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Caulobacter Flagellar Organelle: Synthesis, Compartmentation, and Assembly

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In *Caulobacter crescentus* biogenesis of the flagellar organelle occurs during one stage of its complex life cycle. Thus in synchronous cultures it is possible to assay the sequential synthesis and assembly of the flagellum and hook in vivo with a combination of biochemical and radioimmunological techniques. The periodicity of synthesis and the subcellular compartmentation of the basal hook and filament subunits were determined by radioimmune assay procedures. Unassembled 27,000-dalton (27K) flagellin was preferentially located in isolated membrane fractions, whereas the 25K flagellin was distributed between the membrane and cytoplasm. The synthesis of hook began before that of flagellin, although appreciable overlap of the two processes occurred. Initiation of filament assembly coincided with the association of newly synthesized hook and flagellin subunits. *Caulobacter* flagella are unusual in that they contain two different flagellin subunits. Data are presented which suggest that the ratio of the two flagellin subunits changes along the length of the filament. Only the newly synthesized 25K flagellin subunit is detected in filaments assembled during the swarmer cell stage. By monitoring the appearance of flagellar hooks in the culture medium, the time at which flagella are released was determined.

The flagellar organelle is responsible for cellular motility and chemotaxis in a variety of bacteria (5, 7). The organelle is composed of an external filament, the flagellum, and a complex of structures, including a basal hook, which extend through the cell membrane (1, 2, 5). Little is known about the sequence of assembly of components of the flagellar organelle, although basal structures such as the hook are presumed to be required for elongation of the filaments. The flagellar filament is composed of flagellin subunits that are synthesized internally and transported through the cell membrane to an external site of assembly (5, 7). The polarity of filament assembly has been examined in *Salmonella typhimurium* where incorporation of the amino acid analog, fluorophenyl alanine, leads to a curly flagellar filament (8). *Bacillus subtilis* flagellar assembly has been examined by autoradiography of pulse-labeled isolated flagella (3). In both cases filament assembly was demonstrated to occur by the addition of newly synthesized flagellin to the distal end of the flagellum.

In most bacterial species flagellum synthesis occurs continuously so that, within a population of cells, all stages of flagellar organelle biogenesis are represented. Differentiation in *Caulobacter crescentus,* however, involves the synthesis of a complete flagellar organelle during a discrete stage of cell development (6, 15). The organelle is synthesized by predivisional intermediate cells at the pole opposite the stalk. The subsequent swarmer to stalked cell transition results in the loss of motility and the eventual release of the intact filament and hook into the culture medium (16, 18).

The *Caulobacter* flagellum is a supramolecular structure of intrinsic interest because of its role in cellular motility and its extracellular mode of assembly. Furthermore, in contrast to most bacterial flagella, the filament is composed of two different flagellin subunits which are immunologically cross-reactive (11, 12). *Caulobacter* flagellar synthesis is stage-specific, and the organelle is assembled at a unique cellular location resulting in its segregation with only one of the progeny cells upon cell division (15). Thus, synthesis and assembly of flagellar components in this organism offer an attractive system for the combined analysis of the regulation of gene expression and the asymmetric distribution of cellular components with division and differentiation. We have devised assays to monitor the synthesis, compartmentation, and assembly of *Caulobacter* flagellin and hook subunits during synchronous cell growth.

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MATERIALS AND METHODS

Bacterial strains and media. *C. crescentus* CB15 were grown in HMG minimal medium (17) at 30°C with rotary shaking. Synchronous cells were prepared by the Ludox procedure (4). By this technique, more than 95% of the cells routinely double between 125 and 135 min.

Isotopic labeling and quantitation of protein. Portions (2 ml) of heterogeneous or synchronous cultures containing 2×10^8 to 4×10^8 cells per ml were labeled with 2.5 μCi of ^{14}C-amino acid mixture (Schwarz-Mann reconstituted protein hydrolysate, algal profile) per ml for 8 min at 30°C with rotary shaking. Labeling was terminated by the addition of 50 μl of 10% Casamino Acids at 0°C.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (10) modified as previously described (19). Gels were 15 cm long and contained a 9 to 13% gradient of acrylamide. Radioactive protein profiles were detected by autoradiography using Kodak X-Omat R X-ray film. For quantitation of radioactivity, autoradiograms were exposed for equivalent periods of time and scanned with a Joyce-Loebl double-beam recording microdensitometer MKIIC, equipped with electronic peak integration.

Determination of protein composition of isolated CB15 flagella. Using an improved quantitative protein staining technique (9), and densitometry of slab gels dried on clear backing, the ratio of flagellar components was determined for CB15 flagella. Flagella that had been purified through CsCl equilibrium density gradient sedimentation were composed of flagellin subunits in a ratio of 1:3.5, which is similar to the value determined for CB13Bla flagella (11). CB15 flagella purified only by 30% saturated ammonium sulfate precipitation, however, contained the two subunits in a ratio of 1:6.

Recovery of sheared flagellar fragments. Synchronous cultures, labeled as described above, were sheared by blending in a Vortex mixer at top speed for 90 s. Sheared cultures were centrifuged at 15,000 × *g* for 12 min at 4°C to remove cells. Flagellar fragments were harvested from the supernatant fractions by centrifugation at 140,000 × *g* for 40 min at 4°C. The pellets which contained flagellar fragments were suspended in 50 μl of the SDS disaggregation buffer for SDS-polyacrylamide gel electrophoresis and heated for 5 min in a boiling water bath.

Subcellular fractionation. The cellular fraction was harvested by centrifugation at 12,000 × *g* for 10 min at 4°C. Synchronous cells (2-ml portions) were labeled as described above and flagella were removed by blending in a Vortex mixer at top speed for 90 s. Frozen cell pellets were suspended in 2 ml of 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 6.8); 0.05 ml of 0.4 M ethylenediaminetetraacetic acid (EDTA) (pH 7.0) and then 0.1 ml of 2 mg of lysozyme per ml (Sigma) in water were added and lysis proceeded for 20 min at 30°C. Lysates were sheared by passing them through a 26-gauge needle. A membrane fraction was harvested by centrifugation at 140,000 × *g* for 40 min at 4°C; the supernatant was retained as the cytoplasmic fraction. Membranes were washed once by resuspending them in 1 ml of 10 mM Tris-hydrochloride (pH 6.8) and centrifuging as described above. To insure that the flagellin isolated in the membrane fraction was not a result of nonfunctional adherence of flagellin to the subcellular fractions, cells were mixed with ^{14}C-labeled purified flagellin and fractionated as described above. Contamination was measured by radioimmune assay. Additionally, subcellular fractions were examined for the presence of flagellin filaments by electron microscopy. By either criterion, there was no detectable contamination of subcellular fractions with flagellin.

Cell lysis procedure for detection of cellular flagellin pools. Labeled cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C. The pellets were frozen and suspended by 10 s of blending in a Vortex mixer in 0.5 ml of 10 mM EDTA–10 mM Tris-hydrochloride (pH 7.5) at 23°C. Lysozyme (200 μg) was added and lysis proceeded at 23°C for 15 min. Triton X-100 was then added to a final concentration of 0.5% to solubilize membranes. The resultant lysates were sheared three times by passing them through a 26-gauge needle, and insoluble material was removed by centrifugation at 12,000 × *g* for 10 min.

Radioimmune assay for flagellin. Whole cell lysates or isolated cytoplasmic and solubilized membrane fractions were prepared as described above from cells pulse-labeled with ^{14}C-amino acids. Flagella and flagellin were recovered by a carrier-mediated radioimmune assay. Samples were adjusted to 10 mM EDTA–0.5% Triton X-100–10 mM Tris-hydrochloride (pH 7.5) and 0.15 M NaCl in a volume of 0.5 to 2.0 ml. Anti-flagellin antiserum (25 μl) prepared against electrophoretically purified 25,000-dalton (25K) flagellin (12) was added to the reaction mixture followed by 3 μg of unlabeled carrier flagellar protein. Carrier flagella were partially purified by 30% saturated ammonium sulfate precipitation as previously described (12) and sonically treated for 5 s. The precipitin reaction mixture was first incubated at 37°C for 20 min and then at 4°C for 12 h. The entire reaction mixture was then layered on a solution of 0.5 ml of phosphate-buffered saline (0.15 M NaCl–0.15 M sodium phosphate, pH 7.2), containing 10 mM EDTA, 0.5% Triton X-100, and 1 M sucrose (buffer I), and centrifuged at 12,000 × *g* for 10 min. The supernatant was removed by aspiration and the pellet was washed once by centrifugation in 0.5 ml of buffer I. The precipitin pellet was solubilized by heating in a boiling water bath for 5 min in 50 μl of precipitin disaggregation buffer (0.1 M Tris–0.1 M dithiothreitol–0.1% SDS–20% glycerol–0.001% bromophenol blue) for SDS-polyacrylamide gel electrophoresis.

Radioimmune assay for hook monomer. The protocol described above for the flagellin carrier-mediated radioimmune assay was adapted for the detection of hook monomer: 200 μl of anti-CB13Bla flagella antiserum prepared against intact flagella, purified by CsCl density gradient centrifugation (11), and 3 μg of purified hook protein were used as antibody and carrier, respectively. Immune precipitation of labeled hook was dependent on the inclusion of purified hook carrier in the assay. Purified hooks were isolated from CB15 and CB13Bla after pH 5.3 disaggregation of the flagellar filament by a modification of the technique of Hilmen et al. (5; Lagenaur and Agabian, submitted for publication).

RESULTS

Flagellin synthesis during the cell cycle. Synchronous cells were pulse-labeled with ^{14}C-amino acids, then lysed and assayed by a carrier-mediated radioimmune assay as described above. The synthesis of 27,500-dalton (27K) and 25K flagellins could be simultaneously assayed during the cell cycle since both flagellins are precipitated by antibody generated against the 25K flagellin (12). As shown in Fig. 1, analysis of the relative rates of synthesis of the two flagellin components in whole cell extracts demonstrated that (i) the rates of flagellin synthesis vary markedly during the cell cycle and (ii) the relative rates of 27K and 25K flagellin synthesis vary with respect to each other during the cell cycle. In general, the major period of flagellin synthesis occurred during the predivisional and swarmer cell stages, coincident with the appearance of the flagellar structure and consequent cell motility. Synthesis of the 25K flagellin began in predivisional cells and continued through the first half of the swarmer cell stage. Synthesis of the 27K flagellin, however, was not detected during the swarmer cell stage. A small but significant basal level of 25K flagellin synthesis could be detected during the nonmotile stalked cell stage.

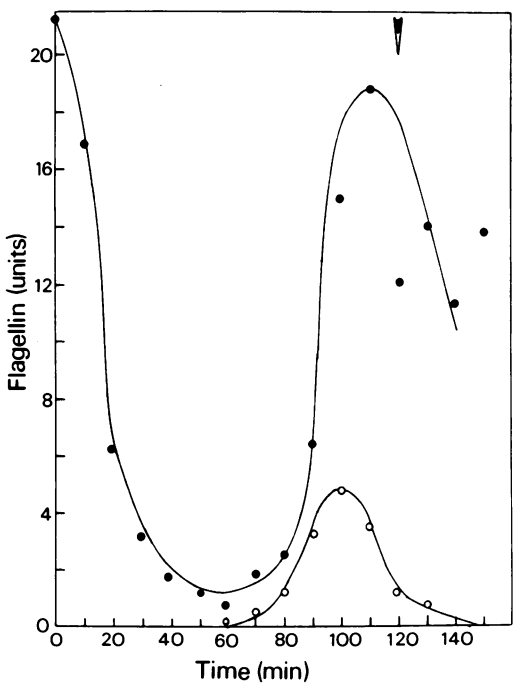

FIG. 1. *Synthesis of flagellin subunits during the cell cycle. Synchronous CB15 cells were pulse-labeled with ^{14}C-amino acids at 10-min intervals and assayed for flagellin synthesis by the radioimmune assay procedure described in the text. Flagellin units were determined by densitometry. ●, 25K flagellin; ○, 27K flagellin. In the experiments described in this study, swarmer stage occurred at 0 to 40 min, stalked stage occurred from 40 to 80 min, and predivisional stage occurred from 80 to 125 min. Arrow indicates cell division.*

The overall pattern of synthesis (Fig. 1) of the two flagellin components in synchronous cultures is similar to that determined by Osley et al. (14), who assayed CB15 flagellins in spheroplast lysates. In our hands, using ^{14}C-amino acid pulse-labeled heterogeneous cells, soluble flagellin, which is preferentially recovered by the spheroplast lysis procedure of Osley et al. (14), accounted for only 24% of the total flagellin recovered in cell lysates prepared by the technique described above. Perhaps for this reason, the synthesis of the two flagellin components shown in Fig. 1 differed quantitatively with that determined previously (14). The total amount of 25K flagellin was in six- to sevenfold excess of the 27K flagellin synthesized during a single cell cycle compared with approximately threefold excess of 25K flagellin calculated from the data cited previously (14). The apparent ratio of the two flagellin components was also dependent on the radioactive precursor used for labeling the proteins. By the radioimmune assay procedure described herein, ^{14}C-reconstituted protein hydrolysate-labeled cells contained 27K and 25K flagellins in a ratio of 1:4.4, whereas cells labeled under identical conditions with [^{35}S]methionine contained 27K and 25K flagellins in an apparent ratio of 1:2. This difference presumably results from an unequal distribution of methionine in the two proteins, as demonstrated for CB13Bla flagella (11).

Assembly of flagellin components. The assembly of flagellin subunits into the external filament was examined by following the appearance of radioactivity in the flagellar filament in synchronously growing cells. Cells were pulse-labeled with ^{14}C-amino acids at 10-min intervals, and flagella were sheared from the cells as described above.

The proportion of flagellin subunits in the assembled structure was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Less than 1% of the sheared cells retained any detectable stubs of flagellar filament, as determined by electron microscopy. Flagellins which remained associated with the cellular fraction were estimated by radioimmune assay. Approximately half of the total flagellin subunits synthesized during an 8-min interval were recovered as external, shear-sensitive flagella (Fig. 2). Neither 27K nor 25K flagellin was detectable

outside the cell during the nonmotile stalked stage, although an appreciable constitutive synthesis of 25K flagellin occurred during this time period. There was an apparent lag of 10 min between the rise of flagellin synthesis detected in sheared predivisional cells and the onset of flagellar assembly. This lag may reflect the time necessary to assemble a filament long enough to be sensitive to shear.

Subcellular localization of flagellins. After removal of assembled filaments by shear, approximately half of the flagellin synthesized at any given time in the cell cycle remained associated with the cell (Fig. 2). To determine the subcellular location of these flagellin pools, cells were sheared to remove flagellin filaments, and the cell membranes and cytoplasm were fractionated and then quantitated by radioimmune assay. The patterns of synthesis of the two flagellin components assayed in the membrane and cytoplasm fractions of synchronously growing cells are shown in Fig. 3. Flagellin synthesis associated with the membrane fraction was distinct from that associated with the cytoplasm in several respects. (i) The membrane fraction contained a larger proportion of the total flagellin synthesized throughout the cell cycle than did the cytoplasm. (ii) The membrane fraction was enriched in 27K flagellin relative to the cytoplasmic fraction. (iii) The low-level synthesis of flagellin components during the stalked cell stage was confined to the membrane fraction. We cannot readily account for the lower recovery of 25K flagellin in isolated membranes relative to the cell body or whole cell fractions from the stalked stage. We have, however, consistently recovered both flagellins by radioimmune assay in the isolated membrane fraction.

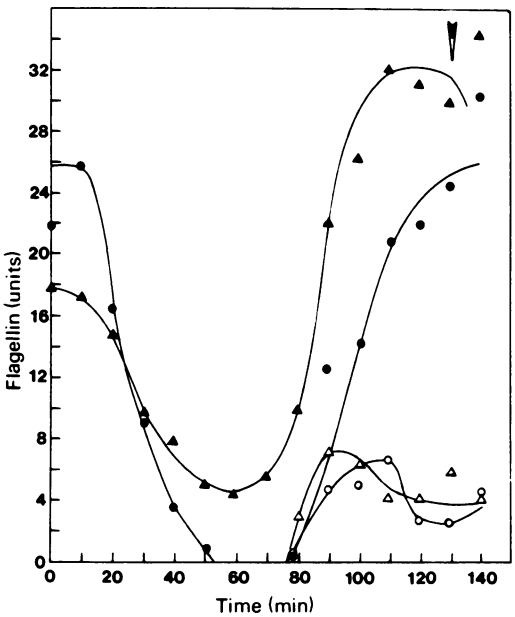

FIG. 2. *Assembly of newly synthesized flagellins. Assembled flagella were sheared from pulse-labeled cells, harvested by ultracentrifugation, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Cell-associated flagellins were assayed by radioimmune assay. 25K flagellin* (●) *and 27K flagellin* (○) *were sheared from cells;* (▲), *25K flagellin and* (△), *27K flagellin associated with cellular fraction. Arrow indicates cell division.*

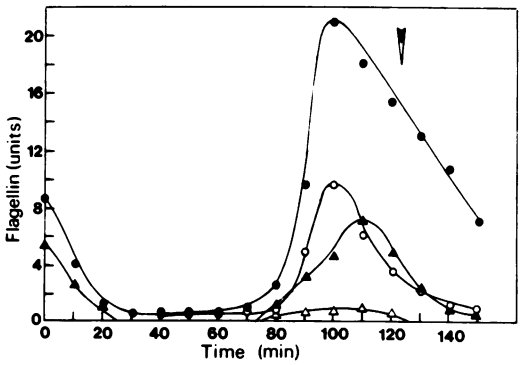

FIG. 3. *Compartmentation of newly synthesized flagellins. Subcellular fractions of synchronized cells were assayed by radioimmune assay. Membrane associated:* ●, *25K;* ○, *27K flagellins. Cytoplasmic:* ▲, *25K;* △, *27K flagellins. Arrow indicates cell division.*

Other flagellar components detected by radioimmune assay. Figure 4 shows an autoradiogram of the proteins precipitated from synchronously growing cells, using the flagellin radioimmune assay. The anti-25K flagellin antibody precipitated both 27K and 25K flagellins (12); however, three other apparently stage-specific proteins were also precipitated in this assay. The 73,000-dalton protein has been identified as the monomeric component of the flagellar hook (Lagenaur and Agabian, submitted for publication). Although anti-25K flagellin antibody is nonspecific and does not cross-react with the hook monomer (12; Lagenaur and Agabian, submitted for publication), the structural association of the hook with the flagellar filament could result in its recovery under the conditions of the assay. Thus, this autoradiograph reveals that newly synthesized hook monomer becomes associated with the immunoprecipitable flagellins between 80 and 110 min of synchronous cell growth. Two other stage-specific proteins coprecipitated in this assay. A 28,500-dalton protein was detected immediately before the onset of 27K flagellin synthesis and a 24,500-dalton protein was coprecipitated during the maximal pe-

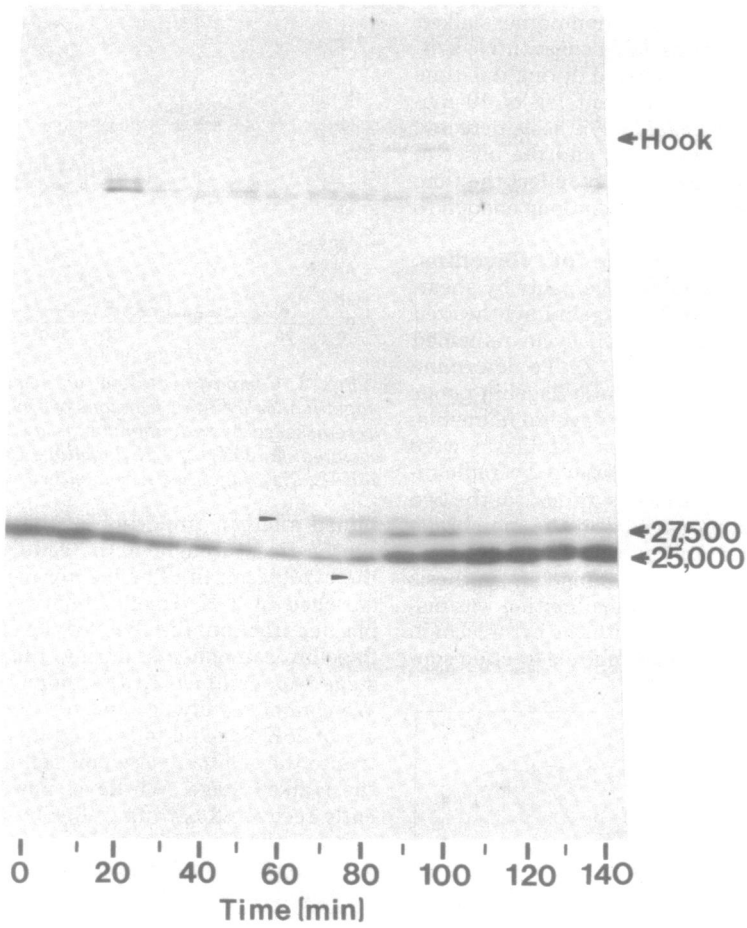

FIG. 4. *Radioimmune assay of flagellins. Autoradiogram of flagellin radioimmune assay. Cells were sheared to remove external filaments, and flagellin associated with the cellular fraction was assayed in synchronously growing cultures as described in the text. Arrows indicate the positions of hook monomer and flagellin subunits. Quantitation of 27K and 25K flagellins from this assay appear in Fig. 2.*

riod of 25K flagellin synthesis. The relationship of these proteins with the flagellar organelle has not been determined; however, a number of nonmotile mutants have been isolated, some of which synthesize a 24,500-dalton protein which is precipitated by anti-25K flagellin antibody (J. Meyer, C. Lagenaur, and N. Agabian, manuscript in preparation).

Synthesis and release of flagellar hook. *Caulobacter* flagella with attached hooks are released into the culture medium at the end of the motile swarmer stage. CB15 and CB13Bla hooks were isolated after pH 3.5 disaggregation of the flagellar filament by a modification of the technique of Hilmen et al. (5; Lagenaur and Agabian, submitted for publication). Purified hooks contained a single protein monomer of 73,000 daltons as determined by SDS-polyacrylamide gel electrophoresis. Antiserum prepared against intact CB13Bla flagella (11) was tested for reactivity with the hook subunit by double immunodiffusion. Intact hooks were disaggregated in 0.1% SDS and analyzed by double immunodiffusion as described previously (11). Either CB13Bla or CB15 purified hooks produced a single precipitin line when tested with this antiserum; hooks from CB13Bla and CB15 cross-reacted completely with one another, but did not cross-react with flagellin. The period of hook synthesis could therefore be detected by a

radioimmune assay analogous to that used to detect flagellins. For the hook radioimmune assay, purified hook was used as a carrier in conjunction with the CB13Bla intact flagella antiserum (11) which contained anti-hook specificity. The temporal relationship between hook and filament synthesis and assembly was assayed in synchronous pulse-labeled cultures of CB15 as described in the legend to Fig. 5. Hook synthesis was first detected in the stalked cell, and maximal synthesis of hook monomer occurred at 90 min (Fig. 5). The major period of hook expression corresponded to the onset of synthesis and assembly of flagellin subunits. This period of hook synthesis was relatively broad when compared with the discrete interval of association of newly synthesized hook subunit with the flagellar filament shown in Fig. 4.

In *Caulobacter* culture media, intact flagella are found with an attached hook. This implies that whole flagella (filament and hooks) are released from the cell after completion of the swarmer stage (15). Flagellar breakage occurs continuously during flagellar outgrowth (Lagenaur and Agabian, unpublished data), and therefore the timing of flagellar release cannot be determined by simply measuring the release of flagellin subunits into the culture medium. The ability to assay the appearance of hook-associated flagellin in the culture fluid, however, allows an accurate measurement of the time in the swarmer to stalked cell transition when the entire flagellum is released. As described in the legend to Fig. 6, synchronous cultures were labeled with radioactive amino acids during maximal hook synthesis, and flagellum release was assayed by monitoring the appearance of hook in the culture medium. Initiation of hook release occurred at approximately 45 min and was completed by 60 min (Fig. 6).

DISCUSSION

Bacterial flagella are usually formed by the polymerization of a single polypeptide subunit as compared with *Caulobacter* flagella which contain two closely related flagellin subunits in a single filament (11, 12). The actual arrangement of the two flagellins within the *Caulobacter* flagellum, however, is unknown. The structural association of the two CB15 flagellins should be reflected in the pattern of their sequential assembly during flagellar outgrowth. During the onset of flagellar assembly by the predivisional cell, newly synthesized 27K and 25K flagellins were assembled into flagella in a ratio approaching 1. This ratio steadily decreased until 27K flagellin synthesis ceased at cell division, whereas 25K flagellin synthesis continued in the swarmer cell (Fig. 2). Assuming that *Caulobacter* flagellar assembly occurs by the addition of subunits at the distal end of the flagellum, the cell-proximal portion of the fla-

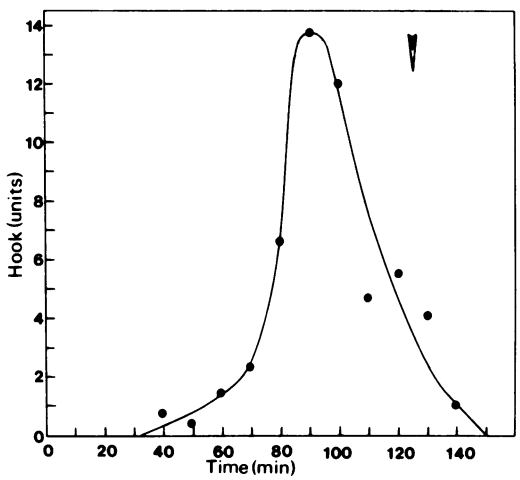

FIG. 5. *Synthesis of hook monomer in synchronous cultures. CB15 cells were synchronized and pulse-labeled with ^{14}C-amino acids as described in the text. Hook monomer was detected by hook radioimmune assay, using antibody raised against intact flagella (11) and hook carrier. Units of hook synthesis represent values determined by densitometry of autoradiograms.*

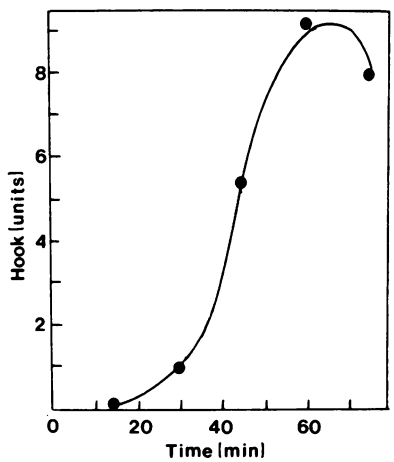

FIG. 6. *Release of flagella into culture media. A 10-ml volume of synchronous CB15 was pulse-labeled with 45 μCi of ^{14}C-amino acids at the time of maximal hook synthesis; cells were allowed to divide, and 2-ml portions were removed at 15, 30, 45, 60, and 75 min after division and centrifuged at 15,000 × g for 12 min to remove cells. Released flagella were recovered by centrifuging at 140,000 × g for 40 min, and samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.*

gellum is polymerized during the predivisional cell stage and the distal portion of the flagellum is then completed by the swarmer cell. The actual distribution of the two flagellar components throughout the length of the flagellum could not be unequivocally determined from these studies. Nevertheless, examination of the change in ratio of newly assembled flagellins during synchronous flagellar outgrowth suggests a model for the completed filament in which the region of the flagellum proximal to the cell is composed of two flagellar subunits in a ratio approaching 1, whereas the distal portion of the flagellum is composed mostly or entirely of the 25K subunit. This interpretation is reinforced by the observation that flagella isolated from pulse-labeled swarmer cells possess both flagellin subunits; however, only the 25K flagellin is radioactive.

As shown in Fig. 2, an appreciable fraction of newly synthesized flagellin does not immediately become associated with shear-sensitive flagella. Radioimmune assay of subcellular fractions (Fig. 3) revealed that the majority of internal, newly synthesized flagellin subunits were associated with the membrane fraction. Membrane-associated flagellins may represent flagellin in transit from the site of synthesis to the site of flagellar assembly. Since the polar membrane region containing the flagellar organelle, pili, and ϕCbK receptors specifically segregates with the swarmer daughter cell, one may speculate that membrane-associated flagellin subunits also segregate with the swarmer cell at the time of cell division, although the fate of membrane-associated flagellins upon cell division is unknown. We have not yet been able to determine if all of the membrane-associated flagellins are ultimately polymerized.

In addition to the two flagellins, proteins of 73,000, 28,500, and 24,500 daltons are detected by the radioimmune assay procedure during discrete periods of the cell cycle (Fig. 4). The 73,000-dalton protein has been identified as a flagella-associated component, hook monomer. The anti-flagellin antiserum used in these studies is monospecific and was prepared against electrophoretically purified 25K flagellin (12). Therefore, it is likely that these additional proteins are flagella-related, as is the case with the flagellar hook protein which is coprecipitated with the filament. The 28,500-dalton protein appears for a short interval before the expression of 27K flagellin; the possibility that it is a precursor of the 27K subunit is being investigated. The 24,500-dalton protein may be a degradation product of the 25K flagellin since it is also found in early swarmer cell stages when there is no 27K flagellin synthesis detected by radioimmune assay. Alternatively, the proteins recovered by this procedure may be other flagellar organelle components such as basal ring structures, which, like hook, are coprecipitated due to a structural association with the filament.

The patterns of synthesis of the two flagellin subunits are most easily explained by differential regulation of 2 flagellar genes. However, both flagellin subunits of CB15 are immunologically identical (12) as are those of CB13Bla (11). It has previously been shown that two-component flagella are not unique to *C. crescentus* CB15 and CB131a, but are in fact a common feature among caulobacter, including strains whose flagella are not immunologically cross-reactive with those of CB15 and CB13Bla (12). The two flagellin components could be products of separate genes with a high degree of sequence homology, such that immunological cross-reactivity of the gene products is maintained. Alternatively, both flagellin components might arise from a single gene. In this case, distinct gene products could result from differential processing of a larger protein or RNA precursor, or from differential transcription of partially overlapping gene sequences. The different functional half-lives of 25K and 27K flagellin mRNA's, as measured by the ability of *C. crescentus* CB15 to synthesize flagellin subunits in the presence of rifampin (14) would seem to indicate that distinct mRNA populations exist for each flagellin component.

C. crescentus CB15 and CB13Bla flagellar hooks are composed of a single monomeric protein subunit of 73,000 daltons. Maximal hook monomer synthesis occurs at 90 min, during the initial period of filament out growth, but prior to maximal synthesis of flagellin subunits. As shown in Fig. 4, newly synthesized hook becomes associated with flagellin (i.e., labeled hook is precipitated by the flagellin radioimmune assay) during a relatively brief interval in the cell cycle; maximal levels of pulse-labeled hook associated with the filament are detected at 100 min. Thus, it appears that the sequence of events in CB15 flagellar organelle assembly begins with the synthesis of hook monomer, presumably followed by assembly of the hook structure itself. Direct assay has shown that the association of hook and flagellin subunits then occurs and flagellin subunits are assembled into an external filament. Some overlap occurs in these events; hook monomer synthesis and association with the flagellins are both detectable during the first 40 min of filament assembly. The most sharply defined event in flagellar organelle assembly is the onset of external filament polymerization. It is unlikely that hook monomer alone acts as a trigger of the assembly process since it is syn-

thesized for 30 min before the assembly of the external filaments. One may speculate that any of a number of other minor components of the flagellar organelle acts as a nucleus for assembly; any of these may be synthesized just prior to the assembly of the external filament.

In addition to the series of events resulting in the assembly of the flagellum, in the *Caulobacter* system, cessation of motility may also require specific gene expression. It was shown that the loss of motility associated with the swarmer to stalked cell transition requires RNA synthesis 25 to 30 min before loss of motility (13). The cessation of motility, however, need not be directly related to the absence of a flagellum. In this study, extrusion of flagella was directly assayed by measuring the release of hook-associated filaments into the culture medium after synchronous cell division. By these criteria, flagellar release occurred at approximately 45 min into the cell cycle. Taking into consideration the differences in generation time between our cells (130 min) and those of Newton (13) (180 min), release of flagella occurs approximately 10 to 15 min after loss of cell motility. This coincides with the first appearance of a stalk by phase-contrast microscopy.

To our knowledge, this is the first biochemical demonstration of the sequence of events in flagellar organelle expression in vivo. The analysis of a large number of mutants defective in motility will permit the identification of additional steps in the regulation of the biogenesis of the *Caulobacter* flagellar organelle.

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