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Flagellar Hook and Basal Complex of *Caulobacter crescentus*

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Intact bacterial flagella possessing a membrane-free hook and basal complex were purified from *Caulobacter crescentus* CB15, as well as from mutants which synthesize incomplete flagella. The basal body consisted of five rings mounted on a rod. Two rings were in the hook-proximal upper set, and three rings (two narrow and one wide) were in the lower set. The diameters of the two upper rings differed, being 32 and 21 nm, respectively. The lower rings were all approximately 21 nm in diameter, although they varied significantly in width. During the normal course of the *C. crescentus* cell cycle, the polar flagellum with hook and rod was shed into the culture medium without the basal rings. Similarly, hooks with attached rods were shed from nonflagellate mutants, and these structures also lacked the basal rings. The hook structure was purified from nonflagellated mutants and found to be composed of a 70,000-molecular-weight protein component.

Caulobacter crescentus contains a single polar flagellum for approximately one-third of the cell cycle (19, 21). The formation, maintenance, and release of this flagellum is coordinated with the cell cycle (15, 18, 22). The flagellin proteins are synthesized at the time of flagellar assembly (15, 18, 22), and evidence has been presented that flagellin gene expression is coupled to the completion of chromosome replication (18). During the transition of the motile swarmer cell to the sessile stalked cell, the flagellum is released into the medium (20), and a stalk structure is assembled at the site previously occupied by the flagellum (10). Flagella purified from the culture medium have both a hook and a rod structure at the proximal end of the filament (15, 22), but do not have the basal ring complex found in intact flagella isolated from both gram-positive and gram-negative bacteria (1, 7-9, 24, 25). In the present study, intact flagella of *C. crescentus* with complex basal structures similar to those of other bacterial species (1, 7-9, 24, 25) were prepared.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. crescentus* CB15 and nonmotile mutants derived from this strain (13) were grown in modified PYE medium (12) with shaking at 33°C.

Preparation of intact flagella from *C. crescentus*. Intact flagella were purified from *C. crescentus*, using a modification of the method of DePamphilis and Adler (8). In a typical preparation of intact flagella, cells were grown to mid-log (optical density at 660 nm = 0.5) phase in 2 liters of PYE broth and harvested by centrifugation at 6,000 × *g* for 15 min. The cell pellet was suspended in 200 ml of 0.1 M Tris (pH 7.8) and centrifuged again. This second cell pellet was suspended in 50 ml of 0.1 M Tris (pH 7.8) containing 20% sucrose and incubated for 2 h at 33°C in the presence of 100 μg of lysozyme per ml and 10 mM EDTA. Triton X-100 was added to a final concentration of 2%, and incubation was continued for 30 min at 33°C with gentle shaking. The viscous lysate was further incubated in the presence of 20 μg of DNase I per ml and 5 mM $MgCl_2$ for an additional 30 min. After removal of cell debris by centrifugation at 8,000 × *g* for 10 min, the resultant supernatant was centrifuged at 100,000 × *g* for 1 h. The pellet was gently suspended in 5 ml of 0.1 M Tris (pH 7.8) containing 0.5 mM EDTA and Triton X-100.

Preparation of flagellar components naturally released into the growth medium. To obtain flagellar filaments with attached hook structures, CB15 cells grown to late log (optical density at 660 nm = 0.9) phase in 5 liters of PYE broth were centrifuged at 5,000 × *g* for 15 min. The supernatant was collected and concentrated 50-fold by Amicon pressure dialysis at 4°C to a final volume of 100 ml. The concentrated culture fluid was centrifuged at 5,000 × *g* for 15 min to remove any remaining cells or cell debris. After centrifugation, ammonium sulfate was added to the supernatant to 20% saturation and mixed at 4°C for 2 h. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min. The resultant pellet was suspended in 5 ml of 15 mM Tris-hydrochloride (pH 7.5) containing 2% Triton X-100 and dialyzed overnight against 6 liters of 15 mM Tris (pH 7.5) containing 2% Triton X-100. The flagellum suspension was then centrifuged at 150,000 × *g* for 1 h, and the resulting pellet, containing the released flagella, was suspended in 15

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mM Tris (pH 7.5) containing 2% Triton X-100.

For the isolation of released hook structures lacking flagellar filaments, the culture fluid of the nonmotile mutants was treated similarly, except that the ammonium sulfate precipitation was omitted and the hook structures were pelleted directly at 100,000 × *g* for 2 h. The pellets were suspended in 10 ml of 20 mM Tris (pH 7.5)–15 mM EDTA–1% (vol/vol) Triton X-100 (TET buffer) and centrifuged for 10 min at 5,000 × *g* to remove any large particulate material. The supernatant was centrifuged again at 100,000 × *g* for 2 h, and the pellet was suspended in 1 ml of TET buffer. Further purification was obtained by the method of Lagenaur et al. (16). Up to 0.5 ml of the hook preparation was layered onto a 15 to 30% sucrose gradient in TET buffer and centrifuged at 100,000 × *g* for 2 h. Hook structures sedimented to the center of the 15 to 30% gradient as determined by electron microscopy and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of fractions (14).

Electron microscopy. Specimens were negatively stained with freshly prepared uranyl acetate and observed in either a Siemens Elmiskop 1A or a JEOL-100B electron microscope. Size measurements reported were obtained from the analysis of at least 20 clearly defined structures.

SDS-polyacrylamide gradient gel electrophoresis. SDS-gradient gels were prepared and run as described by Amemiya et al. (3).

Isoelectric focusing in polyacrylamide gels. Isoelectric focusing experiments were performed in polyacrylamide gels by a procedure developed by S. Craig (personal communication). The gels were made up to 7.5% acrylamide (acrylamide-methylenebisacrylamide, 30:0.8) containing 4% ampholytes (pH gradient of 4 to 6 or 3 to 10; Bio-Rad Laboratories). The anode solution was 0.2% H_2SO_4, and the cathode solution was 0.4% triethanolamine. The gels were overlaid with 100 μl of 4% ampholytes in 5% sucrose and were prerun for 1 h at 200 V. The protein samples were combined with an additional 50 μl of 4% ampholytes made to 15% with respect to sucrose, and layered on top of the gels. Hook structures were prepared for isoelectric focusing by the addition of 5 μl of 0.1 N HCl to 10 μl of purified hooks in TET buffer, incubation for 1 h on ice, and neutralization with 5 μl of 0.1 N NaOH before the addition of ampholytes and sucrose. The samples on the gel cylinders were isofocused for 16 h at 200 V at 4°C. The pH gradient was determined by: (i) slicing a sample gel into 3-mm slices, adding 1 ml of distilled water, and measuring the pH after soaking overnight at 4°C and (ii) isofocusing proteins of known isoelectric points in parallel gels. The gels were washed exhaustively in 45% methanol–10% acetic acid and then stained for 5 h with a 0.2% solution of Coomassie brilliant blue in 45% methanol–10% acetic acid. The gels were destained by soaking in a solution of 5% methanol–7.5% acetic acid.

RESULTS AND DISCUSSION

Intact flagella obtained from lysed cells. Flagella were purified from lysed *C. crescentus* cells as described above, and intact structures were obtained which contained a basal complex in addition to the hook, rod, and flagellar filament found in naturally released flagella (Fig. 1). The fine structure of the hook and basal complex is shown in Fig. 2 and depicted diagrammatically in Fig. 3. The basal body was composed of two outer rings, I and II, and at least three inner rings, III, IV, and V. The outer rings differed in size as follows: the large hook-proximal ring (ring I) was 32 nm in diameter, and ring II just below it was 21 nm in diameter. Rings III and IV had approximately the same diameter as ring II, although they were considerably thinner. The fact that the naturally released hook structures have attached rods (22; see Fig. 4 and 5) not seen when the basal complex is present suggests that, in the intact basal complex, the rod structure is threaded through the outer and inner rings, in agreement with the rod and ring structures described for *Escherichia coli* and *Bacillus subtilis* (6, 7). Ring V had the same diameter as rings II, III, and IV, but was thicker. The major difference between the basal complex of *C. crescentus* and that of other gram-negative structures was the presence of ring V. Since *C. crescentus* differs from other gram-negative bac-

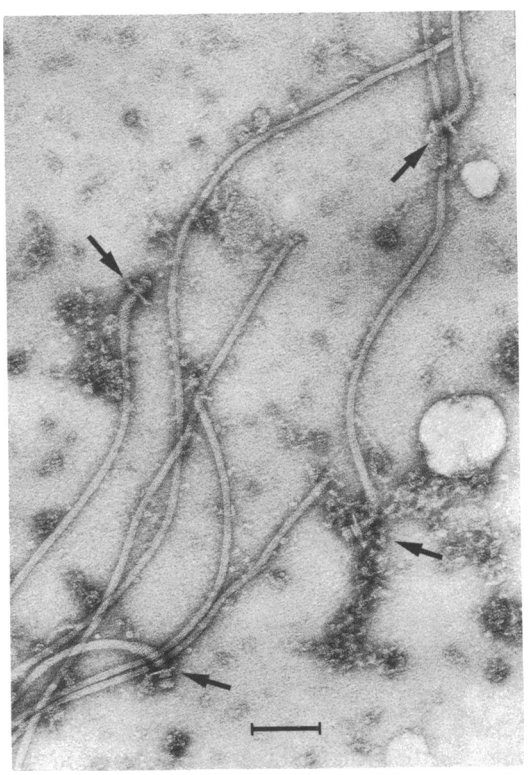

FIG. 1. *Intact flagella from C. crescentus CB15 negatively stained with uranyl acetate, pH 4.5. The arrows indicate complete basal body structures. Bar = 153 nm.*

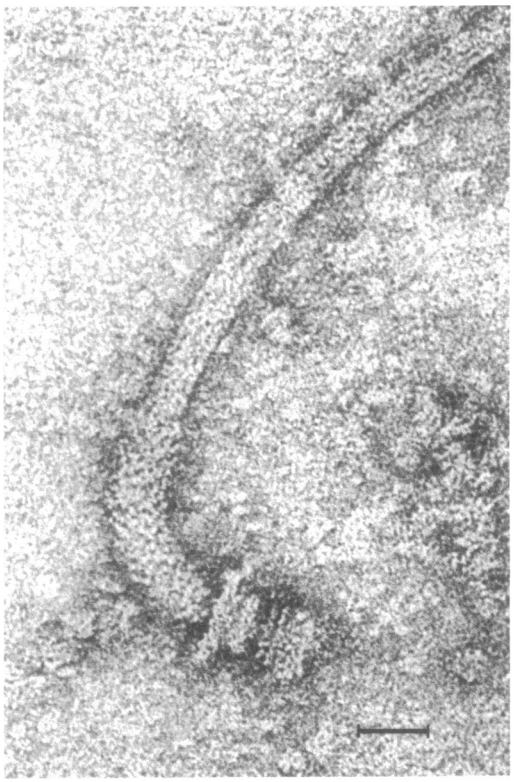

FIG. 2. *Basal body complex of an intact C. crescentus CB15 flagellum stained with uranyl acetate, pH 4.5. The filament, hook, upper rings, lower rings, and rod can be seen. Bar = 30 nm.*

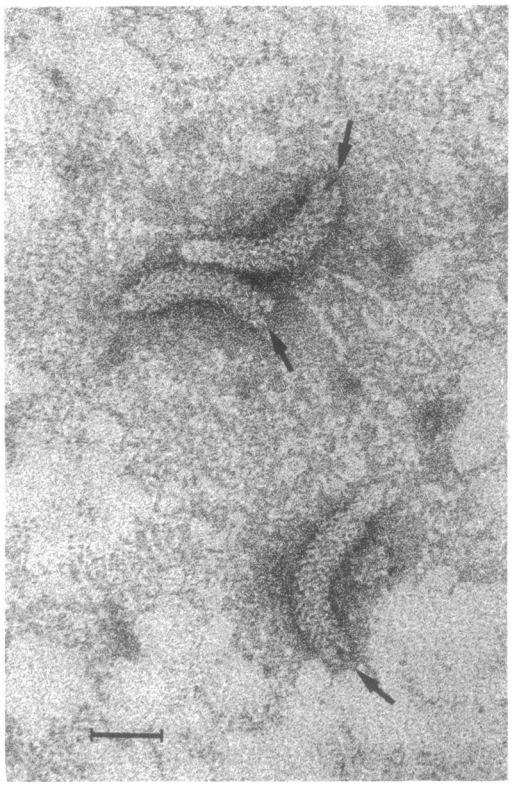

FIG. 4. *Hook and rod structures released from C. crescentus nonflagellate mutant SC305 stained with uranyl acetate. The arrow indicates the notch visible at the rod-distal end of the hook. Bar = 50 nm.*

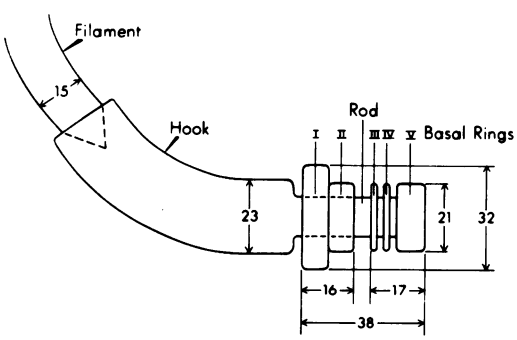

FIG. 3. *Schematic model of the basal body complex of the C. crescentus flagella. Size dimensions are in nanometers.*

teria in that it periodically sheds its flagellum, it may be that this extra ring is involved in the release mechanism. Experiments are in progress to determine whether ring V lies below the level of the cytoplasmic membrane. Alternatively, the extra ring could be involved in the mechanism for rotating the flagellum. An extra ring has been seen occasionally in *E. coli* basal body preparations (M. Simon, personal communication) and may be present in a more stable form in *Caulobacter*.

Incomplete structures isolated from lysed cell membranes of nonmotile mutants. Basal complexes attached to hook and rod structures lacking a flagellar filament were isolated from SC229 (*flaA*) and SC269 (*flaJ*), using the procedures designed for the isolation of basal complexes on intact flagella. In both cases, the positions and the number of rings were the same as in the intact flagella isolated from the parent strain, indicating that the basal complex is assembled normally with the hook and rod structure in these mutants in the absence of a flagellar filament (Fig. 5a and b).

Incomplete structures released from nonmotile mutants. Hook and rod structures lacking a flagellar filament were released from nonmotile mutants into the culture medium during the normal cell cycle and could be isolated

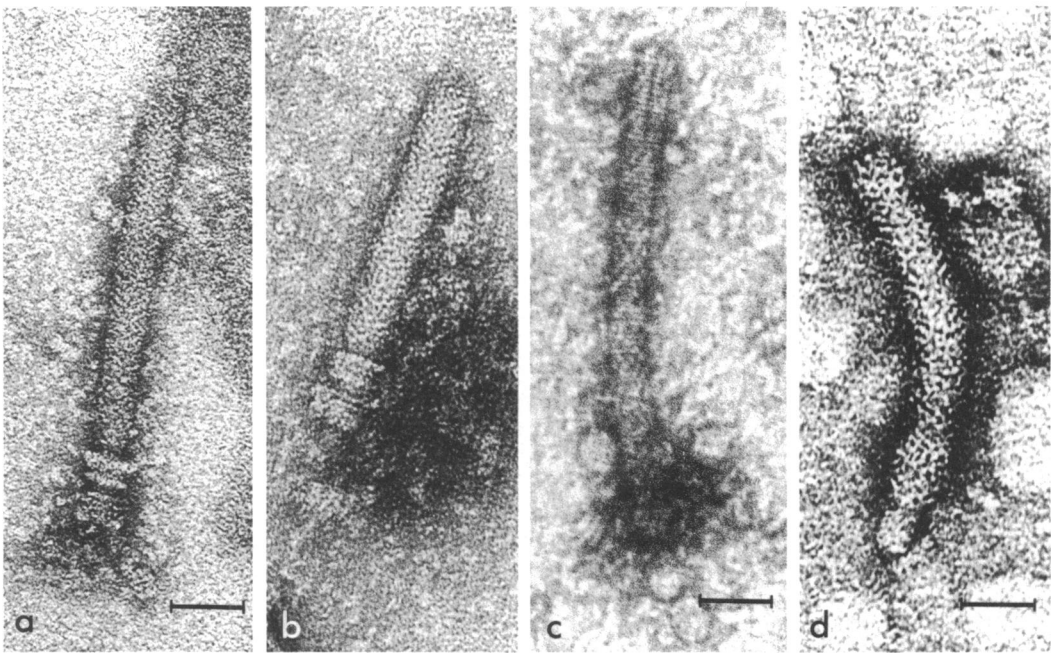

FIG. 5. *Hook and basal body structures from nonflagellate mutants stained with uranyl acetate. Hook and basal body complex obtained from the cell membrane of C. crescentus SC269 (a and b). Bar = 38 nm. Hook and rod structures released from C. crescentus SC269 during the normal course of cell growth (c and d). Bar = 33 nm.*

using the procedures designed for the purification of shed flagella (Fig. 4). Such structures have been isolated from the culture fluid of nonflagellate mutants representing 16 of the known 26 *fla* linkage groups (13) and appear to be structurally identical to hook and rod structures found at the proximal end of released flagella from wild-type cells when observed by electron microscopy. In addition, the hook proteins from these 16 mutant strains are all identical (Fig. 6). Culture fluids from representatives of 9 of the remaining 10 linkage groups (*flaC, -I,-K,-M,-O,-P,-S,-U,* and *-W*) showed no evidence for the presence of hooks when examined by electron microscopy or by SDS-polyacrylamide gel electrophoresis after being subjected to the purification procedure (e.g., Fig. 6, lane 6). The mutant in the remaining linkage group, SC269 (*flaJ*), produced an aberrant hook structure which is described below.

As with the released flagella from wild-type cells (15), the hook and rod structures of the mutants were released from the cell without the array of ring structures seen in artificially released hooks. Free rings were not detected in the culture medium of the wild-type strain of any of the mutants. It may be that the basal rings, which likely function to anchor the flagella to

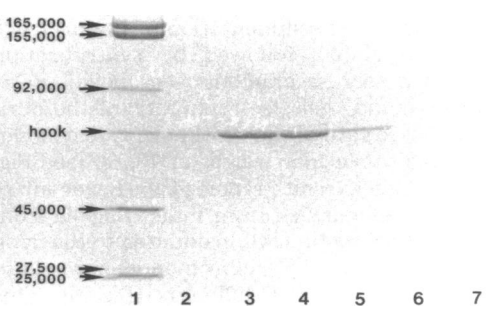

FIG. 6. *SDS-polyacrylamide gel electrophoresis of purified hooks. Lane 1 contains C. crescentus RNA polymerase which has subunit molecular weights of 165,000, 155,000, 92,000, and 45,000 (K. Amemiya, personal communication); C. crescentus flagellin with subunit molecular weights of 27,500 and 25,000 (15); and purified hooks from SC229 (flaA). Lanes 2 through 7 contain purified hooks from SC269 (flaJ), SC280 (flaG), SC517 (flaT), SC305 (flaR), SC239 (flaC), and SC235 (flaQ), respectively. Supernatants from one-liter stationary-phase cultures of each strain were subjected to the procedure for purification of hooks, and 40 μl of the resultant suspension was applied to the gel.*

the cells as well as to mediate filament rotation, remain attached to the cell membranes. Alternatively, they may be disassembled or destroyed (proteolysis) during the process of releasing the flagellum. Since the cell pole which contained the flagellum is the site of stalk formation after release of the flagellum, the ultimate fate of the basal rings during the *C. crescentus* cell cycle remains an interesting question.

Electron micrographs of hook and rod structures shed from incomplete flagella (Fig. 4) or hooks on intact flagella (Fig. 2) show a chevron pattern on the hook surface. The chevron pattern of the hook may result from the superimposed images of out-of-phase upper and lower surfaces of an essentially helical structure (7). In all *Caulobacter* hook specimens studied, the apex of the chevron structure was oriented toward the rod and basal body. In many hook preparations, an electron-dense notch could be seen at the distal tip of the hook (Fig. 4). Similar notches have also been seen in *Bacillus pumilus* (2). It may be that this notch represents an inverted cone-shaped region which is the site of the hook filament attachment. It has been observed with flagella of several bacterial species that broken filaments exhibit V-shaped tips with the point of the V directed toward the hook structure (4, 5, 17, 24).

Purification of hook structures released from nonmotile mutants. Mutants lacking a flagellar filament were used to purify the hook and rod structures. These structures were purified by repeated sedimentation in the presence of Triton X-100, followed by centrifugation through a sucrose gradient (see above). SDS-polyacrylamide gel electrophoresis of the purified hooks indicated that the hook protein monomer has a molecular weight of 70,000 (see Fig. 6), confirming results reported by Lagenaur et al. for hooks purified from intact flagella from the wild-type strain (16). In addition to the hook protein, the major flagellin monomer was also present in very small amounts (Fig. 6). This result was expected since some of the purified hooks appeared to have short pieces of assembled filaments when observed by electron microscopy. A third protein monomer present in purified hook preparations had a molecular weight of 32,000 and was present in trace amounts; it could only be observed when the gels were overloaded for the hook protein. This protein may be a component of the rod structure or, alternatively, a minor component of the hook structure. In *E. coli*, hook structures purified from polyhook (*flaE*) mutants consist of a single protein subunit with a molecular weight of 42,000, and the rod protein has yet to be identified (23). The *E. coli* flagellin protein has a molecular weight of 54,000 (11). Thus, in *C. crescentus*, the hook protein has a molecular weight larger than that of the flagellin monomer, whereas the reverse is true in *E. coli*.

Isolation of hook and rod structures with an altered morphology. In contrast to the mutants described above, one exceptional mutant, SC269 (*flaJ*) produced aberrant hook and rod structures which were usually straight and much longer than those produced by the other mutants (Fig. 5). These mutant structures were isolated from lysed cell membranes with the basal complexes attached (Fig. 5a and b) or from the culture medium as released hook and rod structures (Fig. 5c and d). Thus, the basal complexes appear to be formed normally, even with altered hook and rod structures. As with the hook structures from the other strains, a chevron pattern was observed on the hook surface. The notch present at the distal end of the hook always appeared as a long slit when it was observed by electron microscopy (Fig. 5c).

Purified hook structures from the culture fluid of SC269 were examined by SDS-polyacrylamide gel electrophoresis and had a monomer molecular weight identical to that of the hook protein purified from the other mutants (Fig. 6). After acid dissociation of the hook structures, the hook proteins from SC269 and from the *flaA* mutant SC229 were subjected to polyacrylamide isoelectric focusing, using pH gradients of 3 to 10 and 4 to 6. Gels containing either of the individual proteins and a mixture of the two contained a single band with an isoelectric point of pH 4.6, suggesting that the hook proteins from the two mutants were identical. Furthermore, antibody prepared against either intact wild-type flagella or purified SC269 hooks precipitated hook protein from cell extracts of both wild type and SC269 (R. C. Johnson and B. Ely, manuscript in preparation). When the precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis, the two hook proteins migrated to identical positions on the gel. Therefore, we conclude that the hook protein in SC269 is identical to that of the wild type and that the aberrant hook structures produced by SC269 are most likely due to a defect in termination of assembly of hook structure. A similar explanation has been suggested for the polyhook mutants of *E. coli* and *Salmonella typhimurium* (11, 23).

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