Purification and Characterization of F Pili from *Escherichia coli*†

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ABSTRACT: A purification procedure of F pili from *Escherichia coli* KE328 F8$^+$ cells, which lack both flagella and type I pili on the surface, is described. The method involves removal of F pili from the cell, followed by differential centrifugation, sucrose gradient sedimentation, and equilibrium centrifugation in CsCl of pili. The F pili specimen thus obtained showed a purity higher than 96%, as judged by electrofocusing, centrifugation in a sucrose gradient containing 6 M urea, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Chemical and amino acid analyses indicated that F pilus is composed of protein, phosphate, and hydrocarbon, and that the protein moiety has no trace of cysteine and proline but has a very small amount of histidine and arginine. F pili showed an acidic nature (pI = 3.6) and a highly agglutinative nature in aqueous solution. The molecular weight of the subunit molecule was determined as 11 800 from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both phage-adsorbing activity and morphology of F pili were markedly stable toward protein denaturating agents such as urea and guanidine hydrochloride. Sodium dodecyl sulfate and sodium *N*-lauroyl sarkosinate were the only two agents which induced depolymerization. Circular dichroism spectral analysis revealed that F pilus protein consists of 69% helix and 31% unordered structure in the presence of 10 mM deoxycholate. The secondary structure of pili was strikingly tolerable to 8 M urea.

The filamentous organs, termed "F pili", are found on the surface of male cells and their synthesis is controlled by F factor. F pili exhibit an important role in transfer of genetic elements in bacterial conjugation (Lederberg and Tatum, 1946) and are obligatory for male-specific phage infection (Crawford and Gesteland, 1964; Brinton et al., 1964; Caro and Schnös, 1966).

Purification and characterization of F pili were first carried out by Brinton (1971), using a *traD* mutant of *E. coli*, in which MS2 phage was unable to promote the infection (Willetts and Achtman, 1972). The F pili purified by Brinton showed one major band on NaDodSO$_4$[1]-polyacrylamide gel with a molecular weight of 11 800, and contained one molecule of glucose and two molecules of phosphate per protein subunit. On the other hand, the purification of F-like R pili from *E. coli* R1*drd*19 strain has been established by Beard et al. (1972) and it was observed that the subunit molecule of R pilus was also composed of carbohydrate, phosphate, and protein with the same stoichiometric ratio as reported in F pilus subunit (J. P. Beard, personal communication).

At least two models have been suggested as possible mechanisms for the transfer of nucleic acid in both bacterial conjugation and male phage infection (Brinton, 1965, 1971; Marvin and Hohn, 1969; Curtiss, 1969; Bradley, 1972a-c, 1973, 1974). However, there is at present no conclusive concept of the role of sex pili involved in these phenomena. A reason for this ambiguity may be that much has been left unsolved about the structure and biosynthesis of the organelles.

In this paper, we describe a new method of purification, chemical and physicochemical properties, and sensitivity to chemical agents and heat of F pili produced by *E. coli* strains harboring wild type F.

Materials and Methods

Bacterial Strains. Strain *E. coli* K-12 KE328 F8$^+$ (F-*gal*)/*pro*$^-$ *ahI*$^-$ χ^r *pil*$^-$ *nal*^{r}, which holds a double mutation with respect to the biosynthesis of flagella and a single mutation regarding the biosynthesis of type I pili, was used for isolation and purification of F pili. It produces an average of two F pili on a cell when cultivated in rich media such as L-broth (Lennox, 1955) or L-broth agar. Strain *E. coli* K-12 W4520 F8$^+$ *met*$^-$ was used for the preparation of ^{32}P-labeled MS2 phage.

Materials. Polypeptone, yeast extract, agar, CsCl, Brij 58, and Sarkosyl were purchased from Wako Pure Chemical Ind., Osaka; urea, guanidine hydrochloride, cholate, and DOC were from Nakarai Chemicals, Kyoto; lysozyme was from Sigma; and [^{32}P]orthophosphoric acid was from Commissariat A L'Energie Atomique.

^{32}P-Labeled Bacteriophage MS2. Strain W4520 F8$^+$ was grown in 40 mL of low phosphate M9 medium (Studier, 1973) supplemented with 40 mg of casamino acid and 150 μg of methionine to a density of 3 × 10^8 cells/mL, to which MS2 phages at a multiplicity of 5–10, 0.2 mL of 1 M $CaCl_2$, and 0.2 mCi of [^{32}P]phosphoric acid were added. After the lysis of bacterial cells was complete, usually in 3 h, the culture was worked up to purify phages by the procedure of Yamamoto et al. (1970). Further purification of phages was performed by 5–20% (w/v) sucrose gradient sedimentation using a Hitachi RPS55T rotor at 33 000 rpm at 4 °C for 90 min. Phage particles were stored at 0 °C in an M9 medium–7% sucrose solution with a small volume of chloroform and were used within 2 weeks after preparation. Phages thus obtained had a specific radioactivity of 4–20 × 10^{-8} counts per min per plaque-forming unit.

Unlabeled MS2 phages were obtained by a similar procedure except that [^{32}P]phosphoric acid was omitted from the growing medium.

Assay of F Pili. Phage-adsorbing activity of F pili was measured by the method of Ippen and Valentine (1965) with

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[1] Abbreviations used: NaDodSO$_4$, sodium dodecyl sulfate; Sarkosyl, sodium *N*-lauroyl sarcosinate; DOC, deoxycholate; buffer A, 10 mM Tris-HCl, pH 7.5; buffer B, 10 mM Tris-HCl (pH 8.7)–0.1 M NaCl; CD, circular dichroism.

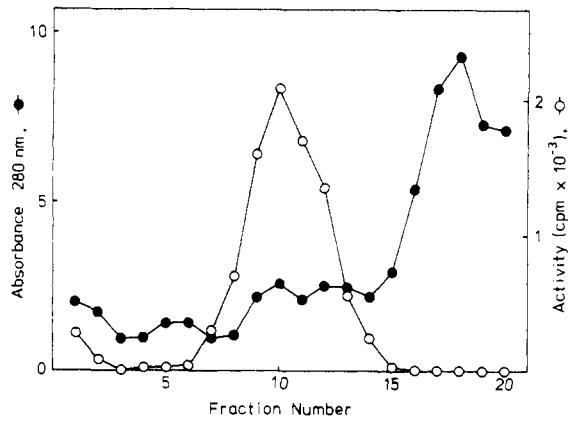

FIGURE 1: Sucrose gradient sedimentation. A total protein of 17 mg from initial supernatant in 9.5 mL of buffer B was layered on 25–70% (w/v) sucrose gradient in buffer B. The gradient was centrifuged in a Beckman SW27 rotor at 27 000 rpm at 4 °C for 4.5 h. Fractions of 1.6 mL were collected by pumping from the bottom of the tube. Fractions 8–12 were collected.

slight modifications as follows: F pili-phage mixture (0.2 mL) containing 10 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 2–5 × 10^{11} plaque-forming units of ^{32}P-labeled MS2 phages, and F pili was incubated at 30 °C for 3 min and then chilled in an ice-water bath. After adding 1 mL of cold water, the mixture was filtered through a Sartrius membrane filter (pore size, 0.45 μm) with gentle suction and washed with 1 mL of cold water four times. The filter pad was dried and immersed in scintillation fluid in a vial, and radioactivity retained on the pad was counted with an Aloka liquid scintillation spectrometer LSC-651. The scintillation fluid contained 4 g of 3,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per L of toluene.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. F pili specimens were analyzed on 0.6 × 8 cm gels containing 12.5% acrylamide and 0.1% $NaDodSO_4$, prepared according to the method of Laemmili (1970). A mixture of 10 to 50 μg of protein in 100 μL of buffer containing 0.1% (w/v) $NaDodSO_4$, 1% 2-mercaptoethanol, 10% (w/v) sucrose, and 0.002% (w/v) bromophenol blue was heated at 80 °C for 1 h, cooled to room temperature, and layered onto the gels. Electrophoresis was carried out at 3 mA per gel for 4 h. The gels were stained with 0.25% (w/v) Coomassie brilliant blue in ethanol–acetic acid–water mixture (5:1:5) for 3 h and destained by a diffusion in the ethanol–acetic acid–water mixture. After the destaining process was nearly complete, the gels were washed with 7% acetic acid and then restained overnight with 0.04% Coomassie brilliant blue in isopropyl alcohol–acetic acid–water (5:2:13), according to the method of Fairbanks et al. (1971). After destaining in 7% acetic acid, scanning of gels at 550 nm was carried out using a Gilford recording spectrophotometer (Gilford Instrument Lab.).

Amino Acid Analysis. F pili samples containing 0.6 mg of pili as protein were added to 6 N HCl containing 0.2% phenol in sealed glass tubes, flushed with pure dry nitrogen gas, and heated at 110 ± 1 °C for 24, 48, and 72 h. The samples were dried in vacuo, dissolved in 1 mL of 0.1 N sodium citrate buffer (pH 2.2), and analyzed on a Hitachi Model CLA-5 amino acid analyzer. The cysteine content of samples was determined after performic acid oxidation and 24-h prolonged hydrolysis in 6 N HCl according to the method of Moore (1964). Tryptophan determination was made spectrophotometrically by the method of Edelhoch (1967).

Electrofocusing. Electrofocusing experiment was performed according to the method of Vesterberg (1970). A column (1.5 × 30 cm) was employed with 1% ampholytes of pH range 3.5–10. Salt-free sample was loaded onto the middle of the column prepared with linear sucrose gradient. Electrophoresis was carried out at 4 °C for 40 h with an average voltage of 300 V. Fractions of 1.3 mL each were collected and their pH was measured at 4 °C with a Radiometer, Type PHM26c. Phage-adsorbing activity of each fraction was determined after the sample was diluted twofold with 0.1 M Tris-HCl, pH 7.5.

Electron Microscopy. Electron microscopic determination was carried out using a JEOL-100C Electron Microscope with samples negatively stained with 0.2% (w/v) uranyl acetate for 30 s on a carbon-coated collodion mesh.

Detection of F Pilus Subunit Molecules. Depolymerization of F pili to their subunit molecules was examined by gel filtration technique using Sephadex G-200. After 50 μg of F pili in 0.2 mL was treated with chemical agents or heat as described later, samples were layered on Sephadex G-200 columns (1.0 × 18 cm) equilibrated well with 10 mM Tris-HCl, pH 7.5, containing the chemical agent with which F pili had been treated, and fractionated with 0.4 mL per tube. The protein content in each fraction was analyzed spectrophotometrically. After treatment of F pili with acid, alkali, or heat, crystalline urea was added to reach 8 M final concentration prior to neutralization or cooling, and the sample was eluted with 8 M urea solution containing 10 mM Tris-HCl, pH 7.5.

Circular Dichroism. Analyses were made on a Jasco J-20 recording spectropolarimeter with CD attachment at 20 °C using a path length of 2 mm: samples contained protein from 50 to 200 μg per mL. The protein concentrations were determined by amino acid analysis. Ellipticities were calculated on a molar of mean residue basis and were corrected for refractive index.

Other Methods. Protein concentrations were determined by the method of Lowry et al. (1951) using egg white lysozyme as a reference compound. Analyses of phosphate and hydrocarbon groups were performed as described by Ames and Dubin (1960) and Dubois et al. (1956), respectively.

Results

All operations of purification of F pili were carried out at 0 to 4 °C.

Initial Supernatant. An overnight culture (about 1 × 10^9 cells per mL; 2 mL) of strain *E. coli* KE328 $F8^+$ in L-broth was spread on a plate (20 × 30 cm) containing 200 mL of L-broth agar (1.2% agar in L-broth), and incubated at 37 °C until the cell growth reached the late exponential phase of growth (about 9 h). The total number of plates used was 100. Two milliliters of 10 mM Tris-HCl, pH 7.5 (buffer A), was added to each plate and cells grown on the plate were harvested. To the ice-cold cell suspension, sucrose was added to reach 30% (w/v) final concentration. After stirring for 3 h, the suspension was centrifuged at 17 000*g* for 20 min. The resulting supernatant was passed through a funnel with a glass wool plug at the tapered end and was recentrifuged at 17 000*g* for 20 min. The supernatant was decanted and saved (fraction of initial supernatant, 340 mL).

Differential Centrifugation. The initial supernatant was dialyzed against buffer A to decrease the concentration of sucrose to as low as 4% (w/v) and centrifuged at 103 000*g* for 60 min using a Beckman 42 type rotor. The pellet was suspended in a small volume of 10 mM Tris-HCl–0.1 M NaCl (pH 8.7) (buffer B) containing 20% (w/v) sucrose to solubilize pili throughly (fraction of differential centrifugation, 9.5 mL).

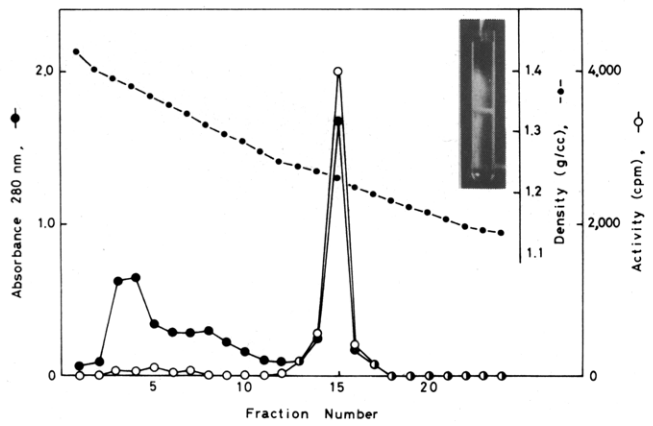

FIGURE 2: Purification of F pili by equilibrium centrifugation in CsCl. A total protein of 4.2 mg from the sucrose gradient sedimentation step was suspended in 10 mL of buffer A containing 3.9 g of CsCl. After the solution was divided into two equal parts, each part was layered on 4 mL of dense CsCl solution (0.52 g of CsCl/mL) and successively 4 mL of less dense CsCl solution (0.25 g of CsCl/mL) was layered on the top. Centrifugation was performed in a Hitachi RPS40T rotor for 20 h at 4 °C. Fractions of 0.5 mL were collected by pumping from the bottom of the tube, diluted with 0.5 mL of buffer A, and assayed for F pili activity. Fractions 14–16 were pooled. In the insert, F pili fraction can be visualized as a white turbid band in the tube after centrifugation.

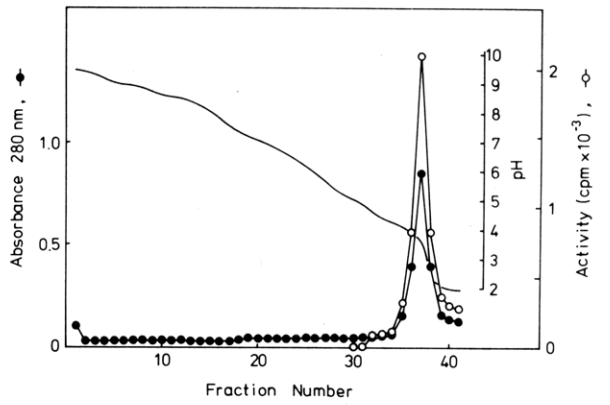

FIGURE 3: Electrofocusing of F pili. F pili, 450 μg, was loaded onto the middle of the column (1.5 × 30 cm) containing 1% ampholine, pH 3.5–10, in a linear sucrose gradient. Electrophoresis was carried out for 40 h at 4 °C with an average voltage of 300 V. Phage-adsorbing activity was assayed as described in the text after 1:1 dilution with 0.1 M Tris-HCl, pH 7.5.

Sucrose Gradient Sedimentation. The fraction of differential centrifugation was applied on a 25–70% (w/v) sucrose gradient (28 mL) in buffer B and centrifuged in a Beckman SW27 rotor at 27 000 rpm for 4.5 h. More than 90% of phage-adsorbing activity, as judged by radioactive MS2 phage assay, appeared as a symmetrical peak at around 35% (w/v) sucrose concentration (Figure 1). Fractions with phage-adsorbing activity were collected and dialyzed overnight against buffer A (fraction of sucrose gradient sedimentation, 12 mL).

Equilibrium Centrifugation in CsCl. F pili were sedimented from the fraction of sucrose gradient sedimentation by centrifugation with a Beckman SW27 rotor at 27 000 rpm for 60 min and were suspended in 10 mL of buffer A containing 3.9 g of CsCl using a Potter-Elvehjem homogenizer. The solution was divided into two parts of equal volume, and each part was layered between 4.5 mL of 0.52 g/mL (at the bottom) concentration and 4.5 mL of 0.25 g/mL (at the top) concentration of CsCl in the same buffer in a Hitachi RPS40T tube. After centrifugation with a Hitachi 55P-3 ultracentrifuge at 30 000 rpm for 20 h, F pili fraction could be visualized in the gradient as a white turbid disc (see an insert in Figure 2). The F pili fractions with a density of around 1.223 (Figure 2) were collected and dialyzed overnight against buffer A to remove CsCl (fraction of equilibrium centrifugation in CsCl, 2.0 mL).

Storage. Material of F pili fractions at the last stage was stored in an ice bath for use within 1 month after preparation. For a longer storage, glycerol was added to the above to reach 50% (w/v) final concentration and the resulting glycerol solution was stored at −20 °C. This solution retained full activity and original fine structure as filaments for at least 6 months.

Summary and Comments on Purification. A summary of the purification of F pili from *E. coli* K-12 KE328 $F8^+$ is presented in Table I. The yield of F pili ranged from 1.5 to 2.0 mg per 67 g wet weight of cells used. The fact that the yield of MS2 phage-adsorbing activity is considerably lower, as expected at the step of differential centrifugation, may be due to the strong agglutinative nature of pili in aqueous solution.

TABLE I: Purification of F Pili from *Escherichia coli.*[a]

Fraction	Vol (mL)	Total protein (mg)	MS2 Phage-adsorbing act. (%)	Purification factor
Initial supernatant	340	409	100	1
Differential centrifugation	9.5	17.0	51.0	12
25–70% sucrose gradient sedimentation	12.0	4.24	59.5	57
Equilibrium centrifugation in CsCl	2.0	1.64	34.0	85

[a] The figures are an average of three preparations for each from 67 g of cells (wet weight).

The key step in the purification procedure of pili was the sucrose gradient sedimentation in 0.1 M NaCl to remove low density substances, which had coaggregated with pili from the crude mixture, prior to purification by equilibrium centrifugation in CsCl.

The optical density ratio 280/260 nm of purified specimen was 1.6 in 0.1% $NaDodSO_4$ or 1% Sarkosyl.

Criteria of Purity. Three methods have been used to determine the purity of F pili specimens obtained after equilibrium centrifugation in CsCl. (1) Electrofocusing Technique. Electrofocusing of the material using pH 3.5–10 ampholine yielded a single symmetrical peak coinciding with both absorbance and phage-adsorbing activity at pH 3.6 ± 0.1 (Figure 3). The recovery of the phage-adsorbing activity of the peak was about 95%. (2) Centrifugation in Sucrose Gradient Containing 6 M Urea. The material was incubated with 8 M urea at 37 °C for 30 min, applied on 5–30% (w/v) sucrose gradient containing 6 M urea, and then centrifuged, taking advantage of the fact that F pili are strikingly stable to 8 M urea in terms of both phage-adsorbing activity and morphological features as judged under electron microscopy, in contrast to the unstable nature of other filamentous surface appendages, e.g., flagella (DePamphilis and Adler, 1971) toward the agent. No other protein peak except those with the specific phage-adsorbing activity was detectable in the gradient (Figure 4). (3) Polyacrylamide Gel Electrophoresis. A gel containing 12.5% acrylamide and 0.1% $NaDodSO_4$ was employed. In this gel system stained with Coomassie brilliant blue, F pilus subunit molecules appeared as a dense band which was, however, often accompanied by a faint side band. On the assumption that the

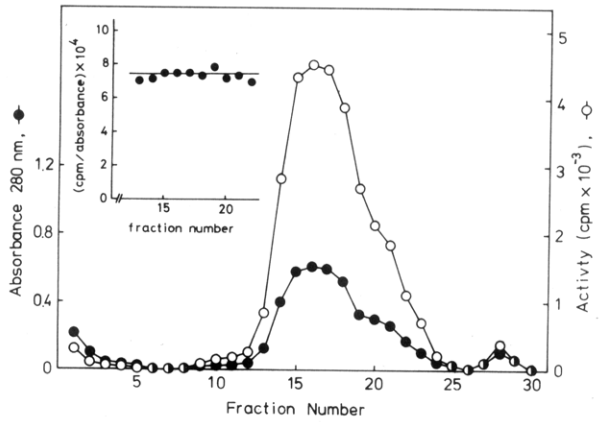

FIGURE 4: Centrifugation of F pili in 5–30% sucrose density gradient containing 6 M urea. Purified F pili (0.8 mg) obtained from the fraction of equilibrium centrifugation in CsCl was incubated with 8 M urea at 37 °C for 30 min and then layered on the top of 5–30% (w/v) sucrose gradient containing 6 M urea. Centrifugation was carried out in a Hitachi RPS40T rotor at 35 000 rpm for 5 h. Fractions of 0.4 mL each were collected.

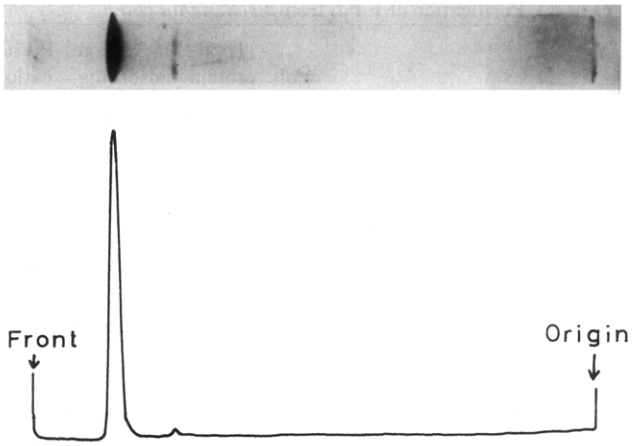

FIGURE 5: Polyacrylamide gel electrophoresis containing 0.1% $NaDodSO_4$ of purified F pili. Protein, 30 μg, was applied on a gel containing 12.5% polyacrylamide. Stained gel is at the top; densitometer tracing, below.

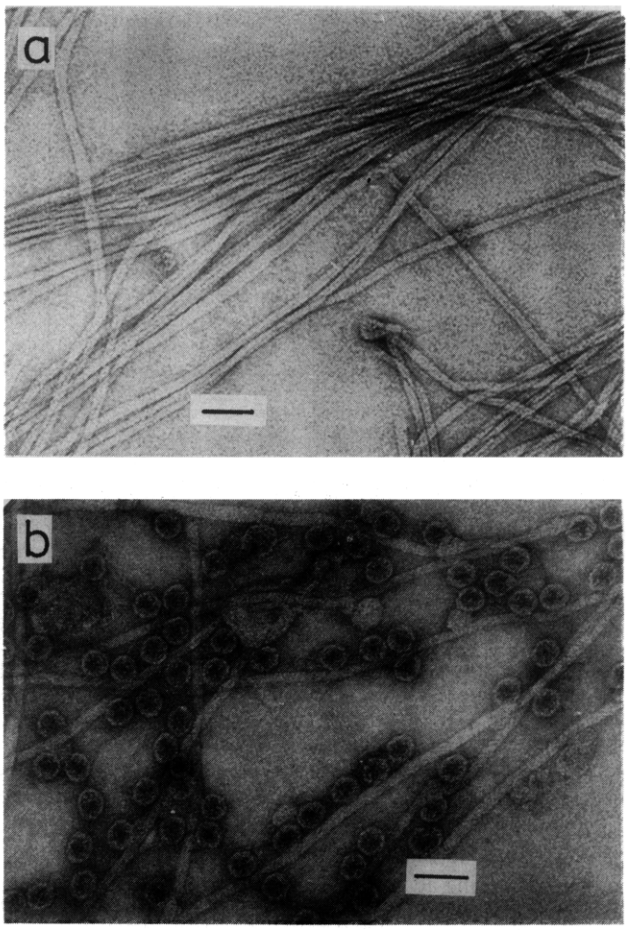

FIGURE 6: Electron microscopy of F pili obtained from the fraction of equilibrium centrifugation in CsCl. (a) F pili; (b) F pili with MS2 phage attached: scale bar 50 nm.

efficacy of staining for each proteinous band is approximately the same (Frasekas et al., 1963), the minor band was found to correspond to less than 4% of the total amount of the material (Figure 5).

Electron Microscopy. Electron microscopy of purified F pili (Figure 6) indicated that pili tend to aggregate or stick to each other. Each pilus showed, as the pili bound to the cell originally did, a 90-nm diameter and occasional presence of an axial line at the center of the pilus.

$NaDodSO_4$ Gel Electrophoresis. The gel electrophoresis of purified pili has suggested that F pili may be composed of one kind of polypeptide. Estimation of the molecular weight of subunit molecules based on the relative mobilities of pili and other proteins of known molecular weight on $NaDodSO_4$ gel (data not shown) gave a value of 11 800 ± 10%. The result was in agreement with the previously reported value for F pili isolated from *traD* mutant of *E. coli* strain by Brinton (1971). Furthermore, the lightly stained minor band in gel showed a molecular weight of approximately 16 000 ± 10%.

Chemical Analysis. The amino acid composition of the F pili we have isolated, together with the data on F pili of the F *traD* mutant reported by Brinton (1971), are presented in Table II. The number of each amino acid was calculated as the nearest integer to agree with the value of 11 800 as the molecular weight of peptide subunit determined by $NaDodSO_4$–polyacrylamide gel electrophoresis. The result showed that the subunit molecule of pilus contains no arginine, histidine, proline, or cysteine. Although the presence of a very small amount of histidine and arginine was suggested, the numbers of both residues were calculated as less than one.

Analysis also showed the presence of 0.4% inorganic phosphate and 1.5% carbohydrate group. Presence of nucleic acid and phospholipid could not be proved.

Buoyant Density. Buoyant density of the purified pili in CsCl showed 1.223 at neutral pH, but 1.243 at alkali (pH 12) in which F pili are well solubilized and hardly aggregate.

Sensitivity to Chemical Agents. Results of effects of various chemical agents on the MS2 phage-adsorbing activity of F pili are presented in Table III. Morphology of pili before and after treatment was examined under electron microscopy, and possible depolymerization of pili into their subunits was checked by Sephadex G-200 gel filtration technique.

It is noteworthy that F pili are strikingly stable to treatment with 8 M urea, 6 M guanidine hydrochloride, and 4 M chaotropic salts. This is in contrast to the high sensitivity of flagella to those agents (DePamphilis and Adler, 1971). In the case of urea, on the contrary, an enhancement of phage-adsorbing activity of pili was observed after treatment with the agent at concentrations higher than 0.5 M.

$NaDodSO_4$ and Sarkosyl were capable of depolymerizing pili into their subunits, and $NaDodSO_4$ was more effective than Sarkosyl in this action. As shown in Figure 7, no phage-adsorbing activity was detected at concentrations higher than 0.3

TABLE II: Amino Acid Composition of *E. coli* F Pili.[a]

Residue	Mole %	Mole per 11 645 g	*traD* mutant[b]
Lys	7.6 ± 0.5	9	10
His	0.15 ± 0.05	0	0
Arg	0.30 ± 0.15	0	0
Asp	6.6 ± 0.6	7	8
Thr	6.4 ± 0.4	7	8
Ser	9.3 ± 0.9	11	11
Glu	3.6 ± 0.4	4	4
Pro	0.0	0	0
Gly	11.6 ± 0.3	13	15
Ala	12.4 ± 0.3	14	15
½-cystine	0.0	0	0
Val	16.7 ± 1.7	19	21
Met	6.9 ± 0.2	8	8
Ile	3.1 ± 0.4	4	4
Leu	7.3 ± 0.5	8	9
Tyr	1.5 ± 0.2	2	2
Trp	1.4 ± 0.2	2	2
Phe	5.3 ± 0.3	6	7

[a] All values except those for half-cystine and tryptophan (see Materials and Methods) are averages computed from determinations of five different samples following 24, 48, and 72 h of hydrolysis. Threonine and serine were determined by extrapolation to zero time of hydrolysis. Valine and isoleucine values are for 72 h of hydrolysis. [b]The values are described as mol per 1 mol of subunit (Brinton, 1971).

TABLE III: Effects of Chemical Agents and Heat on MS2 Phage-Adsorbing Activity and Structure of F Pili from *Escherichia coli*.

Treatment[a]	Condition	Act. (%)[b]	Depolymerization[c]	Change of morphology
No	–	100	–	–
Urea	8 M	170	–	–
Guanidine hydrochloride	6 M	80	–	–
NaSCN	4 M	82	–	–
KSCN	4 M	85	–	–
$NaDodSO_4$	3.5 mM (0.1%)	0	+	+[d]
Sarkosyl	32 mM (1%)	0	+	+[d]
DOC	24 mM (1%)	98	–	–
Cholic acid	70 mM (3%)	77	–	–
Brij 58	1%	128	–	–
Acid	pH 2.2	3	–	–
	pH 1.0	0	–	+[e]
Alkali	pH 13	108	–	–
Heat	65 °C × 30 min	0	–	–

[a]Treatments of F pili were performed at 30 °C for 30 min with the indicated concentration of agent in 10 mM Tris-HCl, pH 7.5, unless otherwise stated. [b]Measurements of MS2 phage-adsorbing activity were carried out after appropriate dilutions (×10–500 dilution) or passing through Sephadex G-200 gel column (guanidine hydrochloride) and/or neutralization (acid and alkali). [c]Depolymerization was checked by passing through Sephadex G-200 gel column. [d]Neither the original nor any deformed structure of pili could be detected. [e]Only vesicle-like structures were detected.

mM for $NaDodSO_4$ and 3 mM for Sarkosyl, at which point depolymerization of F pili began to occur. The minimal concentrations leading to full depolymerization of pili were about 3 mM and 30 mM for $NaDodSO_4$ and Sarkosyl, respectively. Other detergents, e.g., cholate, DOC, and Brij 58, were far less effective in inactivating and depolymerizing F pili.

F pili were more unstable toward acid than alkali; at pH 4, pili lost more than 50% of phage-adsorbing activity; at pH 3–1.6, they lost the activity completely but retained their original filamentous structure, and, at pH 1, all filamentous structures were converted to vesicle-like structure (Brinton, 1971), to which phage cannot adhere. High sensitivity features toward acid have not been found in unpurified or partially purified F pili (Valentine and Strand, 1965; Tomoeda et al., 1975).

F pili were not affected in both structure and activity by heat at 50 °C for 1 h. However, they lost their activity completely when heated at 70 °C for 30 min, while retaining the filamentous structure intact.

Circular Dichroism Spectra. CD spectra of intact F pili were measured in Tris-HCl, pH 7.5, and in the presence of 10 mM DOC. Under these conditions pili are well solubilized retaining in full their morphology and their phage-adsorbing activity. Figure 8 shows a spectrum with negative dichroic bands near 206 and 222 nm; the value of $[\theta]$ at 222 nm was $-23\,000$ deg cm^2 dmol^{-1}. Comparison of the $[\theta]_{222}$ value of the difference curve with the value of $[\theta]_{222} = -31\,500$ deg cm^2 dmol^{-1}, obtained for a complete helical protein (Chen et al., 1972), suggests that F pilus protein consists of 69% helix structure and 31% unordered structure without β structure.

Figure 8 also shows spectra of pili taken in the presence of chemical agents which may affect the secondary structure of F pili. The helical structure of F pili was found to be tolerant against treatment with 8 M urea at 30 °C for 30 min. Guanidine hydrochloride, $NaDodSO_4$, and Sarkosyl can cause a marked decrease of helicity in pili.

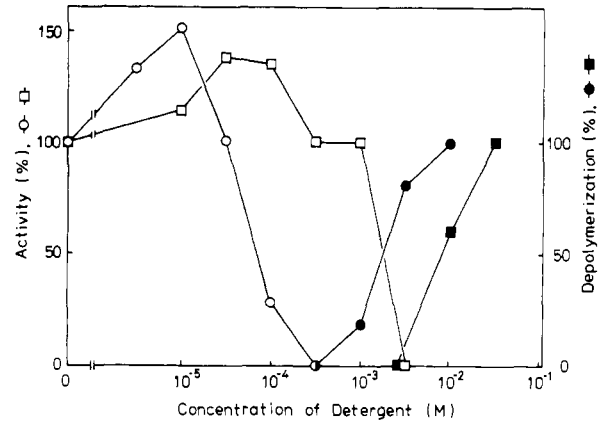

FIGURE 7: Effects of $NaDodSO_4$ and Sarkosyl on the MS2 phage-adsorbing activity and on the depolymerization of F pili. After 40 μg of F pili in 200 μL total volume was incubated with indicated concentrations of $NaDodSO_4$ or Sarkosyl at 30 °C for 30 min, the greater part of the solution (190 μL) was layered on the top of Sephadex G-200 column equilibrated with 10 mM Tris-HCl, pH 7.5, containing detergent with the same concentration as used in incubation of F pili. The proportion of depolymerization was determined as (total protein − protein eluted at void volume/total protein) × 100 (%). The concentrations of protein eluted from the column were measured spectrophotometrically at 220 nm (in the case of $NaDodSO_4$) or by Lowry's method (in the case of Sarkosyl). Another solution (10 μL) was diluted to 500-fold with 10 mM Tris-HCl, pH 7.5, and assayed for MS2 phage-adsorbing activity. (-○- -●-) $NaDodSO_4$; (-□- -■-) Sarkosyl.

Discussion

We have established a procedure for purification of F pili from *E. coli* cells which involves differential centrifugation, sucrose gradient sedimentation, and equilibrium centrifugation in CsCl. The method outlined above has eliminated possible constraints on the preparation of *E. coli* F pili without sacrificing the yield and phage-adsorbing activity. The crucial features of the method are: (a) use of a strain lacking the

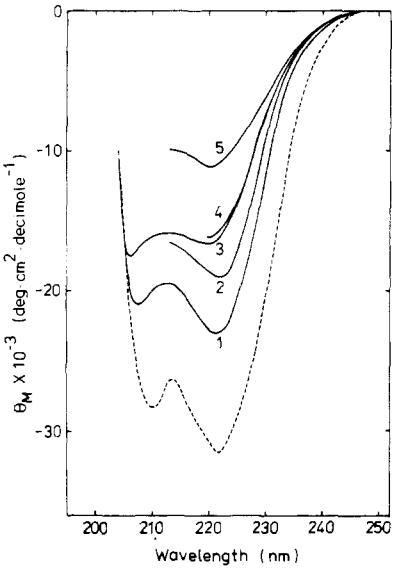

FIGURE 8: Circular dichroism spectra in the far ultraviolet of F pili. Mean residue ellipticities were in 10 mM DOC (curve 1), 8 M urea (curve 2), 3.5 mM $NaDodSO_4$ (curve 3), 3.2 mM Sarkosyl (curve 4), and 6 M guanidine hydrochloride (curve 5). Dashed line shows 100% helix (Chen et al., 1972).

synthetic capability of other filamentous surface appendages, e.g., flagella and type I pili, and (b) high reproducibility of the result. In our method, 67 g of male cells could be routinely processed in 5 days to give 1.5 to 2.0 mg of pure F pili.

One of the key steps of the purification with high reproducibility is the sucrose gradient sedimentation in the medium containing 0.1 M NaCl to separate the pili from other contaminants of low density. The method by Brinton, who first purified the sex pili, includes the crystallization of F *pili* by dialysis against Tris/saline buffer (0.05 M Tris-HCl, pH 8.5, 0.85% NaCl) followed by low speed centrifugation to sediment the pili aggregates (Minkley et al., 1976). The common feature in these two procedures seems to suggest that F pili can be separated from the contaminants in the presence of NaCl. Application of the crystallization and two successive equilibrium centrifugations in CsCl to other strains, except F*lac* JC3273, resulted in a lower yield with lesser purity, as the authors described. According to the method of Brinton, all efforts of application to KE328 strain to obtain highly purified materials with high yield were wasted.

Other sex pili, F-like R pili, have been purified by Beard et al. (1972) from *E. coli* K-12 R1*drd*19, which lacks the capability to produce flagella but possesses type I pili on the cell surface. In the purification procedure, electrofocusing technique was employed as the step to separate the pili from type I pili. Application of electrofocusing to the F pili purification is inadequate since F pili strongly aggregate with contaminants in the solution of low ionic strength used for this procedure.

The chemical and amino acid analyses of the wild type F pili are very similar to those of *traD* mutant cells despite some differences. Both F pili are composed of phosphocarbohydrate–protein and lack cysteine and proline in the protein moiety. But F pili obtained here show less phosphate content (1.7 molecules of phosphate per subunit) and lower isoelectric points than that from *traD* mutant cells (2 molecules of phosphate per subunit, p*I* = 4.15). Furthermore, in contrast to the pili from *traD* cells, our preparation of F pili contains histidine and arginine, although their contents varied in every preparation. There may be a histidine and arginine containing polypeptide in our specimen. Of course, the possibility that those amino acids are from contamination cannot be excluded at this moment. We could not determine what kind of carbohydrate was contained in F pilus.

Three different buoyant densities in CsCl have been reported for F pili: 1.197 (Wendt et al., 1966); 1.257 (Brinton, 1971); and 1.296 (Beard, 1973). F pili which we isolated from wild type $F8^+$ strain showed a density of 1.223 at neutral pH and 1.243 at alkali in which F pili are well solubilized. These conflicting results may reflect variations in the host strains and F factors in each study (Beard, 1973).

The high content of hydrophobic amino acids in F pilus subunit may lead to the hydrophobic nature of filamentous pili. F pili therefore tend to aggregate in solution. Calculation of hydrophobicity of pili using Tanford's free energy values (Bigelow, 1967) indicated that the average hydrophobicity, $H\Phi_{av}$, of F pili is as high as 1129 cal per residue. This value is the highest among fibrous proteins so far reported. It suggests that the main factor for stabilizing subunit interaction in the filamentous structure of F pili may be the hydrophobic bond. The fact, that $NaDodSO_4$ and Sarkosyl could destroy the three-dimensional structure of pili, while urea, guanidine hydrochloride, heat, and acid could not, may be also related to these structural features.

It has been found that sex pili extrude from or retract into the surface of male cells by changes in physiological and other conditions (Marvin and Horn, 1969; Novotny and Lavin, 1971; O'Callaghan et al., 1973; Novotny and Fives-Taylor, 1974). Recently, Fives-Taylor and Novotny (1976) suggested that retraction of pili requires a protein pool, and that the protein in question may be an enzyme necessary for the depolymerization of pili at the cell surface. It appears that extrusion and retraction of pili may occur under the physiological conditions which control an equilibrium of polymerization and depolymerization on the cell surface, presumably the cell membrane. It is hoped that the study of assembly or deassembly of F pili with the aid of membrane fraction may reveal their mechanism.

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Interaction of Hemoglobin with Red Blood Cell Membranes as Shown by a Fluorescent Chromophore†

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ABSTRACT: Hemoglobin quenching of the fluorescence intensity of 12-(9-anthroyl)stearic acid (AS) embedded in the red blood cell membrane occurs through an energy transfer mechanism and can be used to measure the binding of hemoglobin to the membrane. The binding of hemoglobin to red cell membranes was found to be reversible and electrostatic in nature. Using a theory of energy transfer based on Förster formulation, the quantitative data for the binding were derived. The number of binding sites was found to be $1.4 \pm 0.2 \times 10^6$ molecules per cell and the binding constant was 0.85×10^8 M^{-1}.

Plasma membranes readily available from mammalian erythrocytes have been widely used for studies of membrane structure. For such structural studies it was desirable to utilize membranes (ghosts) free of hemoglobin, and several methods of preparing hemoglobin-free membranes have been devised (Dodge et al., 1963; Hanahan and Ekholm, 1974; Steck and Kant, 1974). Nonetheless, it remains difficult to prepare hemoglobin-free red cell membranes.

Another group of workers has studied the putative binding of hemoglobin to the erythrocyte membrane in an effort to establish the physiological meaning of the interaction (Fischer et al., 1975; Mitchell et al., 1965; Hanahan et al., 1973): their studies suggested that the hemoglobin–membrane interaction is reversible, weak, and of uncertain physiological importance.

There is an apparent paradox in these observations: why is it difficult to remove hemoglobin from the membranes if the binding is weak and reversible? One possible explanation for the discrepancy might be the intercalation of hemoglobin within resealed ghosts rather than the existence of high affinity membrane binding of hemoglobin. Techniques which have previously been developed to study the interaction of hemoglobin with the membrane and which have focused on the separation of the membranes from the supernatant could not distinguish between intercalated or membrane-bound molecules.

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