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Stalkless Mutants of *Caulobacter crescentus*

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A stalk, a single flagellum, several pili, and deoxyribonucleic acid (DNA) phage receptors are polar surface structures expressed at a defined time in the *Caulobacter crescentus* cell cycle. When mutants were isolated as DNA phage φCbK-resistant or ribonucleic acid (RNA) phage φCp2-resistant, as well as nonmotile, strains, 5 out of 30 such mutant isolates were found not to possess stalks, but did possess inactive flagella. These stalkless mutants were resistant simultaneously to both DNA and RNA phages and did not possess pili and DNA phage receptors. Motile revertants were isolated spontaneously from five independent stalkless mutants. All motile revertants simultaneously regained the capacity to form stalks and susceptibility to DNA and RNA phages. It is suggested that a single mutation pleiotropically affects stalk formation, flagella motility, and coordinate polar morphogenesis of pili and DNA phage receptors. The stalkless mutants grew at a generation time similar to that of the wild-type strain at 30°C. Cell size and morphology of a stalkless mutant, *C. crescentus* CB13 *pdr-819*, were also similar to those of the wild-type strain, except for the absence of a stalk. In addition, the CB13 *pdr-819* predivisional cells were partitioned into smaller and larger portions, indicating asymmetrical cell division, as in the wild-type strain. From these results, it is suggested that swarmer cells undergo transition to cells of a stalked-cell nature without stalk formation and that the cell cycle of the stalkless mutant proceeds in an ordered sequence similar to that defining the wild-type cell cycle.

Caulobacter is an aquatic stalked bacterium which shows drastic changes of cell morphology during the cell cycle and is currently viewed as a procaryotic system for research on the relation between gene expression and morphogenesis. Typically, this organism is characterized by specific surface differentiation of the two types of cells that occur in a sequential order in the cell cycle (15). The surface differentiation is manifested by the formation of a stalk, flagella, pili, and deoxyribonucleic acid (DNA) phage receptors, which are all polar surface structures and under spatial and temporal control. The flagella, pili, and DNA phage receptors are formed in a coordinate manner at polar proximal sites of a sessile swarmer cell (4, 15). These structures are lost from the swarmer cell upon its transition to a stalked cell, and stalk formation is initiated at a similar polar site of the cell. The transition from swarmer to stalked cell is currently considered to be obligatory in the *Caulobacter* cell cycle.

The stalk appendage is a slender rod, ranging from 0.5 to 3.0 μm in length (6, 11, 18). The membranes and wall of the stalk are continuous with those of the cell (9). The stalk cytoplasm is devoid of DNA and ribosomes and is divided by crossbands, which are correlated with the number of cell divisions or the age of the stalked cell (18). The function of the stalk is not known.

The stalk is synthesized at the junction between stalk and cell (14) and elongates throughout the cell cycle, except at the period of cell division (18). The stalk elongation is affected by a variety of conditions, such as the availability of phosphate and cyclic guanosine 3′,5′-monophosphate (13, 14). Mutants have also been isolated that produce long stalks (Skl mutants, 11) or, alternatively, short stalks (Sei mutants, 12). The regulatory steps for stalk formation, however, are not clear. Certainly an elaborate control mechanism should be exerted for localized stalk formation, which involves the synthesis of membranes, peptidoglycan, and lipopolysaccharide.

We report in this paper the isolation of stalkless mutants of *Caulobacter crescentus* CB13 and demonstrate, from the analysis of the mutants and their spontaneous revertants, that stalkless mutants grow normally and divide asymmetrically, as does the wild-type strain,

and that a single mutation simultaneously affects stalk formation, flagella motility, and coordinate morphogenesis of polar pili and DNA phage receptors.

MATERIALS AND METHODS

Bacteria, phages, and growth conditions. *C. crescentus* CB13B1a wild type and CB13 *str-801*, a streptomycin-resistant mutant from CB13B1a, were used. CB13 *pdr-801*, a stalkless mutant isolated as a DNA phage ϕCbK-resistant and nonmotile strain, CB13 *prr-826*, a stalkless mutant isolated as a ribonucleic acid (RNA) phage ϕCp2-resistant and nonmotile strain, and other stalkless mutants were isolated as described below from CB13 *str-801*. Motile revertants were isolated spontaneously from the stalkless mutants, as described previously (4).

DNA phage ϕCbK (1), flagellotropic DNA phage ϕCp34 (3), and RNA phage ϕCp2 (8) have been described. DNA phage ϕCr22 was kindly supplied by B. Ely.

Bacteria and phages were routinely grown and propagated in peptone-yeast extract (PYE) nutrient broth (10). Phage stocks of high titer were prepared as described previously (3, 8). MC medium contained 1.0 g of $(NH_4)_2SO_4$, 0.023 g of K_2HPO_4, 0.1 g of $MgCl_2$, and 3.0 g of an amino acid mixture of casein composition per 1,000 ml of deionized water. The pH was adjusted to 6.8 with NaOH before autoclaving, and $CaCl_2$ was added to 4.5×10^{-4} M after autoclaving. CB13 strains grow in MC medium at a generation time of 3 h at 30°C.

Isolation of stalkless mutants. CB13 *str-801* cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and nonmotile ϕCbK- or ϕCp2-resistant mutants were isolated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated CB13 *str-801* as described previously (4). Nonmotile, phage-resistant mutants were then screened by electron microscopy for stalkless or flagellated isolates.

Preparation of ^{32}P-labeled ϕCbK and ϕCp34 and assay of ^{32}P-labeled phage adsorption. ^{32}P-labeled phage ϕCbK and ϕCp34 were prepared, and ^{32}P-labeled phage-adsorbing capacities of the cells were assayed as described elsewhere (5).

Flagella purification. CB13 cells were grown in 1,000 ml of PYE medium to the late stationary phase at 30°C and centrifuged at 9,000 × *g* for 10 min. To the supernatant fluid were added 4.5 ml of 2.2 M phosphate buffer (pH 7.1), 1.3 ml of 0.4 M ethylenediaminetetraacetate (EDTA) (pH 7.0), and 142 g of $(NH_4)_2SO_4$. The mixture was kept at 0°C for 4 h and centrifuged at 14,000 × *g* and 2°C for 20 min. The sediment was suspended in 4.0 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride–0.005 M EDTA, pH 7.5 (Tris-EDTA). The suspension was centrifuged at 9,000 × *g* for 10 min to remove remaining cells and mixed with Triton X-100 to reach the final concentration of 0.5% (vol/vol). The flagella suspension was then sedimented through a 20% sucrose layer in Tris-EDTA onto a CsCl cushion (density, 1.5 g/cm^3) in Tris-EDTA at 39,000 rpm and 2°C for 2 h in an Hitachi RPS41 rotor.

Electron microscopy. The basic procedures for electron microscopy were as described previously (4).

RESULTS

Isolation of stalkless mutants. It was shown previously (4) that in *C. crescentus* CB13 a single mutation simultaneously blocks cell motility and susceptibility to DNA and RNA phage infection. Most mutants of this phenotype do not form flagella, pili (RNA phage receptors), and DNA phage ϕCbK receptors, but they do form polar stalks. When a large number of the mutants were examined under an electron microscope, however, some mutant isolates (5 out of 30) were observed to possess polar flagella (Fig. 1b). In addition, all five such mutant isolates did not possess polar stalks. These stalkless mutants were found among nonmotile mutants, which were isolated as either DNA phage ϕCbK- or RNA phage ϕCp2-resistant strains.

Phenotype of stalkless mutants. As the phage resistance indicated, stalkless mutants did not possess pili at polar sites proximal to flagella (Fig. 1e) or adsorb DNA phage ϕCbK and flagellotropic DNA phage ϕCp34. The results with the stalkless mutants CB13 *pdr-819* and CB13 *prr-826* are presented in Table 1. The lack of ϕCp34-adsorbing capacities in CB13 *pdr-819* and CB13 *prr-826* agreed with the inactive nature of flagella in the stalkless mutants, since the cells were observed to be nonmotile under a phase-contrast microscope, and the lack of ϕCbK-adsorbing capacities indicates that ϕCbK receptors are not formed in the stalkless mutants.

Single mutation involved in the formation of stalk and phage receptors and flagella motility. It now became of interest to investigate, by revertant analysis, whether or not a single mutation simultaneously affects the formation of stalk and phage receptors and the motility of flagella. Motile revertants were isolated spontaneously from five independent stalkless mutants and examined for stalk formation under an electron microscope and for phage infectivity and adsorption to the cells. Motile revertants spontaneously isolated from CB13 *pdr-819* and CB13 *prr-826* formed polar stalks and simultaneously regained phage-adsorbing capacities, together with susceptibility to phage infection (Fig. 1f, Table 1). The extent of phage-adsorbing capacities varied depending on revertant isolates. Results with three other stalkless mutants were similar. This revertant analysis strongly suggests that a single mutation simultaneously affects the formation of stalk and

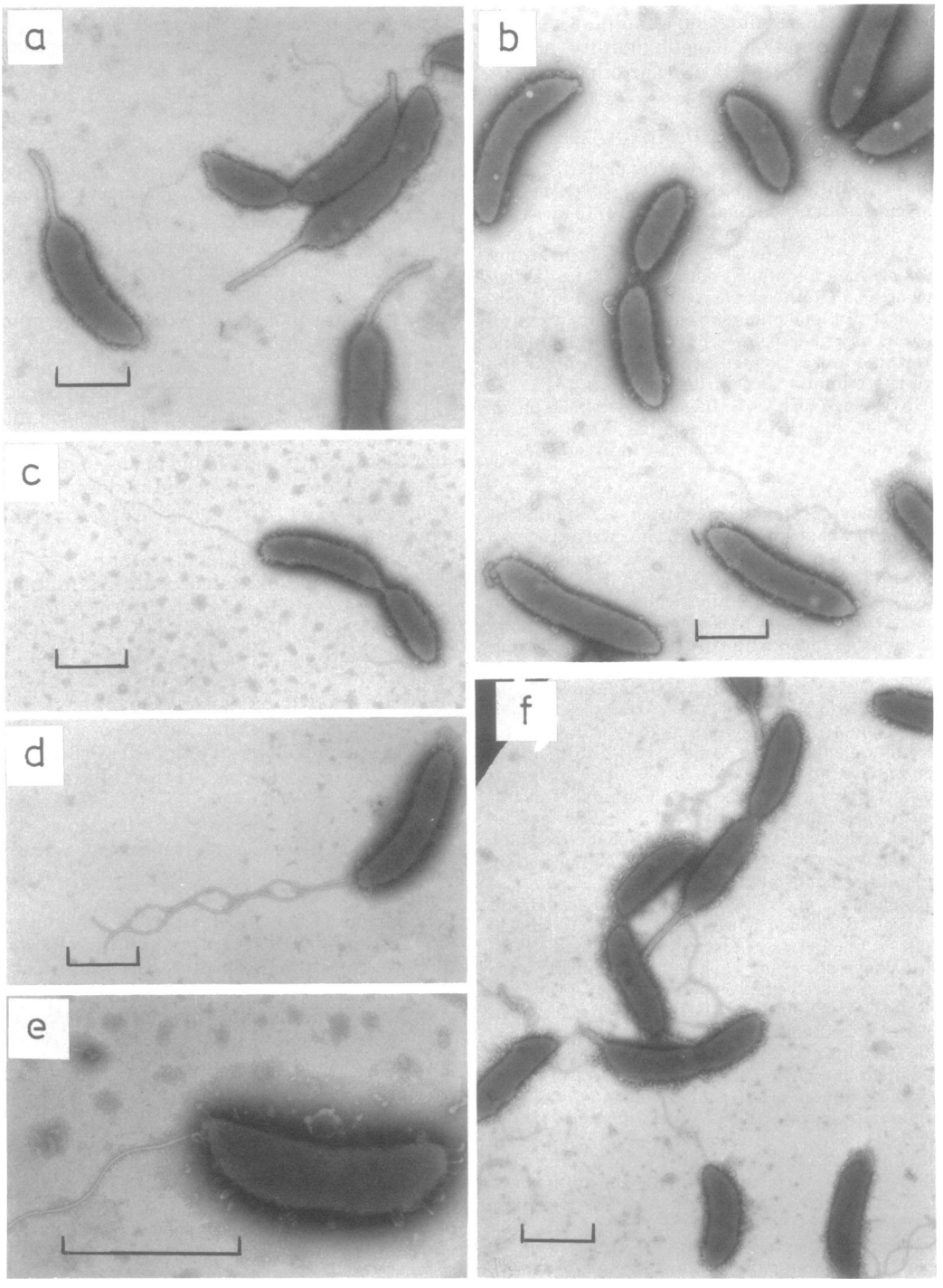

FIG. 1. *Cell morphology of CB13 wild type, CB13 pdr-819 stalkless mutant, and CB13 pdr-819R1 revertant. CB13 cells were grown at 30°C in PYE medium to the early log phase (optical density at 660 nm, 0.1, or 4.4 × 10⁸ cells/ml), stained with 1% neutral sodium phosphotungstate, and photographed under an electron microscope as described in the text. Bar = 1.0 μm. (a) CB13 wild type; (b–e) CB13 pdr-819 stalkless mutant; (f) CB13 pdr-819R1 revertant.*

TABLE 1. *Properties of stalkless mutants and their revertants*[a]

CB13 mutant	Motility	Stalk	Flagella	Phage infectivity				Phage adsorption %	
				φCbK	φCp34	φCp2	φCr22	φCbK	φCp34
Wild type	+	+	+	+	+	+	+	100	100
pdr-819	−	−	+	−	−	−	+	0.2	1.2
*pdr-819*R1	+	+	+	+	+	+	+	108.8	73.6
prr-826	−	−	+	−	−	−	+	0	6.6
*prr-826*R	+	+	+	+	+	+	+	69.0	27.2

[a] Phage infectivity was tested by the spot test. ^{32}P-labeled φCbK adsorbed to cells is presented in a percentage of that on CB13 wild type. φCbK adsorption, 100%, was 8,492 ^{32}P cpm/0.1 optical density unit at 660 nm (OD_{660}) per ml, and φCp34 adsorption, 100%, was 6,674 ^{32}P cpm/ 0.1 OD_{660} per ml. CB13 cells were grown in PYE medium at 30°C. Cell motility was observed under a phase-contrast microscope, and flagella were observed under an electron microscope. CB13 *pdr-819*R1 and CB13 *prr-826*R are spontaneous revertants isolated as described in the text from CB13 *pdr-819* and CB13 *prr-826*, respectively.

phage receptors and the motility of flagella.

Mutants were isolated, as either DNA or RNA phage-resistant strains, that were motile but became resistant simultaneously to both DNA and RNA phages (4). When 30 of these motile and phage-resistant mutants were examined under an electron microscope, all the mutant isolates possessed polar stalks. It thus seems that the mutational change that renders the cells resistant to both DNA and RNA phage infection does not affect stalk formation.

Localized effect of mutation. DNA phage φCr22, unlike the phages (φCbK, φCp34, and φCp2) used above, initiates infection by adsorbing randomly to the cell surface of both swarmer and stalked cells (B. Ely, personal communication). If the above single mutations exert an effect only on the polar surface structure formation, then the stalkless mutants should retain susceptibility to φCr22 infection. The stalkless mutants CB13 *pdr-819* and CB13 *prr-826* were sensitive to φCr22 (Table 1). The other stalkless mutants were sensitive to φCr22 as well. Alternatively, 20 φCr22-resistant mutants were isolated and examined for the loss of cell motility and susceptibility to φCbK, φCp34, and φCp2. All the φCr22-resistant mutants were motile and sensitive to the phages tested and formed stalks. There were no phenotypic variations in φCr22-resistant mutants such as those observed in mutants resistant to phages that initiate infection by adsorbing at polar sites of swarmer cells (4). It thus appears likely that a single mutation that affects flagella motility and the formation of stalks, pili, and DNA phage receptors is involved only in the polar surface function and morphogenesis in the swarmer cell.

Cell size and morphology and asymmetric cell division in the stalkless mutant CB13 *pdr-819*. The transition from swarmer to stalked cell is considered to be obligatory in the *Caulobacter* cell cycle. Is the stalk appendage per se indispensable to the cell cycle? The stalkless mutants CB13 *pdr-819* and CB13 *prr-826* grew at 30°C in PYE medium at a growth rate similar to that of the wild-type strain (generation time, 100 min). It is then possible that the transition from swarmer to stalked cell occurs without stalk formation, or that swarmer cells of the stalkless mutants divide by themselves without undergoing transition to stalked cells. These two possibilities should be distinguished by examining cell size and morphology in the stalkless mutant culture. The stalkless mutant CB13 *prd-819* was grown to early log phase at 30°C in PYE medium, and mutant cell size and morphology were then compared with those of the wild-type cells.

The size of the wild-type swarmer cell was 1.32 ± 0.16 by 0.41 ± 0.03 μm, and the size of the wild-type stalked cell was 1.65 ± 0.21 by 0.43 ± 0.03 μm (Table 2). The size of the stalked cell does not include stalk length. The size estimation was made by scoring more than 30 cells of each type. Similarly, the size of the swarmer portion of the wild-type predivisional cell was 1.25 ± 0.10 by 0.43 ± 0.02 μm, and the size of the stalked-cell portion was 1.59 ± 0.18 by 0.42 ± 0.03 μm (Table 2). Some predivisional cells did not possess single flagella, probably because they were lost during preparation. The unequal partition of the predivisional cell supports the previous notion that the *C. crescentus* cells divide in an asymmetrical manner (19).

The size of predivisional cells in the culture of the stalkless mutant BC13 *pdr-819* was estimated similarly under an electron microscope. The mutant predivisional cells possessed single flagella at either the smaller or larger cell end, or in some cases at both cell ends (Fig. 1c). The size of the smaller portion of the mutant predivisional cell was 1.27 ± 0.14 by 0.42 ± 0.03 μm, and the size of the larger portion was 1.61 ±

TABLE 2. *Estimation of cell size by electron microscopy*[a]

Mutant cell type	Cell size (μm)
CB13 wt	1.25 ± 0.10 × 0.43 ± 0.02 / 1.59 ± 0.18 × 0.42 ± 0.03
CB13 pdr-819	1.27 ± 0.14 × 0.42 ± 0.03 / 1.61 ± 0.22 × 0.43 ± 0.02
CB13 wt	1.32 ± 0.16 × 0.41 ± 0.03
CB13 wt	1.65 ± 0.21 × 0.43 ± 0.03

[a] The wild-type (wt) CB13 and stalkless mutant CB13 *pdr-819* cells were grown at 30°C to the early-log phase in PYE medium. Cells were photographed under an electron microscope at ×7,500 magnification as described in the text. More than 30 cells were scored for size estimation of each cell type.

0.22 by 0.43 ± 0.02 μm (Table 2). These values are quite similar, respectively, to the size of the swarmer-cell and stalked-cell portions of the wild-type predivisonal cell.

Since stalks are missing from the mutant CB13 *pdr-819* cells, it is not feasible to make a distinction among mutant cells of the size of wild-type swarmer and stalked cells. When 103 mutant cells other than elongated and predivisional cells were scored, the size ranged within the size limit of wild-type swarmer and stalked cells (Fig. 2), and the average width was 0.43 μm. Approximately half of these mutant cells possessed single flagella, and the frequency of flagellated cells did not vary with the cell size. When 52 elongated cells of the mutant CB13 *pdr-819* were scored, the size (average length, 2.31 μm) ranged within the size limit of the wild-type elongated cell (Fig. 2). Twenty-three out of 52 elongated mutant cells possessed single flagella at one cell pole. Further, some elongated cells formed more than one flagellum at the same cell pole (Fig. 1d). Flagella could be recovered from the mutant culture after removal of the cells. These flagella released from the mutant cells contained a hook and rod structure (Fig. 3), as did those from the wild-type cells (17).

The results described above suggest that: (i) the culture of the stalkless mutant CB13 *pdr-819* contains various cell types found in the wild-type culture, and these mutant cell types are similar in size and morphology to those of the wild type, except for the absence of stalk; (ii) the stalkless mutant cells divide in an asymmetrical manner, as do the wild-type cells; (iii) the release of flagella from the mutant cells is delayed, probably because of the lack of stalk formation; and (iv) swarmer cells undergo transition to cells of a stalked-cell nature without stalk formation, and the cell cycle of the stalkless mutant CB13 *pdr-819* proceeds in an ordered sequence similar to that defining the wild-type cell cycle (Fig. 4).

DISCUSSION

The polar stalk appendage is a most distinctive surface structure in *C. crescentus* and serves as a good landmark for the study of cell surface differentiation. Stalk formation and its regulation have thus drawn much attention in the past (15). In pulse-chase experiments, using radioactively labeled glucose and autoradiography, Schmidt and Stanier (14) have shown that the formation of stalk structure occurs at the junction between cell and stalk. From its structure (7, 9), stalk formation is expected to involve prior or de novo precursor synthesis, assembly, and/or localization for membranes, peptidoglycan, and lipopolysaccharide, to men-

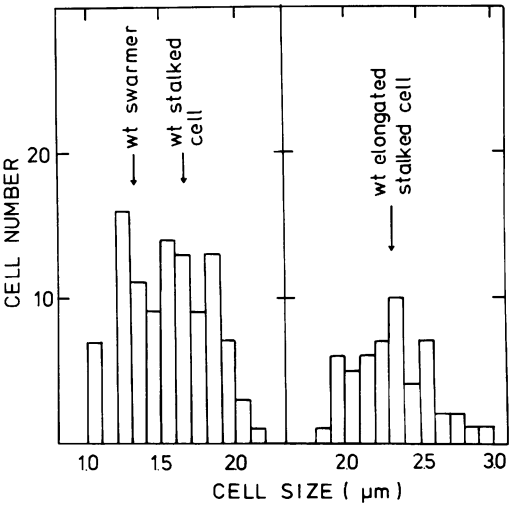

FIG. 2. *Size distribution of stalkless mutant CB13 pdr-819 cells. CB13 pdr-819 cells were grown at 30°C in PYE medium to the early log phase and photographed at ×7,500 magnification as described in the legend to Fig. 1. One hundred-three mutant cells, other than elongated and predivisional cells, and 52 mutant elongated cells were scored, and cell length and width were estimated. The arrows show the average length of the wild-type swarmer cell, stalked cell, and elongated stalked cell.*

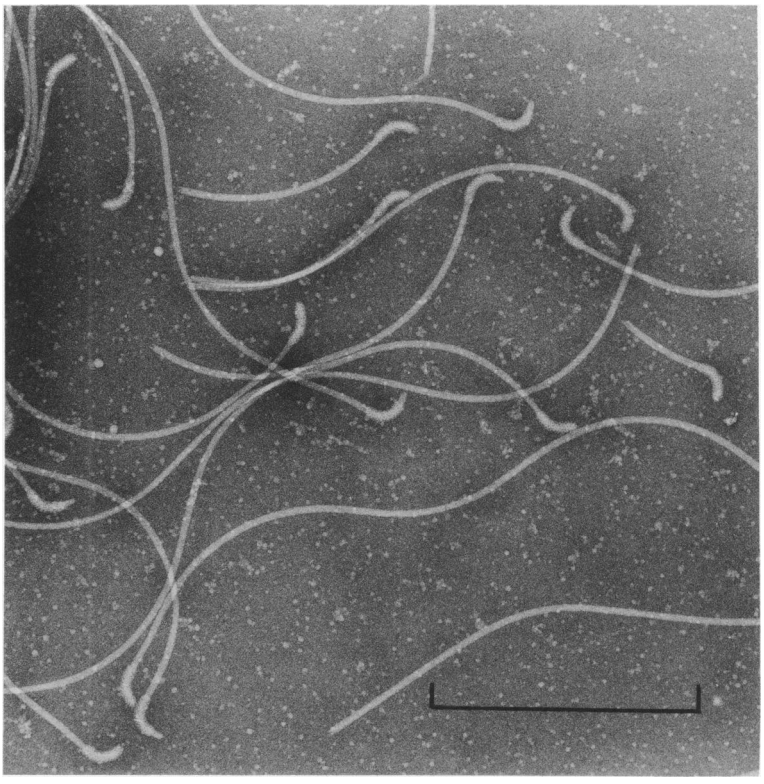

FIG. 3. *Flagella released from the stalkless mutant CB13 pdr-819. Flagella were purified and photographed as described in the text. Bar = 0.5 μm.*

tion only a few. In general terms, the regulation of stalk formation is considered at both initiation and elongation steps. At the moment, however, the biochemical nature of these steps is not defined at all and is a matter of conjecture.

Several lines of evidences indicate that the initiation of stalk formation is dependent on prior cell division. When cell division, but not cell growth, was inhibited at a low concentration of penicillin G, stalk formation was not observed at the nonstalked-cell ends in resulting cell filaments (20). The transition of swarmer to stalked cell, which involves the initiation of stalk formation, was not inhibited by the same drug. It is not clear whether or not penicillin G directly affected the synthesis of peptidoglycan since amino acid compositions appeared to be similar in peptidoglycans of stalked and lateral cell walls (2, 15). As proposed previously (A. Newton, M. A. Osley, and B. Terrana, *Microbiology—1975*, p. 442–459, American Society for Microbiology, Washington, D.C., 1975), the program for stalk formation could be organized before cell division as a branch from a dependent DNA synthesis division pathway. It would then be possible that penicillin G inhibits the synthesis of requisite structural components for cell division and stalk formation. The fact that stalkless mutants divide in an asymmetrical manner, as does the wild-type strain (Table 2), indicates rather that the initiation of stalk formation is a separable phenomenon from cell division.

The stalkless mutants possess flagella which are nonmotile. All motile revertants spontaneously isolated from five independent stalkless mutants simultaneously regained the capacity to form stalks and polar phage receptors. From this revertant analysis we propose that a single mutation pleiotropically affects flagella motility and the formation of polar stalks, pili, and DNA phage receptors.

It was suggested previously that there exists an ordered sequence in the coordinate polar morphogenesis of flagella, pili, and DNA phage receptors (4). A mutation in step I of the sequence gives rise to those cells that are flagellaless and unable to form polar pili (RNA phage receptors) and DNA phage receptors. Since nonmotile but flagellated stalkless mutants were found among mutants of step I, this regu-

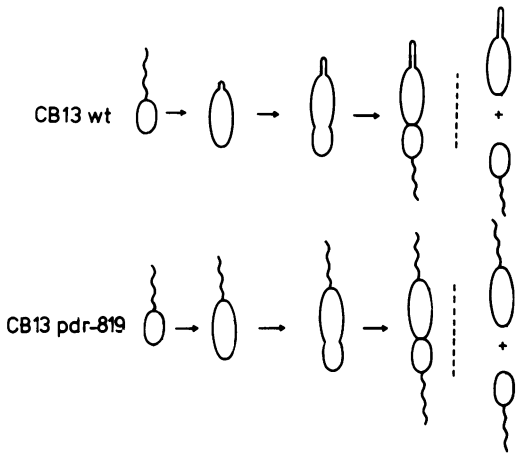

FIG. 4. *Schematic representation of the wild-type CB13 and stalkless mutant CB13 pdr-819 cell cycle. For the stalkless mutant CB13 pdr-819, old flagella are expected to be released slowly during the cell cycle. The instance where old flagella were not released is presented in the scheme.*

latory step could be further divided into at least two distinct steps. It should be pointed out here that no stalkless mutant was found among 30 mutants that are motile and unable to form polar pili and DNA phage receptors (4).

The DNA phage ϕCr22, which initiates infection by adsorbing randomly to the cell surface of swarmer and stalked cells, infects all five independent stalkless mutants and other pleiotropic mutants (4) blocked in the formation of the polar surface structures. Alternatively, phages (ϕCbK, ϕCp34, and ϕCp2) that require polar receptors and motility of polar flagella infect ϕCr22-resistant mutants. It is thus inferred that a mutation that affects polar surface differentiation does not exert a gross effect on the cell surface, or, in other words, a single mutation that affects cell motility and the formation of stalk and phage receptors is involved only in the coordinate morphogenesis of polar surface structures.

It was shown previously that cyclic guanosine 3',5'-monophosphate stimulates stalk formation (13). As possible regulatory factors, cyclic nucleotides were examined for reactivation of flagella and for the formation of stalk and polar receptors in the stalkless mutants. There was no detectable effect of dibutyryl cyclic guanosine 3',5'-monophosphate, or of dibutyryl cyclic adenosine 3',5'-monophosphate on flagella motility and formation of the surface structures in the stalkless mutants grown in PYE medium and in amino acid-supplemented minimal MC medium (A. Fukuda, unpublished data). Cyclic adenosine 3',5'-monophosphate is implicated in the catabolism of lactose in *C. crescentus* (16), but at the moment there is no evidence for a direct involvement of cyclic nucleotides in the *C. crescentus* surface differentiation.

Whatever the mechanism of control for cell motility, and the formation of stalk and polar phage receptors, it should be stressed here that the stalk appendage per se, or the process of stalk formation, is not indispensable to *C. crescentus* cell growth. Stalkless mutants grow normally at 30°C. The comparative study of cell size and morphology described in Results indicates that stalkless cells proceed through a defined sequence in the *Caulobacter* cell cycle, and there was no indication that swarmer cells divide without undergoing transition to cells of a stalked-cell nature. It was shown previously that flagella, pili, and polar DNA phage receptors are not essential for the *Caulobacter* cell cycle (15). Now a most distinctive stalk structure does not seem to be essential either. It would then follow that without expression of stalk, the swarmer cell is able to undergo transition, before the next round of the cell division cycle, to a cell that is of a stalked-cell nature except for the absence of stalk. In this sense the transition from swarmer to stalked cell is still obligatory for the *Caulobacter* cell cycle. Incidentally, unlike the wild-type cells, stalkless mutant cells did not adsorb to a glass surface because of the absence of stalk.

The structure of flagella released from stalkless mutants was observed to be identical to those released from the wild-type strain (Fig. 3; 17). In the wild-type strain, flagella are released upon transition from swarmer to stalked cell. Therefore, the wild-type stalked and elongated stalked cells are not flagellated. In contrast, about half of the stalkless mutant cells of wild-type stalked-cell size and mutant elongated cells possess single flagella. Similarly, about half of the mutant predivisional cells possess single flagella at either the smaller or larger cell end, or even at both cell ends (Fig. 1c). If it is taken into consideration that flagella are easily lost from cells during sample preparation for electron microscopy, probably more than 50% of the mutant cells are flagellated. Does this mean that the mutant cells of a stalked-cell nature can express flagellin and form flagella at the sites where stalks should otherwise be formed, or, more simply, that flagella release is so retarded in the mutant cell cycle that a higher proportion of mutant cells remains flagellated? Certainly, biochemical analysis of flagellin synthesis is necessary to

decide which one of these possibilities is correct. From the above study of mutant cell size and morphology, however, it seems more likely that flagella release is retarded in the mutants because of the absence of concomitant stalk formation or flagella motility.

It was reported previously that when cell division was inhibited at a low concentration of penicillin G, some predivisional cells contained more than one flagellum at the swarmer portion (20). Some stalkless mutant cells were also multiflagellated at one cell pole (Fig. 1d). These cells were invariably longer than the average size of each cell type, and the proportion in the whole cell population was minor. It appears that multiflagellated cells arise from certain physiological aberrations.

ACKNOWLEDGMENTS

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ADDENDUM

Mario et al. (J. Mol. Biol. 107:115-130, 1976) have reported the isolation of a temperature-sensitive stalkless mutant, *C. crescentus* CB13 *skf-501*, which is unable to form stalks at 37°C. CB13 *skf-501*, however, forms pili and retains cell motility at 37°C, and these phenotypes are different from those of the stalkless mutants described in this paper.

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