D1 domain contains other sequence elements characteristic of this protein family, including residues 56 to 61, which correspond well to the preferred primary structure of the γ-turn loop (37).

The D2 domain of PleD, which extends from residue 151 to residue 275 (Fig. 3), displays less similarity to the response regulator family than does the D1 domain. It is 20 to 26% identical to the response regulators shown, and it displays the highest sequence identity (27%) to D1. The D2 domain does contain the characteristic hydrophobic motifs (Fig. 4A) and residues at positions 205 to 210 (Ala-Lys-Asn-Phe-Asp-Gly) that are consistent with the γ-turn loop consensus sequence (37). Only two of the four typically "invariant" residues of the

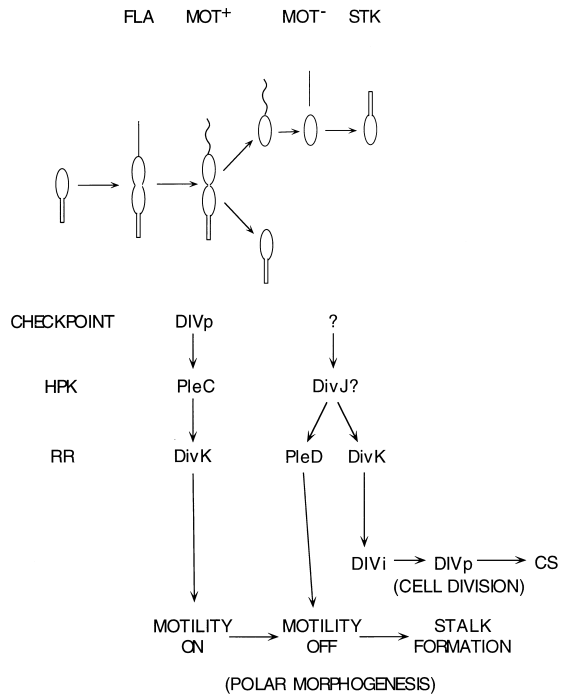

FIG. 5. Proposed functions of PleD, DivK, PleC, and DivJ in cell division and polar morphogenesis. This model proposes that the response regulators (RR) PleD and DivK are controlled at two different times in the cell cycle by two different histidine kinases, PleC and DivJ, which are in turn regulated by the completion of cell cycle checkpoints. Late in the cell cycle DivK responds to the PleC histidine protein kinase (HPK) to initiate motility and events necessary for stalk formation (STK). The PleD protein is required early in the next cell cycle for turning off motility, for the loss of the flagellum (FLA), and for stalk formation in response to another histidine kinase, which we speculate to be DivJ. DivK, which is also shown responding to histidine kinase DivJ, is responsible for regulating an essential cell division step. A cell cycle checkpoint termed division progression (DIVp) has been proposed to regulate the PleC-DivK signal transduction pathway (8, 32, 39) (reviewed in references 12 and 19); an unidentified cell cycle checkpoint is presumed to be responsible for regulating the activities of PleD and DivK during G_1 phase. The developmental events "motility on," "motility off," and "stalk formation" are depicted as a series of dependent steps as described elsewhere (31). DIVi, division initiation; CS, cell separation.

response regulators are conserved in the D1 domain, however. These are Asp-160 and Asp-161, which correspond to Asp-12 and Asp-13, respectively, of CheY (arrowheads in Fig. 4A). A number of residues in and around the active site diverge from consensus: (i) an Asn residue is present in place of an Asp at residue 202, the predicted site of phosphorylation; (ii) Arg-109 replaces the predicted Lys-109; and (iii) the Val-232 and Asp-233 residues are different from the Thr/Ser and nonpolar amino acids, respectively (Fig. 4A).

The C terminus of PleD identifies a novel GGDEF domain. We detected no similarity of the 169-amino-acid C terminus of PleD to known DNA-binding motifs (see Materials and Methods) or other effector domains of previously characterized response regulators (reviewed in references 26 and 35). Analysis by the method of Lipman and Pearson (14) did identify protein sequences from six different bacteria that are highly similar to the C terminus of PleD, however (Fig. 4B). The similarities range from 42% identity to an *R. capsulatus* sequence of 95 amino acids to 29% identity to both an *S. ambofaciens* sequence of 183 amino acids (7) and an *S. griseus* sequence of 167 amino acid (Fig. 4B).

Comparison of the seven sequences reveals four regions (I to IV) of particularly high sequence identity (Fig. 4B). Of the 29 residues included in the consensus sequence for regions I to IV in Fig. 4B, 24 are invariant. To our knowledge, the similarity between these proteins has not been previously reported. We have provisionally designated this sequence family GGDEF, which is a conserved motif present in region III of four of the sequences and corresponds to residues 368 to 372 in PleD. PleD is the only GGDEF protein known to contain a response regulator domain and to be assigned a function on the basis of genetic data.

DISCUSSION

Formation of new swarmer cells in *C. crescentus* results from successive morphogenetic events, including flagellum formation, the turning on of flagellum rotation, and bacteriophage φCbK receptor formation, all of which depend on cell cycle progression (11, 25, 29, 30). Previous results have suggested that the gain-of-motility event in predivisional cells depends on a signal transduction pathway mediated by the histidine kinase PleC and response regulator DivK, apparently in response to completion of late cell division event termed division progression or DIVp (8). The experiments reported here identify the translated *pleD* gene product as a novel compound response regulator that may function after cell division in a swarmer-cell-specific signal transduction pathway leading to loss of motility and stalk formation (Fig. 1A).

Identification of PleD domains. The N-terminal-sequence domain D1 is highly conserved, and identification of the semidominant *pleD301* as a D-53→G change at the predicted site of phosphorylation (27) provides genetic evidence that PleD may be regulated by a histidine kinase. The D2 sequence domain contains conserved motifs necessary for the correct folding of response regulators, but it lacks the conserved residues expected at the active site of response regulators (Fig. 4A). This unique structure indicates that, if this sequence domain is required for PleD activity, it does not function by a conventional transphosphorylation mechanism. The only other compound response regulator reported is the *Myxococcus xanthus* FrzZ protein, which contains two highly conserved response regulator domains and no C-terminal domain (40).

The C terminus of PleD is not similar to the effector domains of any previously described response regulator, but this GGDEF sequence is very similar (29 to 42% identity; Fig. 4B) to the translated amino acid sequences of six other bacterial species. Although no function has been assigned to any of the latter sequences, the modular organization of response regulators into N-terminal regulatory and C-terminal effector domains (reviewed in references 26 and 35) leads us to speculate that the GGDEF sequence in PleD could function as an effector domain or, alternatively, a catalytic domain (see reference 15). Mutational analysis of the PleD domains will help to clarify many of these questions about the structure-function relationships.

Role of PleD in polar morphogenesis. The phenotypes of *pleD* mutants, which are blocked in development before loss of motility and stalk formation, suggest that the PleD protein is part of a signal transduction pathway required for turning off flagellum rotations and other developmental events during the G_1-phase swarmer-to-stalked-cell transition (Fig. 1A and C). PleC (39) and DivJ (21) are the only histidine kinases implicated to date in the regulation of polar morphogenesis in *Caulobacter* spp., and the requirement of PleC for motility and stalk formation is restricted to late S phase (31). Thus, DivJ, whose expression is initiated in the swarmer cell (21), is a more plausible candidate for a G_1-specific kinase regulating PleD (Fig. 5). Supporting this possibility is the observation that double mutants containing the *pleD* null allele and any *divJ* muta-

tion can be constructed but certain *divJ* mutations in combination with the *pleD301* allele cannot (9). The latter results suggest that these double mutants are not viable and raise the possibility of a DivJ-PleD interaction. We have previously proposed that the cell division function of the response regulator DivK depends on the histidine kinase DivJ (8, 12, 19, 21), and as a working model we speculate that DivJ may regulate the activities of both DivK and PleD early in the swarmer cell (Fig. 5).

In this model, PleD is shown to be part of a signal transduction pathway that functions to inhibit motility in the G_1 phase, and the PleC-DivK signal transduction pathway would then start flagellum rotation by relieving this inhibition of motility. Such a mechanism is consistent with the recessive Mot* phenotype of the *pleD*::*kan* disruption (Fig. 2), and it may also explain how *pleD* mutations suppress the nonmotile phenotype of *pleC* strains. If inhibition of motility is not established in a *pleD* mutant, the cells could effectively escape the requirement of the PleC-DivK pathway for turning on motility and thus bypass the Mot phenotype of *pleC* mutations. Several explanations are possible for the semidominance of the *pleD301* allele, including formation of inactive oligomers by wild-type and mutant proteins and the inhibition of the cognate histidine kinase or other signalling partner as a result of the abortive docking of the mutant response regulator.

As an alternative model for *pleD* regulation, we have considered the possibility that PleD functions twice in the cell cycle, once in the G_1 phase, as discussed above, and then a second time during the G_2 phase to control the onset of motility, either in parallel to or as part of a multicomponent signal transduction pathway (4) that includes the PleC and DivK proteins. We favor the formulation in Fig. 5, however, which assumes that PleD acts only during the G_1 phase to switch off motility. This model offers the simplest interpretation of our results and provides a framework for further experiments.

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