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Synthesis and Structure of *Caulobacter crescentus* Flagella

LUCILLE SHAPIRO AND J. V. MAIZEL, JR.

Departments of Molecular Biology and Cell Biology, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York 10461

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During the normal cell cycle of *Caulobacter crescentus*, flagella are released into the culture fluid as swarmer cells differentiate into stalked cells. The released flagellum is composed of a filament, hook, and rod. The molecular weight of purified flagellin (subunit of flagella filament) is 25,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The formation of a flagellum opposite the stalk has been observed by microscope during the differentiation of a stalked cell in preparation for cell division. By pulsing synchronized cultures with ^{14}C-amino acids it has been demonstrated that the synthesis of flagellin occurs approximately 30 to 40 min before cell division. Flagellin, therefore, is synthesized at a discrete time in the cell cycle and is assembled into flagella at a specific site on the cell. A mutant of *C. crescentus* that fails to synthesize flagellin has been isolated.

The synthesis of flagella by the dimorphic *Caulobacter* bacteria is one event in the ordered sequence of morphogenesis occurring during each cell cycle (15, 19). The flagellum appears, along with pili (19) and a deoxyribonucleic acid (DNA) phage receptor site (3), as a stalked cell enlarges and prepares for cell division. Since the flagellum invariably appears at the pole opposite the stalk, only one of the daughter cells is motile after cell division (see Fig. 6). This cell remains motile for a definite period of time, 30 min in nutrient broth (15, 19), or 60 min in minimal media (14, 20), and then loses its flagellum and grows a stalk at the same location. The new stalked cell then repeats the cycle. To study the spatial and temporal control of a well-defined event in cell morphogenesis, we have begun a study of the structure and biosynthesis of flagella in *Caulobacter crescentus*.

MATERIALS AND METHODS

Bacterial strains. The two strains used in this study were *Caulobacter crescentus* CB13Bia and a flagella-less mutant of CB13Bia, CB13 M-2. CB13 M-2 was isolated as a nonmotile strain by microscopy examination from a culture of CB13Bia treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (2). The bacteria were grown at 30 C in either modified PYE nutrient broth (15) containing deionized water, or in minimal media (HMG) containing 0.5 g of NH_4Cl, 1.74 g of Na_2HPO_4, 1.06 g of KH_2PO_4, and 10.0 ml of modified Hutner mineral base (20) per liter of deionized water in the presence of 0.2% glucose.

Purification of flagella. *C. crescentus* (500 ml) was grown to late log phase (optical density of 0.90 at 660 nm) in PYE or HMG media (15) and chilled, and the cells were collected by centrifugation in a Sorvall GSA rotor at 10,000 rev/min for 15 min. The cells were discarded, and the supernatant fluid (culture fluid) was centrifuged in a Spinco 30 rotor at 30,000 rev/min for 90 min. The resulting pellets, containing flagella, were suspended in 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.3 (50 ml). The flagella suspension was centrifuged in the Sorvall GSA rotor at 10,000 rev/min for 15 min to remove any remaining bacterial cells. The supernatant fluid was then centrifuged in a Spinco 40 rotor at 40,000 rev/min for 60 min, and the flagellar pellet was taken up in 0.02 M Tris buffer, *p*H 7.3 (3.0 ml). Linear CsCl gradients of 0.5 to 0.413 g/ml in 0.02 M Tris buffer, *p*H 7.3, were prepared, and 0.5 ml of the suspended flagellar pellet was layered on top. The gradients were centrifuged in a Spinco SW65 rotor at 60,000 rev/min for 1 hr. A single band containing intact flagella and appreciable amounts of what appeared to be membrane vesicles was obtained, and the CsCl was removed by dialysis against 0.02 M Tris buffer, *p*H 7.3 The flagella were then collected by centrifugation in a Spinco 40 rotor at 40,000 rev/min for 60 min. An 8.0-ml amount of dialyzed and concentrated flagella (1 mg) was then mixed with 3.58 g of CsCl and centrifuged in a Spinco 40 rotor for 48 hr at 38,000 rev/min. Two distinct bands were discerned. The upper band contained the purified flagella used in these studies.

Gel electrophoresis. The general procedures for electrophoresis employed in this study have already been described (13). A 0.5-mg amount of protein was made 10% with respect to trichloroacetic acid, and the resulting precipitate was collected, washed twice with 5% trichloroacetic acid and once with acetone. The sediment was dissolved in 0.2 ml of 1.0% sodium dodecyl sulfate (SDS), 0.1% 2-mercaptoethanol, 0.05 M Tris buffer, *p*H 7.8. The solution was heated at 100 C for 1 min to disrupt possible metastable aggregates. The solution was then applied to a cylindrical gel (0.6 by 10 cm) containing 0.1% SDS, 13% acrylamide, 0.4% bis-methylene acrylamide, and 0.35 M Tris buffer, *p*H 8.8, that had been polymerized with 0.05 ml of tetramethylenediamine and 0.05 ml of 10% ammonium persulfate per 100 ml of gel. A constant voltage (90 v) was applied for 4 to 5 hr with a current of 3 ma per gel at the start of electrophoresis, decreasing to 2 ma per gel at the finish. The gel was then removed from the tube by crushing the glass and either stained with 0.2 mg of Coomassie brilliant blue per ml in 50% methanol and 7% acetic acid, or fractionated and counted in a liquid scintillation counter.

Apparent molecular weights were derived by the method of Shapiro et al. (21) by using the following proteins and corresponding molecular weights: β-galactosidase (135,000), bovine serum albumin (67,000), heavy chain of human gamma globulin (50,000), bovine chymotrypsinogen A (24,800), and beef heart cytochrome *c* (11,700).

Cell synchrony. Synchronization of stalked cells was accomplished by differential centrifugation as previously described (18).

Electron microscopy. Bacterial preparations were stained with 1% sodium silicotungstate at *p*H 7.0 in a manner similar to that described by R. W. Horne (10). Flagella preparations were examined by modification of the method of Valentine (22). Thin carbon was evaporated onto a freshly cleaved surface of a sheet of mica. A ⅛-inch (ca. 0.3 cm) square was cut from this composite sheet and pressed carbon side upwards onto a fresh sheet of Parafilm on a flat surface. Five to ten microliters of sample was applied at one edge of the square, and the sample was observed to flow between the mica and carbon film and thus was exposed to a fresh carbon surface. A 200-mesh copper grid coated with a relatively thick fenestrated carbon film having holes approximately 5 to 10 μm in diameter was dropped on top of the sample with the fenestrated carbon downward. The grid and sample were then floated on a droplet of water and a droplet of 2% sodium silicotungstate, air-dried, and examined in succession. A Siemens Elmiskop 1A electron microscope at a voltage of 80 kv was used for this study.

Materials. ^{14}C- and ^{3}H-reconstituted protein hydrolysate from yeast was obtained from Schwarz BioResearch, Orangeburg, N.Y. Cesium chloride was obtained from American Potash and Chemical Corp., Chicago, Ill.

RESULTS

Isolation of flagella. Cell division in the *Caulobacter* genus results in the formation of two dissimilar daughter cells: a stalked cell and a flagellated swarmer cell (see Fig. 6). The swarmer cell contains a single polar flagellum (Fig. 1A) which is lost upon its transition into a stalked cell (17). The transition from one cell type to the other is obligatory, since the swarmer cell is incapable of cell division until it becomes a stalked cell. The new stalk is formed at the site of flagella attachment, and it has been demonstrated that the wall of the new stalk is synthesized at the juncture of cell and stalk (17). An intermediate in the transition, showing a flagellum fragment attached to the end of a newly forming stalk, is shown in Fig. 1B.

The isolation of flagella from *Caulobacter* is facilitated by the fact that flagella are apparently released into the culture fluid during normal growth as swarmer cells differentiate into stalked cells. Flagella concentrated from culture fluid are shown in Fig. 2. Both flagella and membrane vesicles are visible. Since a hook structure can be seen on the majority of filaments, it appears that flagella attached to hooks are released from the cells. Further purification of flagella from the culture medium was accomplished by differential centrifugation and buoyant density flotation in CsCl (see Materials and Methods).

Identification of flagella protein. A nonmotile mutant of wild-type *C. crescentus* CB13Bia, designated CB13 M-2, was isolated (see Materials and Methods). Electron micrographs revealed that the mutant cell lacked a flagellum, and flagella could not be recovered from the medium of a culture of CB13 M-2 grown to late exponential phase. CB13 M-2 had the same growth rate as CB13 in both nutrient (PYE) and minimal (HMG) media. The mutant was an efficient host for the ribonucleic acid bacteriophage φCb5, which has been shown to attach to *Caulobacter* pili (16, 18), as well as for the DNA phage φCbK, whose attachment site resides in the lipopolysaccharide of the cell wall (3, 19). Bacteria, both mutant and wild type, were heated to 100 C in 1% SDS and subjected to electrophoresis in SDS polyacrylamide gels (Fig. 3). A band of protein, which comigrated with the marker chymotrypsinogen, was present in the wild-type cells, but was missing in the flagella-less mutant. Polyacrylamide gel electrophoresis of a purified flagella preparation labeled with ^{14}C-amino acids showed a single major component in addition to minor components (Fig. 4). Figure 5 shows an electron micrograph of a comparable purified flagella preparation. The major band in the gels of purified flagella comigrated with chymotrypsinogen. The molecular weight of the

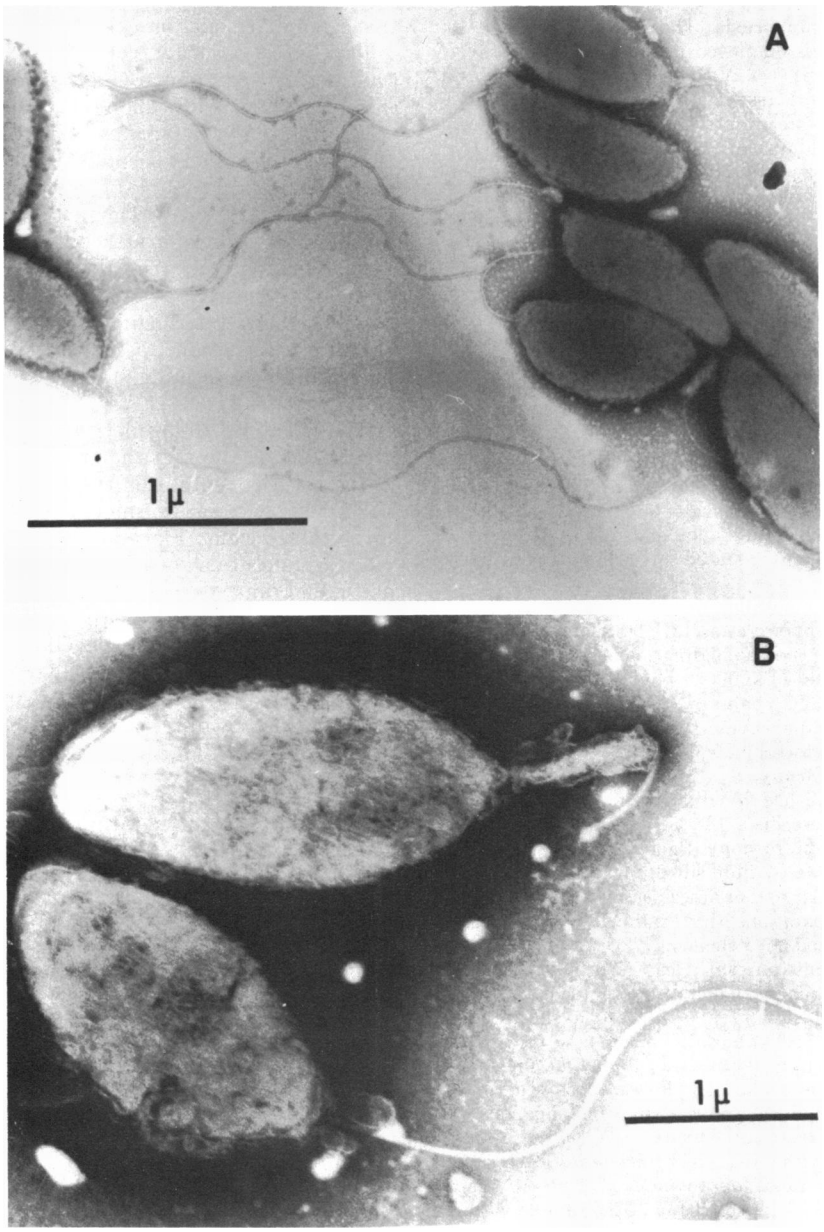

FIG. 1. *Electron micrographs of C. crescentus. Cells grown in HMG media were stained with 0.5% silicotungstic acid at pH 7.0. A, Swarmer cells; B, swarmer cell and a newly forming stalked cell retaining a flagella fragment at the end of the stalk. Bar indicates 1.0 μm.*

Caulobacter flagella subunit, presumed to be flagellin, determined by SDS gel electrophoresis in the presence of known protein markers, is therefore approximately 25,000.

Structure of flagella. Flagella released from the *Caulobacter* cell during normal growth contain a filament, hook, and rod (Fig. 5). No other attached structures, such as rings, were seen.

Flagella attached to the cell are approximately 6 to 7 μm in length and exhibit a wavelength of 0.96 to 1.1 μm. The filaments are 0.02 μm in diameter and are apparently composed of repeating flagellin subunits.

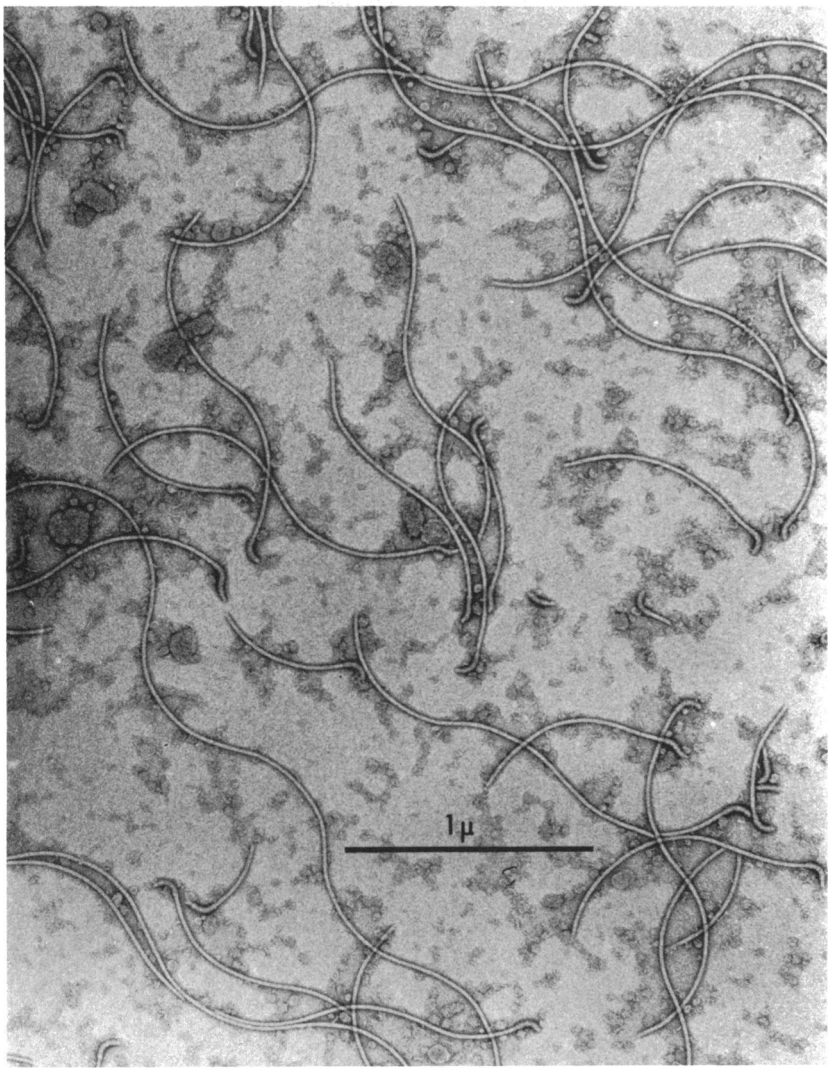

FIG. 2. *Electron micrograph of concentrated flagella prepared from culture supernatant fluid of C. crescentus grown to late exponential phase in HMG media. Bar indicates 1.0 μm.*

A hook-like structure 0.11 μm in length was usually found at one end of the filament (Fig. 5B and C). The hook appeared to be composed of stacked, repeating units. Occasionally a rod-like protrusion of 0.012 μm in diameter was seen to emerge from the end of the hook (Fig. 5D), as has been observed in both intact *Escherichia coli* and *Bacillus subtilis* (6, 9) flagella.

Synthesis of flagella. During a normal *Caulobacter* life cycle a stalked cell elongates, forms a flagellum and pili at the pole opposite the stalk, and then divides, yielding a swarmer cell from the flagellated pole and a stalked cell from the other pole. The swarmer cell then enlarges, grows a stalk, and loses its flagellum. The stalked cell reinitiates a new cell cycle. The purpose of the following experiment was to determine the time of flagellin synthesis in the developing stalked cell. Since the flagellum appeared sometimes between stalked cell formation and cell division, synchronized populations of stalked cells were pulsed with ^{14}C-amino acids. The time span of stalked cell elongation and development to cell division is 80 min (18; Fig. 6). The synchronized cells were pulsed for 10-min periods throughout the 80-min cycle. The cells were then heated in SDS,

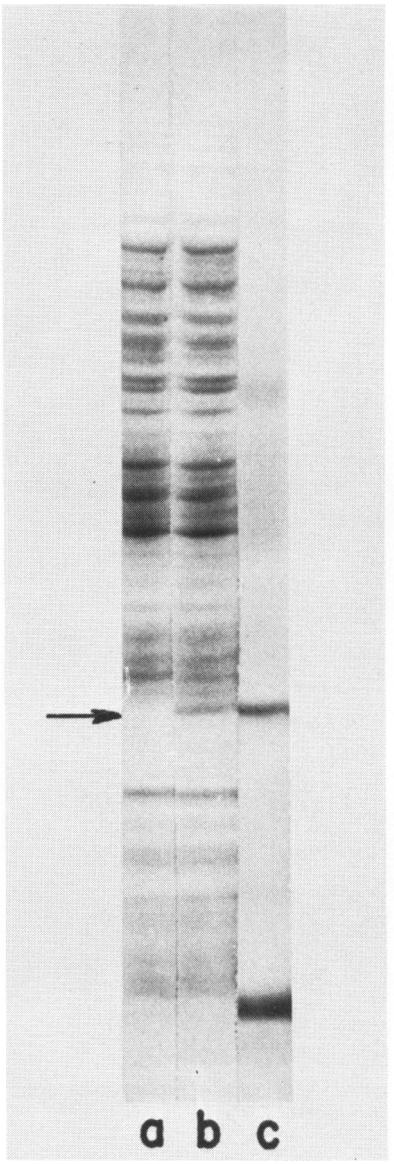

FIG. 3. *Polyacrylamide gel electrophoresis of C. crescentus proteins. Gel electrophoresis was performed by the method of Maizel (13) with 13% acrylamide and 0.1% SDS (see Materials and Methods). Direction of electrophoresis is from top to bottom. (a) C. crescentus, flagella-less mutant CB13 M-2; (b) C. crescentus, wild type, CB13B1a; (c) marker proteins of known molecular weight; β-galactosidase, 135,000; bovine serum albumin, 67,000; gamma globulin, 50,000; chymotrypsinogen, 24,800; and cytochrome c, 11,700.*

mixed with purified ^{3}H-flagella, and applied to 13% SDS polyacrylamide gels. After electrophoresis the gels were sliced and counted. As is shown in Fig. 7, a band of protein labeled with

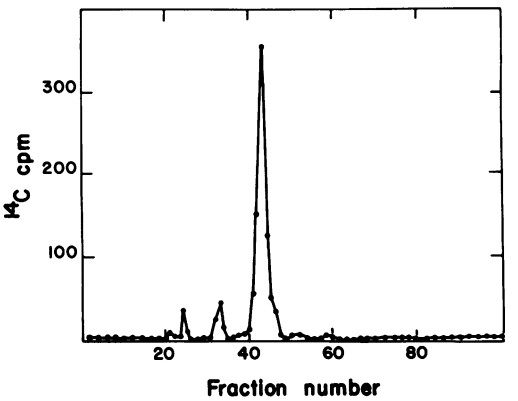

FIG. 4. *Polyacrylamide gel electrophoretic pattern of purified ^{14}C-flagella. Flagella were purified (see Materials and Methods) from culture fluid of C. crescentus CB13 grown to late exponential phase in the presence of ^{14}C-reconstituted protein hydrolysate (1 mCi/liter of cell culture). The sample was analyzed on 0.6 by 20 cm 13% gel for 18 hr at 50 v total, and fractionated as described previously (13). Direction of migration is to the right.*

^{14}C, that comigrated with ^{3}H-flagellin, appeared at 40 to 50 min. A protein band comigrating with a ^{3}H-flagellin marker was absent at 40 to 50 min when the mutant CB13 M-2 was pulsed with ^{14}C-amino acids. At the 40- to 50-min time interval, in the wild-type cell, the flagella protein accounted for approximately 5% of the total counts incorporated into protein during the pulse. The percentage of counts migrating with the flagellin marker decreased as the cell culture progressed toward cell division. In the experiment shown, 3% of the total counts were at the flagellin band at 60 to 70 min. In other experiments the counts at this region dropped to under 1% by 70 min into the cycle. It is not possible to say whether the continued synthesis of low levels of flagellin prior to cell division is due to the degree of asynchrony in the experiment or due to other factors. The degree of synchrony was approximately 80% in these experiments. The pulse experiments were repeated several times, and in one case the cells were pulsed with ^{3}H-amino acids and the marker was ^{14}C-labeled flagellin. In all experiments the synthesis of flagella protein first appeared at approximately 50 min. The data indicate that flagellin is synthesized at a discrete time in the *Caulobacter* cell cycle, and electron micrographs reveal that it is assembled into flagella at a specific site on the cell.

DISCUSSION

Bacterial flagella are usually prepared for study by mechanical shearing followed by dif-

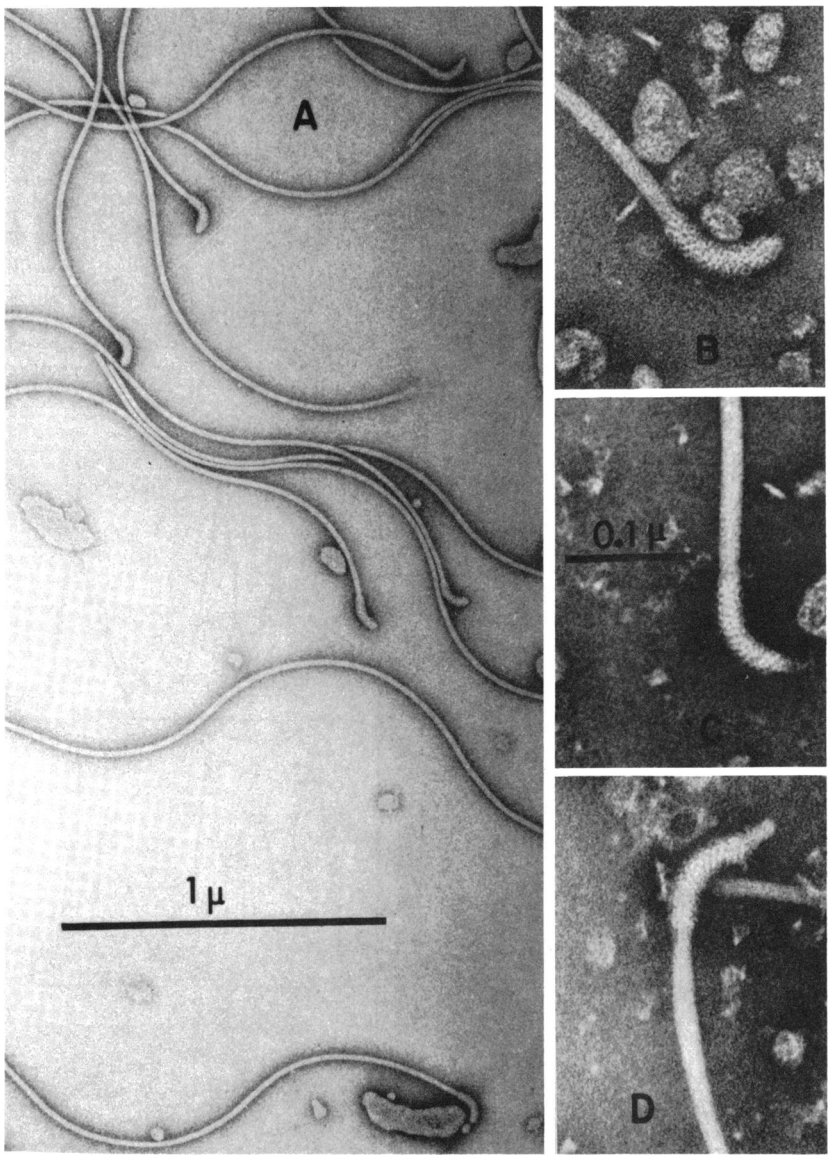

FIG. 5. *Electron micrographs of purified C. crescentus flagella filaments and hooks. A, Intact flagella; bar indicates 1.0 μm. B, C, and D, Flagella hooks; bar indicates 0.1 μm.*

ferential centrifugation (11). Flagella prepared by this method yield only one flagellar component, the filament. Recently, methods have been described for obtaining intact flagella composed of filament, hook, rod, and basal body from lysed bacterial spheroplasts of *E. coli* and *B. subtilis* (6, 9). Purification of flagella containing filament, hook, and rod was easily accomplished by using the culture fluid of *C. crescentus*, since intact flagella are apparently released into the growth medium during the normal cell cycle. The *C. crescentus* filament terminates in a structured, hooklike body. Protruding from the distal end of the hook is a rod whose diameter differs from that of the filament. With the exception of the basal body, the structure of the intact *C. crescentus* flagella is completely analogous to the structure of the *E. coli* flagella complex (6). It has been shown that the basal bodies of *E. coli* and *B. subtilis* flagella are made up of rings which attach to the cytoplasmic membrane, peptidoglycan, and lipopolysaccharide in the former case and to the cytoplasmic membrane in the latter case

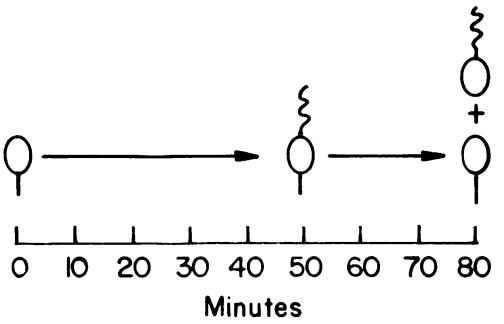

FIG. 6. *Schematic diagram of Caulobacter crescentus life cycle.*

(7). Since basal bodies were never observed attached to the hooks and rods of *C. crescentus* flagella concentrated from the culture fluid, it may be that the basal body remains on the cell during and after the development of the stalk, or that the rings composing the basal body are released into the media. Attempts to prepare flagella complexed with their basal body from spheroplasts of *C. crescentus* were unsuccessful.

The subunit protein of *C. crescentus* flagella (flagellin) comigrated with a marker protein of 25,000 daltons on an SDS-polyacrylamide gel. The apparent molecular weight obtained in this way falls into a spectrum of molecular weights

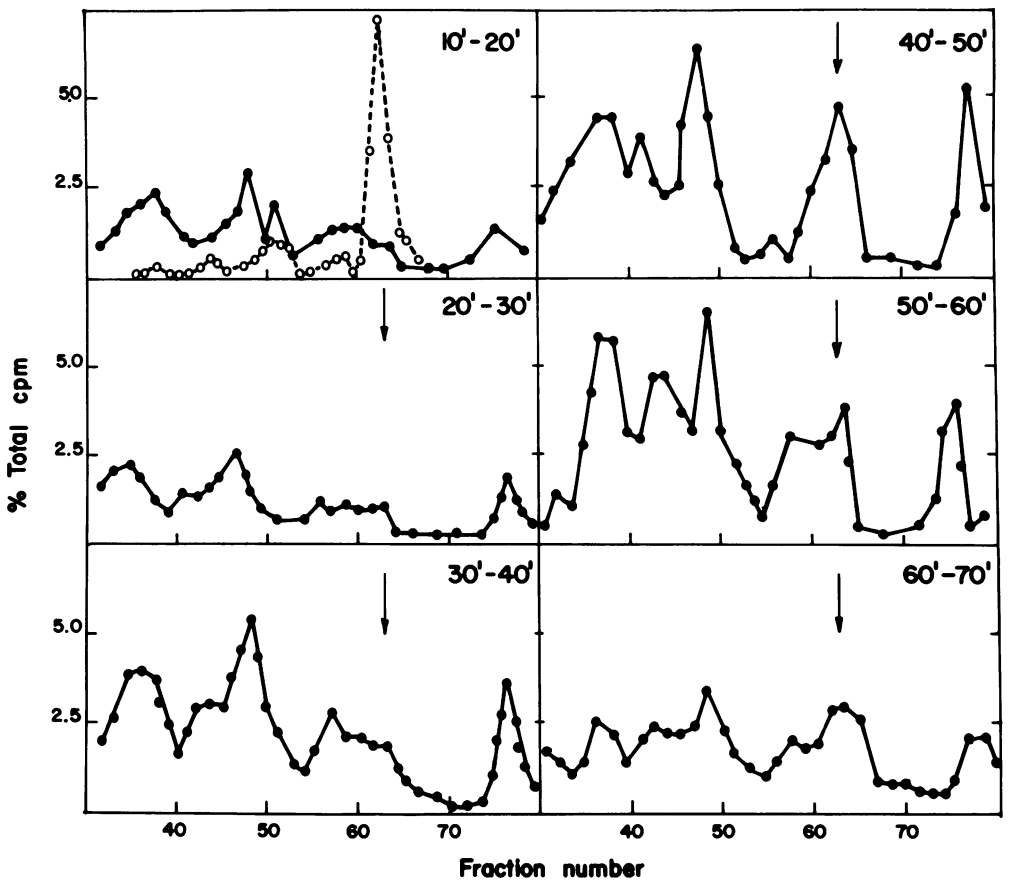

FIG. 7. *Polyacrylamide gel electrophoretic pattern of ^{14}C-proteins in synchronized cultures of C. crescentus. Synchrony was obtained as described previously (18) and initiated with a population of stalked cells growing in PYE media (optical density of 0.2). At 10-min intervals 2-ml samples were withdrawn and added to 100 μCi of ^{14}C-reconstituted protein hydrolysate. At the end of each 10-min pulse, 2 ml of 10% trichloroacetic acid was mixed with the culture, and the samples were then prepared for electrophoresis as described in the legend to Fig. 4. Open circles and dashed line represent ^{3}H-flagella marker. Fractions 30 to 80 out of a total of 100 are shown on each panel. The total counts per minute per gel were: 10 to 20 min, 3,300; 20 to 30 min, 3,148; 30 to 40 min, 2,600; 40 to 50 min, 3,787; 50 to 60 min, 6,800; 60 to 70 min, 5,700.*

estimated for flagellin by other workers, ranging from 14,000 to 40,000 (cf. review by Iino [11] and papers by Burge and Draper [4] and Champness [5]).

By using purified flagellin as a marker it was observed that flagellin was not synthesized in synchronized cultures until 50 min after the formation of the stalked cell. Flagella protein is synthesized, therefore, 30 min before the cell division which yields a flagellated swarmer cell and a stalked cell. A flagellum has been observed, microscopically, on the pole of the predivisional cell opposite the stalk at approximately 50 min in the cell cycle (Fig. 6), and, in fact, a corkscrew type of motility has been observed prior to cell division (17; Shapiro, *unpublished observation*). The precise relationship between the time of synthesis of flagellin and its assembly into visible flagella is currently being studied.

Since the flagella protein products are assembled in a precise location on the *Caulobacter* cell, as well as at a precise time in the cell cycle, the type of controls which govern temporal transcription, as well as the spatial restrictions of the ultimate gene products, have become amenable to study. It was proposed many years ago that a "localized" ribosome system might exist at the base of the flagellum which synthesizes flagellin (12) thereby controlling site specificity. The factors which mediate site specificity in unicellular organisms, however, remain a mystery. Studies with the *Caulobacter* bacteria provide a means for investigating the control of flagellin synthesis and flagella assembly, as well as an approach towards the problems of polarity in a unicellular prokaryote.

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