Cell-Cycle-Dependent Polar Morphogenesis in *Caulobacter crescentus*: Roles of Phospholipid, DNA, and Protein Syntheses

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During swarmer cell differentiation in *Caulobacter crescentus*, morphogenesis at the swarmer pole is characterized by the loss of the flagellum, by the loss of phage receptor activity (PRA) (the ability of the cell to adsorb phage $\phi$CbK), and finally by the initiation of stalk outgrowth at the site formerly occupied by the flagellum and the PRA. We show here that each of these events is a cell cycle-dependent event requiring continuous protein synthesis for its execution but occurring normally in the absence of DNA synthesis or phospholipid synthesis. During stalked-cell differentiation, the flagellum and PRA reappear and the stalk elongates considerably. We show here that these events are also cell cycle dependent, requiring not only de novo protein synthesis but also DNA and phospholipid syntheses. When synchronous cells dividing 160 min after collection were used, PRA reappearance occurred at 110 min. This PRA reappearance was dependent on a phospholipid synthesis-requiring event occurring at 70 min, a DNA synthesis-requiring event occurring at 95 min, and a protein synthesis-requiring event occurring at 108 min. In the absence of net phospholipid synthesis, stalk elongation appeared more or less normal, but the stalks eventually became fragile, and by 240 min, most of the stalks had broken off, leaving only stubs attached to the cell body.

*Caulobacter crescentus*, with its unusual life cycle, has been proposed by several authors as an ideal procaryotic system in which to study the basic principles of differentiation (16, 20, 23). As a result, several morphological and biochemical events have been positioned relative to each other within the cycle. Studies with synchronized populations, single-cell microcultures, cell cycle mutants, and inhibitors of macromolecular synthesis have yielded a picture of the *C. crescentus* developmental program (3, 18, 21, 22). One pattern occurring during the transition from stalked cell to dividing cell (stalked-cell differentiation) involves the assembly of a flagellum, several pili, and receptors for a set of large, DNA bacteriophages at the stalk-distal pole of the cell at various times before cell division. At the same time, the stalk may elongate. Asymmetric cell division then results in a stalked cell which immediately repeats the program of stalked-cell differentiation, leading to the assembly of a set of polar structures at the newer (still stalk-distal) pole of the cell, in preparation for the next cell division. The flagellated (swarmer) cell produced by this asymmetric cell division carries out a different program of events (swarmer-cell differentiation). The swarmer cell loses the entire set of polar structures (the flagellum is shed into the medium, the pili disappear, and phage receptor activity [PRA] is lost) and initiates the growth of a stalk at the site previously occupied by the polar structures. Only then does it begin the program of stalked-cell differentiation, leading to the assembly of a set of polar structures on the (again, newer) stalk-distal pole of the cell in preparation for the next cell division. A particular advantage of the *C. crescentus* system is that the entire program of cellular differentiation is an intrinsic feature of the cell cycle and occurs during exponential growth.

Among the polar structures, the flagellum has been the object of considerable attention (10, 13, 14, 19, 24, 26). In contrast, rather less is known about the periodically expressed PRA; neither the chemical nature of the phage receptors nor the signals that regulate their periodic expression are known (15). The PRA of *C. crescentus* is best characterized for the large, double-stranded-DNA phage $\phi$CbK (2, 15), and one report suggests that phage LC72 may adsorb to the same receptor as $\phi$CbK (11). While the chemical nature of the phage receptor remains unknown, the PRA is a quantifiable property whose cell cycle dependence (15) can be easily analyzed.

The program of polar morphogenesis during *Caulobacter* differentiation can also be considered from the viewpoint of the life history of a cell pole (3). In the first stage, new poles are created as a result of cell division. In the second stage, this newer pole of each daughter is “decorated” with polar structures either immediately (in the stalked daughter cell) or after a delay (in the swarmer daughter cell). In the third stage (occurring in the swarmer cells), the polar structures are lost and are replaced by a stalk, a structure whose envelope layers (inner membrane, peptidoglycan, and outer membrane) are continuous with those of the main cell body but which contains little or no cytoplasm (20). The development of the stalk is a terminal event, and except for the possibility of stalk elongation or the laying down of cross-bands at intervals, no further visible changes occur at such a mature pole.

The data presented here focus mostly on the last two stages in the life history of a cell pole and particularly on the appearance and disappearance of PRA (one of the polar structures) and the growth of the stalk.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *C. crescentus* wild-type CM5000 and CM6000 were obtained from B. Ely (12) and N. Agabian (9), respectively. Both are derived from CB15, and their similarities and differences are discussed elsewhere (4, 17). Strain AE5168 (5) is a glycerol phosphate-requiring mutant derived from CM5000. Growth conditions in the minimal M2-glucose medium described by Ely (12) and the low-phosphate medium BIG are described elsewhere (17). Media for strain AE5168 were supplemented with 1 mM...
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AE5168 lacks glycerol phosphate dehydrogenase activity and thus cannot grow without an exogenous source of glycerol phosphate (5). Since the major phospholipids of C. crescentus are derived from glycerol phosphate (6), phospholipid synthesis is rapidly and completely blocked when strain AE5168 is transferred from glucose minimal medium supplemented with 1 mM glycerol and 1 mM glycerol phosphate to unsupplemented glucose minimal medium (5). The PRA of an exponentially growing culture of strain AE5168 was measured at various times after transfer to unsupplemented glucose minimal medium and was found to decline slowly for about 1 to 1.5 h and then to decline more rapidly (Fig. 2A). A similar result was obtained when phospholipid synthesis in the wild-type strain was inhibited by the addition of the antibiotic cerulenin to an exponentially growing wild-type culture (Fig. 2B). The slower response seen with strain AE5168 reflects its lower growth rate (4-h doubling time versus 3.2 h for the wild type), even when it is supplemented with excess glycerol phosphate.

When DNA synthesis was inhibited by the addition of hydroxyurea, the PRA of the culture again declined after a lag of 40 to 50 min (Fig. 2B). In contrast, the high level of PRA in the culture was maintained for at least 2 h when phospholipid synthesis was inhibited by the addition of chloramphenicol (Fig. 2B).

Maintenance of a given level of PRA in a culture could result from a continued balance between the appearance of PRA on predvisional cells and loss of PRA from swarvers, or it could result from a total arrest of cell cycle events such that PRA was neither lost nor gained by individual cells in the culture. To distinguish between these two possibilities, loss of PRA from swarver cells (swarmer-cell differentiation) and reappearance of PRA on predvisional cells (stalked-cell differentiation) were analyzed separately with synchronous populations.

Bacteriophage and measurement of PRA. Bacteriophage \( \phi \)CbK (1) was propagated by the liquid lysate method (4). PRA was measured as described previously (4) and is defined as the fraction of input phage (multiplicity of infection, \(<0.01\)) that adsorb (i.e., become chloroform sensitive) in 15 min at 30°C.

Antibiotics. Chloramphenicol (Sigma Chemical Co.) and hydroxyurea (Sigma) were used as freshly prepared water solutions. Cerulenin (Calbiochem-Behring) was dissolved at 10 mg/ml in ethanol and diluted 500-fold into the culture for a final concentration of 20 \( \mu \)g of cerulenin per ml and 0.2% ethanol.

Isotopic labeling. Rate of DNA synthesis was measured as the amount of \({ }^3 \text{H}\)guanosine incorporated into alkali-stable material precipitable by 5% trichloroacetic acid (8). Small samples (0.1 to 1.0 ml) were taken from synchronous cultures and mixed with \({ }^3 \text{H}\)guanosine at 100 \( \mu \)Ci/ml (15 Ci/mmol). After a 15-min incubation at 30°C, an equal volume of 0.5 N KOH was added to terminate the incorporation, and this mixture was incubated at 37°C for 16 h. Two volumes of cold 20% trichloroacetic acid was added, and the tubes were allowed to stand on ice for at least 15 min. The resulting precipitate was collected on glass fiber filters, washed with 15 ml of cold 25% trichloroacetic acid and 10 ml of ethanol, and dried under heat lamps. The radioactivity on the filter was measured in a Packard scintillation counter after the filter had been placed in 5 ml of OCS scintillation cocktail (Amersham Corp.).

RESULTS

PRA when macromolecular synthesis is inhibited. Strain AE5168 lacks glycerol phosphate dehydrogenase activity and thus cannot grow without an exogenous source of glycerol phosphate (5). Since the major phospholipids of C. crescentus are derived from glycerol phosphate (6), phospholipid synthesis is rapidly and completely blocked when strain AE5168 is transferred from glucose minimal medium supplemented with 1 mM glycerol and 1 mM glycerol phosphate to unsupplemented glucose minimal medium (5). The PRA of an exponentially growing culture of strain AE5168 was measured at various times after transfer to unsupplemented glucose minimal medium and was found to decline slowly for about 1 to 1.5 h and then to decline more rapidly (Fig. 2A). A similar result was obtained when phospholipid synthesis in the wild-type strain was inhibited by the addition of the antibiotic cerulenin to an exponentially growing wild-type culture (Fig. 2B). The slower response seen with strain AE5168 reflects its lower growth rate (4-h doubling time versus 3.2 h for the wild type), even when it is supplemented with excess glycerol phosphate.

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Polar structures and swarmer-cell differentiation. The PRA of C. crescentus is known to be periodically expressed during the cell cycle, being lost from newborn cells shortly after cell division and reappearing on predvisional cells some time before cell division (2, 4, 15). This timing of loss and reappearance of PRA during the cell cycle is shown for strain CM6000 in Fig. 1, along with the rate of DNA synthesis and the viable count. Although some of these data have been presented before (4) and repeat well-characterized
observations (15), they are repeated here to set a time reference against which other events can be compared. PRA loss was approximately coincident with the onset of DNA replication, and PRA reappearance occurred near the end of DNA replication ($t = 110$ min), some $50$ min before cell division ($t = 160$ min). The distinct temporal separation of loss of PRA from reappearance of PRA allowed these two events to be analyzed as independent markers in the cell cycle.

Protein synthesis was continuously required for the loss of PRA. When chloramphenicol was added to a population of synchronous swarmer cells, PRA was retained by the cells for at least $2$ h (Fig. 3A). The decline in PRA after that time correlates with a slow loss of viability of cells at times greater than $2$ h (data not shown). Addition of chloramphenicol at a time when half the PRA had already been lost ($t = 15$ min) blocked further loss within about $5$ min. Chloramphenicol also blocked loss of motility in these cultures, consistent with the observation by Newton that rifampin blocks the loss of motility from swarmer cells (16). Thus, inhibition of RNA or protein synthesis blocks the process of swarmer-cell differentiation in that neither the loss of polar structures nor the initiation of stalk growth occurs (data not shown).

In contrast, neither inhibition of DNA replication with hydroxyurea (18) nor inhibition of phospholipid synthesis with cerulenin (7) had any effect on the loss of PRA (Fig. 3B and C) or on the loss of flagella (7) (data not shown). Thus, PRA loss, like the other indicators of swarmer-cell differentiation, occurred with normal kinetics in the absence of DNA synthesis or phospholipid synthesis.

**Polar structures and stalked-cell differentiation.** Protein synthesis was also required for the reappearance of PRA. Chloramphenicol was added to synchronous cells at various times after reincubation ($t = 0$), and these cells were incubated in the presence of chloramphenicol until $220$ min to allow expression of PRA to occur. PRA was then assayed.

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**FIG. 2.** Effect of inhibiting macromolecular synthesis on PRA of asynchronous cultures. (A) Strain AE5168 was grown to $2 \times 10^9$/ml in M2-glucose medium supplemented with $1 \text{mM}$ glycerol and $1 \text{mM}$ glycerol phosphate, washed three times, and suspended in unsupplemented M2-glucose. PRA was measured at half-hour intervals. (B) Strain CM5000 was grown in M2-glucose medium to about $2 \times 10^9$/ml. At $t = 0$, cerulenin, hydroxyurea, or chloramphenicol was added (final concentrations, $20 \mu$g/ml, $5 \text{mg/ml}$, and $5 \mu$g/ml, respectively); PRA was measured at various times.

**FIG. 3.** Effects of inhibiting macromolecular synthesis on loss of PRA from synchronous cultures. A synchronous population of CM6000 was prepared, and chloramphenicol was added at $t = 0$ or at $t = 15$ min after growth was reinitiated (A). In an analogous experiment, either cerulenin (B) or hydroxyurea (C) was added at $t = 0$. PRA was measured at various times thereafter. ●, PRA in the corresponding uninhibited controls.
to determine how much additional PRA could be expressed after the addition of chloramphenicol. There was little or no difference between the amount of PRA present when the chloramphenicol was added and the amount of PRA present after the additional incubation in the presence of chloramphenicol (cf. Fig. 1 and 4). If we define the execution point for the last chloramphenicol-sensitive step required for PRA reappearance as the time when 50% of the maximum recoverable PRA is resistant to chloramphenicol, then this execution point occurred at about 108 min. PRA reappearance itself (again measured at the 50% maximum level) occurred at about 110 min. Thus, protein synthesis was required up to the last few minutes for PRA reappearance.

It is well documented both that the appearance of the flagellum and of flagellin synthesis requires that DNA replication proceed beyond a certain point and that this execution point correspond to the time when flagellin synthesis becomes resistant to inhibition by hydroxyurea (24). The last hydroxyurea-sensitive event required for PRA reappearance occurred about 95 min after reincubation of synchronous cells (Fig. 4). This was about two-thirds of the way through the round of DNA replication (Fig. 1), comparable to the point in DNA replication required for flagellin synthesis (24).

Although phospholipid synthesis was not needed for the loss of PRA, the data in Fig. 3B suggested that phospholipid synthesis was required for some event(s) needed for the reappearance of PRA. To determine when the last phospholipid synthesis-requiring event occurred, the execution point for the last cerulenin-sensitive event needed for PRA reappearance was determined. Fifty percent of the recoverable PRA was resistant to cerulenin as early as 70 min after reincubation, at about 40 min before the PRA actually reappeared (Fig. 4). Thus, the execution points for the last cerulenin-sensitive (phospholipid synthesis-requiring), hydroxyurea-sensitive (DNA synthesis-requiring), and chloramphenicol-sensitive (protein synthesis-requiring) events needed for PRA reappearance occurred 70, 95, and 108 min after reincubation, and PRA reappearance occurred at 110 min. Cell division occurred at 160 min.

Although it is clear that a last cerulenin-sensitive step must exist, the nature of this event remains totally unknown. This event, like all events in synchronous populations (e.g., PRA reappearance or cell separation), will display an imperfect synchrony, with precocious cells accomplishing the event earlier than laggards. Thus, we measured the timing of these unknown events in the same way as the more visible events, i.e., by measuring the time when half the population has completed the event.

When "execution points" such as those described above for the cerulenin-sensitive step are being determined, it is important that sufficient time be allowed for the event in question to proceed to completion in the presence of the antibiotic, which may alter the rate of progress through the cell cycle. In the absence of cerulenin, PRA reaches a maximum at about 170 min after reincubation. Even in the presence of cerulenin, all the PRA that could reappear had done so before 220 min after reincubation even if cerulenin was added as early as 65 min after collection (Fig. 5). Thus, although cell cycle events might have occurred somewhat more slowly in the presence of cerulenin, the value of PRA measured at 220 min reflected the maximum reappearance attainable.

**Stalk formation.** Stalk formation, the terminal event in swarmer pole morphogenesis, like all other events in swarmer-cell differentiation, was completely blocked when newborn swarmer cells were treated with chloramphenicol (data not shown). However, stalk formation was initiated normally and at the usual time when newborn swarmer cells were treated with cerulenin at \( t = 0 \) (Fig. 6). Although initiation of stalk growth occurred normally in the absence of net phospholipid synthesis, stalk elongation, part of stalked-cell differentiation, was aberrant. The elongating stalks of the cerulenin-treated cells were apparently more fragile than normal stalks and by \( t = 113 \) min showed deformities such as "blebs" in electron micrographs (Fig. 6). By \( t = 237 \) min, the electron micrographs showed few normal-looking stalks in the cerulenin-treated culture. Rather, the stalks appeared either to be seriously deformed (minority class) or to have broken off near the base, leaving a stubby remainder attached to the cell body (majority). The broken-off stalks were also visible in the micrographs and often seemed attached to a cell by a thin strand of material.
The process of cell division (generation of new cell poles) also seemed to be affected. When swarmer cells were treated with cerulenin at $t = 0$, some cells did begin to constrict in the middle, but none developed the deep constriction typical of predivisional cells, and there was no evidence of cell division, either in electron micrographs like those in Fig. 6 or in wet mounts under phase-contrast microscopy (data not shown).

Effect of phosphate concentration. These experiments were performed with cells grown in a standard, 20 mM phosphate-buffered glucose minimal medium. When cerulenin was added to this medium, even at concentrations as low as 5 mM, _C. crescentus_ cells rapidly lost the ability to form colonies when spread or poured onto broth plates (PYE). In an exponentially growing culture, more than 80% of the colony-forming ability was lost within 30 min and less than 1% remained after an hour in the presence of cerulenin (data not shown). Nevertheless, differentiation proceeded normally for a considerable time (Fig. 5). Adding cerulenin at $t = 65$ min allowed reappearance of PRA 45 min later, even though colony-forming ability had been lost. Thus, these cerulenin-treated cells were still metabolically and morphogenetically active.

To determine whether the cerulenin-sensitive step required for PRA reappearance was related to cell cycle progress or to viability, we determined the last cerulenin-sensitive step required for PRA reappearance in cells grown in low (0.4 mM)-phosphate medium, where colony-forming ability on PYE plates is unaffected for more than an hour and is never reduced below 50% (data not shown). Synchronous cells grown in low-phosphate medium showed a cerulenin-sensitive step for PRA appearance about 40 min before PRA actually reappeared (Fig. 7), exactly as was seen with cells grown in high-phosphate medium. Thus, the cerulenin-sensitive step detected in these experiments reflects the cell-cycle dependence of PRA reappearance rather than the viability of the cells. In the experiment shown in Fig. 7, both the PRA reappearance and the cerulenin-sensitive step occurred later than with cells grown in high-phosphate medium (Fig. 1 and 4). This reflects the fact that strain CM6000 grows more slowly in low-phosphate medium (3.2-h doubling time) than in high-phosphate medium (2.2-h doubling time) and is paralleled by other cell cycle marker events such as initiation of DNA replication (not shown).

**DISCUSSION**

Two events of swarmer-cell differentiation, shedding of the flagellum and initiation of stalk growth, have been shown previously to be independent of both DNA synthesis and phospholipid synthesis (7). The data presented here show that a third event in swarmer-cell differentiation, loss of PRA, is likewise independent of DNA synthesis and phospholipid synthesis. In the presence of cerulenin, an inhibitor of phospholipid synthesis, PRA was lost from swarmer cells with normal kinetics and flagella were shed. Both PRA loss and flagellar release required constant protein synthesis, showing that swarmer-cell differentiation was an active process requiring continual translation rather than a passive decay of swarmer-cell features. Starvation for glycerol phosphate appears to mimic cerulenin addition, suggesting that the phenomena described here result from cessation of phospholipid synthesis rather than some unknown side effect of the antibiotic (see below). In particular, morphological differentiation from swarmer to stalked cell occurred during glycerol phosphate starvation exactly as it did in the presence of cerulenin (7). Furthermore, DNA replication initiated normally in the presence of cerulenin (17).

Reappearance of one of the polar structures, the flagellum, on predivisional cells is known to require DNA replication through a point occurring some time before the polar structures appear (24). The hydroxyurea data in Fig. 6 suggest that PRA also shows this requirement for a specific DNA replication event. Our data (Fig. 5) showed that PRA reappearance became immune to cerulenin addition well before the critical DNA replication event occurred. If cerulenin
neither cell division nor the fully developed constriction seen near the center of predivisional cells occurred when phospholipid synthesis was blocked from the beginning of the swarmercell stage. The execution point (last phospholipid synthesis-requiring step) for constriction is not known. Phospholipid synthesis is periodic in *C. crescentus*, with net phospholipid synthesis restricted to two periods (17), one near the end of swarmercell differentiation and one during the last one-third of the DNA synthesis phase, about the time that constriction would occur. Thus, it is tempting to speculate that one or both of these are required for completion of cell constriction.

Apparently swarmercell are born with a sufficient supply of stored phospholipid precursors (perhaps as the membranous “plug” seen at the prestalk pole of swarmercells [20]) to allow cellular growth, stalk initiation, and stalk elongation up to at least the time of the second period of phospholipid synthesis (which occurs in the newly differentiated stalked cell). The ability of the swarmercell to carry out the entire program of swarmercell differentiation in the absence of DNA or phospholipid synthesis fits well with the presumed role of the swarmercell as a self-contained dispersal unit, designed to move away from the attached stalked cell in order to prevent overpopulation of local surfaces (21).

In contrast to the relatively straightforward size increase of the swarmercell envelope, stalked-cell differentiation involves a considerable reorganization of the growth patterns of the envelope. The current data suggest that the stalked cell deals with its envelope reorganization in the following sequence: stalk initiation and growth (7), some phospholipid synthesis-requiring event(s) affecting the stalk distal pole (Fig. 5), a signal (via DNA replication) for the eventual assembly of the polar structures and division site (19) (Fig. 6), assembly of the polar flagellum and appearance of PRA (19) (Fig. 1), activation of the flagellar machinery (16), and finally cell separation and the appearance of pili (25). The cotemporality of the cerulenin-sensitive step for pole assembly and the second round of phospholipid synthesis during the cell cycle (17) suggests a possible causal connection, but this has not been tested.

In all studies with synchronized populations, it is important to demonstrate that the phenomenon described is not an artifact of the synchronization procedure. In these experiments, the conclusions drawn from an analysis of synchronized cells fit the kinetics of the effects seen in asynchronous populations (Fig. 2B). For example, PRA levels remained high in asynchronous cerulenin-treated cultures for about 40 to 50 min. While PRA loss from swarmers occurred irreversibly (Fig. 3), all cells older than 70 min and younger than 110 min retained the potential to regain PRA of the presence of cerulenin. Thus, for about 40 min, the number of cells gaining PRA about equaled the number losing PRA. After 40 min in the presence of cerulenin, all the cells in the 70- to 110-min interval had regained PRA and cells in the 0- to 70-min interval were reaching the time for PRA appearance. These cells would not regain PRA because of the failure of some cerulenin-sensitive event (Fig. 4). Thus, PRA loss continued, but there was no regaining of PRA to balance the loss, and a net decline in the level of PRA in the culture ensued. A similar argument applies for the hydroxyurea-treated culture.

The maintenance of PRA in chloramphenicol-treated cultures (Fig. 2) is explained by the inability of the cells to lose PRA in the absence of protein synthesis (Fig. 4). Thus, there is a very different explanation for the (long-term) maintenance of PRA in chloramphenicol-treated cultures (inability...
to lose PRA) and the (short-term) maintenance of PRA in hydroxyurea- or cerulenin-treated cultures (balance between PRA loss and PRA reappearance).

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LITERATURE CITED