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Mutational analysis of developmental control in *Caulobacter crescentus*

(cell cycle/flagellin synthesis/differentiation)

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ABSTRACT **The relationship between the cell cycle and control of development has been studied by a genetic analysis of *Caulobacter crescentus*. The behavior of conditional cell division mutants showed that cell cycle events, such as DNA replication and cell division, are organized into a dependent pathway(s), i.e., later steps cannot proceed until earlier ones are completed. The ability of these strains to develop normally under nonpermissive conditions suggested that flagellin synthesis and stalk formation are dependent on the completion of different cell-cycle steps: the periodicity of flagellin synthesis is achieved by coupling it to either DNA chain elongation or completion, and stalk formation is dependent on a later step in the cell-division pathway. These developmental events are not themselves required for cell division, however.**

Differentiation at the cellular level is characterized by the formation of new cell types. This aspect of development is clearest in systems where asymmetric or unequal cell division takes place, e.g., grasshopper neuroblasts (1), blue green algae (2), and *Caulobacter crescentus* (3). We have used *C. crescentus* as a model system to study the control of cellular differentiation because it is a prokaryote with a small genome, the stages of its development can be easily followed, and cell types can be isolated in pure form (4–6). The life cycle of this aquatic bacterium contains two principal developmental sequences: (*i*) synthesis by the stalked cell (Fig. 1A) of the structures—flagellum, pili and holdfast—that will become part of a swarmer cell (Fig. 1C) at division (*swarmer cell development*), and (*ii*) loss of motility and stalk formation by the swarmer cell as it differentiates into a new stalked cell (Fig. 1D; *stalked cell development*).

The direct structural role of unequal cell division in the formation of a new cell type, and the observation that penicillin, a drug which blocks division in many bacteria, also prevents stalk formation in *C. crescentus* (8) suggested that cell-cycle steps may in some way regulate development. We have tested this proposition by applying a mutational analysis similar to one used by Hartwell and coworkers in yeast (9). An analysis of conditional cell-cycle mutants of *C. crescentus* showed that stalk formation and flagellin synthesis are dependent upon the completion of different steps in the cell cycle. This coupling between the cell cycle and developmental events may control the differentiation of new cell types.

MATERIALS AND METHODS

Culture Conditions. *C. crescentus* strains were grown in minimal salts medium M3 (4) or in medium G1 (10) which contained 0.2% glucose and 10 μg of cysteine and casamino acids (Difco) per ml. A nutrient broth medium (Pye; ref. 4) was used in the isolation of mutants.

Isolation of Temperature-Sensitive Mutants. Temperature-sensitive mutants were isolated from strain PC1, a fertile (11) cysteine auxotroph derived from strain CB15 (ATCC 19089), after irradiation with ultraviolet light to produce 0.1–1.0% survival. Colonies from surviving cells were replicaprinted to Pye-agar and supplemented M3-agar plates and incubated at 30° and 37° to identify temperature sensitive mutants.

Screen of Mutant Phenotypes. Mutants were grown at 30° to a density of 10^8 cells per ml in supplemented medium M3, and portions of the cultures were incubated at 30° and 37° for 6–9 hr. The cells were examined for stalk formation and flagella in the electron microscope after staining with 1% uranyl acetate or with 0.25% phosphotungstic acid.

Flagellin Synthesis. Mutant strains grown at 30° and 37° were treated for 10 min with either a ^{14}C-labeled amino acids mixture (New England Nuclear, 100 μCi/ml) or with [^{35}S]-methionine (New England Nuclear, 391 Ci/mM). Total acid-soluble flagellin was extracted from spheroplasts by the method of McGroarty *et al.* (12), and subjected to electrophoresis on sodium dodecyl sulfate/polyacrylamide slab gels as in Laemmli (13). A major flagellin species, flagellin A (26.5 kilodalton), and a minor flagellin species, flagellin B (28 kilodalton), have been described in *C. crescentus* (14; *). A single band comigrating with flagellin A was identified on the polyacrylamide gels, and the rate of flagellin A synthesis was estimated by densitometer tracing of negatives developed after exposure of dried gels to Ilford x-ray film (Ilford, Ltd.).

RESULTS

Identification of conditional cell-cycle mutants

Approximately 400 independent, heat-sensitive strains were isolated from *C. crescentus* strain PC1 by their inability to form colonies at an elevated temperature (36° or 37°). These strains were screened to identify conditional cell-cycle mutants that accumulated at a characteristic phenotype at the nonpermissive temperature (*Materials and Methods*). Strains used were selected according to the following criteria: (*a*) cell division, as determined by particle count on a Coulter Counter, stopped within one generation time at 37° to produce a uniformly filamentous cell culture; (*b*) the rates of growth at 30° and 37° were comparable to those in the parent strain for at least two generations; and (*c*) cell morphology and motility were normal at 30°. Genetic analysis of the mutants showed that the reversion frequencies ranged from 10^{-6} to 10^{-8}, values consistent with single-site mutations (unpublished).

Characterization of conditional mutants

Growth, Polynucleotide Synthesis, and Cell Division. The patterns of growth, as well as DNA and RNA synthesis at 30° and 37° have been examined in detail for 10 filamentous strains

* M. Sheffery and A. Newton, manuscript in preparation.

Table 1. Developmental effects of conditional mutations

Group	Strain	Mutation	Phenotype or cell-cycle step affected	Steps occurring at restrictive temperature					
				Stalk*	DNA_i	DNA_e	Flagellin†	CS	Stalk‡
I	PC1019	*dna-301*	DNA_i	+	−	−	−	−	−
	PC2076	*dna-302*	DNA_i	+	−	−	−	−	−
II	PC1042	*dna-303*	DNA_e	+	nd	−	−	−	−
	PC2153	*dna-304*	DNA_e	+	nd	−	−	−	−
	PC2179	*dna-305*	DNA_e	+	nd	−	−	−	−
III	PC1049	*div-307*	DIV	+	+	+	+	−	−
	PC2244	*div-308*	DIV	+	+	+	+	−	−
IV	PC1040	*div-309*	CS	+	+	+	+	−	+
	PC1029	*div-310*	CS	+	+	+	+	−	+
	PC2269	*div-311*	CS	+	+	+	nd	−	+

Dna_i, DNA initiation; DNA_e, DNA elongation; DIV, division; CS, cell separation. (−), absent; (+), present; (nd), not determined.
* Stalk formation during the *first* cell generation in synchronous swarmer cells shifted to 37°.
† Flagellin synthesis in the first and succeeding cell cycles after shifting swarmer cells to 37°.
‡ Presence or absence of a second stalk on filaments after 6 hr at the restrictive temperature (see *text*).

and the parent strain, PC1 (Table 1; unpublished data, and refs. 5 and 15). All of the mutants grew and synthesized RNA normally after transfer from 30° to 37°, but they differed in their patterns of DNA synthesis. Five of the strains tested showed a defect in either initiation of DNA replication (group I) or DNA chain elongation (group II) as shown in Table 1 (5, 15). Mutants in these groups failed to divide at 37° and formed long, unpinched filaments (Fig. 2b). The other five strains showed normal DNA replication at 37° (Table 1; ref. 5). Because these strains also failed to divide at the nonpermissive temperature, they were classified as *Div*$^-$ and placed in two groups that depended on their morphology. Mutants in group III (Table 1) had an unpinched, filamentous morphology at 37° (Fig. 2c) similar to that of the *Dna*$^-$ strains. The other *Div*$^-$ strains (group IV) formed highly pinched filaments (chains of cells) at 37° (Fig. 2d). They appeared to be blocked later in the cell cycle at a step immediately before cell separation.

Developmental Effects of Conditional Mutations. The effects of conditional cell-division mutations on swarmer cell development and stalked cell development at 37° were determined by assaying for flagellin synthesis and assembly and stalk formation, respectively.

Flagellin Synthesis: At the nonpermissive temperature flagella were not observed (attached or detached) in negatively stained preparations of mutants in group I (DNA initiation) and group II (DNA elongation) (Fig. 2b). Flagellin synthesis also stopped within a generation of the shift to 37° (Fig. 3C). Quantitation of the rates of flagellin synthesis in these strains at 37° by using a radioimmune precipitation assay (16) led to the same conclusion (in preparation)†. The inability of strains in groups I and II to make flagellin at 37° seems to be a direct result of a block in completion of DNA synthesis or in the replication of one segment of the chromosome. This conclusion is supported by experiments with parent strain PC1 showing that chemical inhibitors of DNA synthesis (17) also induce a nonflagellated, filamentous morphology.

In mutants of the *Div*$^-$ groups III and IV, flagellin synthesis continued normally at 37° for at least two generations (Fig. 3B and D). More than 50% of the filaments in these groups had two or more visible flagella, and in approximately 10% of the filaments formed by group IV mutants a flagellum was present within the filament where two cells were joined (Fig. 2c and d). Thus, both flagellin synthesis and assembly (Table 1; Fig. 3b and d) can occur at 37° in the *Dna*$^+$, *Div*$^-$ strains.

Stalk Formation: Strains with defects in the initiation of DNA synthesis (group I, Table 1), DNA chain elongation (group II, Table 1), and one stage of cell division (group III, Table 1) uniformly formed long filaments with a single stalk (Fig. 2b and c). These mutants failed to make a stalk at the end of the filament where a stalk would normally be formed 60 min after cell division, i.e., step C → D in Fig. 1. Because the cells were held at 37° for 6–9 hr, stalk formation at this pole of the cells must be dependent upon a cell-cycle step blocked by these mutations, rather than on cell age alone.

The inability of these mutants to form second stalks at 37° is not due to a defect in genes involved in the synthesis or assembly of stalks. When synchronous swarmer cell cultures of these strains were collected at 30° and shifted immediately to 37°, they lost motility and developed into stalked cells with the same timing as wild type cells (Table 1). The cultures did not divide at this temperature, however, and stalk formation in *subsequent* generations was blocked (see Table 1). Thus, cell-cycle steps in one generation may be required for developmental events in the next generation.

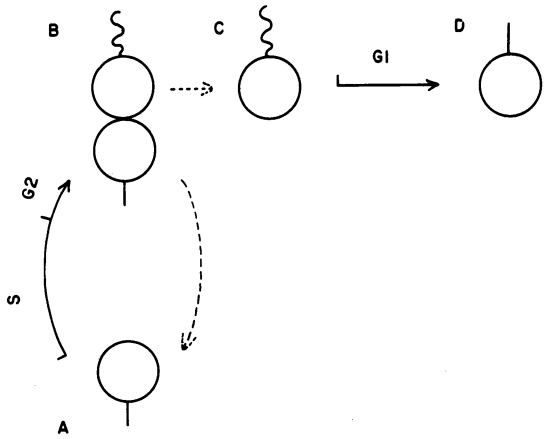

FIG. 1. Life cycle of *C. crescentus*. Nonmotile stalked cell (A); dividing cell (B); motile swarmer cell (C); and new stalked cell (D). The periods of DNA synthesis (S), postsynthetic gap (G2) and the presynthetic gap (G1) are indicated (7).

† M. A. Osley, M. Sheffery, and A. Newton, in preparation.

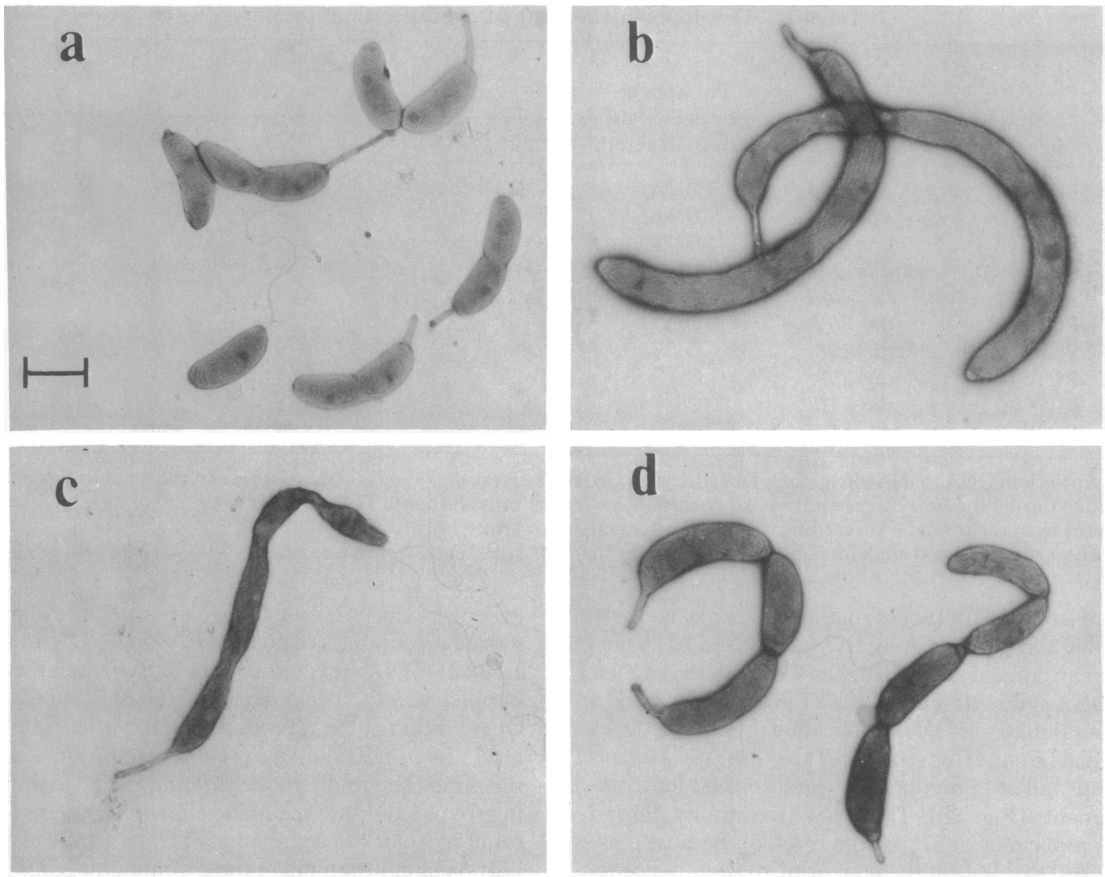

FIG. 2. Photomicrographs of mutants and the parent strain at 30° and 37°. Cells grown in M3 medium at 30° and 37° for 6–9 hr were fixed with 0.4% formaldehyde, stained with 1% uranyl acetate, and photographed on a Jeol electron microscope. The bar indicates 1 μm. A normal morphology was exhibited by PC1 and all mutants at 30°. (a) Parent strain PC1, 30° or 37°; (b) PC1042, 37°; (c) PC1049, 37°; (d) PC1040, 37°.

Stalked cell development occurred only in mutants blocked at a late stage of cell separation (group IV, Table 1): 30–100% of the filaments formed by these strains, dependent upon the number of cell segments, had stalks at both ends, and, in approximately 10% of the cells stained with uranylacetate, a stalk was present within the filaments at the point of maximum pinching.

Are Flagellin Synthesis and Stalk Formation Obligatory Steps in the Cell-Division Pathway? A number of nonmotile strains of *C. crescentus* which divide normally have been isolated (18, 19). The best characterized of these strains contains a temperature sensitive mutation in the structural gene for flagellin (18). At the nonpermissive temperature, this mutant is unable to assemble flagella, but DNA synthesis and cell division occur normally (18). We have extended this investigation to the role of flagellin synthesis in cell division by using a radioimmune precipitation assay to detect flagellin in several nonmotile mutants. One of the nonmotile strains (PCM4) did not make flagella, and it also failed to synthesize detectable crossreacting material to antibody directed against the major species of flagellin (flagellin A; see Table 2); another strain, PCM7, appeared to be temperature sensitive for flagellin production (Table 2). Because none of the nonmotile strains was defective in cell division, it seems likely that flagellin synthesis, like flagellum assembly, is not an obligatory step in the cell cycle.

The data on the relationship of stalk formation to cell division are more preliminary. One mutant (PC1020) isolated in an initial screen for the strains used in this study was defective in the formation of stalks. This stalkless mutant did, however, show a normal pattern of cell division. Another stalkless mutant reported previously by Marino *et al.* (18) also divides normally. Thus, the stalk structure itself is not a requirement for cell division.

DISCUSSION

Mitchison (20) has proposed that progress through the cell cycle might be achieved by organizing cell-cycle events into dependent or independent pathways, and Hartwell and coworkers (9) have developed this idea in their analysis of the yeast cell cycle. We have adapted this approach to our studies of developmental control in *C. crescentus*. The behavior of conditional, cell-division mutants shows that cell-cycle steps, such as DNA replication and cell division, are organized into a dependent pathway(s) in which each step requires the completion of previous steps in the pathway. The additional and novel finding from our studies is that certain developmental events—flagellin synthesis and stalk formation—while not part of the cell-cycle pathway itself, are dependent upon completion of different steps in this pathway. Our working model for developmental control in *C. crescentus* is shown in Fig. 4; a detailed discussion appears elsewhere (5).

The pathway for cell-cycle events was constructed by placing the DNA synthesis and cell-division steps according to the temporal order in which they normally occur in the cell cycle (see legend to Fig. 4). These steps in *C. crescentus* constitute a dependent pathway leading to cell separation, because a block at any one of the steps shown will prevent the subsequent steps

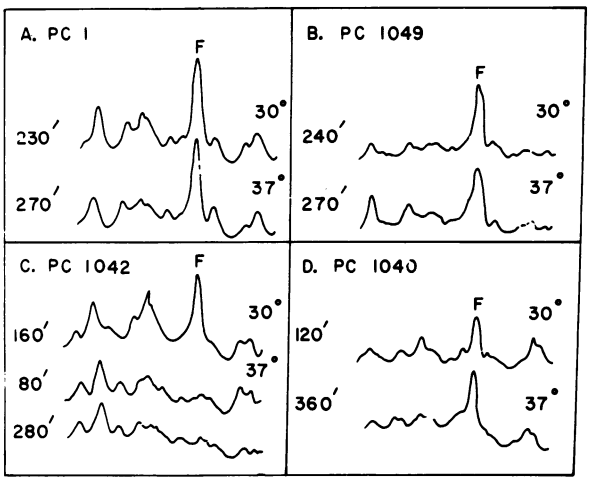

FIG. 3. Flagellin synthesis in mutants and the parent strain. At various times after transfer of cultures grown in supplemented M3 medium from 30° to 37°, samples were treated for 10 min either with 1–4 μCi of ^{14}C-labeled amino acids mix per ml or with 25 μCi of [^{35}S]methionine per ml. Flagellin was isolated, partially purified, and electrophoresed on sodium dodecyl sulfate/polyacrylamide gels as described (*Materials and Methods*). Panels A–D show densitometer tracings of autoradiograms (*Materials and Methods*). The flagellin band (F) was identified by the position of purified flagellin A in this gel system. The times of labeling (min) after the shift to 37° are indicated beside each tracing. (A) ^{14}C-labeled amino acids, 10% gel; (B) [^{35}S]methionine, 10% gel; (C) ^{14}C-labeled amino acids, 10% gel; (D) ^{14}C-labeled amino acids, 12.5% gel.

Table 2. Flagellin synthesis and cell division in nonmotile strains

	Motility†		Flagellin‡ synthesis		Cell division§
Strain*	30°	37°	30°	37°	(30° or 37°)
PC1	+	+	100	100	+
PCM4	−	−	0	0	+
PCM7	+	−	56	18	+
PCM5	−	−	80	160	+

* Nonmotile mutants were isolated from strain PC1 after mutagenesis with ultraviolet light (*Materials and Methods*). Surviving colonies were picked to Pye plates containing 0.35% agar and incubated at 30° and 37° to identify nonmotile strains (19).

† (+), motile; (−), nonmotile; determined by colony morphology on Pye plates containing 0.35% agar and by light microscopy.

‡ Cultures were grown at 30° in minimal medium M3 supplemented with 10 μg each of cysteine and casamino acids per ml and a portion was shifted to 37° for 1 hr before labeling for 10 min with [^{35}S] methionine. Spheroplasts were prepared (*Materials and Methods*) and flagellin was purified from lysed cells by radioimmune precipitation (16). Flagellin A was identified and quantitated on sodium dodecyl sulfate/polyacrylamide gels as described in Fig. 3. The minor flagellin species, flagellin B, was produced by all three nonmotile mutants (data not shown).

§ Determined by light microscopy.

from taking place. In the cell cycles of yeast (9) and *Escherichia coli* (21), there are separate, dependent pathways for DNA synthesis and cell division which converge before cell separation. The possibility of a similar arrangement in *C. crescentus* can be investigated by determining whether *Dna*$^-$ and *Div*$^-$ steps are on the same functional pathway (see refs. 22 and 23).

The pathways for flagellin synthesis and stalk formation shown in Fig. 4 branch at different points from the cell-cycle pathway. The rationale for depicting flagellin synthesis as dependent on DNAe or DNAc is the inability of all of the *Dna*$^-$ mutants tested in groups I and II to make this protein at 37°, whereas the *Dna*$^+$, *Div*$^-$ mutants tested in groups III and IV are able to make flagellin under these conditions. Stalk formation must be dependent on a later step in the pathway since only the mutants in group IV make additional stalks at 37°. The two developmental events are considered to be on independent, branch pathways because neither flagellin synthesis nor stalk formation appears to be a requirement for cell division (see *Results*). An additional reason for placing stalk formation on a branch pathway is the normal occurrence of this particular developmental step *after* cell separation (Fig. 1C and D).

Several results suggest that formation of the flagellum in *C.*

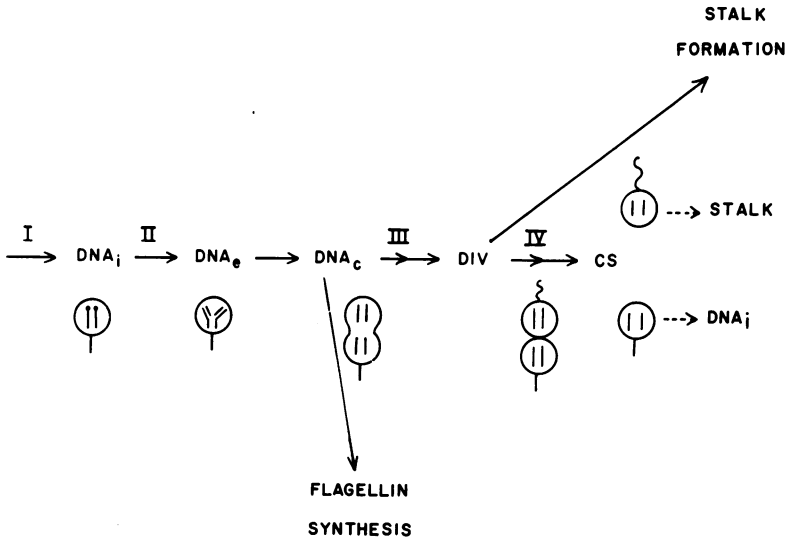

FIG. 4. Proposed organization of cell-cycle pathway and developmental events in *C. crescentus*. Steps in the *C. crescentus* cell cycle are arranged according to the order in which they occur in the cell cycle. The Roman numeral preceding a step refers to the group of mutations which blocks the appearance of that step and subsequent steps in the pathway (Table 1). Thus, the sequence, DNA_i (DNA initiation), DNA_e (DNA elongation), DNA_c (DNA completion), DIV (division), and CS (cell separation), corresponds to a *dependent* pathway in which each step must be completed for later steps to occur. The location of branches for flagellin synthesis and stalk formation is discussed in the *text*.

crescentus requires the synthesis of flagellin at a specified time in the cell cycle (24; *). Our results identify cell-cycle steps that are required for flagellin synthesis, and the model shown in Fig. 4 suggests that the temporal sequence of these steps may determine the periodicity of this developmental event. Some of the other structures associated with swarmer cell development could be under similar cell-cycle control. Because proteins specific to the stalk structure are not available, we cannot determine whether the cell-cycle mutations in *C. crescentus* also affect stalk formation at the level of gene expression. The results do show, however, that the time at which these mutations act is in the generation *prior* to the one in which the stalk structure appears (Table 1). This conclusion is consistent with the idea that the program for stalked cell development is specified within the incipient swarmer cell before cell division has been completed (5).

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