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A histidine protein kinase is involved in polar organelle development in *Caulobacter crescentus*

(differentiation/phosphorylation/cell cycle)

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ABSTRACT **Mutations having pleiotropic effects on polar organelle development (*pod*) in *Caulobacter crescentus* have been identified and shown to occur in at least 13 genes scattered throughout the genome. Mutations at each locus affect a unique combination of polar traits, suggesting that complex interactions occur among these genes. The DNA sequence of one of these genes, *pleC*, indicates that it is homologous to members of the family of histidine protein kinase genes. Members of this family include the sensor components of the bacterial two-component regulatory systems. Furthermore, *in vitro* experiments demonstrated that the PleC protein was capable of autophosphorylation. These results suggest that the PleC protein (and perhaps the proteins encoded by the other *pod* genes as well) regulates the expression of genes involved in polar organelle development through the phosphorylation of key regulatory proteins. The use of a phosphorelay system cued to internal changes in the cell would provide a mechanism for coordinating major changes in gene expression with the completion of specific cell cycle events.**

Differentiation in *Caulobacter crescentus* is observed as an ordered series of events at the poles of the cell. Each cell division results in two cell types, a stalked cell like the parent and a motile swarmer cell. The two new poles formed at cell division lack polar organelles, while the older poles contain polar organelles produced during previous rounds of differentiation. After cell division, DNA replication is initiated immediately in the stalked progeny cell, followed by the assembly of a set of polar organelles including a flagellum, pili, and phage receptors at the newly created pole. The resulting predivisional cell has a different set of organelles at each pole, the original stalk at one end and the flagellum, pili, and phage receptors at the other.

After a period of motility, the swarmer cell releases the flagellum into the culture medium, and the remainder of the polar organelles are replaced by a stalk. An exception is the holdfast material, which is found at the base of the flagellum. The holdfast material is retained and ends up at the tip of the stalk. Consequently, the holdfast material serves as a convenient landmark to demonstrate that the stalk is formed at the flagellar pole. The resulting stalked pole is terminally differentiated and remains unchanged during subsequent cell-division cycles. Thus, newly formed poles undergo two series of differentiation events. First, as part of a predivisional cell, they assemble a flagellum, pili, and phage receptors. Subsequently, as a differentiated pole on a swarmer cell, they lose the first set of organelles and replace them with a stalk.

Several mutations that have multiple effects on differentiation of the polar organelles have been described previously. For instance, *flbT* mutants make bilobed stalks, overproduce the flagellin proteins, are nonchemotactic, and have a reduced ability to release assembled flagellar filaments prior to stalk development (1, 2). Also, *pleC* mutants are resistant to polar bacteriophage, produce a paralyzed flagellum, and are stalkless in the presence of excess phosphate (3–5). In contrast, *pleA* mutants are resistant to polar bacteriophage and do not synthesize a flagellum (3–5). It has been suggested that these genes encode regulatory elements that control the expression of genes involved in polar development (1, 2, 5).

To provide evidence that these genes encode regulatory elements, we have cloned, sequenced, and analyzed one of these genes, *pleC*.§ Comparison of the derived amino acid sequence of the PleC protein to known sequences suggests that it is a histidine-specific protein kinase similar to the sensor proteins involved in two-component regulatory systems. The kinase activity of the PleC protein was demonstrated by showing that the C-terminal portion of the protein was capable of autophosphorylation. Thus, we propose that the PleC protein is involved in a phosphorelay system that functions to regulate polar organelle development. To identify additional genes involved in this regulatory system, we isolated a collection of mutants with altered polar organelle development.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The bacterial strains used in this study are all derivatives of the *C. crescentus* wild-type strain CB15. Growth conditions and genetic techniques have been described (6–8). Bacterial cells were observed by phase-contrast light microscopy, in the light microscope after staining (9), or by electron microscopy (10). Resistance to bacteriophage ϕCbk was determined by spotting dilutions of a phage lysate on lawns of bacteria in PYE soft agar (11).

Molecular Techniques. Recombinant DNA experiments were performed by using techniques described by Sambrook *et al.* (12). Single-stranded DNA for sequencing was obtained from derivatives of the Bluescript plasmids, and sequencing reactions were performed with Sequenase according to the directions of the manufacturer (United States Biochemical). Western analyses and pulsed-field gel electrophoresis were performed as described (2, 13).

Autophosphorylation Assay. *Escherichia coli* cells containing the *lacZ*::*pleC* fusion were grown at 37°C to an optical density at 600 nm of 0.6, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the

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Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M91449).

culture was incubated for an additional 2.5 hr. The resulting cells were collected by centrifugation, washed with phosphate-saline buffer (14), and resuspended in phosphate saline buffer in 2.5% of their original volume. After storage at −70°C overnight, the cells were disrupted by two passages through a French pressure cell at 20,000 psi (1 psi = 6.89 kPa), and the unbroken cells and cell debris were removed by centrifugation at 1900 × *g*. The cellular proteins were separated by SDS/polyacrylamide gel electrophoresis and purified as described by Hager and Burgess (15). The PleC fusion proteins were renatured and phosphorylated essentially as described by Jin *et al.* (16). To test the stability of the phosphorylated fusion proteins, the acrylamide gels containing the phosphorylated proteins were treated with either 0.5 M NaOH for 2 hr at room temperature or with 5% trichloroacetic acid for 2 hr at 68°C. The treated gels were neutralized with 0.1 M Tris (pH 7.5), rinsed with water, and exposed to the Kodak x-ray film.

RESULTS

***pleC* Gene Encodes a Histidine Protein Kinase.** A cosmid clone (pLSG7) containing the *pleC* gene was identified by complementation of SC618 (*pleC147*) (17). Additional experiments demonstrated that a subclone of pLSG7, pSCC25, containing a 4.4-kilobase (kb) *Xho* I–*Xho* I fragment complements SC618 and that the smaller *Xho* I–*Eco*RI fragments could not (Fig. 1). Thus, DNA on both sides of the central *Eco*RI site is required for complementation. The location of the *pleC* gene was confirmed by use of Southern hybridization experiments demonstrating that all of the *pleC*::Tn*5* insertions were located in a small region that flanked the *Eco*RI site (data not shown).

The DNA sequence of the entire 4.4-kb region flanking the *Eco*RI site was determined by the strategy in Fig. 1. Computer analysis of the sequence revealed the presence of three coding regions. The central coding region was designated the *pleC* gene because it includes the *Eco*RI site demonstrated to be important in the complementation experiments. The ATG at position 588 is the most likely start codon for the *pleC* gene, and it is preceded by a ribosome-binding site. The stop codon for the *pleC* gene is located at position 3114, generating a protein with 842 amino acid residues (M_r 88,915). The region upstream of the *pleC* gene contained the 3′ end of a gene transcribed in the same direction as *pleC* and separated from *pleC* by 167 base pairs (bp). The third coding region was 67 bp downstream of the *pleC* gene and could encode another protein of 326 amino acid residues.

To address the role of the *pleC* gene, the deduced amino acid sequence derived from the *pleC* gene was used in a homology search of the GenBank data base. The results indicated that the PleC protein shares a high level of homology in the C-terminal region with the products of the histidine protein kinase gene family. Proteins in this gene family are capable of autophosphorylation at a conserved histidine (16, 18). PleC contains an appropriate histidine within a highly conserved sequence centered at amino acid 610 (Fig. 2). The proteins with histidine protein kinase regions most homologous to PleC were the family of sensor proteins from two component regulatory systems, such as *Bordatella pertussis* BvgS (19) and *Escherichia coli* RcsC (18) (Fig. 2). The sensor proteins are often transmembrane proteins responsible for receiving a signal from either outside or inside a cell. Once the sensor receives the signal, it is autophosphorylated by its histidine protein kinase region and then transfers the phosphate moiety to another protein called the response regulator. The regulator then usually activates transcription of a set of coordinately regulated genes. Besides their conserved C-terminal regions, the sensors often contain membrane-spanning domains in their N-terminal region. Examination of the deduced amino acid sequence of PleC revealed two hydrophobic domains in the N-terminal region that stretch from the valine at position 29 to Val-50 and from Gly-283 to Ile-303. These two regions could serve as membrane-spanning regions generating a periplasmic domain of ≈230 amino acid residues. Thus, PleC appears to be a membrane-bound histidine protein kinase.

C-Terminal Domain of PleC Is Sufficient for Histidine Protein Kinase Activity. To confirm that the *pleC* gene

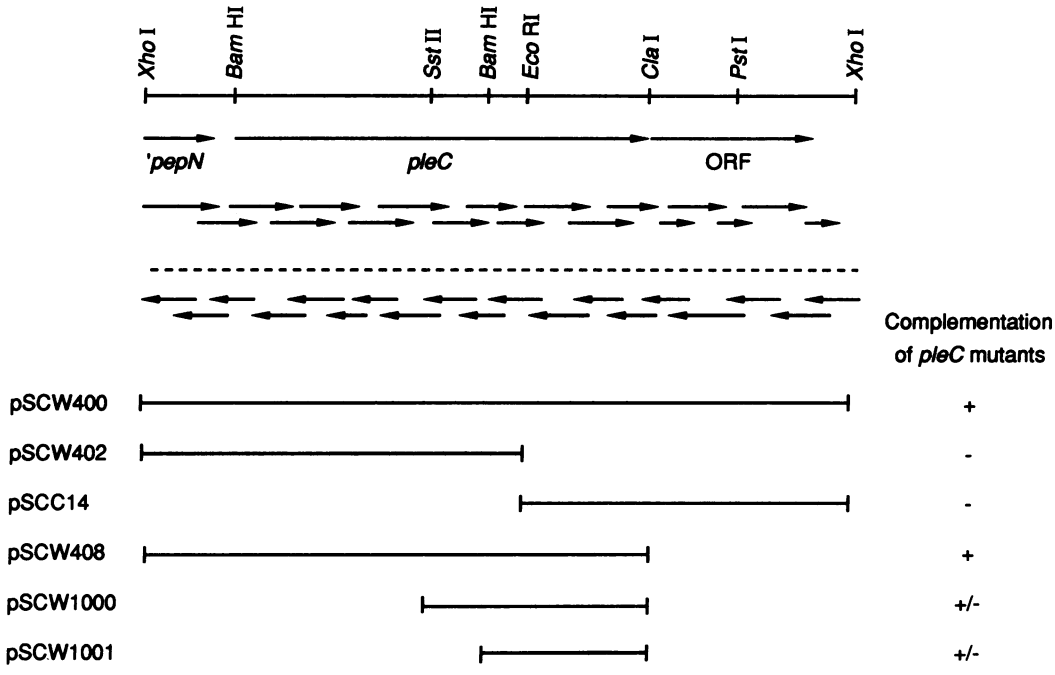

FIG. 1. Restriction map and sequencing strategy for the *pleC* gene. Arrows indicate the length and direction of the sequenced DNA fragments. The positions of subclones are shown along with the complementation of *pleC* mutants. A positive complementation result (+) indicates that the clone restored the wild-type phenotype. A negative complementation result (−) indicates that the clone did not alter the pleC phenotype. An intermediate result (+/−) indicates that the clone provides partial complementation.

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Ec.RcsC  ..........  .........V SSRVKMEESL QEMAQ.....  ..AAEQASQS  453
Cc.PleC  ..........  RNEEQLQNAV AGLERSQEQL AELARKYETE  KVKAESANKA  600
Bp.BvgS  HVYQWTIPYG DSLGELKGII GGWIDITER. AELLRELHDA  KESADAANRA  719

Ec.RcsC  KSMFLATVSH ELRTPLYGII GNLDLLQTKE L.PKGVDR..  .LVTAMNNSS  499
Cc.PleC  KSEFLANMSH ELRTPLNAIN GFSEIMMNEM FGPLGDQRYK  GYSQDIHSSG  650
Bp.BvgS  KTTFLATMSH EIRTPMNAII GMLEL...AL LRPTDQEPDR  QSIQVAYDSA  766

Ec.RcsC  SLLLKIISDI LDFSKIESEQ LKIEPREFSP REVMNHITAN  YLPLVVRKQL  549
Cc.PleC  QHLLALINDI LDMSKIEAGK MNLKFESMHL EDVAEDAVRL  VRN..RAEAA  698
Bp.BvgS  RSLLELIGDI LDIAKIEAGK FDLAPVRTAL RVLPEGAIRV  FDGLARQKGI  816

Ec.RcsC  GLYCFIEPDV PVALNGDPMR LQQVISNLLS NAIKFTD.TG  CIVLHVRA..  596
Cc.PleC  GLKLDIDFPQ LPEIEADYRA VKQVLLNLLS NAIKFTPRAG  SVTVRVEVRR  748
Bp.BvgS  ELVLKTDIVG VDDVLIDPLR MKQVLSNLVG NAIKFTT.EG  QVVLAVTARP  865

Ec.RcsC  ..DGDYLSIR VRDTGVGIPA KEVVRLFDPF FQV.GTGVQR  NFQGTGLGLA  643
Cc.PleC  DPFGDLIKVS VTDTGIGIAK EDLARLAKPF EQV.ESQFSK  TTQGTGLGLA  797
Bp.BvgS  DGDAAHVQFS VSDTGCGISE ADQRQLFKPF SQVGGSAEAG  PAPGTGLGLS  915

Ec.RcsC  ICEKLISMMD GDISVDSEPG MGSQFTVRIP LYGAQYPQKK  GVEGLSGKRC  693
Cc.PleC  LTKSLITMHD GVLEMHSTPG EGT..TVSFT L.........  ..........  826
Bp.BvgS  ISRRLVELMG GTLVMRSAPG VGT..TVSVD L.........  ..........  944
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FIG. 2. Comparison of the histidine protein kinase region of the pleC gene to that of two previously identified sensor proteins. Ec.RcsC, *E. coli* RcsC protein (18); Cc.PleC, *C. crescentus* PleC protein; Bp.BvgS, *B. pertussis* BvgS protein (19). Vertical lines indicate identical amino acids. Colons indicate similar amino acids as defined by the GCG program (20). The conserved histidine believed to be the site of autophosphorylation is highlighted by shading.

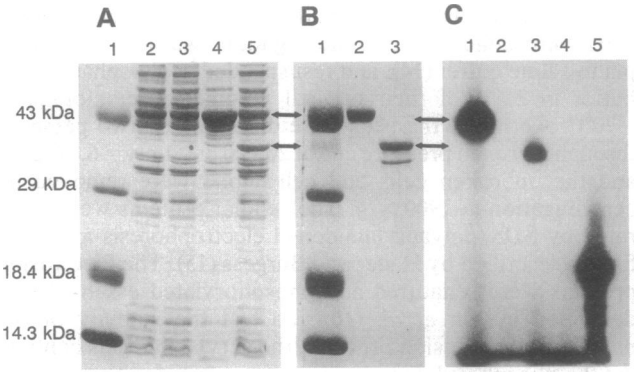

FIG. 3. Autophosphorylation of LacZ-PleC fusion proteins. (*A*) Expression of the *lacZ-pleC* fusions in *E. coli* strain XL-1 Blue (Stratagene). Total cellular proteins, obtained by SDS lysis of logarithmic-phase cultures, were separated on a SDS/15% polyacrymide gel, stained with Coomassie brilliant blue R-250, and destained with 5% methanol containing 7% acetic acid. Lanes: 1, molecular mass standards; 2, pBluescriptII SK(−) without induction; 3, pBluescriptII SK(−) induced by IPTG; 4, pSW1000 induced with IPTG to generate a 45-kDa protein; 5, pSW1001 induced with IPTG to generate a 35-kDa protein. (*B*) Polyacrylamide gel electrophoresis of the purified proteins. Lanes: 1, molecular mass standards; 2, 45-kDa SW1000 fusion protein; 3, 35-kDa SW1001 fusion protein. (*C*) Autophosphorylation of the LacZ-PleC fusion proteins. Lanes: 1, SW1000 fusion protein with [γ-^{32}P]ATP; 2, SW1000 fusion protein with [α-^{32}P]ATP; 3, SW1001 fusion protein with [γ-^{32}P]ATP; 4, SW1001 fusion protein with [α-^{32}P]ATP; 5, phosphorylated myelin.

encodes a histidine protein kinase, the DNA encoding the conserved C-terminal portion of PleC was fused to the β-galactosidase gene, and the resulting hybrid protein was purified and tested for autokinase activity. The gene fusions were constructed by cloning either the 1382-bp *Sst* II–*Cla* I fragment or the 1029-bp *Bam*HI–*Cla* I fragment into the pBluescriptII SK(−) vector (Stratagene) to create pSW1000 and pSW1001, respectively. The DNA sequence of both of the fusion junctions was determined to confirm that in-frame gene fusions had been constructed. The results indicated that pSW1000 would encode a fusion protein containing the first 18 amino acid residues of β-galactosidase fused to the C-terminal 431 amino acid residues of PleC and that pSW1001 would encode a fusion protein containing the first 32 amino acid residues of β-galactosidase and the last 313 amino acid residues of PleC. In both cases, the fused genes were under the control of the *lac* promoter, and regions encoding the membrane-spanning regions of PleC were not present. The 45-kDa and 35-kDa fusion proteins were overexpressed in *E. coli* by induction of the *lac* promoter and were found to be the predicted size when examined by SDS/polyacrylamide electrophoresis (Fig. 3*A*). The appropriate protein bands were purified from the gel (Fig. 3*B*), renatured, and tested for autophosphorylation. The results indicated that both PleC fusion proteins were labeled in the presence of [γ-^{32}P]ATP but not with [α-^{32}P]ATP (Fig. 3*C*). This autophosphorylation was examined further by treating the phosphorylated proteins with alkaline and acidic solutions. The labeled phosphate was readily removed by 5% trichloroacetic acid but was stable in 0.5 M NaOH, indicating a phosphoramidate bond. In contrast, a control protein containing a phosphoester bond at a serine residue was labile under alkaline conditions and stable in the presence of acid (data not shown). Taken together, these results indicate that the C-terminal portion of PleC is sufficient for autophosphorylation and that the phosphorylation probably occurs at a histidine residue. A similar result has been obtained for the VirA protein of *Agrobacterium tumefaciens* (16). Quantitatively, the larger C-terminal fragment appears to be autophosphorylated more efficiently (Fig. 3*B*).

To test the biological function of the truncated PleC proteins, the plasmids containing the gene fusions were transferred into SC3487 (*pleC147 recA526*). The resulting merodiploids produced stalks and were both motile and sensitive to polar bacteriophage (Fig. 1). However, the stalks were not as long as those of the wild type, the swarms were smaller than those of the wild type, and the phage seemed to be less virulent than when it infected wild-type cells. In each case, pSW1000, containing the larger fragment of *pleC*, complemented SC3487 more efficiently than pSW1001. Thus, these *lacZ-pleC* fusions can partially complement a *pleC* mutant even though it does not contain the DNA that codes for the N-terminal region containing the membrane-spanning regions. On the other hand, the N-terminal region does seem to be important for PleC to function with maximum efficiency.

Additional Mutants with Altered Polar Organelle Development. Since the *pleC* gene appears to encode a histidine protein kinase similar to those of the two-component regulatory systems, it is critical to identify other genes involved in the regulation of polar organelle development (*pod*), which may interact with PleC. An additional 22 *pod* mutants have been obtained (3, 6, 10) that are altered in specific aspects of polar organelle development (Table 1). Logarithmic-phase cultures of each of the *pod* mutants were examined by phase-contrast microscopy, both in wet mounts and after being subjected to a flagellar stain (9). Each was examined for motility, the presence of a flagellum, the length of the stalks, the length of the cell, and the production of holdfast material as evidenced by the ability of the stalks to aggregate at the tips to form rosettes. In addition, each mutant was tested for sensitivity to polar bacteriophage. The 22 mutants could be grouped into four classes based on the properties of the stalk.

The class I mutants, represented by *pleC*, did not produce detectable stalks when grown under conditions of excess phosphate (Table 1). The other properties examined varied considerably among the class I mutants, suggesting that each regulated a unique set of genes involved in polar organelle differentiation. In fact, the *flbU* gene has been shown to encode a σ-54 protein and therefore corresponds to the *rpoN* gene of the enteric bacteria (Y. Brun and L. Shapiro, personal communication). This σ factor is required for the synthesis of a number of structural components of the flagellum (22–27) and for stalk formation as well (Y. Brun and L. Shapiro, personal communication).

The class II *pod* mutants, typified by *flbT* (1, 2), frequently fail to release their flagellum prior to stalk formation and were observed to have a flagellum attached to their stalk (Table 1). Both the *flbT* and the *podQ* mutants also produce branched stalks. The class III mutants have altered stalk lengths compared with the wild type. SC246 (*podO*) produces stalks that are longer than those of the wild type regardless of the phosphate concentration, and SC1046 (*podE*) and SC2194 (*podT*) produce stalks that are shorter than those of the wild type. The class IV mutants produce stalks that are the same size as those of the wild type. As with the class I mutants, the other properties of the class II, III, and IV mutants varied considerably, underscoring the complexity of the interactions controlling polar organelle development.

The genetic map location of each of the *pod* mutations was determined by using pulsed-field gel electrophoresis (13). Three of the *pod* mutations were located in the *pleC* gene (Table 1). The remainder were located in one of 12 genes scattered throughout the genome. Mutations occurring at the same chromosomal location always resulted in the same phenotype and were presumed to be in the same gene. In the case of the *rpoN* mutants, all four Tn*5* insertions were shown to occur in a 1.7-kb *Bam*HI fragment containing the *rpoN* gene (Y. Brun and L. Shapiro, personal communication; D. L. Zhang, and B. Ely, unpublished results).

DISCUSSION

Polar differentiation in *C. crescentus* requires precise temporal and spatial control of gene expression throughout the cell cycle. To obtain more information about these regulatory mechanisms, we determined the DNA sequence of the *pleC* gene. *pleC* mutants lack stalks, have paralyzed flagella, are incapable of forming rosettes, and are resistant to polar bacteriophage. Analysis of the deduced amino sequence indicated that the PleC protein is homologous to the histidine protein kinase family of proteins. The C-terminal portion of the protein was shown to be autophosphorylated. Therefore, it appears likely that the PleC protein contributes to the regulation of polar organelle development through autophosphorylation in response to some cell cycle signal, followed by the transfer of the phosphoryl group to one or more additional regulatory proteins. When amino acid sequences were compared, the strongest homology was observed between PleC and several sensor proteins that are members of the family of two-component regulatory systems. These sensor proteins

Table 1. Characterization of strains with defects in polar organelle development

Mutant class	Gene	Map position*	Strains†	Stalk length‡	Motility§	Flagellin synthesis¶	Rosette formation‖	Polar phage**	Cell morphology††
	wt		CB15	N	wt	+	+	S	N
I	*pleC*	3195	SC618, SC1035, SC1056, SC1136	O	Mot	+	−	R	N
	rpoN (flbU)	2050	SC1055, SC1137, SC1138, SC3534	O	Fla	R	+	S	L
	podW	710	SC1029, SC3526	O	Fla	R	+	R	L
II	*flbT*	280	SC276	NBF	Che	++	+	S	N
	podJ	3630	SC1119, SC1124	NF	Che	+	−	R	N
	podQ	980	SC263	LBF	Che	+	+	S	N
III	*podE*	3120	SC1046	S	wt	+	+	S	N
	podO	2440	SC246	L	Che	+	+	S	L
	podT	3390	SC2194	S	wt	+	+	R	N
IV	*pleA*	3430	SC296, SC611, SC614	N	Fla	R	+	R	N
	podR	3510	SC2184	N	wt	+	+	R	L
	podS	3610	SC2663	N	Fla	R	+	R	L
	podU	2540	SC2185	N	Fla	R	+	R	L

wt, Wild type.

*Numbers indicate the approximate position of the gene on the standard genomic map (6).

†All of the strains designated SC1029 or higher contain Tn*5* insertions. The remainder were obtained as spontaneous mutants. Strains SC276, SC296, SC611, SC614, and SC618 have been characterized previously (1, 2, 10, 21) and are included for comparison.

‡N, normal stalks the same length as the wild-type strain when grown in the presence of excess phosphate; O, no stalk is formed unless phosphate is limiting; S and L, shorter and longer stalks, respectively; B, 10–30% of the stalked cells have branched stalks; F, 10–30% of the stalks have a flagellum at their tip.

§wt, Wild-type motility; Fla, no flagellum is produced; Che, motile cells are formed but the strain does not swarm in semisolid media; Mot, the strain produces a paralyzed flagellum.

¶+, Wild-type flagellin synthesis; R, reduced levels of flagellin synthesis; ++, overproduction of flagellin proteins.

‖+ or −, presence or absence of rosettes, respectively.

**S, sensitivity to the polar bacteriophage ϕCbK; R, resistance.

††N, wild-type cell morphology; L, most cells significantly longer than those of the wild-type strain.

are autophosphorylated in response to the binding of a signal molecule. Most sensor proteins of two-component regulatory systems described in other bacteria generally respond to external signals in the environment. However, in the case of PleC, it is likely that the signal would be some internal change in the cell that is indicative of its position in the cell cycle, since differentiation is independent of changes in the environment. This hypothesis is supported by the fact that truncated *pleC* genes encoding proteins that lack the membrane-spanning regions partially complement *pleC* mutants. Alternatively, the N-terminal portion of PleC may inhibit the protein kinase activity until a signal is bound. Thus, the absence of the N-terminal region would eliminate the need for the signal.

Once the autophosphorylation of the sensor protein occurs, the phosphoryl group is transferred to an effector protein. This second phosphorylation activates the effector protein so that it can regulate the expression of the appropriate genes. Although we have not yet identified any effector proteins that interact directly with the PleC protein, the work of Sommer and Newton (5, 28) suggests several candidates. They demonstrated that *pleD*, *divJ*, *divL*, and *divK* are suppressors of a *pleC* mutant. Recent results indicate that the *divJ* gene also encodes a histidine protein kinase that has 48% identity to the C terminus of the PleC protein (N. Ohta, T. Lane, E. Ninfa, J. M. Sommer, and A. Newton, personal communication). Thus, DivJ may function in parallel to PleC, and the mutant DivJ protein may be able to phosphorylate the effector proteins that would have been phosphorylated by PleC. These results suggest that protein phosphorylation may be a primary mechanism for the regulation of polar organelle development in *C. crescentus*.

To identify additional genes involved in these phosphorelay systems, we isolated mutations that affect polar organelle development by screening for colonies with an altered ability to swarm. The majority of these mutants have defects in flagellum biogenesis or chemotaxis (3, 6, 10). However, ≈20% of these mutants contain mutations that affect multiple aspects of polar organelle development. Genetic experiments demonstrated that although several of these mutations occurred in the *pleC* gene, the majority were located in 12 other genes scattered throughout the genome. In fact, additional *pod* genes probably exist, since 8 of the 13 genes were identified by a single mutation. One group of the new mutants that lack both flagella and stalks recently has been shown to contain a Tn*5* insertion in the *rpoN* gene, which encodes the σ-54 protein (Y. Brun and L. Shapiro, personal communication; D. L. Zhang and B. Ely, unpublished data). The mutant phenotype indicates that σ-54 is required for both flagellum and stalk biogenesis. The proteins encoded by the remainder of the newly identified genes have not been determined. However, since each of the *pod* genes affects multiple aspects of polar organelle development in a unique way, the identification of 13 *pod* genes suggests that the regulatory interactions controlling this differentiation are likely to be quite complex.

Taken together, the results presented here suggest that a series of protein phosphorelays may be responsible for coordinating polar differentiation with events in the cell-division cycle. These phosphorelays would respond to internal changes that occur during the cell cycle and thus would coordinate major changes in gene expression with the completion of specific events during the cell cycle. For instance, the completion of some aspect of cell septation might trigger a change in the cell pole that would activate a phosphorelay system responsible for the initiation of DNA replication in stalked cells. Also, in swarmer cells, the accumulation of a protein could activate a different phosphorelay system that is responsible for the loss of the polar organelles and their replacement with a stalk. In each case, a particular cell cycle event would activate a phosphorelay system that in turn would regulate the expression of a variety of genes involved in differentiation. Thus, phosphorelay systems activated by membrane-bound sensor proteins that are capable of detecting specific cell cycle events may be the key elements in the long-sought "clock" that governs the timing of gene expression during the *C. crescentus* cell cycle.

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