Physical Characterization of Caulobacter crescentus Flagella

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Preparations of intact flagella isolated from Caulobacter crescentus CB13B1a were found to contain two protein species of apparent molecular weights 28,000 and 25,000. Both proteins cross-reacted completely with each other and with purified flagella in Ouchterlony double-immunodiffusion assays. The amino acid compositions of the isolated proteins were similar to one another but precluded any precursor-product relationship. Absence of both the 25,000- and 28,000-molecular-weight proteins from a number of nonmotile mutants and the simultaneous reappearance of these proteins in a motile revertant provide further evidence of the relationship of these two proteins to flagellar structure.

In the sequence of morphological changes that characterize differentiation in Caulobacter crescentus, there is a temporally and spatially controlled synthesis of several surface structures (12, 15). The synthesis of a polar flagellum in the developing swarmer cell and the loss of motility associated with the development of a stalked cell were among the earliest recognized stage-specific events in the Caulobacter cell cycle (4). Flagellin biosynthesis is correlated with the onset of motility, and biochemical analysis has demonstrated that flagellin is not synthesized in the mature stalked cell (16). An interesting feature of swarmer cell development is that the synthesis and cellular location of several polar surface structures is coordinated with flagella expression, i.e., the polar ϕCbK deoxyribonucleic acid bacteriophage receptor site (1) and the assembly of pili, which are receptors for ribonucleic acid bacteriophages (14, 15). As the swarmer cell develops into a stalked cell, the flagellum is extruded into the culture fluid along with its hook and rod structures (13, 16) in a step that requires ribonucleic acid synthesis (10). Flagellar extrusion likewise appears to be coordinated with the loss of deoxyribonucleic acid bacteriophage receptor sites and pili during swarmer cell maturation. In the course of studies of the control of various aspects of flagellar biosynthesis and release, we have attempted to identify the structural components of the flagellum in detail. We present, in this report, physical, immunological, and genetic evidence that the flagella of C. crescentus are composed primarily of two proteins of apparent molecular weights 28,000 and 25,000, which are found in a ratio of 1:3.3 in intact, functional flagella, and thus further extend the observation of Shapiro and Maizel (16).

MATERIALS AND METHODS

Bacterial strains and growth conditions. C. crescentus strains CB13B1a and CB15 were routinely grown in modified (1) peptone-yeast extract (12). Mutants SW1 fla-297, SW2 fla-298, SW3 fla-204, SW4 fla-212, SW5 fla-215, SW6 fla-223, and SW7 fla-210 were isolated as nonmotile colonies on 0.35% peptone-yeast extract agar plates containing ϕCbK (10⁶ plaque-forming units/ml). Motile revertants (e.g., SW8 fla-211) were selected from pellicles formed in test tube cultures grown without shaking at 30°C. Mutant AE1 fla-501 was a generous gift from L. Shapiro.

Purification of flagella. Cultures were grown with shaking to late exponential phase in peptone-yeast extract at 30°C, and cells were removed by centrifugation at 15,000 × g for 10 min at 4°C. The supernatant fluid, which contained release flagella, was adjusted to 10 mM ethylenediaminetetraacetic acid, 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.2. Solid ammonium sulfate was added rapidly to 30% saturation, and the solution was stirred at 4°C for 60 min. The precipitated flagella were harvested by centrifugation at 22,000 × g for 12 min. CsCl equilibrium density gradient centrifugation was used for additional purification. Five-milliliter gradients containing 35% (wt/vol) CsCl (Harshaw Chemical Co.) were centrifuged at 75,000 × g for 50 h in a Beckman SW50.1 rotor. Fractions were collected dropwise from the bottom of gradients; flagella formed a visible band at a density of 1.29 g/cm³. Preparations were assayed by electrophoresis and electron microscopy at each step of purification.

Electron microscopy. Intact flagella were adsorbed to Formvar-coated grids and stained with 1% phosphotungstic acid, pH 7.0. Samples were examined in a Phillips EM 201 electron microscope at 60 kV.

Immunological analysis. Intact flagella, purified through CsCl as described above, were used to immunize one New Zealand White female rabbit according to a modification of the immunization sched-
ule of Kenney (6). A total of 1 ml of 0.5 mg of protein per ml was injected intramuscularly with an equal volume of complete Freund adjuvant. A series of four intravenous injections was given at 3-day intervals, starting 20 days after the first injection. Intravenous injections contained 0.1, 0.2, 0.3, and 0.5 ml, respectively, of the above protein solution without Freund adjuvant. Blood was collected 1 week after the last injection.

Serum was partially purified by precipitation with 50% saturated ammonium sulfate followed by dialysis against 0.15 M NaCl and 0.01 M sodium phosphate (phosphate-buffered saline), pH 7.0. Serum was stored frozen at −20°C. Double-immunodiffusion assays were a modification of the technique of Ouchterlony (11). Immunodiffusion was carried out on glass slides covered with 1% agarose (Gallard-Schlessinger) containing 1% Triton X-100 in phosphate-buffered saline. Routinely, the antibody well contained 20 μl of serum, and the antigen well contained 1 to 5 μl of sample. Flagella antigens were disaggregated in 0.1% sodium dodecyl sulfate (SDS). Intact flagella could not enter agarose, and the SDS solubilization ensured that the antibodies reacted with monomeric antigen. Triton X-100 (1%) in the immunodiffusion plates prevented precipitation of antiserum by SDS at concentrations of the detergent up to 2%. Immunodiffusion plates were incubated in a moist chamber at 30°C for 24 h. Using purified flagella as standard, as little as 0.5 μg of protein could be readily assayed. Preimmune serum did not react with any Caulobacter flagella preparations or whole-cell homogenates.

SDS-polyacrylamide gel electrophoresis (PAGE). Routine assays for flagellin during purification used cylindrical 15% polyacrylamide disc gels, according to the general procedure of Laemmli (7) modified as described previously (20).

Semipreparative SDS-PAGE techniques for separation of flagellar proteins used 10 to 15% polyacrylamide gradient gels, which were poured into a slab gel apparatus as described by Studier (19). For application to either gel system, flagella samples were suspended in SDS sample buffer (0.5 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 6.8, 1% SDS, 1% mercaptoethanol, 10% glycerol, 0.01% bromphenol blue and then heated for 3 min in a boiling-water bath, cooled to room temperature, and applied to the gel. If immunological studies were to be carried out on the separated proteins, boiling was omitted. It should be noted, however, that brief boiling in SDS changed neither the migratory behavior in gels nor the immunological properties of either of the two flagella-associated proteins. Slab gels were run at 17 mA/gel.

Proteins were stained by immersing the gel in 0.25% Coomassie brilliant blue in methanol-acetic acid-water (6:1:5) for 1 h at 60°C, or overnight at 25°C, and destained first in methanol-acetic acid-water (5:1:5) for 1 h at 60°C and then in methanol-acetic acid-water (1:1:18) until desired decoloration was attained.

Electrophoretically purified flagella-associated proteins were prepared using the method of Lazarides (8). Isolated protein bands were cut from stained gradient gels and placed in cut-off Pasteur pipettes. Gel slices were held in place with glass wool, and a short length of dialysis tubing, attached to the anodic end of the tube, retained the electrophoretically eluted (5 mA/tube, 5 to 6 h) protein.

Amino acid analysis. Electrophoretically purified protein was hydrolyzed in 6 N HCl at 110°C for 48 h. Analysis was carried out with a Beckman model 120C analyzer and a Durrum D500 amino acid analyzer using the technique of Spackman et al. (18).

Protein determination. Protein was determined by the microbiuret technique (22).

Densitometry. Coomassie brilliant blue-stained SDS-PAGE slab gels were dried on clear cellulose membrane backing (Bio-Rad) and scanned with a Joyce-Loebel double-beam recording microdensitometer MKIIC with electronic peak integration.

RESULTS

Purification of flagella. Flagella, assayed at each stage of purification by SDS-PAGE, are shown in Fig. 1. The cell-free supernatant (Fig. 1A) obtained from late log-phase Caulobacter cultures provides an enriched source of flagella, since the intact structures are released from the swarmer cells during the swarmer-to-stalked cell transition (13, 16). The culture fluid contains three major protein species of apparent molecular weights 20,000, 25,000, and 28,000 and a variety of minor, high-molecular-weight proteins. Several of these high-molecular-weight proteins and the 20,000-molecular-weight species appear to be derived from the outer cell membrane and are released, under normal growth conditions, into the culture fluid (Lagenaur and Agabian, unpublished data). A crude flagella fraction (Fig. 1B), prepared by ammonium sulfate precipitation, is composed predominantly (>90%) of two protein species of 25,000 and 28,000 molecular weight. CsCl equilibrium density gradient centrifugation provides a flagella fraction (Fig. 1C), which again contains these two protein species and traces of a protein of approximately 70,000 molecular weight. Electron microscopy of the CsCl preparation reveals only intact flagella with attached hooks; in some cases, the rod is also visible (Fig. 2). The flagella filament diameter was 13 to 15 nm.

Densitometric analysis of the relative amounts of the two flagella-associated proteins indicated that the 28,000- and 25,000-molecular-weight proteins are present in a ratio of 1:3.3 throughout the purification procedure. In addition to co-purification in constant ratio, a physical association between these proteins and flagella could be surmised from their recovery in the same ratio after centrifugation under conditions in which intact flagella, but no protein of 20,000 to 30,000 molecular weight, is sedi-
Fig. 1. Purification of flagella-associated proteins from C. crescentus CB13B1a. Flagella were purified from cell culture fluid as described in Materials and Methods and displayed on 10 to 15% SDS-PAGE slabs: (A) 200-fold concentrated culture fluid; (B) ammonium sulfate-precipitated flagellar proteins; (C) CsCl-banded flagella; (D) electrophoretically purified 28,000-dalton protein; (E) electrophoretically purified 25,000-dalton protein; (F) protein standards.

Counts sedimented (30,000 rpm, 2 h). Attempts to separate the proteins by gel filtration or sedimentation velocity centrifugation through sucrose likewise allowed the recovery of both protein species. This constant ratio of the two flagella proteins throughout a variety of preparative procedures suggest that the 28,000-molecular-weight species is not present simply as a persistent contaminant of flagellin. This conclusion was reinforced by the finding that related C. crescentus strains also possess this flagellar-protein doublet on SDS-PAGE (data not shown).

To determine the relationship between these two flagellar proteins, we have compared them with respect to their amino acid composition and genetic and immunological properties. The two proteins (Fig. 1D and E) have been electrophoretically purified (see Materials and Methods); the 25,000-dalton protein (Fig. 1E) corresponds in molecular weight to the previously
reported *C. crescentus* CB13B1a flagellin (16).

Amino acid composition of flagellar proteins. The electrophoretically purified flagella-associated proteins were prepared for amino acid analysis as described in Materials and Methods; their amino acid composition is shown in Table 1. Both proteins possess a complement of amino acids that is typically found in gram-negative bacterial flagella (17). In addition, the two proteins are remarkably similar to one another. The average of a large number of molecular weight determinations have yielded values of 27,900 and 24,800, on which the amino acid compositions reported in Table 1...
are based. It was surmised at first that a precursor-product relationship might exist between the 28,000- and 25,000-molecular-weight species; however, amino acid analyses revealed that the smaller component contained a greater number of aspartyl (and/or asparaginyl), threonyl, alanyl, isoleucyl, and leucyl residues than the larger protein, thus precluding the possibility that one is a simple precursor of the other.

**Immunological analysis.** To investigate the relationship between the two flagella-associated proteins, the antibody generated against intact flagella was first characterized by Ouchterlony double immunodiffusion. Flagella preparations were disaggregated in 0.1% SDS to optimize the antigen-antibody reaction, since it was determined that intact flagella could not penetrate the agarose gel. Ouchterlony double immunodiffusion of the crude flagella (ammonium sulfate, fraction shown in Fig. 1B) against antibody prepared against CsCl-purified flagella is shown in Fig. 3. Three Caulobacter-specific antibodies could be detected. Of the three corresponding antigens, only one, antigen A, was detected in flagella that had been purified through CsCl. Since CsCl-purified flagella were used for the original immunization, the two remaining antigens (Fig. 3B and C) must represent highly antigenic trace contaminants, which are largely removed by CsCl isopycnic centrifugation. In Fig. 4, flagellin antibody has been reacted against CsCl-purified flagella; only one precipitin line, corresponding to antigen A, can be detected (wells 1 and 2). One of the contaminants, antigen B (Fig. 3), was identified as CB13B1a strain-specific lipopolysaccharide by testing the antibody against lipopolysaccharide purified by the procedure of Westphal (21). Antigens A and B could be detected in CB13B1a cell extracts that had been solubilized in 0.1% SDS and briefly sonicated. Antigen C could only be detected in crude flagella purified through the ammonium sulfate step; its identity remains unknown. Presumably, it is present in whole cells in such small quantities that it cannot be readily detected by double-immunodiffusion techniques. Flagella from the related Caulobacter strain, CB15, purified by ammonium sulfate precipitation, produced precipitin lines in Ouchterlony double immunodiffusion that fused completely with antigens A and C. The electrophoretically purified, 28,000-molecular-weight, flagella-related protein also produced a single precipitin line, which fused completely with the single line produced by the 25,000-molecular-weight protein (Fig. 5). These species both correspond to antigen A seen in purified intact flagella. This antigenic relatedness strongly suggests that both the 28,000- and 25,000-molecular-weight proteins are flagella associated, since they together account for more than 98% of the protein present in CsCl-purified flagella preparations.

**Mutational analysis.** The relationship of the two flagella-associated proteins to bacterial motility was investigated by analysis of a number of nonmotile mutant strains selected as described in Materials and Methods. In addition to being nonmotile, electron microscopy revealed that the mutants were nonflagellated. The culture fluid from late exponential-phase cultures was concentrated by lyophilization, and the compositions of the preparations were examined by SDS-PAGE. The protein profiles are shown in Fig. 6. Both protein bands associated with wild-type flagella (Fig. 6A) were absent in each of the nonmotile mutants (Fig. 6B–I). SW8, a spontaneous motile revertant of SW7 (Fig. 6J), regained both proteins simultaneously. Thus, the presence of an intact functional flagellum seems to be strongly correlated with the presence of both proteins. These studies are consistent with the type of regulation observed in Escherichia coli, where little or no flagellin is synthesized in mutants that are defective in flagella assembly, even though the structural gene for flagellin is intact (3).

**DISCUSSION**

Purification and characterization of flagella pose a special biochemical problem, since these organelles can no longer be tested for their

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**Table 1. Amino acid composition of flagella-related proteins**
primary physiological function — cell motility — once they are separated from the cell body. Thus, a definition of components that constitute the minimal units of the flagellar structure must be approached indirectly by a combination of genetic, immunological, and biochemical techniques.

In general, gram-negative bacterial flagella are composed of a single monomeric protein, flagellin, which accounts for greater than 95% of the flagellar mass (2). In the purification of intact Caulobacter flagella, we have found two proteins of apparent molecular weights 28,000 and 25,000. These proteins co-purify and remain in a ratio of 1:3.3, respectively. The following evidence suggests that these proteins represent structural components of Caulobacter flagella. (i) The proteins co-purify in constant ratio, through a variety of preparative techniques, and can only be separated under conditions that cause denaturation of the intact flagellar structure. (ii) The amino acid composition of both proteins is typical of that found for bacterial flagellins in general and appears to preclude a simple precursor-product relationship. (iii) The two electrophoretically purified proteins cross-react completely with antiflagellin antibody. (iv) Both proteins are found in preparations of flagella from a related strain, CB15; flagella from both strains are immunologically identical. (v) Mutations that result in a loss of motility result in a concomitant loss of both proteins. (vi) Spontaneous reversion of nonmotile mutants results in the simultaneous reappearance of both proteins.

Gram-negative bacterial flagellar filaments which fall into two general classes have been found: most commonly, those that are composed of a single protein and those that have an accessory filament structure or sheath (5). The presence of a sheath usually is reflected in the diameter of the flagellar filament, although in-
crements in flagellar width are minimal in some cases (9). Our measurements of Caulobacter flagellar filaments (13 to 15 nm) are more in keeping with the diameter of unsheathed flagella.

Although these data support the conclusion that both proteins are constituents of Caulobacter flagella, they do not necessarily indicate that each flagellum contains both proteins. It is possible that individual flagella contain either one or the other protein. If either protein is used in a single filament and expressed within the population at a frequency of 1:3.3, one could draw analogy with the phase variations found in the expression of Salmonella flagella. However, it should be mentioned that alternatively expressed Salmonella flagellins are immuno-logically distinct (5).

Genetic studies to date confirm the relationship of the 28,000- and 25,000-dalton proteins to flagellar structure in that they appear to be under coordinate control and exist only in cases where there is an intact and functionally motile flagellum. In the E. coli system, it has been shown that defects in a variety of genes required for motility (other than the flagellar filament structural gene) result in a failure to synthesize flagellin (3, 17). Thus, the loss of both flagellar proteins in nonmotile mutants of Caulobacter could indicate similar control mechanisms, again supporting the contention that both proteins are elements of the flagellar structure.

In addition to the highly complex regulation of flagella synthesis and assembly of the sort demonstrated in E. coli (3, 17), Caulobacter flagella are further regulated in their stage-specific appearance and extrusion in the life cycle of this organism, thus providing an interesting system for the study of bacterial motility.

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FIG. 5. Identification of flagellar antigens. Center well contains antiflagella antibody. Well 1 contains electrophoretically purified 28,000-dalton protein; well 2 contains electrophoretically purified 25,000-dalton protein; wells 3, 4, and 6 contain ammonium sulfate-precipitated flagella dissociated in 0.1% SDS.
Fig. 6. SDS-PAGE of culture fluid from nonmotile mutants. (A) Wild-type CB13B1a, (B) AE1 fla-501, and (C-J) SW 1-7 as described under Materials and Methods; (J) SW8, a motile revertant of SW7. Culture fluids are 200-fold concentrated by lyophilization.
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LITERATURE CITED