Sequential Regulation of Developmental Events during Polar Morphogenesis in *Caulobacter crescentus*: Assembly of Pili on Swarmer Cells Requires Cell Separation

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Pili, along with the flagellum and DNA bacteriophage receptors, are structural markers for polar morphogenesis in *Caulobacter crescentus*. Pili act as primary receptors for a number of small, *C. crescentus*-specific DNA and RNA bacteriophages, and the timing of pilus-dependent adsorption of bacteriophage ϕ Cb5 in synchronized cell populations has led to the general conclusion that pili are formed coordinately with the flagellum and other polar surface structures in the predivisional cell. The use of rotary platinum shadow casting and electron microscopy as a direct assay for formation of flagella and pili in synchronous cell cultures now shows, however, that when expressed as fractions of the swarmer cell cycle, flagella are assembled on the predivisional cells at approximately 0.8 and that pili are assembled on the new swarmer cells at approximately 0.1 of the next cell cycle. Adsorption of pilus-specific bacteriophage ϕ Cb5 prevented the loss of pili from swarmer cells during development, which suggests that these structures are retracted at the time of stalk formation. Examination of temperature-sensitive cell division mutants showed that the assembly of pili depends on completion of cell separation. These results indicate that the stage-specific events required for polar morphogenesis in *C. crescentus* occur sequentially, rather than coordinately in the cell cycle, and that the timing of these events reflects the order of underlying cell cycle steps.

Morphogenesis in the aquatic bacterium *Caulobacter crescentus* is characterized by stage-specific assembly and loss of polar surface structures. During each cell cycle, the nonmotile stalked cell assembles a flagellum and receptor sites for DNA bacteriophages ϕ CbK and ϕ LC72 at the cell pole distal to the stalk. These structures become part of the motile swarmer cell at division and are lost again when the swarmer cell differentiates into a stalked cell (for reviews, see references 15 and 25). The pili, which are also assembled at the flagellated cell pole (22), are another stage-specific marker for polar morphogenesis. The pili are flexible filaments with a diameter of 4 nm and a length from 1 to 4 μ m and are similar in appearance to the common polar pili of *Pseudomonas aeruginosa* (1). The *C. crescentus* pilin has a molecular weight of 8,000 and an amino-terminal sequence similar in hydrophobicity to the *P. aeruginosa* pilin (31), which is in turn homologous to the chromosomally encoded pilins of a number of unrelated organisms, including *Neisseria gonorrhoeae* and *Moraxella nonliquefaciens* (11, 19).

The pili act as primary receptors for a number of small DNA and RNA bacteriophages that are specific for the caulobacters (22, 23). The time of the pilus-dependent RNA phage ϕ Cb5 adsorption in synchronized cell populations is restricted to the period that includes cell division and the early swarmer cell stage (26-28). This observation has led to the general conclusion that pili are formed coordinately with the flagellum and other polar surface structures in the predivisional cell (24, 30).

To date, a direct structural assay for the presence of pili has not been used to confirm the stage specificity and timing of pilus assembly in the cell cycle. This is due in part to the unreliability of conventional methods of uranyl acetate and phosphotungstate staining for the detection of *C. crescentus* pili by electron microscopy (31; unpublished observations).

Using a technique of rotary platinum shadow casting, however, we have been able to consistently resolve pili by electron microscopy, and we have applied this technique to determine the pattern of pilus assembly in synchronous cultures of *C. crescentus*.

The results reported in this paper demonstrate that pilus formation is indeed periodic in the *C. crescentus* cell cycle and that this structure is assembled in the swarmer cell immediately after cell division. Consistent with this pattern of assembly, we also show that the final cell division event, cell separation (CS) (18), is required for the formation of pili. Thus, the assembly of pili is regulated differently from the assembly of the other major developmental events at the swarmer cell pole, including flagellum biosynthesis and activation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. crescentus* CB15 was obtained from the American Type Culture Collection, Rockville, Md. (ATCC 19089). Cell division mutants PC1053, PC2244, PC1029, and PC1040 have been described previously (17, 18). The cells were routinely grown at 30°C in M3 minimal salts medium containing 0.2% glucose (20), supplemented with cysteine and Casamino Acids (Difco Laboratories) as described previously (18). Synchronized cultures of strain CB15 and of the cell cycle mutants were obtained by the plate release technique (3). Synchronous CB15F swarmer cell populations were obtained by centrifugation on a Percoll (Pharmacia Fine Chemicals) density cushion as previously described (7).

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 (pore size, 0.2 μ m; Millipore Corp.) distilled water and spotted onto Formvar-coated electron microscope grids (200

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mesh; E. F. Fullam). Air-dried grids were stained by rotary platinum shadow casting. We found that the evaporation of ca. 3.8 cm of platinum wire (0.008 in. diameter; Polysciences Inc.) at a distance at 5.0 cm from the center of a rotating platform and at a relatively low angle of 8 to 10° allows very consistent detection of *Caulobacter* pili. Up to 50 grids were shadow cast simultaneously. The grids were examined on a JEOL 100C transmission electron microscope at 80 kV.

RESULTS

Timing of assembly of pili. We have used a platinum shadow casting technique to detect *C. crescentus* pili by electron microscopy. With this procedure, every cell examined could be unambiguously scored for the presence or absence of pili. We have observed only one type of pili in the wild-type *C. crescentus* CB15; it measures 4 nm in diameter and 0.5 to 4 µm in length (Fig. 1A).

Initial observations of exponential cell cultures showed that from 40 to 50% of the swarmer cells were piliated, whereas predivisional cells with pili were rarely found (<0.2%). To determine the time of pilus formation and loss in the *C. crescentus* cell cycle, we examined synchronous cell populations. Figure 2 shows the kinetics of flagellum and pilus assembly in a synchronous culture of swarmer cells prepared from strain CB15F by density gradient centrifugation. Although many of the purified swarmer cells were already piliated at the beginning of the synchrony experiment, there was an initial increase in the number of cells with pili. Subsequently, cells lost the pili when the flagellum was shed, and stalk formation occurred at 40 to 50 min in the cell cycle; pili were formed again in the next cell cycle on the progeny swarmer cells soon after cell division (Fig. 2A). The results also showed that pili were assembled well after flagellum biosynthesis, which occurs on the predivisional cell (Fig. 2B).

The assembly of pili by the newly divided swarmer cell was more apparent when cells were synchronized by the plate release technique (Fig. 3A). This procedure (3) allows the collection of a uniformly younger population of swarmer cells and also avoids the possible shearing of pili from swarmer cells during the density gradient centrifugation procedure. As observed in the previous experiment, the assembly of pili in the second cell cycle occurred within 10 min after cell division and about 30 min after flagellum formation (Fig. 3B).

Role of cell division in expression of pili. Penicillin G in low concentrations blocks the earliest identifiable cell division event in *C. crescentus*, DIV_i (initiation of cell division), without inhibiting cell growth (18, 34). Under these conditions, successive rounds of flagellum assembly and polar DNA bacteriophage receptor formation take place, producing long filamentous cells with multiple flagella at one cell pole after several generations. Pilus formation, however, was prevented when cell division was blocked by the addition of penicillin (Fig. 2A). Inhibition of DNA synthesis by the addition of hydroxyurea to these cultures was also shown to block pilus formation (data not shown), presumably by preventing cell division (18) and assembly of the pilin subunit. Although DNA synthesis is required for flagellum biosynthesis (29) and all cell division events in *C. crescentus* (4, 18), hydroxyurea reportedly does not inhibit the synthesis of the pilin protein in these cells (30).

Unlike the expression of pili in *Escherichia coli* (16, 33), the production of *C. crescentus* pili is not dependent on growth temperature. This allowed us to examine the require-

TABLE 1. Effect of blocking cell cycle steps on the formation of pili in *C. crescentus*

Strain and condition	Cell cycle step blocked	Pili ^a	Flagella ^a
CB15 (wild type)			
30 or 37°C	None	+	+
Hydroxyurea (3 mg/ml) ^b	DNA _e	—	—
Penicillin (240 U/ml) ^b	DIV_i	—	+
PC1053	DIV_i	—	+
PC2244	DIV_p	—	+
PC1029	CS	—	+
PC1040	CS	—	+

^a Flagella and pili were examined as in Fig. 2 after cells had grown for two generations in PYE (20) medium. At least 50 cells were examined in each sample. Symbols: +, the number of pili or flagella was at least 50% of the number in the 30°C control culture; —, no pili or flagella were detected.

^b Final concentration.

ment of individual cell cycle steps for pilus formation in temperature-sensitive mutants that are blocked at different stages of cell division at the elevated temperature (Table 1). The steps defined by these mutants are organized into the dependent sequence $DIV_i \rightarrow DIV_p$ (progression of division) $\rightarrow$ CS (18). All of the cell division mutants produced pili at the permissive temperature (30°C), but none of them assembled pili at the nonpermissive temperature (37°C). The failure of CS mutants PC1029 and PC1040 to form pili at 37°C is particularly interesting, since these CS mutants complete DIV_p and are able to execute other swarmer-cell-specific steps, including loss of the flagellum and stalk formation (6).

Requirement of CS for pilus formation. The possible dependence of pilus formation on CS was further examined in a synchronous culture of mutant PC1040. The execution point for the temperature-sensitive step in this strain is at the time of stalk initiation, or approximately 60 min into the swarmer cell cycle (18). When synchronous cells of this mutant were shifted to 37°C at the beginning of the swarmer cell cycle, pili were assembled in swarmer cells, but no pili were detected at the time of normal cell division (Fig. 3A). On the other hand, in a synchronous culture that was first incubated at 30°C for 90 min (to allow most cells to complete the step required for CS) and was then shifted to the nonpermissive temperature, a substantial fraction of the cells assembled pili at 37°C (Fig. 3A). We conclude from these results that the block in pilus formation is a direct result of the failure of the cells to undergo CS, rather than a nonspecific effect of the elevated temperature.

Retraction of pili. The loss of pili by the swarmer cell coincides with the loss of the flagellum and stalk formation. Unlike released flagella, however, pili are not detected in the growth medium of *C. crescentus* cultures. They may therefore be retracted rather than shed into the medium. In *E. coli* and *P. aeruginosa*, retraction of pili can be prevented by the addition of pilus-specific bacteriophages (1, 9). In similar experiments, when *C. crescentus* cells were grown in the presence of RNA phage ϕ Cb5 for 1 to 2 h at a multiplicity of infection of 10, many of the stalked cells carried pili attached to the stalk tip (Fig. 1B and C), which is not observed in uninfected cell cultures. Presumably, the spherical phage particles, which adsorb to the sides of the pili, block the retraction of pili when the swarmer cell differentiates into a stalked cell.

DISCUSSION

Our analysis of pili formation in *C. crescentus* CB15 confirms the earlier observation that the production of these

FIG. 1. Electron micrograph of pili on *C. crescentus* swarmer cell and the effect of ϕ Cb5 binding on pilus retraction in stalked cells. (A) CB15F swarmer cell grown at 30°C, fixed with glutaraldehyde, and stained by rotary platinum shadow casting (see Materials and Methods). (B and C) The loss of pili from the stalked cells is prevented when an exponentially growing culture of strain CB15 was infected with the pilus-specific RNA bacteriophage ϕ Cb5 at a multiplicity of infection of 10. Samples were examined as described above after 1 h (B) and 2 h (C). The cells shown here are typical of those observed in these cultures. Bar = 0.5 μ m.

FIG. 2. Kinetics of pilus and flagellum formation in synchronous *C. crescentus* cells and the effect of penicillin. Synchronous swarmer cells of strain CB15F were isolated as described (6) and incubated at 30°C. Penicillin G was added to one subculture at 240 U/ml at 20 min. The percentage of cells in the control culture with flagella ($\circ$) and pili (Δ) was estimated by examining at least 50 cells in each sample by electron microscopy. The percentage of cells with flagella ($\bullet$) and pili ($\blacktriangle$) in the penicillin-treated culture was determined in the same way. Newly divided stalked cells were not considered in calculating the percentage of flagellated and piliated cells. Cell division was monitored by measuring the cell number ($\square$) in a Coulter Counter model ZBI.

structures is stage specific in the cell cycle. The results from synchronous cell cultures prepared by two different methods indicate that pili are assembled shortly after cell division at the flagellated pole of the new swarmer cell, which is later in development than inferred from earlier results based on phage adsorption assays (26, 27). Since pili are formed immediately after cell division, it would be difficult to conclude that assembly occurs on swarmer cells without the direct morphological observations carried out in these studies.

Smit and Agabian (30) have reported that the pilin subunit is made continuously in the stalked and predivisional cells of *C. crescentus*, but that it is not synthesized in the swarmer cell. Since pili are produced only by the swarmer cell, these structures must be assembled from pilin synthesized in a previous cell cycle. The analysis of cell division mutants strongly suggests that the assembly step is triggered by completion of a late cell division event, specifically CS. Thus, pilin synthesis and assembly appear to be indepen-

FIG. 3. Pilus and flagellum formation in the CS mutant PC1040. Synchronous swarmer cells of strain PC1040 were obtained by the plate release technique within 5 min after division at 30°C, and samples were immediately incubated at 30 or 37°C. The percentage of cells with flagella at 30 (○) and 37°C (●) and with pili at 30 (△) and 37°C (▲) was measured by examining at least 50 cells in each sample by electron microscopy. One subculture (▲) was incubated at 30°C for 90 min and then shifted to 37°C, when most cells had completed the execution point for CS (see text; 18). Cell division was monitored by measuring the cell number as described in the legend to Fig. 2.

dently regulated. There is also evidence for the separate control of synthesis and assembly of fimbrial subunits in other systems (8, 21).

An important question about morphogenesis is how different developmental events are regulated in the cell cycle. The results reported here suggest that the stage-specific events leading to differentiation of the new swarmer cell occur sequentially, rather than coordinately in the *C. crescentus* cell cycle; when expressed as fractions of the swarmer cell cycle, the flagellar filament is assembled at 0.8

(24), the flagellum is activated at 0.9 to 0.95 (14; unpublished observations), and as shown here, the pili are assembled at 0.1 in the next cell cycle.

The sequential timing of developmental events in *C. crescentus* appears to reflect their control by different steps in the cell cycle. The pole-specific events examined in this study occurred shortly after the completion of the required cell cycle steps (Fig. 4); expression of the major *fla* genes and flagellum assembly requires a mid-to-late stage of DNA chain elongation (17, 29); cell motility, or activation of the

FIG. 4. Sequential regulation of developmental events by the cell cycle. Previous results have shown that *fla* gene expression and flagellum assembly require chromosome replication (29) and that flagellum activation and cell motility require completion of DIV_p (6). The results in Fig. 3 show that CS is required for the assembly of pili. The organization of DNA synthetic steps (DNA_i, initiation of chromosome replication; DNA_e, DNA chain elongation; DNA_c, completion of chromosome replication) in a dependent, circular pathway and the organization of the cell division events on a dependent pathway that requires DNA_c have been discussed previously (15, 18).

flagellum, requires completion of cell division event DIV_p, and pilus assembly requires completion of CS (Fig. 2 and 3). These results are consistent with the earlier suggestion that the cell division cycle of *C. crescentus* is one component of a developmental clock that determines the timing of morphogenetic events in these cells (18).

The pili of several bacteria are known to be assembled from large membrane or periplasmic protein pools through a mechanism of polymerization and depolymerization at the base of the pilus, which allows controlled outgrowth and retraction of the filaments (5, 12, 13). There is also evidence in *C. crescentus* for a cytoplasmic pool of pilin (32). The observation that adsorption of bacteriophage ϕ Cb5 to the *C. crescentus* pili prevented the loss of these structures at the time of stalk formation (Fig. 1B and C) suggests that the pili are retracted in *C. crescentus* as one of the stage-specific events that precedes formation of the stalked cell. This mechanism could also explain why pili have not been detected in *C. crescentus* culture medium along with the flagellar filaments that are shed in the process of stalk formation (10).

The function of *C. crescentus* pili is unknown. They have not been implicated in bacterial conjugation, and it is possible that they serve a novel function in this differentiating organism. Based on the timing of pili formation, it is possible that they are involved in substrate adhesion of the developing swarmer cell. A holdfast which is initially located at the base of the flagellum (2, 20) is responsible for stable attachment of the stalked cell to the substrate. Pili on the developing swarmer cell could be involved in the initial step of adhesion, and the retraction of pili could then promote the interaction of the holdfast with the substrate surface.

In summary, we have demonstrated that while the flagellum is assembled on the predivisional cell at 0.8 of the swarmer cell cycle, pili are assembled on swarmer cells, at approximately 0.1 of the next cell cycle. The analysis of temperature-sensitive cell cycle mutants suggests that pili formation is dependent on completion of CS and that the sequential timing of flagellum formation, flagellum activation, and pilus assembly in *C. crescentus* reflects the order of underlying cell cycle steps.

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