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Turning Off Flagellum Rotation Requires the Pleiotropic Gene *pleD*: *pleA*, *pleC*, and *pleD* Define Two Morphogenic Pathways in *Caulobacter crescentus*

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We have identified mutations in three pleiotropic genes, *pleA*, *pleC*, and *pleD*, that are required for differentiation in *Caulobacter crescentus*. *pleA* and *pleC* mutants were isolated in an extensive screen for strains defective in both motility and adsorption of polar bacteriophage φCbK; using temperature-sensitive alleles, we determined the time at which the two genes act. *pleA* was required for a short period at 0.7 of the swarmer cell cycle for flagellum biosynthesis, whereas *pleC* was required during an overlapping period from 0.6 to 0.95 of the cell cycle to activate flagellum rotation as well as to enable loss of the flagellum and stalk formation by swarmer cells after division. The third pleiotropic gene, *pleD*, is described here for the first time. A *pleD* mutation was identified as a bypass suppressor of a temperature-sensitive *pleC* allele. Strains containing this mutation were highly motile, did not shed the flagellum or form stalks, and retained motility throughout the cell cycle. Since *pleD* was required to turn off motility and was a bypass suppressor of *pleC*, we conclude that it acts after the *pleA* and *pleC* gene functions in the cell cycle. No mutants defective in both flagellum biosynthesis and stalk formation were identified. Consequently, we propose that the steps required for formation of swarmer cells and subsequent development into stalked cells are organized into at least two developmental pathways: a *pleA*-dependent sequence of events, responsible for flagellum biosynthesis in predivisional cells, and a *pleC-pleD*-dependent sequence, responsible for flagellum activation in predivisional cells and loss of motility and stalk formation in progeny swarmer cells.

Caulobacter crescentus is an asymmetrically dividing bacterium in which a nonmotile stalked cell divides repeatedly to produce a new, motile swarmer cell. Formation of the swarmer cell and its subsequent differentiation into a stalked cell occur by a series of cell cycle-dependent events. The first of these is the formation of a single polar flagellum at the stalk distal pole of the dividing stalked cell. This event is followed by the appearance of DNA bacteriophage receptors at the same pole and the activation of flagellum rotation just before cell separation. This developmental sequence continues in the newly divided swarmer cell, with the assembly of polar pili (27), loss of motility, shedding of the flagellum, and formation of a cellular stalk (see reviews references 18 and 24).

The best-studied developmental event in *C. crescentus* is flagellum biosynthesis. At least 30 flagellar (*fla*) genes are required for assembly and function of the flagellum (5, 14). All of the *fla* genes examined to date are periodically expressed in the cell cycle at a time that corresponds generally to the time at which these gene products are assembled into the flagellar structure. The order of *fla* gene expression appears to be regulated at the transcriptional level by their organization in a hierarchy in which expression of genes at one level of the hierarchy is required for expression of genes at lower levels (2, 19). An analysis of cell cycle mutants has shown that many *fla* genes are not expressed when DNA synthesis is blocked. This result has suggested that the periodic transcription of *fla* genes may be controlled by coupling expression of genes at the top of this regulatory hierarchy to chromosome replication (10, 21).

Much less is known about the regulation of the developmental events that occur after flagellum biosynthesis except that they are dependent on completion of specific events in the cell division pathway: initiation of cell division, progression of cell division, and cell separation. Conditional mutants blocked early in cell division undergo DNA synthesis and assemble flagella with normal periodicity (26), but all subsequent developmental events are blocked (10). These studies have shown that progression of cell division is required for both activation of the flagellum and stalk formation (10), whereas cell separation is required for the assembly of polar pili (27). On the basis of these observations, we have proposed that polar morphogenesis in *C. crescentus* is organized into at least two developmental sequences: a DNA replication-dependent pathway that leads to the formation of the flagellum and bacteriophage receptor sites, and a cell division-dependent pathway that leads to activation of the flagellum and subsequent developmental events at that cell pole (10).

If the morphogenetic events in *C. crescentus* are organized in two pathways as proposed above, then it should be possible to identify regulatory genes in these pathways by mutations that cause multiple and characteristic developmental defects. Two types of pleiotropic (*ple*) mutations described previously conform to this expectation. *pleA* mutants do not assemble a flagellum or polar φCbK receptors (8, 13), whereas *pleC* mutants form an inactive flagellum, but they are also φCbK resistant and fail to complete subsequent developmental events, including stalk formation (5, 7). Thus, the *pleA* phenotype is similar to that of cells blocked in DNA synthesis, and the *pleC* phenotype is similar

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TABLE 1. Bacterial strains

C. crescentus strain	Genotype	Source, derivation, or reference
CB15	Wild type	ATCC 19089
CB15F	Synchronizable density variant	B. Ely
PC1053	*divA301*	20
PC2244	*divB301*	20
PC5225	*pleC301*::Tn*5*	Tn*5* insertion mutagenesis
PC5227	*pleA301*::Tn*5*	Tn*5* insertion mutagenesis
PC5230	*pleC303*::Tn*5*	Tn*5* insertion mutagenesis
PC5231	*pleC304*::Tn*5*	Tn*5* insertion mutagenesis
PC5233	*pleC306*::Tn*5*	Tn*5* insertion mutagenesis
PC5241	*pleC309*::Tn*5*	Tn*5* insertion mutagenesis
PC5243	*pleC349*::Tn*5*	Tn*5* insertion mutagenesis
PC5244	*pleC310*::Tn*5*	Tn*5* insertion mutagenesis
PC5255	*pleC314*	UV induced
PC5257	*pleA316*	UV induced
PC5262	*pleC319*	UV induced
PC5264	*pleC321*	UV induced
PC5270	*pleC323*	UV induced
PC5281	*pleC330*	UV induced
PC5283	*pleC343*	UV induced
PC5286	*pleC334*	UV induced
PC5316	*pleC319 pleD301 divJ302*	Spontaneous revertant of PC5262
PC5318	*pleC319 divK301*	Spontaneous revertant of PC5262
PC5330	*pleC319 hunE112*::Tn*5*	SC1588 × PC5262
PC5333	*pleC319 pleD301 divJ*$^+$ *zzz-350*::Tn*5*	Derived from PC5316
PC5334	*pleA316 zio-301*::Tn*5*	PC5336 × PC5257
PC5336	*pleA*$^+$ *zio-301*::Tn*5*	This study
PC5342	*pleA316 zio-301*::Tn*5*, synchronizable	PC5334 × CB15F
PC5345	*pleC319 pleD301 divJ302 zhf-341*::Tn*5*	PC5348 × PC5316
PC5346	*pleC319 pleD301 divJ302 hunE112*::Tn*5*	SC1588 × PC5116
PC5348	*zhf-341*::Tn*5*	This study
PC5349	*pleD301 zhf-341*::Tn*5*	PC5345 × CB15
PC5375	*pleD301 zhf-341*::Tn*5*, synchronizable	PC5345 × CB15F
PC6074	*zhf-334*::Tn*5*	This laboratory
SC1069	*argA107 str-179*	B. Ely
SC1389	*hisD101 rif-149*	B. Ely
SC1588	*hunE112*::Tn*5 str-152*	5
AE6002	*pigA501 hclA501 fatA506*	9

to that of cells blocked in cell division except that *ple* mutants divide normally.

To obtain additional evidence for developmental pathways controlling polar morphogenesis in *C. crescentus* and to study their regulation, we have undertaken an extensive screen of Tn*5* insertion, UV-induced, and spontaneous mutants that are simultaneously nonmotile and resistant to the polar bacteriophage φCbK. All of the mutations isolated in this screen map to either *pleA* or *pleC*, and among them we have identified temperature-sensitive alleles of these two genes. Temperature shift experiments show that *pleA* activity is required for flagellum formation and motility only during a brief period from 0.7 to 0.8 of the swarmer cell cycle, whereas *pleC* is required for an overlapping period that extends from 0.6 to 0.95 of the swarmer cell cycle for flagellum activation and subsequent developmental events. We also describe for the first time a third pleiotropic gene, *pleD*, which is required for loss of motility and stalk formation. Thus, both *pleC* and *pleD* are required for a sequence of events that lead to formation of swarmer cells and differentiation into stalked cells. These results support a model in which the events required for polar morphogenesis in *C. crescentus* are organized into at least two developmental pathways, one for flagellum formation and phage receptor assembly and the other for flagellum activation, loss of motility, and stalk formation.

MATERIALS AND METHODS

Media and cell growth. Strains were routinely grown in PYE (22) medium. Temperature shift experiments were performed in M2 minimal salts medium containing 0.2% glucose (12). Cell number was determined by using a model ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) after dilution of the samples 1,000-fold in a 0.9% saline–3.7% formaldehyde solution.

Isolation of *ple* mutants by Tn*5* insertion mutagenesis. Random Tn*5* insertions were produced as described by Ely and Croft (4) in a streptomycin-resistant CB15 strain plated into soft agar (PYE broth [22] plus 0.35% agar). Nonmotile insertion mutants were purified and tested for resistance to the DNA phage φCbK (15) by streaking a cell suspension across the phage on an agar plate. φCbK-resistant cells were also tested for resistance to the pole-specific DNA phage φLC72 (10) and the pilus-specific RNA phage φCb5 in plaque-forming assays. Approximately 2.5% of random Tn*5* insertions in the *C. crescentus* chromosome resulted in nonmotile mutants. Among 1,300 nonmotile Tn*5* insertion mutants examined, 10% were at least partially resistant to φCbK and approximately 3.5%, or 45, were completely phage resistant. All of the partially resistant mutants examined by electron microscopy were found to lack flagella. Since a considerable reduction in efficiency of phage φCbK

TABLE 2. Phenotypes of representative *ple* mutants[a]

Strain	Mutation	Flagellum assembly	Motility	φCbK receptor formation	Pilus assembly	Loss of flagellum[b]	Stalk formation[b]	Division
PC5227	*pleA301*::Tn*5*	0	0	0	0	ND	+	+
PC5257	*pleA316*	ts	0	ts	ts	ND	+	+
PC5225	*pleC301*::Tn*5*	+	0	0	0	0	0	+
PC5262	*pleC319*	+	ts	ts	ts	ts	ts	+
PC5349	*pleD301*	+	+	+	+	0	0	+
PC5382	*pleC319 pleD301*	+	+	ts	ND	0	0	+
PC5316	*pleC319 pleD301 divJ302*	+	+	+	ND	0	0	cs

[a] +, Formation; 0, does not occur; ts, temperature sensitive; cs, cold sensitive; ND, not determined.
[b] Observed under standard growth conditions in PYE medium (see Discussion).

adsorption in the absence of a flagellum had been observed previously by Johnson and Ely (13), R. Bender (personal communication), and us (unpublished data), these mutants were not analyzed further. All of the *ple* mutants tested had a plating efficiency for φCbK, φLC72, or φCb5 of less than 10^{-6}.

Isolation of temperature-sensitive *ple* mutants. CB15 wild-type cells were grown in liquid PYE medium and irradiated with UV light to produce approximately 0.1% survival. Irradiated cells were further grown in PYE broth at 37°C for 3 h before being plated at a density of 10^7 cells per ml into soft agar containing 3×10^7 φCbK per ml. After the agar solidified, the plates were immediately incubated at 37°C for 2 to 3 days. This procedure allowed us to directly isolate nonmotile cells that were phage resistant. Among 590 such strains isolated at 37°C, 41 displayed some degree of motility when screened at 24 or 30°C, and 7 isolates exhibited wild-type motility and phage φCbK sensitivity at 30°C. An additional 180 nonmotile, phage-resistant strains were isolated at 37°C as spontaneous mutants from soft agar containing φCbK. Of these, two were temperature sensitive for both motility and phage resistance. Two temperature-sensitive mutants (PC5257 and PC5262) and the Tn*5* insertion mutants used in these studies are described in Table 1.

Motility assay. Cell motility was assayed by a method described previously which measures the rate at which *C. crescentus* cells collide and stick to a glass surface (17). The average number of cells adhering to a glass coverslip over a 30-s time period divided by the optical density at 650 nm of the culture at that time is taken as an index of motility. Cell motility in the *pleD* mutant was determined by video microscopy as described in the legend to Fig. 8.

Cell synchrony experiments. A Tn*5* insertion genetically linked to the *pleA* locus in strain PC5257 was used to move the temperature-sensitive *ple* allele into strain CB15F, a synchronizable density variant of the CB15 wild-type strain. Synchronous swarmer cells from this strain (PC5342) were obtained on Percoll gradients as described elsewhere (11).

Radioimmune precipitation assay. The radioimmune precipitation assays were performed essentially as described elsewhere (25) except that no unlabeled carrier cells were added to the samples and all of the centrifugation steps were performed in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, N.Y.). The *Staphylococcus aureus* cells (Pansorbin) were obtained from Calbiochem-Behring, La Jolla, Calif.

Electron microscopy. Cells were fixed by the addition of an equal volume of 0.8% glutaraldehyde in 50 mM cacodylic acid, pH 7.4, and incubated for at least 30 min on ice. Samples were centrifuged and gently suspended in filtered (0.2-mm-pore-size filter; Millipore Corp., Bedford, Mass.) distilled water and spotted onto Formvar-coated electron microscope grids. The samples were either stained with phosphotungstate as described elsewhere (10) or air dried and stained by rotary platinum shadow casting in order to detect polar pili (27). Grids were examined on a JEOL 100C transmission electron microscope at 80 kV.

RESULTS

Isolation and characterization of *ple* mutants. We isolated 45 mutants that are simultaneously defective in motility and resistant to infection by the polar phage φCbK after Tn*5* insertion mutagenesis. An additional 42 point mutants with the same phenotype, including 7 temperature-sensitive mutants, were isolated after UV-induced mutagenesis or as spontaneous mutants (see Materials and Methods). Over 50 of these *ple* mutants were examined by electron microscopy and shown to display one of two morphologically distinct phenotypes (Table 2):(i) a nonflagellated type, identified as *pleA*, which lacks flagella and pili but makes stalks and (ii) a stalkless type, identified as *pleC*, which lacks pili and stalks but possesses one or more inactive flagella (Fig. 1C). A third class of pleiotropic mutants, which are also stalkless and multiflagellated (*pleD*, Fig. 1D) was subsequently identified in an analysis of extragenic suppressors of a *pleC* mutant as described below. Unlike *pleC* mutants, however, *pleD* mutants are motile and phage φCbK sensitive and assemble pili (Table 2).

The observation that *pleA* and *pleC* mutants fail to assemble pili may reflect a requirement of the φCbK receptors for pilus formation. Consistent with this idea is the observation that none of 12 independent mutants isolated solely on the basis of φCbK resistance was found to assemble pili (data not shown); in a similar study of spontaneous φCbK-resistant mutants, Fukuda et al. (8) showed that all of these mutants were also resistant to the pilus-specific RNA phage φCp2.

Characterization and genetic mapping of *pleA* mutants. Seventeen Tn*5* insertion and five point mutants were found to be of the nonflagellated type, a phenotype resembling that of *pleA* and *pleB* mutants described by Ely et al. (5). All of our nonflagellated *ple* mutations were assigned to a single linkage group based on the low frequency of motile recombinants produced when phage φCr30 grown on one mutant was used to transduce a second mutant on soft agar (6). The relatively small number of motile flares obtained in these crosses indicated that the mutations were tightly linked. Nine of the Tn*5* insertions and three point mutants were examined for linkage by transduction to auxotrophic markers *hisD101* (SC1389) and *argA107* (SC1069). All of the mutations showed approximately 40% linkage to *hisD* and

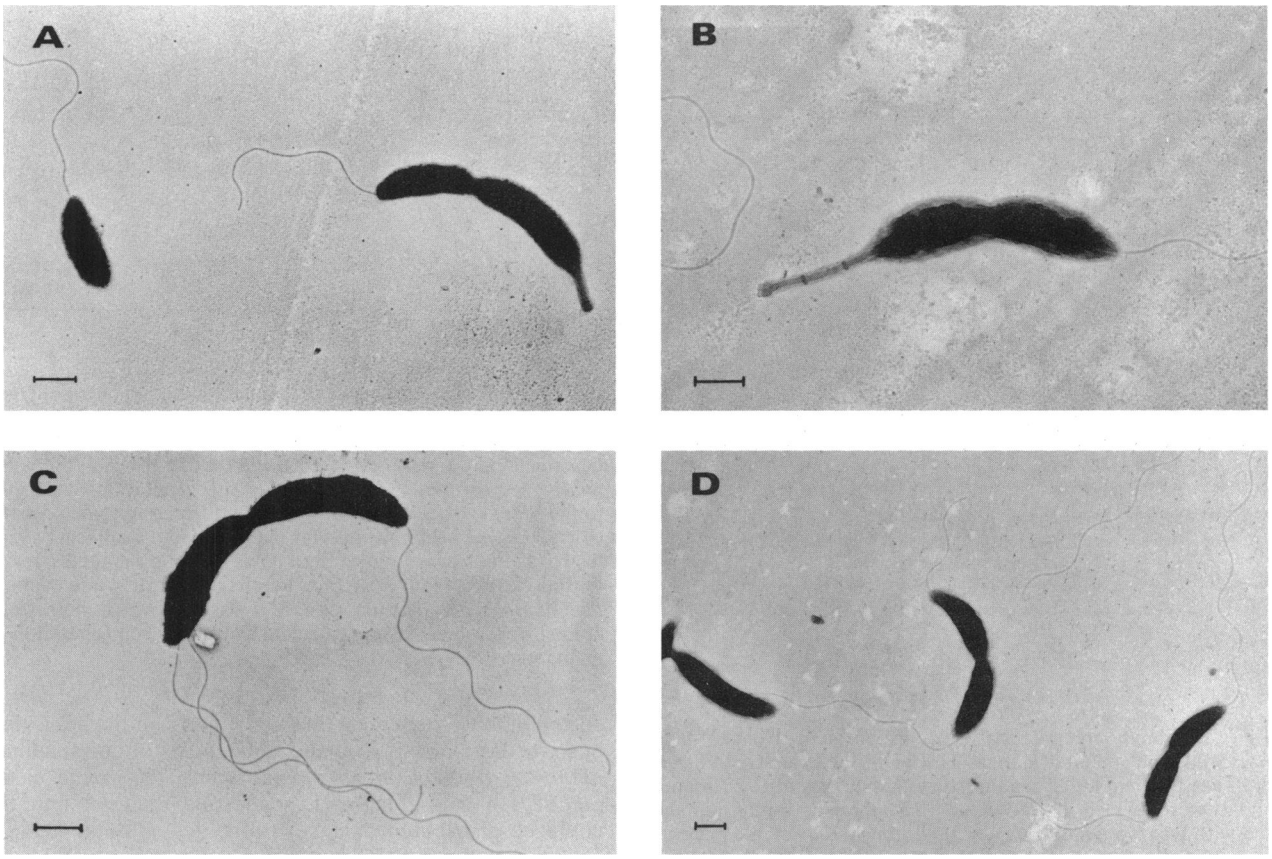

FIG. 1. Electron micrographs of *C. crescentus* wild-type cells and of *pleC* and *pleD* mutants. (A) CB15 wild-type swarmer and predivisional cells; (B) strain PC5262 (*pleC319*) grown at the permissive temperature (30°C); (C) PC5262 grown at 37°C, showing the stalkless, multiflagellated phenotype characteristic of *pleC* mutants; (D) strain PC5349 (*pleD301*). Samples were fixed and stained by rotary platinum shadow casting as described in Materials and Methods.

15% linkage to *argA*, and we conclude from this result that they map to the *pleA* locus described previously (5).

Function of *pleA* in flagellar gene expression. We examined the effect of the *pleA*::Tn*5* insertion mutation of strain PC5227 on the synthesis of flagellin and hook protein, the major structural proteins of the flagellum, by radioimmunoassay (Fig. 2). Compared with findings for the wild-type strain, synthesis of the 27- and 25-kilodalton (kDa) flagellins, which assemble into the hook-proximal and hook-distal segments of the flagellar filament, respectively, was reduced at least 20-fold. This result confirms the findings of Fukuda et al. (6) and Johnson and Ely (13). The 70-kDa hook protein was not expressed in the *pleA*::Tn*5* insertion mutant examined (Fig. 2, lane 4), and the residual flagellin made in this mutant was not assembled, since any flagellin assembled into a flagellar structure would have been precipitated by the anti-hook serum (19). The *pleA* mutant did, however, synthesize 29-kDa flagellin at about the same rate as did wild-type cells (Fig. 2, lanes 1 and 2). This is an unusual synthetic pattern among *fla* mutants, since most strains defective in 27- and 25-kDa-flagellin synthesis produce elevated levels of 29-kDa flagellin (19).

Time of *pleA* function for flagellum assembly and motility. *pleA* point mutants PC5256 and PC5257 are temperature sensitive, showing the *ple* phenotype at 37°C but not at 30°C. The time of *pleA* activity in the cell cycle was determined by shifting an exponentially growing culture of strain PC5257 (*pleA316*) from 30 to 37°C and measuring the time required for loss of motility by a cell adhesion assay (see Materials and Methods). Under these conditions, a temperature shift of a wild-type culture had no significant effect on cell motility (data not shown). The initial loss of motility in strain PC5257 occurred 50 min after the shift to the nonpermissive temperature (Fig. 3). Given that new swarmer cells remain motile for about 20 min after division, we calculated that *pleA* is last required about 30 min before cell division, or 0.8 of the 145-min swarmer cell cycle. In experiments in which the mutant cells grown at 37°C were shifted to the permissive temperature, significant motility was observed only after a delay of 30 min. This result indicates that at least 30 min is required for the assembly of an active flagellum after the *pleA* function is present. The rapid accumulation of motile cells after this initial lag period also indicated that a large fraction of these exponentially growing cells had been blocked at the *pleA*-dependent step. Apparently, cells at any stage late in the cell cycle can assemble a flagellum and gain motility when *pleA* function is restored. If only a narrow age distribution of cells were able to initiate flagellum formation after the shiftdown, a more gradual accumulation of motile cells would be expected.

We determined the exact time in the cell cycle at which *pleA* can function for flagellum formation and motility in the synchronizable strain PC5342, which carries the same temperature-sensitive *pleA316* allele examined above in exponential cultures. Swarmer cells were incubated at 37°C, and at various times of development cultures were shifted to the

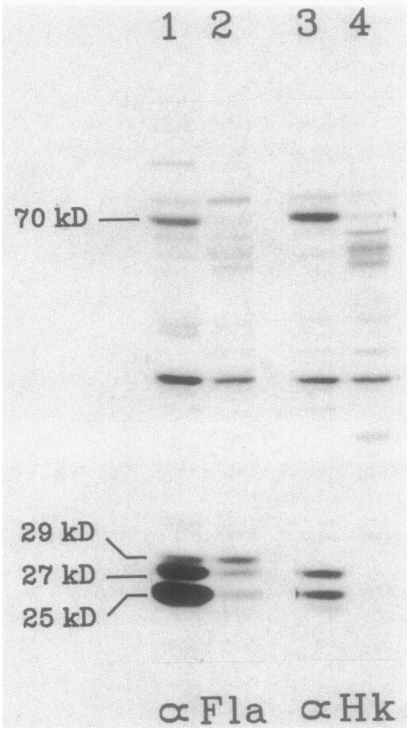

FIG. 2. Immunoprecipitation of flagellar proteins from *C. crescentus* wild type and *pleA* mutant. The wild-type strain CB15 (lanes 1 and 3) and the *pleA301*::Tn*5* insertion mutant strain PC5227 (lanes 2 and 4) were labeled in vivo with [^{35}S]methionine for 10 min and lysed with lysozyme and EDTA (25). Triton X-100-soluble material was incubated with anti-flagellin immunoglobulin G (lanes 1 and 2) or anti-hook protein serum (lanes 3 and 4), and the immunoprecipitates were resolved on a sodium dodecyl sulfate–10% polyacrylamide gel. The flagellin proteins have apparent molecular masses of 25, 27, and 29 kDa, and the hook protein migrates at 70 kDa.

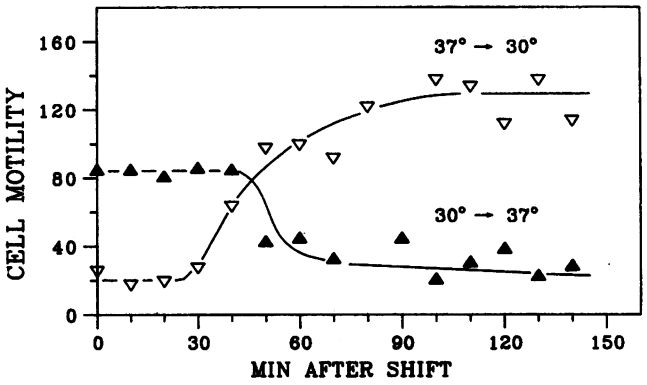

FIG. 3. Temperature shift experiment in an exponentially growing culture of the temperature-sensitive *pleA* mutant PC5257. Motility was assayed after a shift to the nonpermissive temperature as described in Materials and Methods (▲). Cells grown at the nonpermissive temperature (37°C) for at least five generations were shifted to the permissive temperature (30°C) and tested for gain of motility (▽). Since these cells are growing exponentially, the onset of gain or loss of motility was used to determine the time required after shift for these events to take place. A doubling time of 120 min in minimal medium (M2 salts plus 0.2% glucose) was observed at both temperatures, from which we can calculate a swarmer cell generation time of 145 min (28).

permissive temperature for a 10-min period and then returned to 37°C. A 10-min shift to the permissive temperature between 110 and 140 min (Fig. 4, lines d to f; 0.6 to 0.8 of the cell cycle) resulted in almost full cell motility by the time of cell division. Thus, only a very short period of *pleA* function was sufficient for motility. However, cultures shifted before 110 min (Fig. 4, lines a to c) gained very little motility, which indicates that the first time that *pleA* can function in the cell cycle is around 0.6.

Although there is a some leakage for flagellin synthesis at 37°C in the temperature-sensitive *pleA316* mutant, it was still possible to estimate the time of gene function required for flagellin synthesis in these experiments. A 10-min period at 30°C at later time points was sufficient to induce up to 60% of the flagellin protein synthesis seen in the control culture grown continuously at 30°C. We also observed that *pleA* activity could be delayed until shortly before cell division with little effect on the cumulative amount of flagellin produced compared with shifts at earlier time points but that these cultures were only partially motile (Fig. 4, lines g and h). Thus, *pleA* function may be required earlier in the cell cycle for assembly of a fully functional flagellum or, alternatively, cells shifted late in the cell cycle gain full motility only after 170 min, the time at which motility was assayed.

Mapping of *pleC* mutations. All of the stalkless *ple* mutants, including both Tn*5* insertion and point mutants, were assigned to a single linkage group by transduction. The close linkage of 30 point mutations to the *hunE112*::Tn*5* allele of strain SC1588 indicates that they map to the *pleC* locus described previously (5). The frequencies of cotransduction of *pleC* with *hunE*::Tn*5* ranged from 77% in the case of *pleC323* to 96% (*pleC334*), suggesting that the mutations could be distributed over several genes. Complementation analysis of these *pleC* mutations using cloned DNA fragments indicates that the mutations are in a single transcription unit (B. Ely, personal communication).

To facilitate genetic analysis of the *ple* mutations examined in this study, we mapped the *pleC* gene cluster relative to adjacent genetic markers by bacteriophage ϕCr30 transduction (Fig. 5). Previous results had shown that *pigA501*, a mutation causing the release of a red pigment into the culture medium (9), and a division gene cluster containing *divA* and *divB* (A. Newton, L. Kulick, and M. Bucuk, unpublished data) are linked to *hunE* by transduction. We have shown that *hunE* is 22% linked to *pigA* but only 5% linked to *divB*. Since *divB* is tightly linked to *zhf-334*::Tn*5*, which is in turn 37% linked to *pigA*, these results suggest the map order *divB-pigA-hunE* (Fig. 5). The 22% linkage of *pigA* to *hunE* and 11% linkage to the *pleC301*::Tn*5* insertion of strain PC5225 establishes the order *pigA-hunE-pleC* (Fig. 5).

Time of *pleC* activity. Our results and those published previously (7) show that *pleC* is required for activation of the flagellum, loss of the flagellum, and stalk formation. To define the time in the cell cycle at which *pleC* gene activity is required for these events, we analyzed two temperature-sensitive *pleC* mutants, PC5255 (Fig. 6A) and PC5262 (Fig. 6B). Wild-type swarmer cells gain motility 5 to 10 min before cell division and lose motility 30 min later (17). When exponentially growing cultures of strains PC5255 and PC5262 were shifted from 30 to 37°C, the number of motile cells detected in these cultures decreased gradually over a period of 30 to 40 min (Fig. 6) after the shift. This result suggested that no new motile swarmer cells were produced after the shift to the nonpermissive temperature and that the *pleC* gene product was not required to maintain swarmer cell motility once the flagellum had been activated. We calculate from this result that *pleC* is last required at the time of

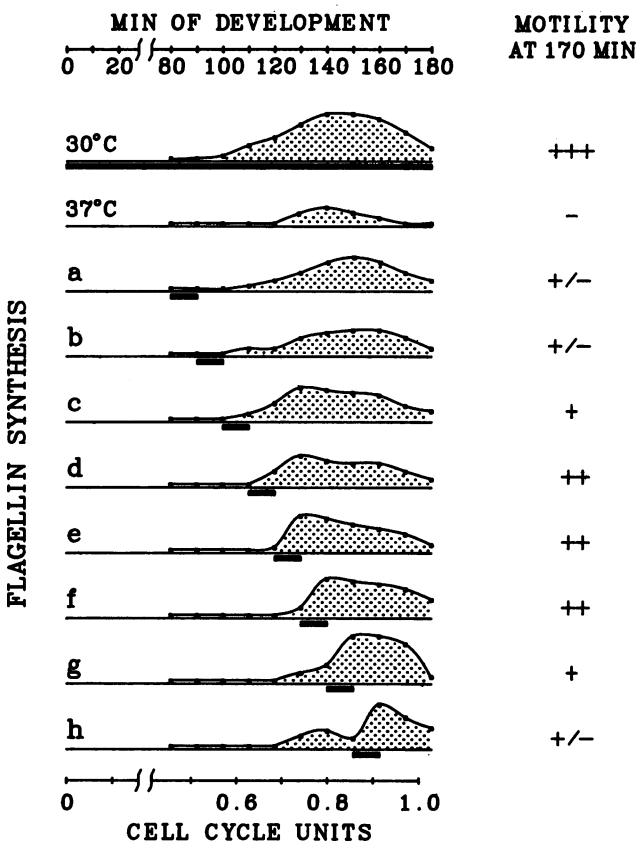

FIG. 4. Flagellin synthesis and cell motility in a temperature-sensitive *pleA* mutant. Strain PC5342 was grown in minimal (M2 salts plus glucose) medium at 30°C. Synchronized swarmer cells were incubated at 37°C and shifted to the permissive temperature (30°C) either continuously or for a 10-min period at the times indicated by the solid bars. In this experiment, *pleA* activity was measured by monitoring flagellin synthesis: 0.5 ml of cells was labeled with [^{35}S]methionine for 5 min at the appropriate temperature and then killed by the addition of NaN_3 and chloramphenicol. Flagellin was precipitated with a mixture of monoclonal antibodies prepared against purified flagellin (Sommer and Newton, unpublished data) and Pansorbin (Calbiochem-Behring). The immune complexes were washed on a dot blotting apparatus (Schleicher & Schuell, Inc., Keene, N.H.) and resolved on a sodium dodecyl sulfate–10% polyacrylamide gel. Synthesis of the 25- and 27-kDa flagellins at each time point was calculated from densitometric data of the autoradiographs. The shaded area under each curve represents the cumulative production of flagellin in that particular shift experiment. Cell motility was determined at 170 min and is indicated on the right of the figure. Cell number was determined in a Coulter Counter, and the midpoint of cell division was at 170 min.

flagellum activation of 0.95 of the swarmer cell cycle. In the shiftdown experiments (37 to 30°C), there was a lag of 40 min before motility was first observed in exponential cultures, which places the initial time of gene action at approximately 50 min before cell division, or 0.6 of the swarmer cell cycle. These results suggest that *pleC* mutants grown at 37°C and shifted to 30°C must assemble a new flagellum before they can regain motility. Thus, from the data obtained by using the two different *pleC* alleles, we conclude that this gene is required from 0.6 to 0.95 of the cell cycle (temperature-sensitive period), which includes the time of flagellum biosynthesis and activation.

We also examined the time required for stalk formation in

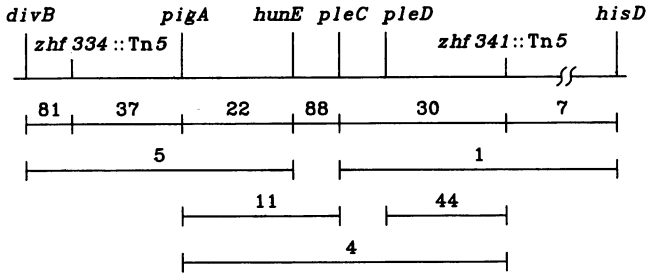

FIG. 5. Genetic map of the *divB*-to-*hisD* region of the *C. crescentus* chromosome. Linkage distances were determined by bacteriophage φCr30-mediated transduction. Mapping of *divB*, *pigA*, *hunE*, *pleC*, and *pleD* is described in the text. The insertions *zhf-334*::Tn*5* and *zhf-341*::Tn*5* were isolated from a pool of random Tn*5* insertions in the genome of wild-type strain CB15 and identified by their linkage to *divB* or *pleC*. In general, between 100 and 400 recombinants were analyzed to determine the cotransduction frequencies shown.

the two *pleC* mutants in these same shiftdown experiments. Estimating the time of stalk formation by electron microscopy is difficult because the stalks in wild-type cells vary in length and stalk formation is somewhat leaky in the *pleC* mutants. However, the time required for stalk formation could be estimated as 80 min in strain PC5262 and 120 min in strain PC5255 after the shift to the permissive temperature (Fig. 6). The formation of stalks of shorter than normal length by strain PC5255 at the permissive temperature may account for the longer time before stalks were detected after the shift to 30°C. Nevertheless, these results place the time of *pleC* activity required for stalk formation in the previous cell cycle. *pleC* activity is therefore required for both flagellum activation and stalk formation during the last third of the cell cycle.

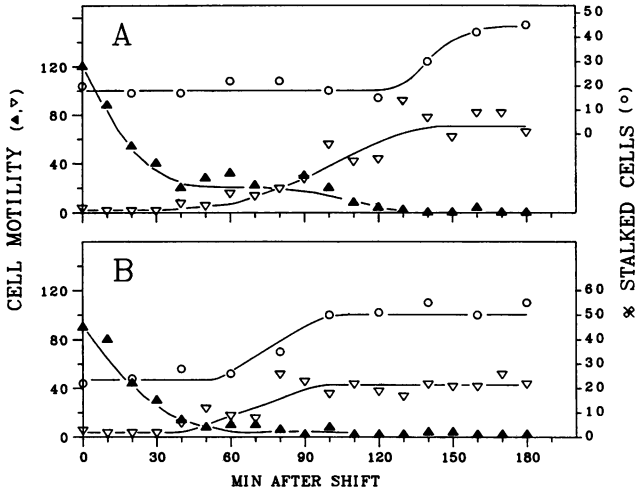

FIG. 6. Temperature shift experiments in *pleC* mutants. Motility was assayed as described in Materials and Methods in exponentially growing cultures of the *pleC* mutants PC5255 and PC5262 after a shift to the nonpermissive temperature (▲). Cells grown at the nonpermissive temperature (37°C) for at least five generations were shifted to the permissive temperature (30°C) and tested for gain of motility (▽). Stalk formation (○) after a shift to the permissive temperature was measured by examining at least 40 cells by electron microscopy. On the basis of the doubling time of 120 min at an optical density of 650 nm, the swarmer cell cycle was estimated to last 145 min (28).

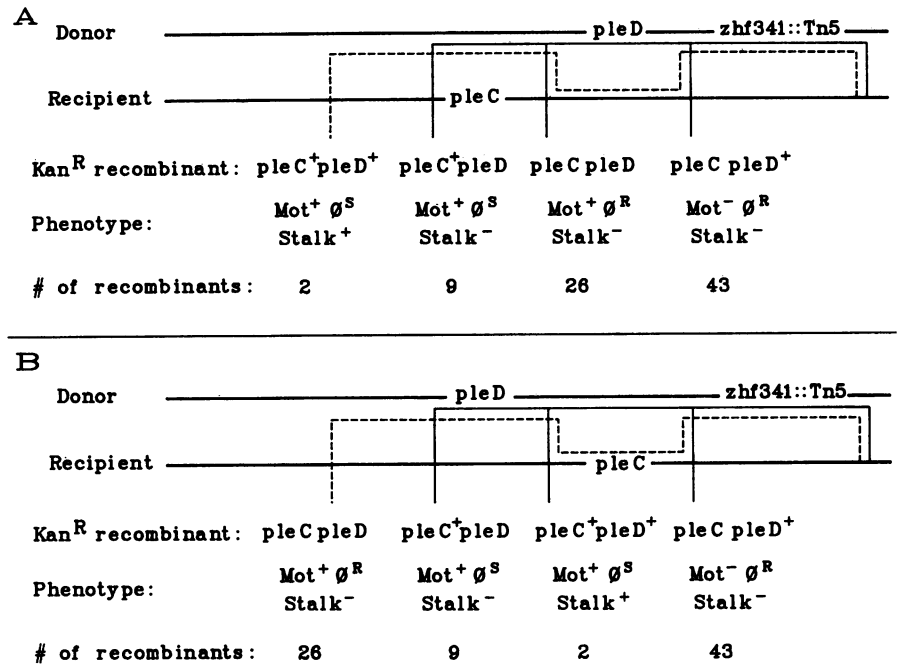

FIG. 7. Relative map order of *pleC*, *pleD*, and *zhf*-341::Tn*5*. A lysate on strain PC5349 (*pleD301 zhf-341*::Tn*5*) was used to transduce the temperature-sensitive *pleC319* allele of strain PC5262. Kanamycin-resistant recombinants were assayed at 37°C for motility, phage resistance, and stalk formation. Shown are the two possible orders of *pleC-pleD-zhf-341*::Tn*5* (A) and *pleD-pleC-zhf-341*::Tn*5* (B). – – –, Quadruple-crossover events.

Identification and mapping of *pleD*. We previously isolated extragenic suppressors of the temperature-sensitive allele *pleC319* which restore motility and phage sensitivity at 37°C and at the same time confer a cold-sensitive cell division phenotype. Many of these unlinked suppressors map to the cell division gene *divJ*, whose product appears to interact directly with the *pleC* gene product (J. Sommer and A. Newton, manuscript in preparation). Replacement of the *divJ* suppressor allele by the wild-type *div*$^+$ allele in most of these cold-sensitive revertants restored the normal cell division phenotype and the temperature-sensitive motility phenotype of the original *pleC319* mutant. The cold-sensitive revertant PC5316 did not behave in this manner, however. Genetic analysis of this strain showed that in addition to the cold-sensitive suppressor mutation *divJ302*, it also contained a second suppressor of *pleC319* now designated *pleD301* (Table 2). Thus, replacement of *divJ302* with the wild-type allele restored normal cell division but not the temperature-sensitive motility defect of the *pleC* mutation: cells of the resulting recombinant strain PC5333 were motile and phage resistant at 37°C. The phenotype of this *pleC319 pleD301* recombinant was characterized as supermotile, since most of the cells appeared motile by light microscopy at 30 and 37°C, as compared with approximately 20% motile cells found in wild-type cultures. Initially, these results suggested that the revertant PC5316 either did not contain the original *pleC319* mutation or had acquired a second suppressor mutation that affected motility.

We demonstrated that the original *pleC319* allele was still present in the *pleC* revertant PC5316 by integrating *hunE*::Tn*5*, which is approximately 90% linked to *pleC*, into this strain, preparing a ϕCr30 lysate on the recombinant strain (PC5346), and transducing wild-type strain CB15 to kanamycin resistance. Of the resulting recombinants, 55% displayed the temperature-sensitive motility and bacteriophage ϕCbK-resistant phenotype expected of the *pleC319* allele and 30% had the supermotile, phase-resistant phenotype of the *divJ*$^+$ *pleC319 pleD301* recombinant described above (Table 2). The latter class of recombinants suggested that the mutation responsible for the supermotile phenotype could be cotransduced with the *hunE*::Tn*5* marker along with *pleC319*. Among 80 recombinants analyzed, 1 kanamycin-resistant, supermotile, but phage-sensitive recombinant was recovered from this cross, which indicated that the suppressor mutation had been separated from the *pleC319* allele and that the supermotile phenotype was a characteristic of the suppressor allele, subsequently designated *pleD301*.

The low frequency with which *pleD301* was recovered in the cross described above suggested the gene order *hunE-pleC-pleD* (Fig. 5). To confirm this conclusion, the insertion *zhf-341*::Tn*5*, which is approximately 30% linked to *pleC* and maps between *pleC* and *hisD* (Fig. 5), was inserted into the revertant strain PC5316. When a ϕCr30 lysate made on this recombinant strain, PC5345 (*pleC319 pleD301 zhf-341*::Tn*5*), was used to transduce wild-type strain CB15 to kanamycin resistance, the supermotile, phage-sensitive *pleD301* phenotype was recovered at a high frequency. A lysate on this latter recombinant (*pleD301 zhf-341*::Tn*5*) was used to transduce strain PC5262 (*pleC319*). Analysis of the frequency of recombinants obtained from this cross confirmed the proposed order of *pleC*, *pleD*, and *zhf-341*::Tn*5* on the genetic map. Figure 7 diagrams the two possible map orders. The cotransduction frequency of *pleD* with *zhf-341*::Tn*5* was 43%, whereas the linkage distance between this Tn*5* and *pleC* point mutants was approximately 30%. We therefore concluded that the Tn*5* insertion maps to the right of *pleC* and *pleD* (Fig. 5). If the gene order were *pleC-pleD-zhf-341*::Tn*5*, a quadruple crossover would be required to generate the *pleC*$^+$ *pleD*$^+$ recombinant, a phenotype that was

obtained at a frequency of only 2.5% (Fig. 7A). Alternatively, if the order were *pleD-pleC-zhf-341*::Tn*5*, a quadruple crossover would produce the *pleC319 pleD301* double mutant, a phenotype that was recovered at a frequency of 33% (Fig. 7B). Based on these results and the previous finding that quadruple crossovers in *C. crescentus* occur at a frequency of less than 5% (1), we conclude that the correct order of loci is *hunE-pleC-pleD-zhf-341*::Tn*5* (Fig. 5).

Evidence that *pleD301* is a bypass suppressor of *pleC*. We tested *pleD301* for ability to suppress other *pleC* alleles by transducing the mutation linked to *zhf-341*::Tn*5* into *pleC* point mutants PC5264, PC5281, and PC5283. In addition, the *pleC*::Tn*5* insertions in strains PC5225 (*pleC301*::Tn*5*), PC5230 (*pleC303*::Tn*5*), PC5231 (*pleC304*::Tn*5*), PC5233 (*pleC306*::Tn*5*), PC5241 (*pleC309*::Tn*5*), PC5243 (*pleC349*::Tn*5*), and PC5344 (*pleC310*::Tn*5*) were transduced into the *pleC319 pleD301* revertant strain PC5316, thus replacing the *pleC319* allele. In all cases, supermotility was observed in the *pleC pleD* double mutants, which suggested that *pleD301* is not allele specific and acts as a bypass suppressor for motility.

Requirement of *pleD* for turning off motility and for stalk formation. Morphologically, the *pleD301* mutant is indistinguishable from *pleC* mutants in that it does not form stalks and possesses multiple flagella (Fig. 1D). Unlike the *pleC* mutant, however, the *pleD301* strain is motile and φCbK sensitive and assembles polar pili (Table 2). The initial observation of the supermotile phenotype of the *pleD301* mutant may have resulted from the failure of swarmer cells to lose motility at the normal time in the cell cycle. We confirmed this possibility by assaying cell motility in a synchronizable derivative of *pleD301* (strain PC5375, Table 1). A normal pattern of cell motility was observed in the parent strain CB15F, which lost motility within 30 min of swarmer cell development and gained motility shortly before cell division (Fig. 8A). Strain PC5375 (*pleD301*), however, did not lose motility during development (Fig. 8B). Furthermore, the percentage of motile cells remained unchanged after cell division, which indicates that both of the daughter cells are motile in the *pleD* mutant.

The production of two motile progeny cells in the *pleD* mutant might suggest that these swarmer cells do not differentiate into a stalked cell type. This does not appear to be the case, because the time of the first and second cell cycles in the *pleD* mutant occurred at the same time as the first (G1 plus S plus G2) swarmer cell cycle and second (S plus G2) stalked cell cycle in the wild-type strain CB15. These results indicate that the underlying swarmer and stalked cell cycles are not affected in the *pleD301* mutant, and we conclude that the *pleD* progeny cell corresponding to the stalked cell must carry an active flagellum for at least one generation after cell division.

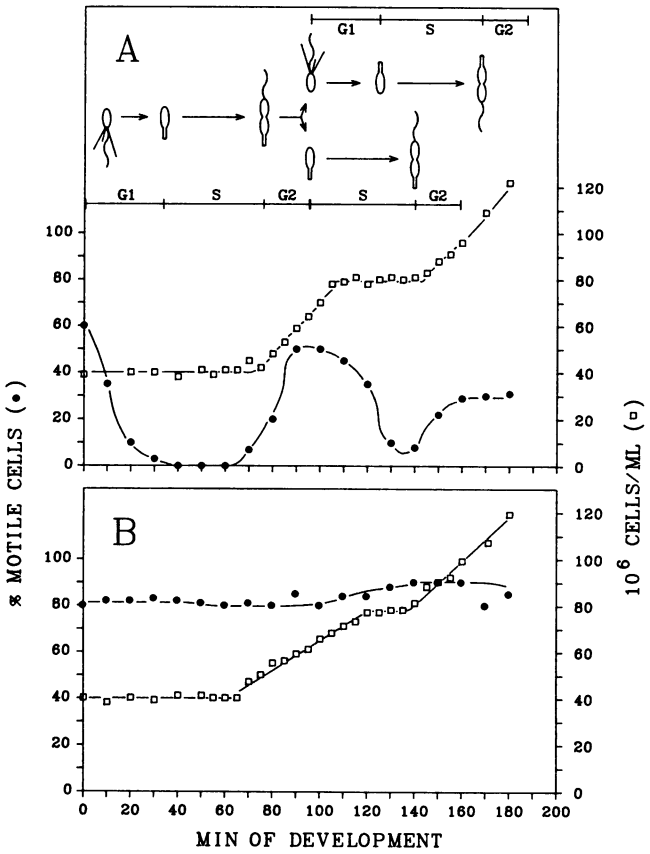

FIG. 8. Cell motility in the *pleD* mutant. Synchronous swarmer cells of strains CB15F (wild type) (A) and PC5375 (*pleD301*) (B) were obtained by purification of swarmer cells on a Percoll density gradient (11) and incubated at 30°C in PYE (22) medium. Cell number (□) was determined in a model ZBI Coulter Counter. Cell motility (●) was assayed at the indicated times by recording the cell movement observed by phase-contrast microscopy over a period of 1 min on videotape and estimating the percentage of motile cells. The predominant cell type during development of the wild-type culture is indicated at the top. The swarmer cell cycle comprises G1, S, and G2 periods, whereas the stalked cell produced by cell division follows an S-plus-G2 cell cycle (3).

DISCUSSION

To identify genes regulating polar morphogenesis in *C. crescentus*, we have completed an extensive screen of pleiotropic mutants that simultaneously affect motility and the assembly of the polar bacteriophage φCbK receptors. Mutations in only two genes were identified in this screen: *pleA*, which is required for the assembly of the polar flagellum and bacteriophage receptors, and *pleC*, which is required for activation of the flagellum and subsequent developmental events, including loss of the flagellum and stalk formation. Point mutations in *pleA* and *pleC*, as well as deletion mutants that display a nonflagellated, φCbK-resistant phenotype (*pleB*), have been reported previously (5, 7, 8). Mutants that are simultaneously defective in flagellum formation and stalk formation were not isolated in these studies or in the work reported here. This result is consistent with the idea that *pleA* and *pleC* define two pathways required for polar morphogenesis and may indicate that there is no "master" gene controlling both *pleA*- and *pleC-D*-dependent functions. Some mutations in regulatory genes could have gone undetected, however, either because these genes are redundant in *C. crescentus*, like the flagellin structural genes (16), because they give a phenotype not examined in our mutant screen, or because they are required for an essential cell cycle function. The latter possibility is suggested by an analysis of motile revertants of *pleC*. Among the suppressors of *pleC*, we have identified a cold-sensitive cell division mutation, *divK301*, which appears to be allelic with *pleD301* (Sommer and Newton, in preparation). The identification of *pleD*, a gene which is required for the loss of motility, suggests that other genes are required for developmental events after flagellum activation (e.g., loss of

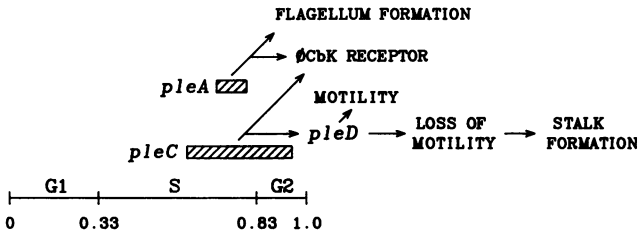

FIG. 9. *pleA*, *pleC*, and *pleD* activity required for flagellum formation, polar phage ϕCbK receptor formation, cell motility, and stalk formation. The time of *pleA* and *pleD* activity in the cell cycle is indicated (▨). The time of *pleD* activity has not been determined, but *pleD* is proposed to act after *pleC* in the developmental pathway leading to stalk formation.

motility, shedding of the flagellum, and stalk formation) and that many of these genes remain to be identified.

The temperature-sensitive alleles identified among *pleA* and *pleC* mutants allowed us to determine the temperature-sensitive periods of these two genes in the cell cycle. These results suggest that the *pleA* product acts normally for a brief period around 0.7 of the swarmer cell cycle, which corresponds closely to the time of initiation of flagellum biosynthesis. This time also corresponds to the time of *pleA* transcription, which has been estimated by nuclease S1 protection assays to peak at 0.65 of the cell cycle (Sommer and Newton, unpublished data). The temperature shift experiments (Fig. 4) also indicate, however, that the *pleA* gene product can execute its function during a broad window from 0.65 of the cell cycle until the time of cell division.

pleC is required during an overlapping period that extends from 0.6 to 0.95 of the cell cycle for activation of the flagellum and subsequent developmental events. Although a temperature-sensitive allele of *pleD* was not isolated and the time of *pleD* activity during the cell cycle could not be determined, the function of *pleD* as a bypass suppressor suggests that the gene may be required after *pleC* in this sequence of developmental events for loss of motility and for stalk formation. On the basis of these results, we propose a model in which the developmental events required for polar morphogenesis in *C. crescentus* are organized into two pathways that are initiated in predivisional cells: a *pleA*-dependent sequence leading to flagellum biosynthesis and a *pleC-pleD*-dependent sequence leading to flagellum activation and differentiation of swarmer cells to stalked cells (Fig. 9). Although *pleA* and *pleC* are required for the successive developmental events leading to flagellum formation and flagellum activation, the conclusion that they are on independent pathways is based on the formation of stalks by *pleA* mutants and flagella by *pleC* mutants (Fig. 9). *pleC pleD* double mutants are phage resistant and supermotile (Table 2). The behavior of *pleD301* as a bypass suppressor of motility but not phage ϕCbK sensitivity also suggests that *pleD* functions after *pleC* in a pathway leading to cell motility and stalk formation (Fig. 9).

Polar morphogenesis in *C. crescentus* is influenced by growth conditions as well as by genetic regulation. Strain CB15 grown at a low P_i concentration (5×10^{-6} M) forms stalks up to 10 μm long (23), and *pleC* mutants grown under these conditions form short stalks but retain the nonmotile phenotype (data not shown; Ely, personal communication). *pleD* mutants grown in low-phosphate medium form short stalks; in addition, they lose the multiflagellated, supermotile phenotype and display a wild-type motility phenotype. It could be argued from these results that stalk formation itself is responsible for the loss of the flagellum and motility. However, the observation that the flagellum is shed normally in synchronous swarmer cells when stalk formation is blocked by high concentrations of penicillin G indicates that flagellum loss is controlled independently of stalk formation (P. Nathan and A. Newton, unpublished data). We suggest that the primary defect in *pleC* and *pleD* mutants is in the control of motility and that when the regulatory defect preventing stalk formation is partially overcome by phosphate limitation, loss of the flagellum results from the rearrangement of the polar cell surface during outgrowth of the stalk.

In summary, we have analyzed a large number of point and Tn*5* insertion mutants which are simultaneously nonmotile and ϕCbK resistant. All of these mutations map to either the *pleA* or the *pleC* locus of *C. crescentus*. On the basis of the analysis of these mutants and the newly identified *pleD* mutant, which also maps to the *pleC* gene cluster, we propose that the events of polar morphogenesis in *C. crescentus* are organized into at least two pathways: a *pleA*-dependent sequence of flagellum biosynthesis and a *pleC-pleD*-dependent sequence of events leading to flagellum activation and differentiation of the swarmer cell pole, which finally results in stalk formation.

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