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Flagellar Assembly Mutants in *Escherichia coli*[1]

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Genetic and biochemical analysis of mutants defective in the synthesis of flagella in *Escherichia coli* revealed an unusual class of mutants. These mutants were found to produce short, curly, flagella-like filaments with low amplitude (~0.06 μm). The filaments were connected to characteristic flagellar basal caps and extended for 1 to 2 μm from the bacterial surface. The mutations in these strains were all members of one complementation group, group E, which is located between *his* and *uvrC*. The structural, serological, and chemical properties of the filament derived from the mutants closely resemble those of the flagellar hook structure. On the basis of these properties, it is suggested that these filaments are "polyhooks", i.e., repeated end-to-end polymers of the hook portion of the flagellum. Polyhooks are presumed to be the result of a defective cistron which normally functions to control the length of the hook region of the flagellum.

Biochemical and genetic analysis of mutants defective in the assembly and functioning of bacterial flagella has been undertaken in a number of laboratories with the goal of using this system as a model for studying the mechanism involved in gene regulation and expression (14, 22), protein structure and function (6, 7, 15, 20), subcellular organelle assembly (4, 16, 18), and primitive behavioral systems (3, 12). *Escherichia coli* mutants defective in the assembly of flagella can be obtained easily by selecting clones resistant to the flagellotropic phage χ (21, 23). However, obtaining biochemical information from these mutants is usually difficult for two reasons: (i) most of these mutants possess no flagella and are phenotypically indistinguishable; (ii) the assembly of flagella probably involves many structural proteins which lack a specific activity that can be easily measured.

During the course of genetic analysis of *fla* mutants in *E. coli*, we became aware of a class of mutants with an unusual phenotype. Although these mutants were nonmotile with respect to translational motion, they showed a rapid spinning motion. They produced curly filaments with a wavelength of ~0.14 μm which were 1 to 2 μm in length. What appeared to be a normal flagellar filament was often attached distal to this curl filament. Four independent mutants showed this phenotype and were all found to be members of complementation group E (Silverman and Simon, *in manuscript*). In this paper we report the results of an investigation of the nature of the filaments produced by these mutants. Our experiments indicate that they are composed of a subunit protein very similar to the flagellar hook protein. We suggest that a defect in a cistron which normally codes for a protein that terminates the hook structure results in this filament which we now call polyhook filament.

[1] A preliminary report of these findings was presented at the Annual Meeting of the American Society for Microbiology, 21 April 1972.

MATERIALS AND METHODS

Media. Tryptone broth contained per liter of distilled water: 10 g of tryptone (Difco), 5 g of NaCl, and 0.1 g of thymine. L broth contained per liter of distilled water: 10 g of tryptone, 10 g of NaCl, 5 g of yeast extract (Difco), and 0.1 g of thymine. L agar plates were prepared by adding 1.5% agar (Difco) to L broth. Motility plates were prepared by adding 0.35% agar to tryptone broth. Minimal medium contained per liter of distilled water: K_2HPO_4, 11.2 g; KH_2PO_4, 4.8 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $Fe_2(SO_4)_3$, 0.5 mg; glucose, 5 g; and thiamine, 1 mg. The $MgSO_4 \cdot 7H_2O$ glucose and thiamine were added aseptically after autoclaving. Amino acids and thymine, if required, were added to a final concentration of 100 mg/liter. Minimal plates were prepared by adding 1.5% agar to minimal medium.

Bacteria. The *flaE* mutants described in this study were derived from MS1350. MS1350 is *his*, *argE*, *thy*, *thi*, *sup*$^+$, *galU*, *uvrC*, *str*^{R}, *fla*$^+$ and was constructed by M. Silverman in M. Simon's laboratory from the K-12 strain AB1884 which was obtained from J. Adler. Two of the *flaE* mutants are suppressible by a Φ80 d sup_{III} transducing phage supplied by J.

Abelson. These two are *flaE234*, and *flaE694*. The other two *flaE* mutants are *flaE1011*, and *flaE1071*.

Isolation of mutants. Mutants in flagellar assembly were selected for their resistance to the flagellotropic phage χ after mutagenesis with ethyl methanesulfonate (EMS). The procedure of M. Wright was used for EMS mutagenesis (25) except that minimal medium was used and 0.05 ml of EMS was added to 2.5 ml of cell concentrate. Phage resistance selection was accomplished on L agar plates at 37 C with a soft agar overlay consisting of a mixture of 2.5 ml of motility agar, plus 0.1 ml of exponential phase cells grown to allow the mutations to segregate, and 0.1 ml of phage, for a final multiplicity of infection of approximately 1. Survivors were restreaked twice and then tested for motility. We obtained paralyzed mutants (*mot*), flagellar mutants (*fla*), and *flaE* mutants (polyhook mutants) by this procedure. Glucose must be excluded from the media used to cultivate flagellated cells (2, 26) or cells with curl filaments (Silverman and Simon, *in preparation*), since the synthesis of these structures is subject to catabolite repression.

Electron microscopy. Exponential phase cells grown in tryptone broth at 37 C were washed gently two times with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.8, and spread on carbonized, Formvar-coated grids. Excess fluid was removed after 5 min. Antibody at 1/100 dilution, if added, was flooded on the grid after the cells and allowed to react for 3 min. One wash with 0.05 M Tris buffer followed. Antibody binding can be easily observed as the increase in the apparent width of the filament. Grids were stained with 2% phosphotungstic acid and examined with the Phillips 200 electron microscope.

Purification of curl filaments. The filaments obtained from *flaE* mutants are referred to as curl filaments or polyhooks. Curl filaments could be removed from cells by shearing a 100-fold concentrate of cells, harvested in late log or early stationary phase, for 1 min at 13,000 rev/min in an Omnimixer (Virtis Co., Inc., Gardiner, N.Y.). Cells were removed by two low-speed centrifugations at 10,000 × *g* for 5 min. The suspended filaments were then collected by centrifugation at 100,000 × *g* for 90 min. The pellet was resuspended overnight in 0.05 M Tris-hydrochloride, *p*H 7.8. A low-speed clearing spin removed insoluble material. Flagellin filaments were removed from the curl filament preparation by incubating the mixture at 56 C for 15 min. This treatment disaggregated the flagellin filament but had no apparent effect on the curl filament. After a low-speed spin to remove denatured protein, the curl filaments remaining were pelleted by high-speed centrifugation, leaving the flagellin subunits in suspension. Flagellin filaments could be prepared by shearing and concentrating flagella from MS1350 grown in tryptone broth. For amino acid analysis, it was necessary to purify flagellin more extensively and this was done by ammonium sulfate precipitation followed by isopycnic centrifugation in cesium chloride (8). Homogeneity was tested by sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis and electron microscope examination.

Purification of hook protein. Hook structure was purified by a modification of the procedure of Abram et al. (1). The flagellin filaments in 20 mg of a preparation of sheared wild-type flagella were disaggregated by heating at 56 C for 15 min. Remaining flagellar components were collected by centrifugation at 100,000 × *g* for 180 min and resuspended in 0.05 M Tris, *p*H 7.8. Electron microscope examination showed only hook-like structures remaining. Flagellin protein was still present, but about 50 μg of hook protein enriched by more than 100-fold remained. This was used for electrophoretic comparison on SDS acrylamide gel electrophoresis with curl filament protein.

Preparation of whole flagella. Flagella with basal structures were obtained by following the procedure of DePamphilis and Adler (5).

Antisera. Six-month-old rabbits were injected intramuscularly and in the toe pads with 0.5 mg of antigen in complete Freund's adjuvant every week for 3 weeks. One month later another injection was administered, and the following week the animal was bled and serum prepared. Anti-curl filament antibody had some anti-flagellin filament activity. This activity was removed by absorption with a preparation of flagellar filaments which contained less than 1% contamination by hook protein as determined by gel electrophoresis. Anti-curl filament antibody uncontaminated with anti-flagellin filament activity was also prepared by injecting curl filaments derived from *flaE flaF* double mutants. *flaF* (*hag*) is the complementation group which controls the structure of the flagellin protein (Silverman and Simon, *in press*).

Complement fixation assay. The thermal stability of the flagellin and curl filaments could be measured by following the loss of complement-fixing activity as a function of temperature (8). The resulting subunits did not bind complement in the presence of specific antibody. Each time point was held at the given temperature for 15 min and then cooled to 0 C. The procedure of Wasserman and Levine was used for complement fixation (24).

SDS gel electrophoresis. The curl and flagellin filaments as well as the hook structures were disaggregated according to the method of Dimmitt and Simon (9). The electrophoresis was performed according to the method of Gelfand and Hayashi (11). Gels were dialyzed overnight in 5% trichloroacetic acid and stained for about 12 hr with 0.25% Coomassie blue in 5% trichloroacetic acid. Gels were then destained in 7% acetic acid.

Amino acid analysis. Amino acid determinations were performed according to the method of Fuller and Doolittle (10). In order to detect tryptophan, *p*-toluenesulfonic acid hydrolysis was used (19).

Construction of double mutants. *flaE flaF* (*hag*) double mutants were obtained by using P1 transduction to contransduce a *uvr*$^+$ *flaF* (*hag*) fragment into a *uvrC flaE* recipient. The *hag* gene is more closely linked to the *uvrC* locus than *flaE* (Silverman

and Simon, *in press*), so the double mutant can be constructed by using *uvrC* as the selective marker. The method of Armstrong and Adler (3) was used to select uvr^+ recombinants with the exception that cells were plated on minimal medium agar for uvr^+ selection. Double mutants could be identified by their inability to give fla^+ recombinants with either *flaE* or *flaF* donor lysates whereas they give fla^+ recombinants with wild-type or other fla^- lysates.

RESULTS

Electron microscopic examination. All four group E mutants displayed unusual filaments ~1 to 2 μm long with a wavelength of ~0.14 μm and an amplitude of ~0.06 μm. Their width was slightly greater than a flagellin filament, or about 0.02 μm (Fig. 1A, 1B). Often a flagellin filament was attached distal to what we shall tentatively call a curl filament (Fig. 1C). The curl filament appeared to be attached directly to the basal assembly (Fig. 1D, 1E). The arrangement of subunits on the curl filament appeared to be helical whereas that of the flagellin filament appeared more like parallel rows. The wavelength of these filaments is much less than that reported for curly flagella which result from the polymerization of altered flagellin molecules (13). Further studies were carried out on mutant *flaE694*.

Antigenicity of curl filaments. *flaE* mutants produce curl filaments and flagellin filaments. Often the flagellin filament is found attached to the curl filament. Antibody specific for wild-type flagella binds only the flagellin filament and not the curl filament region. Upon electron microscope examination, this binding can be seen as an enhancement of the width of the filament (Fig. 2A). Conversely, anti-curl filament antibody binds only the curl filament region (Fig. 2B). Significantly, this anti-curl

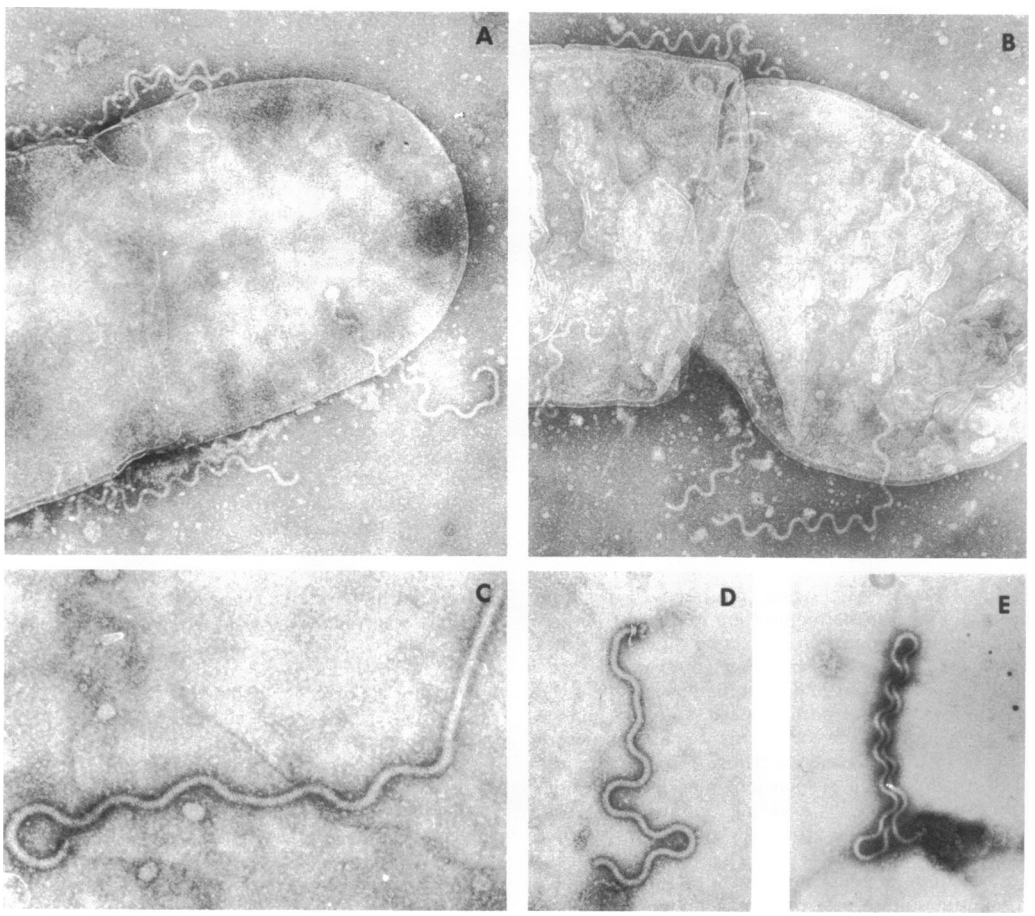

FIG. 1. *Curl filaments on Escherichia coli flaE694. A and B, Filaments are attached to cells. C, A flagellin filament is seen attached to the curl filament. D and E, The curl filament is attached directly to the basal flagellar structures.*

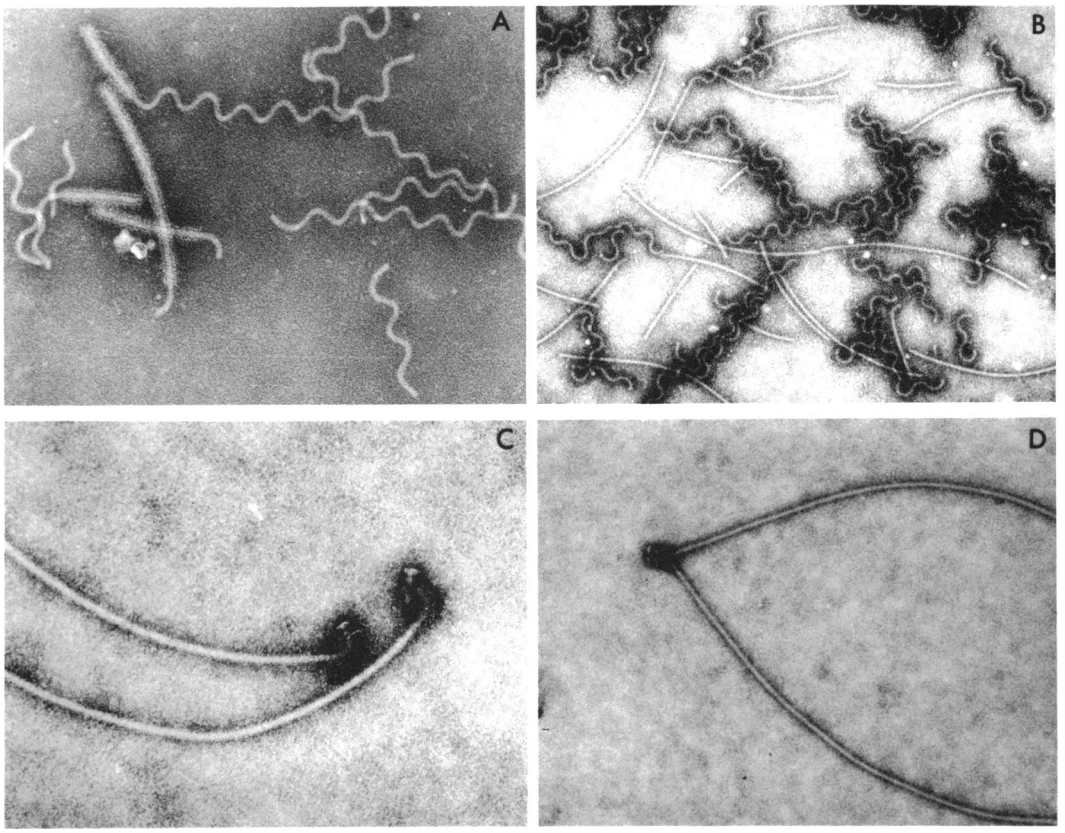

FIG. 2. *Antibody binding to curl and flagellin filaments and whole flagellar structures. A, Anti-flagellin filament antibody added to filaments from mutant flaE694. B, Anti-curl filament antibody added to filaments from mutant flaE694. C and D, Anti-curl filament antibody added to whole flagellar structures from Escherichia coli.*

serum also binds to the hook region of whole wild-type flagella (Fig. 2C, 2D).

Thermal stability of the curl filament. These initial observations suggested that the curl filaments are not composed of flagellin and appear to be related to the hook structures. Hooks have been shown to be more stable to heating than flagellin (1, 7, 9). We therefore tested the curl filament for its relative heat stability. Curl filaments do not disaggregate when heated at 46 C, which is the temperature at which flagellar filaments disaggregate. They begin to lose antigenic activity at 65 C and are completely disaggregated after heating at 72 C (Fig. 3). Their relative thermal stability allowed the separation of the curl filament from the flagellar filament by heating at 56 C to remove residual flagellar filament structures.

SDS acrylamide gel electrophoresis. In order to further compare the properties of flagellin with the hook protein subunit and the protein component of the curl filament, all were examined by SDS acrylamide gel electrophoresis. The mobility of curl filament protein relative to flagellin indicated that it is about 20% smaller than the flagellin molecule (Fig. 4). On the other hand, curl filament protein had the same mobility as hook protein derived from a preparation of wild-type flagella. The bulk of the other protein in the hook protein preparation has the same mobility as flagellin (Fig. 5) and represents residual flagellin.

Amino acid analysis. Curl filament protein is considerably different from flagellin with respect to its amino acid composition (Table 1). Specifically, curl filament protein has more residues per molecule of proline, methionine, and phenylalanine than flagellin (Table 1). No tryptophan or cysteine is present in either molecule. It was not possible to obtain enough hook protein to do amino acid analysis.

Double mutants. *flaE flaF* (*hag*) double mutants were prepared to test the relationship between the flagellin gene and the expression of

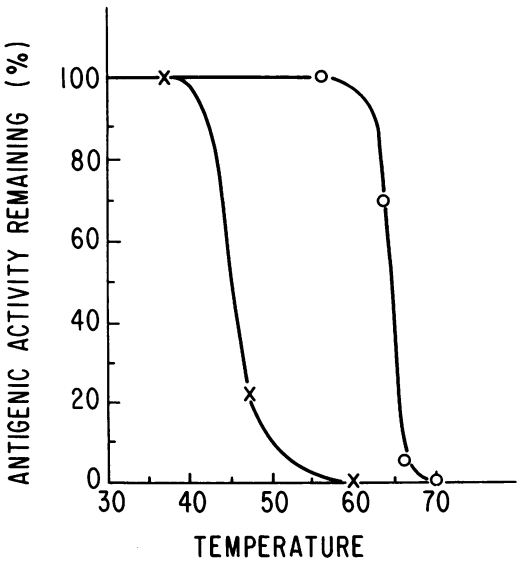

FIG. 3. *Thermal stability of curl and flagellin filament. Flagellin filament from wild-type bacteria, ×; curl filament from mutant flaE694, O.*

the curl filament phenotype. The result of introducing the *flaF* (*hag*) defect (several different *flaF* mutants were used) was to remove the flagellin filament distal to the curl filament. No change in the appearance, serology, or physical properties of the curl filament resulted. *flaE* mutants show no translational motion but do spin in tryptone broth. The *flaE flaF* double mutants are motionless or show slight jerking motions. Binding the double mutants together by means of anti-curl antibody results in violent counter-rotation of the bound pair of cells. The question of the relative contribution of the curl and flagellin filaments to the motion of the cells will be the subject of a subsequent report (Silverman and Simon, *in preparation*).

Antigenic specificity. The *flaE flaF* double mutant served as a source of curl filament devoid of flagellin filament contamination. Antisera prepared with filaments from the *flaE flaF* mutant react with low concentrations of curl filament and also react with high concentration of flagellin filaments sheared from wild-type cells (Fig. 6A, 6C). This reaction could result from the activity of partial hook structures associated with the flagellin filaments.

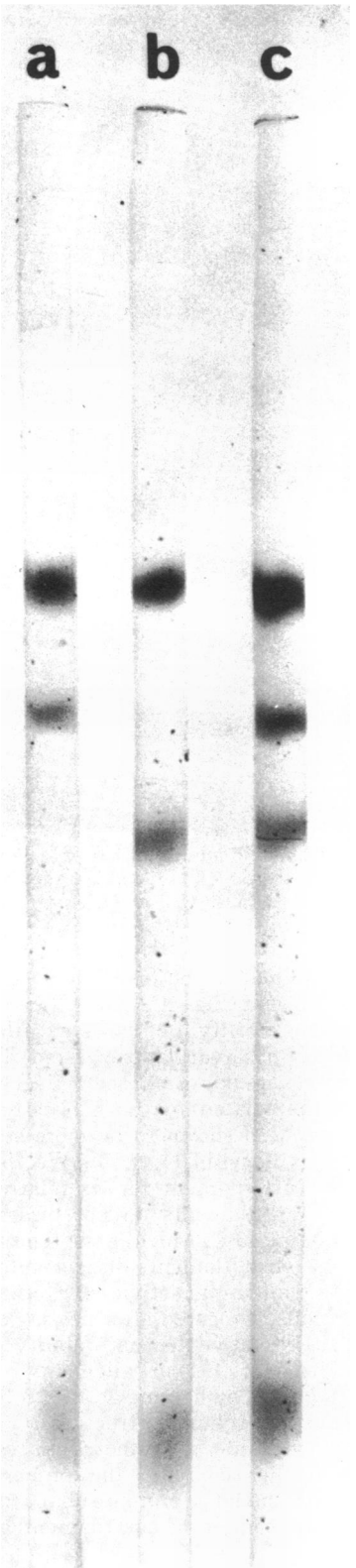

FIG. 4. *SDS acrylamide gel electrophoresis. Anode is at bottom. In all gels, top band is bovine serum albumin reference and bottom band is lysozyme reference. A, 25-μg flagellin filament protein from wild-type Escherichia coli. B, 25-μg curl filament protein from mutant flaE694. C, 25-μg curl protein plus 25-μg flagellin protein.*

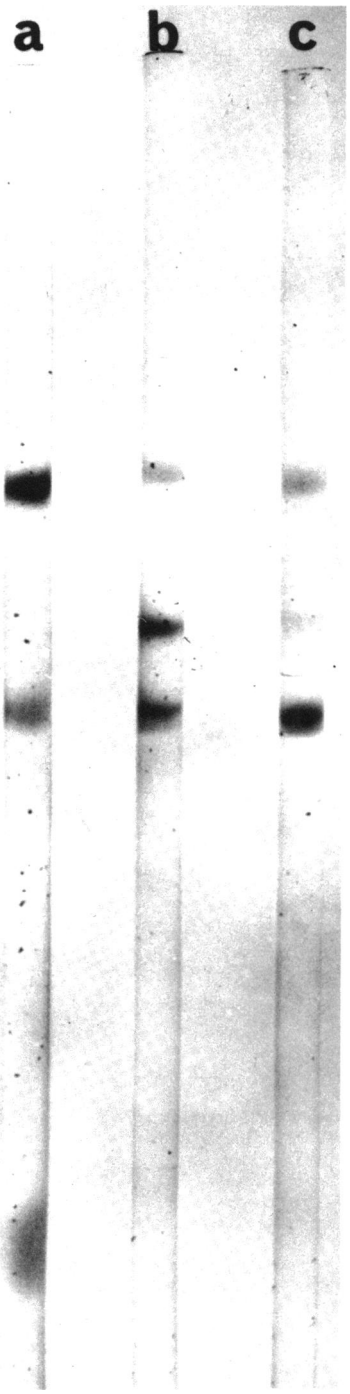

FIG. 5. *SDS acrylamide gel electrophoresis. Anode is at bottom. In all gels, top band is bovine serum albumin reference. Bottom band (not visible in B and C) is lysozyme reference. A, 25-μg curl protein from mutant flaE694. B, 50-μg protein from hook preparation enriched from wild-type Escherichia coli flagella. C, 10-μg curl filament protein and 20-μg hook protein preparation.*

TABLE 1. *Amino acid composition of flagellin and curl filament protein*[a]

Amino acid	Flagellin (mole %)	Curl filament (mole %)
Alanine	12.1	10.0
Arginine	3.3	2.0
Aspartic	17.0	17.8
Glutamic	8.5	8.9
Glycine	9.5	10.1
Histidine	0.4	0.6
Isoleucine	4.2	3.6
Leucine	7.6	7.7
Lysine	7.2	3.1
Methionine	1.0	2.6
Phenylalanine	1.4	4.4
Proline	1.4	2.6
Serine	8.6	9.1
Threonine	9.9	10.0
Tyrosine	2.3	2.9
Valine	5.8	4.4

[a] After 24-hr acid hydrolysis.

Similar reactions were observed between antisera prepared against *B. subtilis* hook structures and sheared flagella (9). The purified flagellin filaments were heated to 56 C for 15 min, and this heating abolished the reaction of sheared flagellin filaments with anti-flagellar antibody (Fig. 6B) but had no effect on the reaction with anti-curl filament antibody (Fig. 6C). The anti-curl filament antibody reaction with sheared flagellar filaments is apparently due to residual hook structures.

DISCUSSION

Several explanations for the nature of the curl filament and the genetic defect which produced it were entertained: (i) the curl filament arose from the polymerization of an altered flagellin molecule; (ii) the curl filament arose from the polymerization of a defective hook component; (iii) the curl filament might be the result of defective termination of the hook structure.

It is unlikely that the curl filament results from the polymerization of an altered or mutant flagellin for several reasons. The curl filament defect does not map with the flagellin gene nor does its complementation behavior make it a member of the *flaF* (*hag*) group (Silverman and Simon, *in preparation*). The curl filament often has a normal flagellin filament attached distal to it so the capacity to produce flagellin remains unaltered. The curl filament is antigenically dissimilar to the flagellin filament and shows greater thermal stability than the flagel-

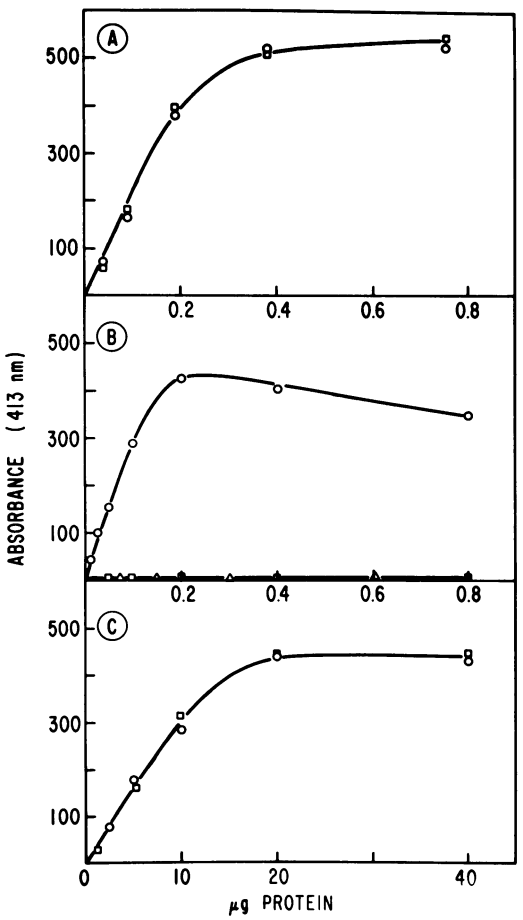

FIG. 6. *Reactivity of specific anti-curl filament antibody. A, Curl filament plus anti-curl filament antibody (○); curl filament 56 C for 15 min plus anti-curl filament antibody (□). B, Sheared flagellin filament plus anti-flagellin filament antibody (○); sheared flagellin filament 56 C for 15 min plus anti-flagellin filament antibody (□); curl filament plus anti-flagellin filament antibody (△). C, Sheared flagellin filament plus anti-curl filament antibody (○); sheared flagellin filament 56 C for 15 min plus anti-curl filament antibody (□).*

lin filament. It is also 20% smaller than the flagellin protein. The amino acid analysis showed more residues of proline, methionine, and phenylalanine per molecule than flagellin protein. The curl protein could not, therefore, be derived from the flagellin molecule in any simple manner. Furthermore, the presence of curl filaments on *flaE flaF* double mutants demonstrate that native flagellin molecules are not necessary for the expression of the curl filament phenotype.

There are considerable similarities between curl filament proteins and hook proteins in serological behavior and on SDS acrylamide gel electrophoresis. Furthermore the curl filament also shows higher thermal stability and greater resistance to disaggregation at low *p*H (Silverman and Simon, *in preparation*) than the flagellar filament. The hook structures have been reported to have similar properties (1, 6, 8). In addition, the curl filament is situated between the basal assembly and the flagellin filament, the place where the hook structure is always found. The hook structure appears identical to a one-half wavelength section of curl filament. Furthermore, it is unlikely that the curl filament protein is polymerized from an altered hook gene product since two of the four *flaE* mutants are ambers and might be expected to produce no recognizable protein rather than a protein so similar to the hook protein. The four *flaE* mutants all have the same phenotype, and it is improbable that four independent mutations in the same gene should all produce such a specific effect.

These arguments lead us to believe that the curl filament results from an abnormal termination of the hook structure. Thus, the curl filament is a continuous polymer of hook protein molecules, i.e., this filament is a polyhook. We conclude that the group E gene codes for the production of a protein which controls the termination of the hook structures, and a defect in this gene results in a polyhook.

Mutants of bacteriophage T4 form polysheaths from the polymerization of sheath protein in the absence of core protein (17). We are currently examining the possibility that there exists in the hook structure a minor component which controls termination and is defective in group *flaE* mutants. Isolation of basal body-hook complexes and basal body-curl filament complexes may answer this question.

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