JOURNAL OF BACTERIOLOGY, Dec. 1977, p. 1027-1030

Vol. 132, No. 3
Printed in U.S.A.

Reconstitution and Purification of Flagellar Filaments from *Caulobacter crescentus*

MICHAEL SHEFFERY AND AUSTIN NEWTON*

Department of Biology, Princeton University, Princeton, New Jersey 08540

Received for publication 26 August 1977

Filaments from isolated flagella of *Caulobacter crescentus* have been purified by successive dissociation and reconstitution. After the second and third reconstitutions from subunits in 0.8 M sodium citrate, filament preparations contained only two proteins, flagellin A (26,000 daltons) and flagellin B (28,000 daltons). There was some enrichment for flagellin A during reconstitution by this procedure, since isolated flagella contained flagellin A and flagellin B in a ratio of approximately 3.8:1 and filaments after the third reconstitution contained the two proteins in a ratio of 5.0:1.

The flagellum is a useful marker for the study of developmental control in *Caulobacter crescentus* because it is one of several localized surface structures formed at one pole of the stalked cell just before cell division. The constituent proteins of the filament are also synthesized periodically in the cell cycle at the time of flagellum formation (9, 12). Another advantage of the flagellum is that it can be readily isolated and purified: the filament, hook, and rod are released into the medium when the motile swarmer cell develops into the nonmotile stalked cell, and the flagella can be collected from the culture medium and purified by density gradient centrifugation (12). Lagenaur and Agabian have reported that filament preparations isolated by this procedure contain two proteins (molecular weights of 25,000 and 28,000) in a ratio of 3.3:1 (6). We report here a method for the in vitro reconstitution of intact filaments from dissociated protein subunits. This procedure provides a rapid and efficient purification of the flagellin proteins, referred to below as flagellin A and flagellin B, and the results of reconstitution experiments confirm the conclusion that the flagella of *C. crescentus* contain both of these proteins.

Bacterial filaments are generally dissociated into protein monomers by lowering the pH of the filament solution to 2.0 (1). In *Salmonella* the protein subunits can then be reassembled into filaments by "salting out" at neutral pH with ammonium sulfate or sodium citrate at concentrations higher than 0.5 M (4, 14). Only under these conditions will assembly occur without short pieces of filaments to act as seeds. For the study of reassembly of filament proteins of *C. crescentus*, we have determined that the optimal conditions for reconstitution are 0.8 M sodium citrate and pH 7.5.

Flagella were isolated from a 4-liter culture of *C. crescentus* strain CB15 (ATCC 19089) that had been grown to late log phase in a medium made of equal parts of a peptone-yeast extract medium (PYE; 10) and an imidazole-buffered medium, G1 (11). Cells were removed from the culture fluid by two successive centrifugations at 11,790 × *g* for 20 min; except where noted, all steps were carried out at 4°C. Sodium azide (10 mM) was added to the supernatant fraction, the flagella were collected by sedimentation at 105,650 × *g* for 90 min, and the pellets were suspended in 3.0 ml of buffer A [0.02 M tris(hydroxymethyl)aminomethane-10 mM NaN_3, pH 7.5]. This suspension was then centrifuged at 12,000 × *g* for 10 min to remove the remaining cells. The cell pellet was washed once with 0.5 ml of buffer A and centrifuged as described above. The combined supernatant fractions (concentrated culture fluid; see Table 1) were the starting material for the first reconstitution procedure. The flagella in this concentrated solution were depolymerized by adjusting the solution to pH 2.0 with 1 M HCl and stirring for 1 h. The solution was then centrifuged at 110,900 × *g* for 100 min to remove any remaining particulate matter. Filaments were reconstituted from the flagellin subunits by dialyzing the acidified supernatant fraction overnight at room temperature against 1 liter of 0.02 M tris(hydroxymethyl)aminomethane-10 mM NaN_3-10% glycerol-0.8 M sodium citrate, pH 7.5. The precipitate was then separated from the dialysate by centrifugation at 17,370 × *g* for 20 min. The opaque, whitish pellet was collected, resuspended in 3.0 ml of buffer A, and centri-

TABLE 1. *Flagellin A and flagellin B in reconstituted filaments*

Sample	Flagellin[a]			Total protein[b] (mg)	Recovery[c] (%)	Total recovery (%)
	A	B	A/B[d]			
Concentrated culture fluid	5,160	1,350	3.8	8.4		
First reconstitution	9,640	2,520	3.8	6.9	82	82
Second reconstitution	9,460	2,370	4.0	1.2	38	14
Third reconstitution	7,330	1,460	5.0	0.28	23	3.3

[a] Flagellin was determined by scanning negatives of gels that had been fixed in 10% glacial acetic acid and stained with Coomassie brilliant blue, as described in the legend to Fig. 2. The negatives were scanned with a Joyce-Loebel recording microdensitometer. The relative amounts of flagellin A and flagellin B determined by this procedure were shown to be linear with total flagellin applied to the gel in the range of 2 to 15 μg; all determinations were carried out at 10 μg of protein.

[b] Protein was determined by the method of Lowry et al. (7).

[c] Recovery was calculated individually for each reconstitution.

[d] Average of two flagellum preparations.

fuged again at 110,900 × *g* for 90 min. The transparent pellet, which contained reconstituted filaments, was suspended in buffer A and used as the starting material for two additional cycles of depolymerization and reconstitution by the above procedure (Table 1).

Figure 1A is an electron micrograph of negatively stained flagella that were used as the starting material for reconstitution. Hooks and rods could be observed on the ends of many filaments (arrows). Only filaments were present after each of the three reconstitutions. Figure 1B is an electron micrograph of a preparation after one cycle of dissociation and reconstitution. The filaments were shorter than those in the starting material, and structures with the characteristic thickness and morphology of hooks or rods could not be detected. The wavelength of most reconstituted filaments was comparable to those of filaments isolated from the culture medium (Fig. 1A); some short straight filaments were also observed.

Fractions from a reconstitution experiment were analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels; a stained gel is shown in Fig. 2. Several contaminating proteins were concentrated from the culture supernatant along with the filaments (column 1). After acidification of the concentrated culture fluid, most of the contaminating proteins were removed in the precipitate (column 3) while flagellin remained acid soluble (column 2). After the first reconstitution (column 4), only trace contaminants were present, and only flagellins A and B could be detected after the second and third reconstitutions (column 5 and 6). As much as 30 μg of filaments obtained from the second and third reconstitutions was analyzed on gels without observing contaminating proteins (data not shown).

The lower-molecular-weight protein (flagellin A) has a molecular weight of 26,000, and the higher-molecular-weight protein (flagellin B) has a molecular weight of 28,000, as calculated from protein standards in the same gel system (data not shown). These values correspond closely to those determined earlier for the two proteins present in filament preparations purified from *C. crescentus* strain CB13B1a by density gradient centrifugation (6).

The protocol for the purification of flagellin is shown in Table 1. The recovery of flagellin after each polymerization generally varied from 40 to 80%, depending on the protein concentration; a higher total yield of flagellin should result by starting with larger amounts of flagella and by reducing the volumes used for repolymerization. The average ratio of flagellin A/flagellin B found in isolated flagella was 3.8:1. There is some indication that this ratio increases with repeated polymerizations, partially as a result of the greater solubility of flagellin A at acid pH.

The presence of flagellin A and flagellin B in filaments after successive reconstitutions confirms the report (6) that the flagellar filaments of *C. crescentus* contain two proteins. Although *Escherichia coli* filaments have only one protein subunit (2), there are at least two other bacteria with multiple species of flagellin. *Salmonella typhimurium* synthesizes two immunologically distinct flagellins with separate structural genes; since only one of the two genes functions at a time, individual filaments contain a single flagellin species (3). Smith and Koffler have reported that *Bacillus pumilis* filaments also contain two subunits in a ratio of 7:3 (13). Their data suggest that individual *B. pumilis* cells produce both flagellins, but this has not been conclusively shown. It is also unclear in *C. crescentus* whether the two flagellins are produced by the same cell and are thus constituents of the same filament, since the reconstitution re-

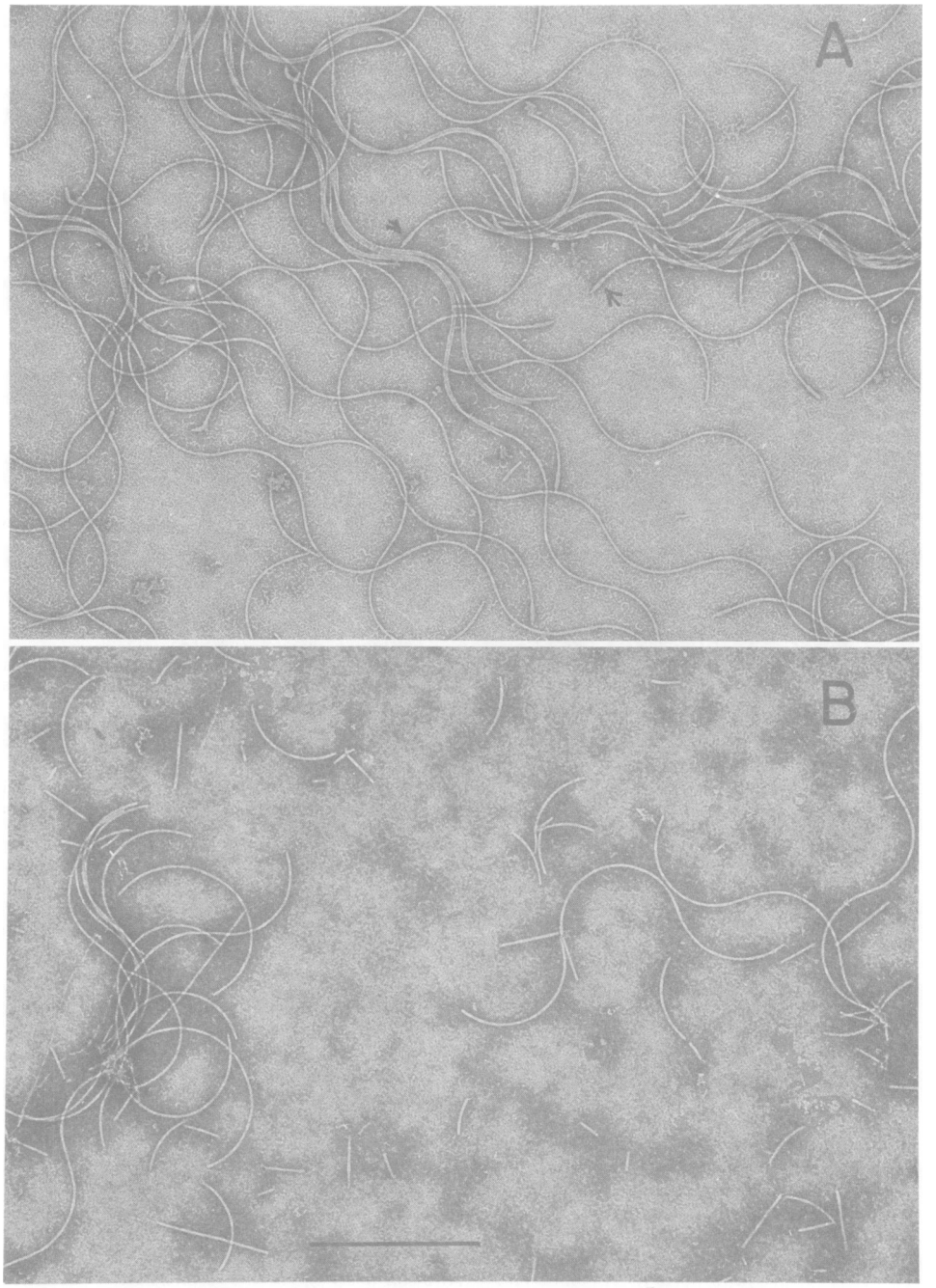

FIG. 1. *Electron micrographs of isolated flagella and reconstituted filaments. Portions of concentrated flagella (A; see Table 1) and reconstituted filaments (B, first reconstitution; see Table 1) were diluted, fixed in 0.5% formaldehyde, and spotted on Formvar-coated copper grids (T. Pella Co.). After 30 min the grids were washed three times with cytochrome c (1 mg/ml) and stained with 1% phosphotungstic acid in 0.5% ammonium acetate, pH 7.5. Grids were examined in a JOEL 100C electron microscope at an instrumental magnification of ×8,300. Arrows indicate hook and rod structures in panel A, and the bar length is 1 μm.*

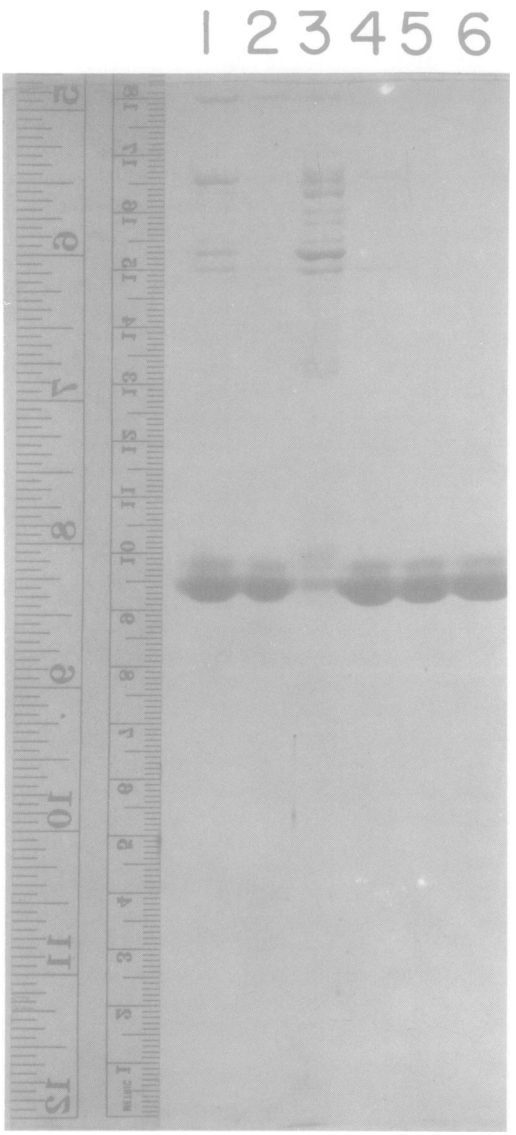

FIG. 2. *Reconstitution and purification of filaments. Flagella and reconstituted filaments (see Table 1) were analyzed by electrophoresis. Fifteen micrograms of proteins from fractions obtained in a reconstitution experiment was applied to a 12.5% sodium dodecyl sulfate-polyacrylamide gel (5). Flagella were harvested as described in the text to obtain the concentrated culture fluid (well 1). This material was adjusted to pH 2.0 and centrifuged to obtain the acid-soluble (well 2) and acid-insoluble (well 3) fractions. Filaments were reconstituted from the acid-soluble material as described in the text: first reconstitution (well 4), second reconstitution (well 5), and third reconstitution (well 6). The gel was stained with 0.25% Coomassie brilliant blue.*

sults (above) can be explained by the incorporation of flagellins A and B into the same or separate filaments. The isolation of mutants that synthesize only flagellin B and do not make flagella (8) suggests that at least flagellin A is required for assembly of filaments. It is possible, however, that flagella are missing in these strains because the expression of other genes required for flagellum formation is also affected.

This work was supported by grant BSMS70-00422 from the National Science Foundation, by Public Health Service grant GM22299 from the National Institute of General Medical Sciences, and by the Whitehall Foundation.

LITERATURE CITED

1. **Abram, D., and H. Koffler.** 1964. *In vitro* formation of flagella-like filaments and other structures from flagellin. J. Mol. Biol. **9**:169–185.
2. **Depamphilis, M. L., and J. Adler.** 1971. Purification of intact flagella from *Escherichia coli* and *Bacilis subtilis*. J. Bacteriol. **105**:376–383.
3. **Iino, T.** 1969. Genetics and chemistry of bacterial flagella. Bacteriol. Rev. **33**:454–475.
4. **Iino, T.** 1974. Assembly of *Salmonella* flagellin *in vitro* and *in vivo*. J. Supramol. Biol. **2**:372–384.
5. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**:680–685.
6. **Lagenaur, C., and N. Agabian.** 1976. Physical characterization of *Caulobacter crescentus* flagella. J. Bacteriol. **128**:435–444.
7. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
8. **Osley, M. A., and A. Newton.** 1977. Mutational analysis of developmental control in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. U.S.A. **74**:124–128.
9. **Osley, M. A., M. Sheffery, and A. Newton.** 1977. Regulation of flagellin synthesis in the cell cycle of *Caulobacter*: dependence on DNA replication. Cell **12**:393–400.
10. **Poindexter, J. S.** 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. **28**: 231–295.
11. **Schmidt, J., and R. Y. Stanier.** 1966. The development of cellular stalks in bacteria. J. Cell Biol. **28**:423–436.
12. **Shapiro, L., and J. V. Maizel, Jr.** 1973. Synthesis and structure of *Caulobacter crescentus* flagella. J. Bacteriol. **113**:478–485.
13. **Smith, R. W., and H. Koffler.** 1971. Bacterial flagella. Adv. Microb. Physiol. **6**:219–338.
14. **Wakabayashi, K., H. Hotani, and S. Asakura.** 1969. Polymerization of *Salmonella* flagellin in the presence of high concentrations of salts. Biochim. Biophys. Acta **175**:195–203.