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New Method for Isolation of Immunologically Pure Pili from *Escherichia coli*

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A new technique for purification of bacterial pili was developed and applied to *Escherichia coli* strains isolated from the urine of patients with symptomatic urinary tract infections. After mechanical detachment from the bacterial cells, the pili were concentrated by precipitation with ammonium sulfate, dialyzed, and solubilized in buffer containing deoxycholate. The fraction containing the pili was purified further by ultracentrifugation in a sucrose gradient and by elution through a Sepharose 4B column in 6 M urea buffer. The pilus filaments were not dissociated by concentrated urea and were eluted in the void volume of the column. The purified pili had a molecular weight of 17,000. The isoelectric point of the pili from one of the strains was 4.9, and about 43% of the amino acids were hydrophobic. Hyperimmunesera raised in rabbits against the purified pili did not contain detectable antibodies to the lipopolysaccharide O antigen or to the capsular polysaccharide K antigen of the homologous strain. The pili obtained by this purification procedure are free from other detectable bacterial surface antigens, and the purified pilus filaments are of relatively homogeneous size. This procedure enables purification of the pili also from flagellated strains.

Bacterial pili, or fimbriae, are nonflagellar filamentous appendages on the bacterial surface (16, 26). The pili are polymers of pilin subunits and consist mainly of protein with a high content of hydrophobic amino acids (2, 30). Due to deficient purification methods, the classification of *Escherichia coli* pili has been based on electron microscopy and on data from erythrocyte agglutination with whole bacteria. Thus, the type 1 pili are described as being 7 nm in width and causing mannose-sensitive agglutination of guinea pig erythrocytes (2, 8, 30). The colonization factor antigens of *E. coli* resemble the type 1 pili morphologically, but cause mannose-resistant agglutination of human and bovine erythrocytes (11, 12). K88 and K99 are pilus-like antigens on the *E. coli* strains enteropathogenic for pig, calf, and lamb (17, 36, 37).

The significance of the pili for bacterial survival and pathogenicity in vivo is uncertain. The pili are thought to confer on bacteria of several species the capacity to attach to mammalian cells (2, 6, 7, 28, 35, 39). Binding of isolated pili paralleling attachment of whole bacteria has been shown for the type 1 pili from *E. coli* (31) and for gonococcal pili (27). The pili, being extremely hydrophobic proteins, are easily contaminated by other bacterial surface antigens such as outer membrane proteins and lipopolysaccharide (LPS). The presence of contaminants, especially LPS, has not been excluded in earlier reports on isolation of *E. coli* pili (2, 9, 30).

Immunologically pure pilus preparations are vital to the clarification of the role of pili for bacterial adhesion and virulence. The present paper describes a new method for pilus purification in which the other bacterial surface antigens are removed by using deoxycholate and concentrated urea. The method was tested on *E. coli* strains isolated from patients with acute pyelonephritis. For such strains the capacity to attach to human urinary tract epithelial cells in vitro correlates with virulence in vivo (38) and to the presence of pili on the bacterial strains (39).

(The method was presented in part at the Host-Parasite Interaction Symposium in Umeå, Sweden, May 1979.)

MATERIALS AND METHODS

Bacteria. Strains 3048 and 6013 of *E. coli* were isolated from the urine of two patients with acute pyelonephritis. Strain 3048 (serotype O4K3) was richly piliated and weakly flagellated; strain 6013 (serotype O6K13) was richly flagellated and weakly piliated. For pilus purification, the cells were grown for 48 h in static Luria broth (21) in 3- to 10-liter Erlenmeyer flasks. The medium volume was 1/10 of the volume of

the flasks, and the inocula were passaged three times under similar conditions before the cultivation.

Chemicals and buffers. All chemicals were of analytical grade unless otherwise specified. Specially pure sodium dodecyl sulfate (SDS) from BDH (Poole, England) was used without further purification. Ampholines were obtained from LKB, Bromma, Sweden. Buffers used were tris(hydroxymethyl)aminomethane (Tris) buffer (10 mM Tris-hydrochloride, pH 7.5, and 0.05% NaN_3), DOC buffer (Tris buffer containing 0.5% [wt/vol] sodium deoxycholate from Merck Co., Darmstadt, Germany), and urea buffer (50 mM Tris-hydrochloride, pH 7.0, and 6 M urea from Merck Co.). Urea was crystallized twice from ethanol, and fresh buffer was prepared immediately before gel filtration.

Electron microscopy. The samples were applied onto copper grids coated with Formvar and carbon. After removal of excess liquid, the samples were immediately stained with 1% (wt/vol) phosphotungstic acid adjusted with KOH to pH 6.5 or 7.0. The grids were examined in a Jem 100B electron microscope at an operating voltage of 80 kV.

Ultracentrifugation. Sucrose gradients for ultracentrifugation were prepared by layering 1 ml of 60% (wt/wt), 2 ml of 50% and 3 ml of 40, 30, 20, and 10% sucrose in DOC buffer. In larger tubes the volumes were 5 ml of 60% and 6 ml of 50, 40, 30, 20, and 10% sucrose solutions. The gradients were stored overnight at 4°C, and 1.5 ml (in larger tubes, 3 ml) of the pilus solution was layered on the gradients. The gradients were run in a Beckman L3-50 ultracentrifuge for 20 h at 22,000 rpm in an SW27 rotor at 4°C and then fractionated into fractions of 15 drops. The refractive indexes and ultraviolet absorbances of the fractions were measured with an IRF-22 refractometer and a Zeiss MQ3 spectrophotometer. In routine pilus separations, the pilus fractions were visualized by illuminating with concentrated light in the dark and collected with a Pasteur pipette.

Gel filtration. Gel filtrations were performed at 4°C with Sepharose 4B in a K9/15 column (Pharmacia, Uppsala, Sweden) and urea buffer. The elution speed was 6 ml h^{-1}, and fractions of 1 ml were collected. The void volume was measured with blue dextran 2000 (Pharmacia), and the total volume was measured by the elution of NaCl. Chloride was determined with an ion-specific electrode (Orion, Helsinki, Finland). Elution volume of lysozyme (Merck Co., molecular weight 14,300 [14.3 k]) was used as an approximate marker for the pilin subunits.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in SDS (SDS-PAGE) was performed in 1-mm-thick slab gels (gel concentration, 15%) by the system of Laemmli (20). Standard proteins were bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), lactic acid acid dehydrogenase (Boehringer, Mannheim, West Germany), trypsin (Merck Co.), and hemoglobin (Sigma).

Ultrafiltration. Protein concentrations were performed in an Amicon ultrafiltration cell model 52 with X50 membrane.

Isoelectric focusing. Isoelectric focusing was performed in a 110-ml focusing column of LKB (29). The ampholines were pH 3.5 to 10.

Gas chromatography. Fatty acids in O4 LPS from an O4K12 *E. coli* strain and in the purified pilus preparation were determined as described by Saraste and Korhonen (32). O4 LPS was purified by the hot phenol water method (40).

Amino acid analysis. Samples were hydrolyzed in 6 M HCl at 110°C for 24 h under nitrogen. Amino acid analysis was performed on a Jeol JLC-5AH amino acid analyzer with sodium citrate buffers. No corrections for losses of threonine and serine were made. Results represent an average of two separate determinations.

Protein estimation. Protein was estimated by the method of Markwell et al. (22) with bovine serum albumin as the standard.

Preparation of antisera and quantitation of antibodies. Anti-pilus antibodies were induced by subcutaneous immunization of rabbits. Each rabbit received 30 μg of pili without adjuvant divided among six injection sites. This was repeated four times at 3-week intervals. Antibodies were quantitated by the enzyme-linked immunosorbent assay (10). The antigens used were the purified pilus proteins, O4 LPS from an O4K-12 *E. coli* strain, and O6 LPS from an O6K-12 *E. coli* strain (40). K3 capsular polysaccharide from an O_{nt}K3 and K13 from an O12K13 *E. coli* strain were isolated by Cetavlon precipitation (18, 34). The anti-pilus antibody level is given as the dilution of antibody preparation giving an extinction value of 0.2 above the baseline at 400 nm after incubation for 100 min.

RESULTS

Pili purification procedure. After growth for 48 h in static Luria broth, *E. coli* 3048 formed pellicles on the culture surfaces and the cells were heavily piliated (Fig. 1). The cells (wet weight, 35 g) from 7 liters of medium were harvested by centrifugation for 15 min in a Janetzki K26 centrifuge (4°C, 4,000 × *g*) and suspended in 250 ml of cold Tris buffer. Pili were detached from the bacterial cells by using an Ato-Mix homogenizer (Measuring & Scientific Equipment Ltd.) for 5 min at half speed. After centrifugation the supernatant was saved. The Ato-Mix treatment was repeated twice for 30 s, and the supernatants were combined (volume = 750 ml). This treatment did not cause significant cell breakage. All subsequent steps were performed at 4°C unless otherwise stated.

Crystalline ammonium sulfate was added to the supernatant to 50% saturation, and the suspension was allowed to stand overnight. The precipitate was collected by centrifugation in a Sorvall RC2-B centrifuge (1 h, 10,000 × *g*), suspended in 50 ml of Tris buffer, and dialyzed for 48 h against Tris buffer with several changes of the buffer. After dialysis, sodium deoxycholate was added to the suspension to a final concentration of 0.5% (wt/vol), and the suspension was dialyzed against DOC buffer (600 ml) for 48 h. Shorter dialysis times were found to cause difficulties in the subsequent steps of the purifica-

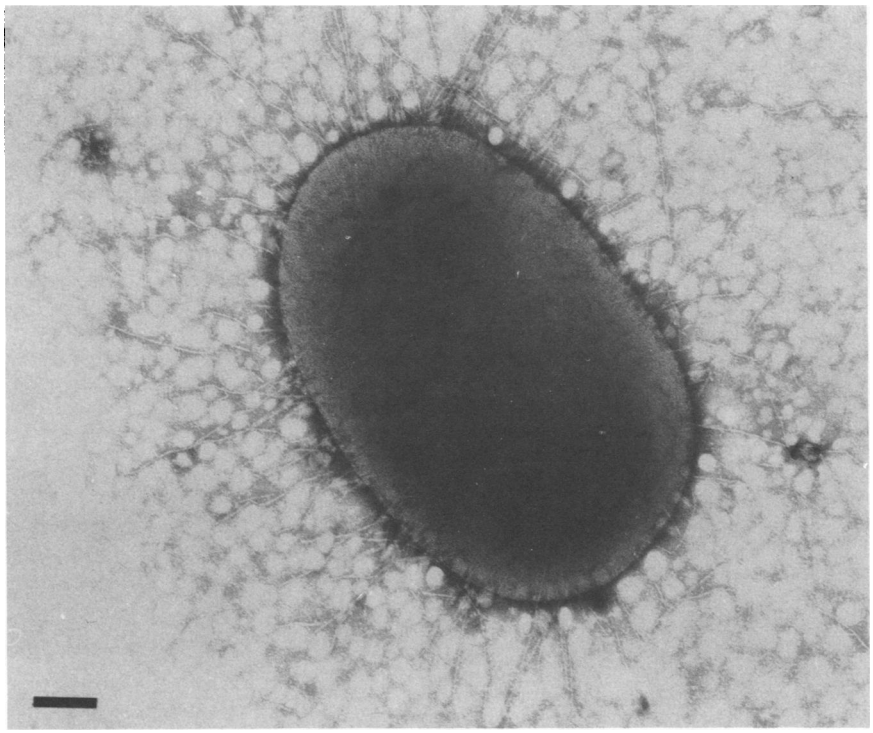

FIG. 1. *E. coli 3048 after growth for 48 h in static, aerobic Luria broth. The bar represents 200 nm.*

tion process. Deoxycholate was found by electron microscopy to disaggregate pili-pili and pili-vesicle aggregates. The suspension was centrifuged in a Sorvall RC2-B centrifuge for 10 min at 10,000 × *g*, and the supernatant was collected. This material is referred to as DOC-soluble material, and the pellet is called DOC-insoluble material. No pilus protein remained in DOC-insoluble material, whereas most of the outer membrane proteins remained in this fraction (Fig. 2a, b, and c). The supernatant was concentrated to 6 ml in an Amicon ultrafiltration cell at room temperature.

The pilus solution was divided into four ultracentrifugation tubes containing 10 to 60% sucrose gradients in DOC buffer, and the gradients were run for 20 h at 22,000 rpm in a SW27 rotor in a Beckman L3-50 ultracentrifuge and fractionated. Ultraviolet absorbance measurements revealed three main fractions (Fig. 3). The band at the density of 1.21 g cm^{-3} was probably deoxycholate micelles since it was also formed in the control tubes (gradient and DOC buffer) and was free of protein. The critical micelle concentration of deoxycholate is 0.2 to 0.3% (15). The pili banded at the density of 1.10 to 1.15 g cm^{-3} (fractions 12 to 16 in Fig. 3). These fractions were combined and dialyzed against Tris buffer for 48 h with several changes of the buffer. After dialysis, the preparation was concentrated again to 3 ml, and deoxycholate was added to a concentration of 0.5%. The sample was kept in the buffer for 2 h, and ultracentrifugation and dialysis were repeated.

After dialysis, the pilus sample was concentrated to 2 ml in a rotary evaporator. Crystalline urea was added to a final concentration of 6 M, and the solution was kept at room temperature for 2 h. It was then eluted through a Sepharose 4B column with urea buffer as the eluant (Fig. 4). Most of the pilus proteins did not penetrate the gel and eluted in the void volume of the column. From the elution volumes of lysozyme and NaCl, the elution volume of the pilin subunits was estimated as about 30 ml, but no pilin subunits were found. Longer incubation with urea of up to 4 h at room temperature or 16 h at 4°C did not change the elution diagram. Also, the material eluted at the volumes of 15, 23, and 26 ml contained pili as judged by SDS-PAGE. These probably represent filaments fragmented during the purification procedure.

Fractions 8 to 12 were combined and dialyzed against distilled water for 48 h with several changes of water. For analysis, the purified pili were concentrated to 2 ml in a rotary evaporator. Average yield of pure pilus protein from 35 g (wet weight) of 3048 cells was 4 to 6 mg.

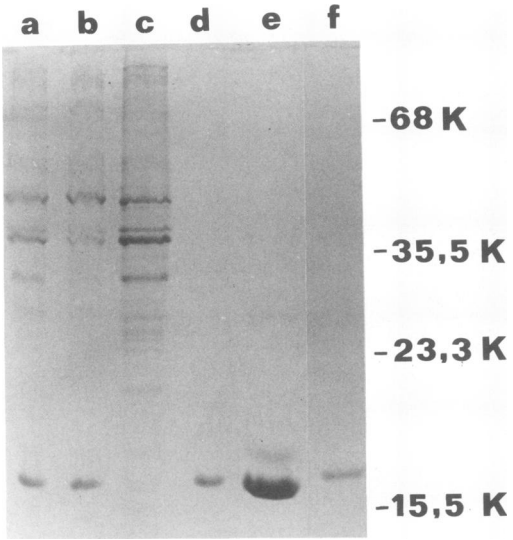

FIG. 2. *SDS-PAGE analysis of the E. coli 3048 pili solubilization by deoxycholate and of the purified pilus preparations. The 3048 pilus preparation was dialyzed for 48 h against 0.5% deoxycholate buffer, and the deoxycholate-insoluble material was removed by centrifugation at 10,000 × g for 10 min. (a) the 3048 pilus preparation after precipitation with ammonium sulfate, 25 μg; (b) the deoxycholate-soluble fraction, 25 μg; (c) the deoxycholate-insoluble fraction, 25 μg; (d) the purified 3048 pilus preparation, 17 μg and (e) 130 μg; (f) the purified 6013 pilus, 15 μg. Reference proteins indicated on the right were bovine serum albumin (68K), lactic acid dehydrogenase (35.5K), trypsin (23.3K), and hemoglobin (15.5K).*

Characterization of the 3048 pilus protein. The pili retained their native morphology in the purification process (Fig. 5). Their width varied between 5 and 7 nm, and their length varied between 0.5 and 1 μm. No contaminating membrane vesicles were seen in electron microscopy.

The purified pili gave only one band in the SDS-PAGE with a molecular weight of 17K (Fig. 2d). When the gel was overloaded with the pilus preparations, a minor band of 18K became visible (Fig. 1e). This protein was not abundant in the starting material (Fig. 1a) and seemed to have been enriched in the purification process.

Isoelectric focusing of the purified 3048 pili revealed only one fraction with an isoelectric point of 4.9.

Table 1 gives the amino acid composition of the 3048 pilus protein. Hydrophobic amino acids comprised 43% of the total amino acid composition.

Possible LPS contamination was checked chemically by gas chromatography of the fatty acids in the pilus preparation. Purified O4 LPS used as standard contained lauric, myristic and β-hydroxymyristic acids. None of these fatty acids could be detected from 1 mg of the 3048 pili. The detection limit for this method was about 10 μg of LPS (1% contamination).

Application of the procedure to the *E. coli* strain 6013. The procedure was used in purifying pili from a richly flagellated and only weakly piliated *E. coli* strain, 6013. The purified pili (molecular weight, 17K) were free from flagella and outer membrane proteins, as shown by the SDS-PAGE (Fig. 1f). The yield from 370 g (wet weight) of cells was 16.5 mg of pili.

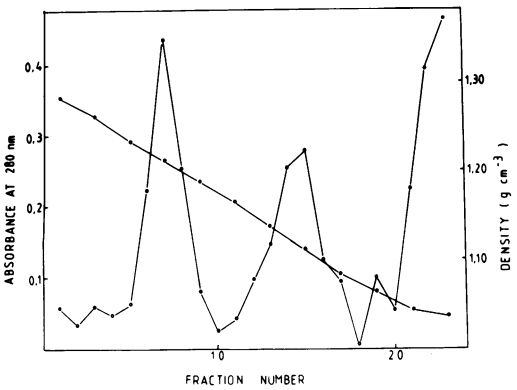

FIG. 3. *Ultracentrifugation of the 3048 pilus preparation in sucrose gradient in deoxycholate buffer. The pilus preparation (1.5 ml) was layered on top of the 10 to 60% (wt/wt) sucrose gradient in deoxycholate buffer, and the gradients were run for 20 h at 22,000 rpm in a SW27 rotor in a Beckman L3-50 ultracentrifuge and fractionated. Symbols: ○, Absorbance at 280 nm; ●, the density at 20°C (g cm⁻³).*

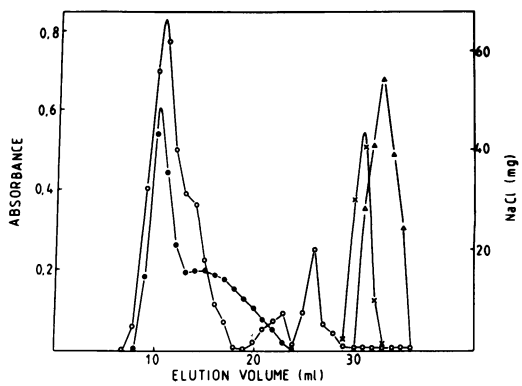

FIG. 4. *Gel filtration of the 3048 pilus preparation in Sepharose 4B in 6 M urea buffer. The pilus sample was treated with 6 M urea for 2 h at room temperature and eluted through the Sepharose 4B column. Symbols: ○, the pilus sample, absorbance at 280 nm; ●, blue dextran 2000 (4 mg), absorbance at 600 nm; ×, lysozymes (1 mg), absorbance at 280 nm; △, NaCl (200 mg).*

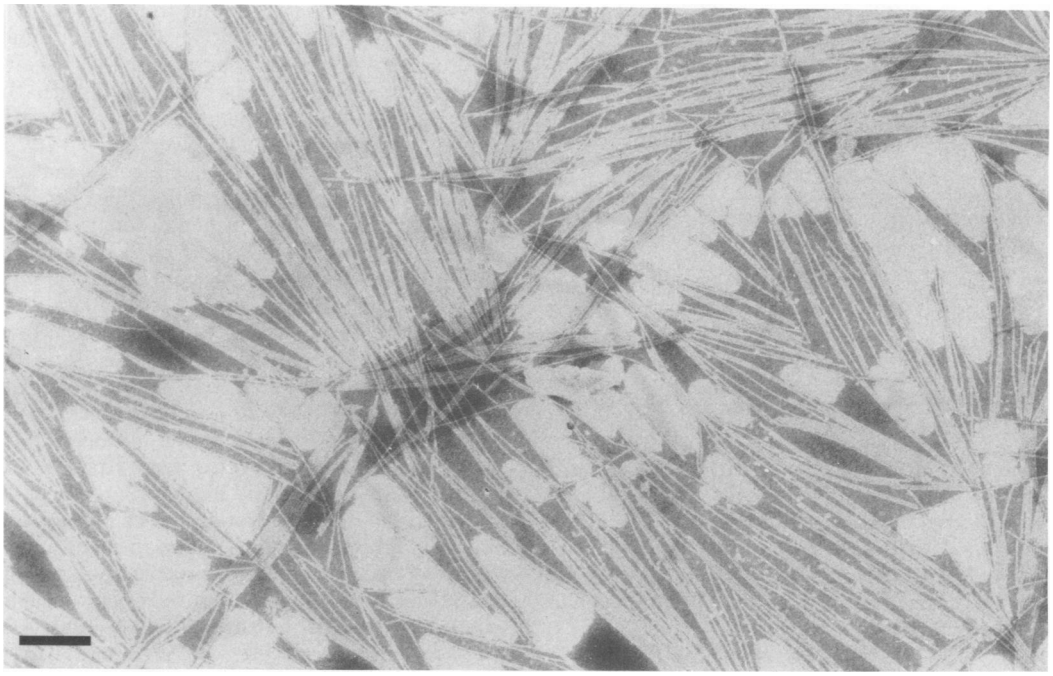

FIG. 5. *Purified pili from E. coli 3048. The bar represents 200 nm.*

TABLE 1. *Amino acid composition of the pili purified from E. coli 3048*

Amino acid	Residues per molecule[a]
Aspartic acid	22
Threonine	22
Serine	12
Glutamine	14
Proline	4
Glycine	18
Alanine	32
Cystine (half)	ND[b]
Valine	16
Methionine	Tr
Isoleucine	6
Leucine	12
Tyrosine	Tr
Phenylalanine	8
Lysine	6
Histidine	2
Arginine	4

[a] The percentile proportion of proline, alanine, valine, methionine, isoleucine, leucine, and phenylalanine hydrophobic was 43%. The calculated molecular weight was 17,767.

[b] ND, Not detected.

Immunoresponse and quantitation of antibodies. The enzyme-linked immunosorbent assay was succesfully adapted for the measurement of anti-pilus antibodies. For optimal coating of the polystyrene tubes, the minimal concentration of the pilus antigen required was 10 μg/ml, and the coating time at 37°C was 5 h (data not shown). The preimmune sera contained only low amounts, if any, of anti-pilus antibodies. A significant rise in anti-pilus antibodies was obtained with each immunization (highest levels shown in Table 2). The rise in antibody titers to LPS was insignificant and did not increase when rabbits were reimmunized with pili. No rise was observed in antibodies to capsular polysaccharide. The antiserum raised against the 3048 pili reacted with the purified 6013 pili also and vice versa (Table 2). These data suggest antigenically similar parts on the pili of these two *E. coli* strains.

DISCUSSION

To clarify the significance of the pili for bacterial adhesion requires the availability of pilus preparations that are free from other bacterial surface antigens. The present paper reports for pilus preparation a new method that uses deoxycholate and 6 M urea to separate the pili from other antigens. The resulting pili are morphologically intact and free from outer membrane protein, LPS, capsular polysaccharide, and flagella. The pili also retain their biological activities (19a).

Bacterial pili which are hydrophobic proteins have a strong tendency to aggregate with membranous material during purification; pilus preparations are thus easily contaminated with other bacterial surface antigens. Data et al. (3) showed

TABLE 2. *Antibody levels in rabbit serum before and after immunization with purified pili from E. coli 3048 or E. coli 6013*

Immunization antigen	Antibody level[a]		
	Solid-phase antigen	Preimmune serum	Hyperimmune serum
Pili from *E. coli* 3048	Pili from 3048	<10	32,000
	LPS O4	<10	40
	Capsule K3	<10	<10
	Pili from 6013	<10	2,500
Pili from *E. coli* 6013	Pili from 6013	<10	50,000
	LPS O6	<10	13
	Capsule K13	<10	<10
	Pili from 3048		10,000

[a] Enzyme-linked immunosorbent assay.

recently that F pili from *E. coli* are resistant to various detergents, including deoxycholate, and to 8 M urea. Also type 1 pili from *E. coli* did not migrate into the isoelectric focusing gel in the presence of 6 M urea (30). These observations led us to test the combination of deoxycholate and urea in pilus purification.

Not only did deoxycholate solubilize pili very efficiently, but it also disaggregated pilus-cell wall complexes. Thus, the deoxycholate-insoluble fraction contained no pili, but most of the outer membrane proteins remained in it (Fig. 2). The outer membrane protein Ia, difficult to remove from pili by current purification procedures (30), was removed efficiently. Our results agree with those of Mutoh et al. (25) and show that deoxycholate functions similarly to Triton X-100 which does not solubilize outer membrane proteins (5, 33). After solubilization, it was possible to separate the pili from the cell wall material by ultracentrifugation in a sucrose gradient (Fig. 3).

The 6 M urea buffer used in gel filtration of the pilus fraction through a Sepharose 4B column did not dissociate the pili into subunits (Fig. 4). Equally resistant to urea were the pili from *Salmonella typhimurium* purified by the same procedure (T. K. Korhonen, unpublished data). It thus seems that their stability in concentrated urea is a common property of enterobacterial pilus filaments. Flagella of *E. coli* dissociate in 8 M urea (4), and those of *Bacillus subtilis* and *Spirillum serpens* dissociate in 4 M urea at pH 4.3 (23). The gel filtration in urea thus separates the pili from the flagella and fractionates the pili into filaments of a relatively homogeneous size. This enables the purification of pili also from the flagellated strains which has been difficult to achieve with current methods. The strain 6013 used here was heavily flagellated.

When analyzed in SDS-PAGE, both of the pilus preparations were found to be pure and to have a molecular weight of 17K (Fig. 2). A minor contaminant of a molecular weight 18K became visible when the gel was overloaded with the 3048 pili. This protein, enriched during purification, might represent another pilus present on the strain or one of the multiple conformations in SDS solution recently postulated for the type 1 pili from *E. coli* (24). Since outer membrane proteins were not solubilized by deoxycholate, the 18K band is not likely to represent the outer membrane protein III (14).

The amino acid composition of the 3048 pilus protein is similar to that of the type 1 pilus (2, 30) with the exception that no cysteine was found (Table 1). However, the isoelectric point of 4.9 of the 3048 pilus is about 1.0 higher than that reported for the type 1 pilus from *E. coli* K-12 (2) but close to the isoelectric point of 4.8 reported for the colonization factor antigens pilus (13). The colonization factor antigens and *E. coli* 3048 pili resemble each other morphologically, and both cause mannose-resistant agglutination of human erythrocytes (11, 19a). Also the adherence of *E. coli* 3048 to human urinary tract epithelial cells is resistant to mannose (39) and the adherence of *E. coli* to human uroepithelial cells has been correlated with mannose-resistant agglutination of human erythrocytes (19). There may thus be a group of pili with common properties which are important for the interaction between enteric bacteria and human tissues.

The purified pili were effective immunogens and gave rise to antisera free from measurable antibodies to other bacterial surface antigens (Table 2). The small rise in LPS antibodies was not considered significant since it did not increase when boostered. Nonspecific mitogenic response to pilus antigens propably explains the small rise in anti-O antibodies. Furthermore, no fatty acids specific for LPS were found in 1 mg of the 3048 pilus preparation. The cross-reactivity of the two pilus proteins observed in the enzyme-linked immunosorbent assay (Table 2) might indicate the presence on the pili of a common antigen. The present method of pilus purification will be useful for further elucidation of the role of pili and anti-pilus antibodies in infections via mucous surfaces.

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