Characterization of the Proteins of the *Caulobacter crescentus* Flagellar Filament

**PEPTIDE ANALYSIS AND FILAMENT ORGANIZATION***

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During each cell division cycle, the bacterium *Caulobacter crescentus* synthesizes a single polar flagellum and then releases this flagellum into the culture medium. Two flagellins, Fla A (25,000 daltons) and Fla B (27,500 daltons), are recovered from the total population of released flagellar filaments in a ratio of 4:1, respectively. These two proteins have been shown to be immunologically cross-reactive (Lagenaur, C., and Agabian, N. (1976) *J. Bacteriol.* 128, 438-444) and to have similar amino acid compositions (Fukuda, A., Koyasu, S., and Okada, C. (1978) *FEBS Lett.* 95, 70-75). Two questions are addressed here: 1) are the two flagellins the products of different genes, and 2) are both flagellins present in each filament or, as in *Salmonella* phase variation, is only one flagellin assembled into a given filament?

Tryptic peptide maps were prepared from the two purified flagellins. Pairs of peptides from Fla A and Fla B, accounting for 33% of their sequences, had identical amino acid compositions, and therefore, probably identical sequences. Pairs of peptides accounting for an additional 20% of the sequence of the two proteins showed compositions related by simple amino acid substitutions, most of which could be explained by a single base change in the DNA sequence. The amino acid sequences of one such pair of peptides were determined and the specific amino acid differences in the otherwise identical sequences were demonstrated. These results suggest that *C. crescentus* has two genes for the flagellins, proteins Fla A and Fla B, and that the two genes likely arose by duplication of an ancestral sequence.

Flagellar filaments of different lengths were prepared by sonication and then immunoprecipitated using hook-specific antibody. Protein analysis of these hook-associated filaments showed a preponderance of Fla B in filaments of short length. A filament about one-fifteenth the full length was composed of 80% Fla B. In contrast, filaments from two different nonmotile mutants that only assemble short filaments contained over 90% Fla A. Thus, both flagellins are found in each filament, the region of the filament proximal to the hook is highly enriched for Fla B, and the ordered polymerization of both Fla B and Fla A may be required for the assembly of a normal filament.

The single polar flagellum of *Caulobacter crescentus* is made up of a filament, hook, rod, and five basal rings (1), and thus is similar in structure to flagella of other gram-negative bacteria (2). The flagellum is synthesized and assembled at one cell pole prior to cell division and is then shed into the culture medium when the flagellated cell differentiates into a stalked cell (3, 4). When the protein composition of the total population of shed flagella is analyzed, two filament subunits (or flagellins), Fla A (25,000 daltons) and Fla B (27,500 daltons), are consistently found in a ratio of about 4:1, respectively (5, 6). The amino acid composition of the two *Caulobacter* flagellins are similar to one another (7) and similar to those of other bacterial flagellins (for review see Ref. 8). The ability of each flagellin to reaggregate in vitro to form a filament indistinguishable from filaments formed in vivo indicates that both proteins function as filament subunits (7).

Although most *Caulobacter* species have two flagellins (9), such polymorphism has been shown in only three other bacterial flagellins, those from *Bacillus pumilis*, *Vibrio parahaemolyticus*, and *Salmonella* sp. The two *B. pumilis* flagellins are found in a ratio of about 7:3, and appear to be similar based upon the amino acid composition and tryptic peptide maps (8, 10). Antibody prepared against either of the two, however, did not cross-react with the other. Decoration of the entire filament with antibody prepared against either *B. pumilis* flagellin indicates that both flagellins are present in each individual filament, and thus, that each cell synthesizes both proteins (10). The two flagellins from *V. parahaemolyticus* are also antigenically distinct. However, any given filament contains only one flagellin, although both are found in the same cell: one of the flagellins makes up the single polar sheathed flagellum and the other is the subunit of the multiple peritrichous flagella (11, 12). In contrast, only one of the two antigenically distinct *Salmonella* flagellins is synthesized and assembled into the flagellar filaments of an individual cell. The genes for the *Salmonella* flagellins, H1 and H2, are widely separated on the chromosome (13), and are expressed in a mutually exclusive fashion. The expression of only one flagellin gene at a time, termed phase variation, results from inversion of a segment of DNA containing the promoter for

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* The abbreviations used are: Fla A and Fla B, two flagellins of 25,000 and 27,500 daltons, respectively; SDS, sodium dodecyl sulfate; dansyl, 5-dimethylaminonapthalene-1-sulfonyl.
the H2 gene and for the gene of a protein which represses the expression of the H1 gene (14, 15).

The two flagellins in Caulobacter differ from these three cases in that they are immunologically cross-reactive. From previous observations that the rates and durations of synthesis of Fla A and Fla B are different, it has been argued that the two flagellins are products of individual genes. The peak rate of synthesis of Fla A is ten times greater than the peak rate of Fla B synthesis, and only Fla A continues to be synthesized in the swarmer cell (16, 17). Furthermore, the apparent mRNA half-life for each of these proteins is different, 6.5 min for Fla A as opposed to 2.5 min for Fla B mRNA (17). By amino acid composition and sequence analysis of individual tryptic peptides isolated from purified Fla A and Fla B, we provide direct evidence that the Caulobacter Fla A and Fla B proteins are products of two separate genes, which likely arose from a gene duplication. The constant relative proportion of Fla A and Fla B in a given population of cells after relatively few generations of growth (5, 6) implies that phase variation (18) does not regulate the synthesis of Caulobacter flagellins and thus their distribution in the filaments. We present evidence here that both Fla A and Fla B are present in each flagellar filament and that Fla B is preferentially located in the hook-proximal region. Our analysis of non-motile mutants that form short filaments further suggests that the ordered assembly of both Fla A and Fla B is required to form a normal filament.

EXPERIMENTAL PROCEDURES

RESULTS

Purification of Fla A and Fla B—Flagellar filaments were dissociated into subunits by treatment with acid and Fla A and Fla B were then separated by DEAE-cellulose column chromatography (Fig. 3). Column fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The Fla A protein elutes as two peaks; however, when the column was run at a faster flow rate the amount of Fla A eluted in 5 mM NaCl was reduced to less than 10% of that in the wash-through peak. It has been reported that flagellin from Proteus vulgaris also elutes from DEAE-cellulose in multiple peaks (31). These were presumed to result from different aggregation states because the multiple peaks were reduced to a single peak when the column loading was decreased and their amino acid compositions were indistinguishable (31). A similar explanation may be valid for the multiple peaks of Fla A.

Analysis of Fla A and Fla B—The amino acid compositions of Fla A and Fla B isolated from C. crescentus CB15 are similar to each other (Table I) and agree with the compositions determined previously by Marino et al. (32) and Fukuda et al. (7). Two major differences between Fla A and Fla B are apparent: Fla B contains proline and the unusual amino acid e-N-trimethyllysine, whereas Fla A contains neither. Although e-N-mono- and e-N-dimethyllysine have been found in other bacterial flagellins (33, 34), e-N-trimethyllysine has not. Our data do not exclude the possibility that e-N-monomethyllysine is also present, nor do they indicate that a single lysine residue in the sequence of Fla B is trimethylated, rather than fractional modifications at several different sites. The NH2 terminus of both proteins appears to be heterogeneous in that both alanine and glycine were detected as their dansyl derivatives. Fla A, however, appeared to have a higher proportion of alanine, while Fla B had a higher proportion of glycine.

Antibody raised against homogeneous Fla A cross-reacted completely with Fla B in double diffusion agar (data not shown), agreeing with the results reported by Lagenaur and Agabian (5) and Fukuda et al. (7). Although Fla A was present as a very small contaminant of Fla B preparations, the amount was too small to account for the strength of the precipitin line seen. The immunological cross-reactivity between these proteins suggests, however, that they are very similar, although the presence of antigenic sites unique to each flagellin cannot be excluded. To compare the primary structures of these proteins and thus deduce whether they come from one or two genes, we isolated tryptic peptides from both proteins and determined their amino acid compositions, and in one instance their amino acid sequences. From the amino acid compositions, 22 tryptic peptides are expected from Fla A and 22 peptides from Fla B. Tryptic peptide maps showed 21 peptides from Fla A and 22 peptides from Fla B (Fig. 4). The two maps, when compared, showed at least 11 examples of peptides with similar mobility (peptides 1 to 4, 6 to 9, 12, 19, and 21 in each map). The number of peptides unique to the map of a single flagellin was greater than would be expected if Fla B were a precursor to Fla A. If one of the flagellins was modified post-translationally, on the other hand, and the modification were 100% efficient, then the given result could be reconciled with a precursor-product relationship between two flagellins. This possibility was tested by determining the amino acid composition of peptides whose mobilities differed in the maps of Fla A and Fla B. If the above post-translational modification hypotheses were correct, and if the modifications were labile to acid hydrolysis, then peptides from Fla A and Fla B with

![Fig. 3. DEAE-cellulose column chromatography of Fla A and Fla B.](http://www.jbc.org/)
the same amino acid composition but with different mobilities should be found. If the post-translational modifications were not acid-labile, then the compositions of unmatched peptides should be identical, except for slight differences involving the modified residues. Of 13 pairs of peptides analyzed, no examples of this type were found. Nine of these peptide pairs had similar mobilities and identical amino acid compositions (Table II). Two other peptide pairs, isolated from preparative thin layer plates, had similar chromatographic or electrophoretic mobilities and similar, but not identical, amino acid compositions (Table III), suggesting that they were related by simple amino acid substitutions. Peptides A1 and B1 had the same mobilities with an apparent serine (Fla A)-threonine (Fla B) substitution. Peptides A5 and B5 had different chromatographic mobilities but the same electrophoretic mobility, with compositions consistent with several amino acid substitutions. The above data argue against the post-translational modification hypothesis as an explanation for the unmatched peptides and support the hypothesis that these two proteins arise from two separate genes.

Among additional tryptic peptides purified by ion exchange chromatography and reversed phase high pressure liquid chromatography, several identical and similar pairs were obtained (Tables I and III), some previously identified in the peptide maps (peptide pairs A5, B5 and A16, B13) and others not identified on the maps (A23, B23) (Fig. 2). Comparison of the amino acid compositions in these peptide pairs showed the possible amino acid substitutions in each pair (although not necessarily in the order given): aspartic acid, glycine, and alanine (A5) for two serines and leucine (B5); aspartic acid and glycine (A16) for threonine and serine (B13); threonine and alanine (A23) for serine and valine (B23). In order to determine whether, in fact, these peptide pairs had identical sequences with specific amino acid substitutions, we sequenced the A16, B13 peptide pair (Table IV). The 16 amino acid residues in each peptide were found to have the identical amino acid sequence, except that, at residue 4, an alanine was present in the Fla B peptide (B13) in place of a glycine; at residue 7, a threonine replaced an aspartate; and at residue 9, a serine replaced an alanine in Fla A peptide, A16. These results are totally consistent with the amino acid substitutions implicated by the amino acid compositions, and suggest that the two flagellins come from similar, but not identical genes.

Organization of Fla A and Fla B in the Flagellar Filament—There are three possible arrangements of the two flagellins in flagellar filaments: (a) only Fla A or only Fla B in each filament, (b) both Fla A and Fla B uniformly distributed in each filament, or (c) both Fla A and Fla B in each filament, but in a nonuniform distribution along the length of the filament. To distinguish among these possibilities, we generated, by sonication, filaments of various lengths that remained attached to the flagellar hook, and then isolated them by immunoprecipitation using hook-specific antibodies. Their lengths were measured and their protein compositions determined by SDS-gel electrophoresis. The filament proteins in the hook immune precipitates show a striking difference in the relative amounts of Fla A and Fla B in filaments of different lengths (Fig. 5). Although the total population of flagella contained 82% Fla A (Fig. 5A), the hook immune precipitate contained only 67% Fla A (Fig. 5B). The filaments that were attached to hooks in this sample had an average length of 0.90 μm. After only 5 s of sonication, the average filament length in the hook immune precipitate was decreased to 0.34 μm, and the precipitate now contained 81% Fla B (Fig. 5C). Further decrease in the average length of the filament did not change the proportion of Fla B (Fig. 5D). Both possibilities a and b predict a constant ratio of Fla A and Fla B regardless of the length of the filament attached to the hook. Because the Fla A/Fla B ratio was not constant in the above experiments and was not the same as in the total population of flagella, it is most likely that each filament contains both Fla A and Fla B and that the region of the filament proximal to the hook, with a minimum length of about 0.3 μm, is composed predominantly of Fla B.

The presence of both flagellins in each filament and their organized arrangement along the length of the filament suggests that normal assembly and function of the flagellum may require both flagellins. However, in agreement with Fukuda et al. (17), we found that each flagellin reaggregated in vitro to form filaments with sine waves of the same frequency and
Flagella from linkage groups represented by flaX137 and flaY129, which have filaments less than one-sixth the length of the wild type, were compared to sonicated wild type flagella of equivalent size. In the electron microscope, the mutant's short flagella showed no obvious abnormality in structure other than the abbreviated filament length. However, flagella preparations from each of these mutants contained over 90% Fla A. When the mutant flagella were precipitated with hook antibody, the hook proximal filament (with an average length of 0.32 μm; for distribution, see Fig. 7) was also found to be composed of over 90% Fla A (Fig. 7, A and B). This protein composition is in marked contrast to that in the wild type strain, where the hook-proximal 0.32-μm region of the filament would be composed of 80% Fla B (Fig. 8) in a total filament length of 6 to 7 μm. One of the mutants, flaY129, is temperature-sensitive for motility (19). At the permissive temperature, however, the filaments were still composed of over 90% Fla A. The hook immune precipitate of these flagella also showed the preponderance of Fla A (Fig. 7C). The average length of the filament attached to the hook obtained from flaY129 grown at the permissive temperature was 0.7 μm. The length distribution of filaments attached to hooks showed that one-half were shorter than 0.50 μm (Fig. 7C). Even if all of the shortest filaments were the products of aberrant assembly, the longer filaments presumably correspond to the flagella from the motile cells. If these flagella had been of normal composition, then about 30% Fla B would have been detected in the immune precipitate. These observations suggest that both flagellins appear to be required for efficient assembly of flagellar filaments of normal length.

**DISCUSSION**

**Two Genes for the C. crescentus Flagellin**—Nine pairs of flagellins are the products of two genes which may have appeared by duplication of an ancestral gene. Multiple genes for one particular protein are uncommon in bacterial. In *Escherichia coli*, the naturally occurring examples are two genes for the monomers of the trimeric enzyme, ornithine carbamoyltransferase (argl and arg2) and two genes for elongation factor Tu (ttuA and tufB) (for review, see Ref. 33). In both of these cases, the two genes are separated on the genome. The presence of multiple inverted repeat sequences in the *E. coli* chromosome and the consequent structural similarity of regions of the chromosome to transposons has led to the proposal that the inverted repeat sequences have participated in the duplication of regions of the *E. coli* genome (35, 36). Since *Caulobacter* has been shown to have a significant population of inverted repeat sequences (4% of the total genome) (37), transpositions could explain the duplication of the flagellin genes.

Although the location on the chromosome of these flagellin genes is not known, it can be argued that they are close together. The rates of both Fla A and Fla B synthesis double during the initial period of flagellin synthesis (17). In synchronized cultures of *Bacillus subtilis*, the rates of enzyme (de-
hydroquinase and phosphoglucomutase) and flagella synthesis and the transduction frequencies of purA6, hisA1, and leu6 were found to double during DNA synthesis (38). The relative order of appearance of doubled rates and increased transductants corresponded to the order of the genes on the genetic map, suggesting that the replication of the DNA segment on which the gene was located caused the observed increase in gene expression. Since the synthesis rates of both Fla A and Fla B apparently double simultaneously, this would argue that their genes are very close together, if not adjacent. Analysis of cloned flagellar genes and genetic mapping is currently in progress in order to define further the genetic organization of the genes for Fla A and Fla B in relation to the 28 known loci (19) responsible for the synthesis and localized assembly of the flagellum.

**Ordered Assembly of the Filament**—Analysis of the flagellin content of filaments sonicated to various lengths and precipitated with anti-hook antibody showed that the two flagellins are arranged in a specific order along the length of each filament: Fla B predominates proximal to the hook and Fla A predominates distal to the hook. Among the published compositions of bacterial flagellar filaments, the *C. crescentus* filament is unique in the distribution of its constituent proteins. Because both flagellins apparently are present in each flagellar filament, both flagellin proteins must be synthesized in a given cell.

An alternative explanation for the enrichment for Fla B in the hook immune precipitates is that phase variation occurs in *Caulobacter* and the filaments composed of Fla A are more sensitive to sonication. This appears unlikely, however, for the following reasons. (a) An additional claim about the stability of the two types of filaments is necessary. The Fla A filaments would have to be less stable than the Fla B filaments even when subjected to the purification procedure, because the isolated unsonicated flagella already show an enrichment for Fla B in the filaments attached to hooks (Fig. 5B). (b) The phenotype of the two mutants, flaX137 and flaY129, are inconsistent with this explanation. If there were an assembly defect, both flagellins should be equally affected (in the simplest case) by the defect and there should not be a vast predominance of Fla A in the assembled filaments. If the low amount of Fla B detected in extracts of the culture were due
to a decreased efficiency of expression of the Fla B phase, then there is no reason why Fla A does not form functional filaments of appropriate length. In one mutant, flaY129, Fla A forms functional filaments which are not fragile. If this were due to a mutation in the Fla A structural gene that increased the stability of the filaments, then there is no explanation for the lack of Fla B filaments. (c) If expression of the two flagellin genes were controlled by phase variation then the rate of change between the two phases in Caulobacter would have to be much more rapid than the rate of change seen in Salmonella. If logarithmic growth were continuous (which it is not) throughout the entire 4-day period during which Caulobacter is grown from a single colony to a 1-liter culture, then only 64 generations would have elapsed. Although a maximal value, this is far below the several hundred generations required to give a constant proportion of flagellins in Salmonella. According to Stocker's (18) formula for calculation of the forward and reverse "mutation rate" in Salmonella phase variation (rate = proportion of mutants per number of generations), the rate of mutation in Caulobacter would have to be 0.064 or 0.012 (starting from a Fla B-producing cell) and 0.2/64 or 0.0031 (starting from a Fla A-producing cell) in order to achieve the observed 4:1 ratio of Fla A to Fla B. The latter rate is about equal to the fastest rate of phase switching in Salmonella, but is 10 to 100 times faster than the majority of the observed switching rates.

Three-dimensional reconstruction of the hook by optical diffraction analysis (39) has indicated that the filament end of the hook is concave, perhaps forming a nucleation site for the polymerization of the first monomers of flagellin. These first monomers are likely to be Fla B. A strict requirement for Fla B would argue that Fla B was the ancestral gene and that in gene duplication Fla A may have lost the ability to polymerize efficiently onto the hook. The three-dimensional reconstruction of the hook showed a hole in the center that was large enough to allow passage of flagellin on its way to assembly at the growing end of the filament (39). The results of experiments with E. coli and Salmonella flagella suggest that in vivo their filaments elongate from the distal end (40, 41). If, in Caulobacter, the subunits are assembled as soon as they are synthesized, results of experiments measuring the 4:1 ratio of flagellin synthesis in synchronized cultures imply that Fla A and Fla B are mixed in about equal proportion at the hook proximal end of the filament and that the proportion of Fla A then increases until Fla A alone is found at the tip of the filament. This prediction, which has been made previously by Lagenaur and Agabian (5), is partly substantiated by the results presented here. The proportion of Fla A in the filament does increase in the regions further from the hook until Fla A becomes the predominant component. However, the results presented here additionally indicate that the initial one-fifteenth of the filament is composed predominantly of Fla B (Fig. 8). This initial one-fifteenth of the filament length cannot contain the entire amount of Fla B present in flagella released into the culture medium. Exclusive segregation of Fla B to the hook proximal region would predict a length of 1.2 to 1.4 μm for this region in a natural filament length of 6 to 7 μm (4). If, in Caulobacter, the subunits are assembled as soon as they are synthesized, then it is conceivable that Fla B could be totally confined to hook proximal regions despite the 4:1 ratio. However, an excess of short filaments was not found when an unsonicated preparation of filaments was examined. Thus, there must be a region of the filament...
composed of a mixture of Fla A and Fla B. However, it is still unknown whether there is a region of pure Fla A as suggested by the incorporation of only labeled Fla A into the flagella of swarmer cells (16). The production of antibodies specific for Fla A and Fla B and subsequently testing them for a differential reaction with the populations of flagellates is the most direct way to determine the arrangement of the flagellins in each filament. Because of the similarity of the two proteins, however, such antibodies have been difficult to obtain.

Although in vitro experiments have shown that Fla A and Fla B can each assemble into filaments or can elongate short fragments that are composed of the other protein (7), the flagellins do not seem to be interchangeable in vivo. The absence of the short region of Fla B adjacent to the hook in the nonmotile mutants flaX137 and flaY129 suggests that this region is required for the proper assembly of a normal length filament. Fukuda et al. (42) have independently come to the same conclusion based on (a) their isolation of mutants that assemble short filaments and have only Fla A intracellularly and (b) their preparation of hooks with short, attached filaments that contain equal amounts of Fla A and Fla B. The filament phenotype of flaX137 and flaY129 cannot be explained by the absence of Fla B synthesis because both classes have intracellular Fla B. 3 Fla A appears to be normal in these mutants as judged by a normal tryptic peptide map. That functional filaments apparently composed of Fla A are formed at 23°C by flaY129 suggests that under some conditions Fla A can polymerize into functional filaments in vitro. The structure of the flagellum in nonmotile mutants of Salmonella indicates that the flagellum will assemble up to the point of the component for which the structural gene is mutated (43). If Fla B is indeed required for assembly of the filament, then the analysis of Suzuki et al. (43) suggests that the defect in these two mutants is in Fla B. For example, Fla B could be unmethylated, could have an altered sequence, or could be synthesized at the wrong time during the cell cycle, or could be synthesized in amounts too low to be assembled into a filament. Any of these alterations could interfere with the coordinated assembly of Fla A and Fla B into a functional flagellum. However, two other explanations for Fla X and Fla Y phenotype are possible. (a) The structure of Fla A could be altered, and this would interfere with the assembly of Fla B. However, tryptic peptide maps of Fla A isolated from cultures of flaX137 and from flaY129 were found to be identical with those of Fla A from the wild type strain. Although a point mutation in the Fla A gene cannot be ruled out, the simplest conclusion is that Fla A is not altered in these strains. (b) Both Fla A and Fla B could be normal and could be synthesized at the correct time in the cell cycle, but the assembly machinery could be abnormal.

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1 Johnson, R. C., unpublished data.

REFERENCES
Prolinolys in Proteins of the Caulobacter Flagellar Filament

Supplementary Material: "Characterization of the Proteins of the Caulobacter Flagellar Filament Protein Analysis and Filament Organization" by H. McNair, R. Stollm, and E. Shep, following

EXPERIMENTAL PROCEDURE

Materials: All chemicals were reagent grade unless otherwise noted. Trypsin and the sample buffers and solutions were kept on ice throughout the experiment. Samples were subjected to electrophoresis at a constant current of 20 mA in a vertical gel tank filled. Acrylamide, Acrylamide, Acryl ammonium, and Gelatin were purchased from Fischer Scientific. Other reagents were Schenck, Schenck, or Eastman. All other chemicals were obtained from Fisher.

Reagents and Collected Conditions: Gelatin (Bovine, Type II 20 g/mL) was used to construct the gel. Gels were prepared as follows: 20 g/mL gelatin was dissolved in 2 mL of 10% acetic acid and then cooled. The gelatin solution was added to 100 mL of 10% acetic acid, and then boiled until the gelatin solution was clear. The solution was then cooled to room temperature. The solution was then cooled to room temperature.

Preparation of flagellin and flagellar filament. A solution of flagellin was prepared as follows: 10 mL of 10% acetic acid was added to 100 mL of 10% acetic acid, and then boiled until the solution was clear. The solution was then cooled to room temperature. The solution was then cooled to room temperature.

RESULTS

The PAGE gels were run at 10 kV for 2 h in a gel tank filled. The gelatin solution was clear. The PAGE gels were run at 10 kV for 2 h in a gel tank filled. The gelatin solution was clear.
Proteins of the Caulobacter Flagellar Filament

Table I

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Table II

Amino acid composition of Fla A and Fla B peptides

Table III

Amino acid composition of similar cryptic peptides

Table IV

Amino acid sequence of Fla A peptides A11 and Fla B peptide B13
Characterization of the proteins of the Caulobacter crescentus flagellar filament.
Peptide analysis and filament organization.
A Weissborn, H M Steinmann and L Shapiro