Purification of the *Escherichia coli* Type 1 Pilin and Minor Pilus Proteins and Partial Characterization of the Adhesin Protein

MARK S. HANSON,† JOHN HEMPEL, 2 AND CHARLES C. BRINTON, JR.†

Departments of Biological Sciences 1 and Microbiology, Biochemistry and Molecular Biology, 2 University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Received 19 May 1987/Accepted 9 May 1988

Type 1 pili of *Escherichia coli* contain three integral minor proteins with apparent molecular weights (*M*) of 28,000 (28K protein), 16,500, and 14,500 attached to rods composed of *M* 17,000 pilin subunits (Hanson and Brinton, Nature [London] 322:265–268). We describe here an improvement in our earlier method of pilus purification, which gives higher yields and higher purity. Also reported are methods allowing fractionation of intact type 1 pili into rods of pure pilin and free minor proteins, as well as fractionation of the 28K tip adhesion protein from the 16.5K and 14.5K proteins. We have determined the amino acid composition and amino-terminal sequence of the adhesin protein. This sequence shows limited homology with the amino-terminal sequences of several *E. coli* pili, including type 1.

Pili are filamentous protein surface appendages occurring on many pathogenic bacteria (3-5, 7, 10, 14, 30). Pilus genes are carried both on chromosomes (9, 40, 50, 51) and on plasmids (10). The expression of many pilus genes is controlled by a genetic switching mechanism of phase variation (3, 16, 40). The piliated phases of pathogenic bacteria have a group of properties that change markedly when switching to a nonpiliated phase occurs (5). These include attachment to specific host receptors, resistance to phagocytosis, surface translocation by twitching motility, specific immunogenicity, increased colonizing ability, and increased virulence. The coordinate control of these properties and piliation by phase variation strongly supports an important role for pili and phase variation in bacterial disease.

Antibodies to pili can neutralize many of these functions. Pilus vaccines developed in this laboratory have been found to be protective against both experimental and natural diseases caused by *Escherichia coli* (5, 8, 19, 41, 43), *Neisseria gonorrhoeae* (6, 11), *Pseudomonas aeruginosa* (6; A. Levine, Ph.D. thesis, University of Pittsburgh, 1980; J. Fusco, Ph.D. thesis, University of Pittsburgh, 1987), *Moraxella bovis* (D. Vilella, M.S. thesis, University of Pittsburgh, 1981), and *Haemophilus influenzae* (R. B. Karasic, D. J. Beste, S. C. To, W. J. Doyle, K. Tanpowpong, C. D. Bluestone, and C. C. Brinton, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 328, 1987; R. B. Karasic, S. C. To, K. Tanpowpong, C. D. Bluestone, and C. C. Brinton, Annu. Meet. Am. Pediatr. Soc. Soc. Pediatr. Res., Washington, D.C., 1988, abstr. no. 1028). In order to design the most effective pilus vaccines, it is important to identify and understand the several mechanisms by which pilus antibodies protect against disease. It has often been assumed that pilus vaccines must induce antibodies that block adhesion by piliated bacteria in order to be effective. Recent results from this laboratory indicate that neutralization of receptor-specific adhesion is not likely to be the principal protective mechanism of certain intact pili vaccines. The specificity of protection by these vaccines shows a strong correlation with the serological specificity of the main rod protein, pilin. On the other hand, our studies on the adhesion specificity of these pili (22; C. C. Brinton, Jr., E. Goyarts, and A. Tagare, manuscript in preparation) show that for a number of different pili families, receptor-specific adhesion is mediated not by pilins but by a class of minor pilus proteins whose serological specificity and structure are more conserved within pilus families than pilins and that do not cross-react with them. If antibody neutralization of adhesion were the major protective mechanism, vaccine protection would not correlate with main rod protein specificity in these families.

We have also shown that the several adhesion proteins tested are not strongly antigenic in intact pili (22). This result provides an explanation for the lack of an adhesion-blocking response correlating with the protection elicited by these intact pilus vaccines. It also suggests that if ways could be found to enhance the low natural antigenicity of the pilus adhesins, protective efficacy might be increased and broadened. Purification and concentration of the adhesin proteins is one such strategy. Methods for the purification of pili adhesins should also allow the separate evaluation of protection resulting from the antiadhesin effects of pilus antibodies and from binding of pilus antibodies to the main rod protein.

*Escherichia coli*, a ubiquitous pathogen, can produce some 14 or more families of pili, distinguishable by their morphological, serological, chemical, and receptor-binding properties (4, 8, 13, 14, 19, 30, 33, 46, 52). Among these, the first identified (7), the type 1 family (4), remains the best characterized and most widely studied. The popularity of these pilis as research subjects is owed, among other reasons, to their occurrence on essentially all *E. coli* strains (8), the availability of a convenient adhesion assay system, mannose-inhibitable hemagglutination (44), the novel mechanisms involved in the control of their expression (3, 16), and their unusual chemical stability, which facilitates purification (4, 19).

Type 1 pili were first chemically and structurally characterized in this laboratory and found to be helical assemblies of identical protein subunits (4). For some time they were thought to be composed solely of a single protein, pilin, of *M* 17,000, in about 1,000 or so copies per rod (18, 48). Our recent biochemical evidence has shown this to be incorrect.
as in addition to pilin, three minor proteins present in about three to six copies each have been identified as integral components of the E. coli type 1 pilus (22). We have shown that one of them, of Mr 28,000, is the actual mannoside-binding adhesin (22), consistent with the genetic data of others (31, Goteborg, removal of the minor pilus proteins and for the purification of the 28,000-Mr (28K) adhesin. The chemical composition and partial sequence of this novel protein are also reported.

(Preliminary reports of these findings have been presented [Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, abstr. D113, p. 84; IVth International Symposium on Pyelonephritis, Göteborg, Sweden, June 1986].)

**MATERIALS AND METHODS**

**Bacteria and growth conditions.** E. coli B strain American (Bam) is a laboratory-passaged strain originally isolated from sewage and obtained from E. Kellenberger. This strain does not express flagella or sex pilin under any growth conditions yet tried. Pilin other than type 1 were not detectable by electron microscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), antiserum agglutination, or hemagglutination.

**Growth of bacteria and purification of type 1 pilin.** E. coli were phase-cloned for pilination by selecting characteristic compact rounded colonies (3) on minimal glucose agar [1 g of sodium citrate, 2 g of dextrose, 7 g of KH₂PO₄, 3 g of KH₂PO₄, 2 g of (NH₄)₂SO₄, 0.1 g of MgSO₄, 0.01 g of agar per liter]. The presence of type 1 pilin was verified by hemagglutination of guinea pig erythrocytes and agglutination of bacterial cells with anti-type 1 serum as described below. Bacteria were harvested from minimal glucose plates and used to inoculate aluminum trays containing Z agar (8 g of NaCl, 12 g of Tryptone [Difco], 1 g of yeast extract, 1 g of dextrose, and 17 g of agar per liter) for production of pilin.

**Bacteria were harvested in phosphate-buffered saline (PBS): 4 mM sodium phosphate, 0.85% NaCl (pH 7.2), and pilin were sheared off, precipitated, and purified by a modification of the procedure described by Brinton (4). Briefly, pilin were aggregated into paracrystalline needles by addition of MgCl₂ to 0.1 M and sedimented at 30,000 × g for 60 min at 4°C. Pellets were solubilized in phosphate buffer (PB) overnight at 4°C, and particulate debris was sedimented in the same manner. Three or four such crystallization and solubilization cycles gave pilin that were greater than 99% pure at a yield of 1 to 1.5 mg/g (wet weight) of cells. Minor membrane contamination, evidenced by appearance of membrane vesicles in dark-field or electron microscopy and a doublet band at 36K (OmpCF) on SDS-PAGE, was removed by suspending pelleted pilin in 5 mM EDTA-10 mM Tris (pH 7.2) by incubation overnight at 4°C. Triton X-100 was added to 0.2%, the pilin were stirred for 1 h at 4°C, and then 1/20 volume of saturated ammonium sulfate was added to a final 5% saturation. After crystallization for 1 h at 4°C, pilin were pelleted by centrifugation at 30,000 × g.

**Purification of pilin rods and the 28K adhesin protein.** Pilin thus purified are homogeneous by SDS-PAGE and silver staining. That is, at 1 mg of protein per ml, only pilin (17K) and three tightly associated minor (28K, 16.5K, and 14.5K) proteins are detectable. Trace contaminants which would be concentrated later were removed by suspending pilin to about 5 mg/ml in 4% SDS–10 mM dithiothreitol (DTT)–10 mM Tris, pH 8, and stirring for 1 h at room temperature. Type 1 pilin intact under these conditions and are insoluble (see Results). Pilin were sedimented by centrifugation at 24,000 × g for 1 h. Pilin were again suspended in the 4% SDS buffer, reducing the total volume to give a concentration of about 10 mg of protein per ml, and boiled for 5 min to solubilize the minor proteins. The 17K pilin subunit protein remained in its polymerized rod form and was sedimented by centrifugation at 100,000 × g for 1 h at 10°C. Centrifugation at lower temperatures tends to precipitate SDS and make withdrawal of the supernatant more difficult. The pellet contains pure pilin in the form of rods, devoid of the minor proteins. Allowing the hard pellet to soak in distilled water overnight at room temperature accomplished resuspension; by using small volumes of water, concentrations of 50 to 100 mg of pilin per ml were achieved, yielding a thick, viscous suspension.

After the pilin were boiled in SDS, the resultant supernatant contained the minor proteins concentrated 10-fold and small amounts of pilin rod aggregates and fragments. Larger rod aggregates were removed by filtration through two filters (0.45 and then 0.2 μm pore sizes). The minor proteins were concentrated by precipitation in a Corex glass tube with 19 volumes of acetonitrile-triethylamine-acetic acid (85:5:5) (25) at 4°C, followed by centrifugation at 13,200 × g in an IEC 28K (hanging bucket) rotor for 15 min. This precipitation also removed the detergent. Residual triethylamine and acetic acid were removed by washing the pellet with acetone and air-drying. Residual pilin rod fragments not removed by filtration were depolymerized by the addition of 40% formic acid (1 to 2 ml/mg of protein) and incubation at room temperature for 30 min. The 28K protein was largely insoluble at this stage, while pilin and the other minor proteins were partially soluble. Centrifugation at 24,000 × g (4°C) for 30 min sedimented the majority of the 28K protein, leaving much of the others in the supernatant. The pellet was aceton-washed and dried as before. The pellet, enriched for 28K protein, was suspended by boiling in 1% SDS–10 mM DTT–10 mM Tris (pH 8) for 2 min.

The 28K protein was separated from the other minor proteins by gel filtration chromatography in 0.1% SDS–10 mM DTT–20 mM Tris (pH 8) with 1 mM EDTA and 0.02% NaN₃, as bacteriostats. Typically, 0.3 to 0.5 mg of the 28K-enriched fraction was loaded on a Sephadex G-75 (1.5 by 110 cm) or Sephadex G-100 (1.5 by 80 cm; Pharmacia) column; the 28K protein elutes in the void volume, with the 17K, 16.5K, and 14.5K proteins partially resolved in later fractions. Fractions of interest were pooled and concentrated with a Centricon-10 (Amicon) microconcentrator.

**Preparative SDS-PAGE.** The 28K protein was also purified by preparative SDS-PAGE. A 10% acrylamide concentration was the lowest concentration that gave good separation of the 28K protein from the dye front. Protein bands were visualized by soaking gels in 0.25 M KCl at room temperature to precipitate SDS within the gel (21) and were then cut out with a clean razor blade. After the gel slices were briefly soaked in distilled water to resolubilize the SDS, protein was removed from the gel slices by electrodialysis. The most efficient apparatus tested for this purpose proved to be the International Biotechnologies Inc. analytical electrodialator. Protein was eluted into a 100-μl cushion of 7.5 mM ammonium acetate–6 M urea for 1 h at 200 V. Standard SDS-PAGE running buffer (see below) was used as the reservoir buffer. The salt-urea-protein mixture was collected from the bridge channel and dialyzed extensively to remove urea and salt. An alternative method used for electrodialution, which

Downloaded from http://jb.asm.org/ on October 29, 2017 by guest
increases the solubility of the recovered protein, is similar to that described by Anderson et al. (1). Briefly, 0.5 ml of stacking gel was polymerized in a 1.5-ml microfuge tube, and a dialysis bag, open at one end, was attached to the conical microfuge tube after removal of its tip. This collection apparatus was connected to the upper reservoir of a tube gel electrophoresis cell so that the dialysis bag completed electrical contact with the lower reservoir. The reservoir buffer was the standard running buffer. Gel slices and tracking dye were placed above the gel plug support. Protein was eluted into the bag in 30 to 40 min at 400 V.

SDS-PAGE and staining. The gel system used was that of Laemmli (35) with minor modifications. Slab gels (10 by 14 by 0.05 cm) or minislab gels (6 by 9 by 0.05 cm) were used. Gels contained 12.5 or 15% acrylamide for analytical purposes or 10% for preparative purposes. Staining was done with Coomassie brilliant blue R-250 (BioRad Laboratories) (35) or with silver (42). Molecular weight standard proteins were purchased from Sigma Chemical Co. Apparent molecular weight assignments were those of Weber and Osborn (54); bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), sperm whale skeletal myoglobin (17,200), and lysozyme (14,300). Gels were dried onto Whatman 3MM chromatography paper for storage.

Pilus HA assays. The hemagglutination (HA) strength of bacterial suspensions was determined by mixing 50 µl of cells in PBS with an equal volume of 2% guinea pig erythrocytes and shaking on a serological ring slide at 100 rpm for 20 min at room temperature. Guinea pig blood was collected by us or by a local supplier via cardiac puncture and mixed with an equal volume of Alsever solution (12). A 1:25 dilution of this 50:50 stock in PBS was referred to as a 2% erythrocyte suspension.

Serum agglutination tests were done similarly, with a 1:100 dilution (in PBS) of rabbit anti-Bam type 1 pilus serum instead of blood. This serum had an enzyme-linked immuno- sorbent assay (ELISA) titer of >1,000,000 and a bacterial cell agglutination titer of >500,000. Production of the serum has been described elsewhere (22).

Pilus crystal HA and soluble pilus passive HA activity assays were conducted by determining activity endpoint titers with a twofold dilution series of pili mixed with guinea pig erythrocytes as described elsewhere (22).

Protein concentration assays. Conventional (37) assays of pilus concentration have been found to give results with as much as 100% error (19). Concentrations of whole pili were determined from a UV light absorbance scan from 350 to 240 nm, corrected for light scattering (17), with an extinction coefficient determined by dry weight (19). The concentration of minor pilus proteins was determined from densitometry of Coomassie blue-stained gels by using pilin of known concentration (see above) as an internal standard. Densitometry was performed with a Beckman DU-8 spectrophotometer at 550 nm with a slit of 0.1 by 4 mm. Peak area calculations and baseline subtractions were done with a computer program provided by the manufacturers. Concentrations of protein on test-of-purity (silver-stained) gels were estimated by using known amounts of carbonic anhydrase as calibration standards. The concentration of 28K protein was also estimated by the UV absorbance method of Scopes based on an A280/A205 ratio (49).

Amino acid composition and sequence analysis. Protein from preparative SDS-PAGE gels was dialyzed against PBS and then against distilled water. For amino acid analysis, the 28K protein was lyophilized and then hydrolyzed in vacuo in 6 N HCl at 110°C for 24 h. For analysis of cysteine, hydrolysis was done in the presence of 5% dimethyl sulfoxide. Amino acid analyses were done in a Durum D-500 analyzer by William Brown, Carnegie-Mellon University, Pittsburgh, Pa. Attempts at sequencing 3 to 4 nmol of these samples were made on an Applied Biosystems model 478 gas-phase sequenator.

Protein purified by gel filtration for chemical analyses was eluted from the column with 0.1% SDS–20 mM N-ethylmorpholine (pH 8) rather than in the elution buffer containing primary amines. After dialysis against 0.01% SDS, the protein was lyophilized and peptide bonds were hydrolyzed in vacuo with 100 µl of 6 N HCl-0.5% phenol for 24 h at 110°C. Compositions were determined on a Beckman 6300 analyzer. Automated Edman degradation of about 2 nmol of protein soluble in 0.01% SDS was performed in a Beckman 890M liquid-phase sequenator. A 0.1 M quadrol peptide program was used for degradations in the presence of glycine-precycled polybrene (28) and phenylthiodyantoin were identified by high-pressure liquid chromatography (HPLC) (23).

RESULTS

Purification of intact pili and pure pilin rods. The method developed by Brinton (4), modified in this report, for the purification of type 1 pili from E. coli is a gentle procedure allowing recovery of HA-active pili in high yield. Figure 1 shows the nonpilus material removed from pili during the crystalization and solubilization cycling procedure. HA activity was always found associated with the pili and not with the particulate (lanes 6, 8, and 10) or soluble (lanes 5, 7, 9, and 11) impurities. Little soluble protein impurities persisted past cycle 2. Small amounts of membrane contaminants that remained aggregated with the pili were released by Triton-EDTA treatment. These remained soluble in the supernatant (lane 12) after pili were precipitated with (NH4)2SO4. The inability of nondepolymerized pilin to enter

FIG. 1. Crystallization and solubilization cycling of pili. Samples of material taken at various steps during the purification of type 1 pili were treated with dilute HCl (pH 2, 100°C, 2 min) to depolymerize the pilin monomer assemblies, run on SDS-PAGE (15% acrylamide), and silver stained. Lane 1, Whole pili standard. Lanes 2 and 15, Molecular size standards (in kilodaltons). Lane 3, Harvest of pilated cells. Lane 4, Pilus-containing supernatant after sedimenting blended cells. Lanes 5, 7, 9, and 11, Supernatants containing 0.1 M MgCl2-soluble contaminants from four successive cycles. Lanes 6, 8, and 10, Pellets containing PB-insoluble material from three successive cycles. Lane 12, Triton-EDTA-soluble material after (NH4)2SO4 precipitation of pili. Lane 13, Depolymerized purified pili. Lane 14, Nondepolymerized purified pili.
FIG. 2. Removal of pilus minor proteins by gel filtration after heating in SDS. Pili were heated in 2% SDS–10 mM DTT–PB for 30 min and then passed over a gel filtration column (see Table 1). Fractions from the void volume were treated to depolymerize the pilin assemblies (lanes 3, 5, and 7) or left untreated (lanes 2, 4, and 6). Lane 1 shows molecular size standards (in kilodaltons).

SDS gels was demonstrated by comparing pili boiled at pH 2 (lane 13) with those that were not so treated (lane 14). Pilin assemblies that were not depolymerized did not substantially penetrate the stacking gel (not shown). The 28K, 16.5K, and 14.5K proteins penetrated the running gel with or without pilin depolymerization. The 16.5K and 14.5K bands were obscured by pilin (17K) in depolymerized samples (lane 13). The minor proteins in purified pilus preparations were difficult to visualize unless silver staining or radiodination and autoradiography (22) were used. Immunoblotting with antipilin serum indicated that the bands below pilin in depolymerized samples were acid hydrolysis products of the pH 2, 100°C treatment (data not shown). An important point is that soluble pili were inactive as hemagglutinins until aggregated by some means, and portions taken during the purification cycling that contained nonaggregated pili were HA inactive. The requirement for pilus aggregation and the parameters of HA by such pili have been described previously (M. S. Hanson, M.S. thesis, University of Pittsburgh, 1983).

The final HA-active pure pili (Fig. 1, lanes 13 and 14) contained about 98% pilin subunit and about 2% pilus-associated proteins (see below). Attempts at removing these minor proteins with concentrated urea, guanidine hydrochloride, or nonionic detergents were unsuccessful. Removal of the three minor proteins was achieved by using SDS, but only in conjunction with elevated temperature. Pili heated in 2% SDS were passed over a gel filtration column, and the eluant fractions were assayed by SDS-PAGE to determine the extent of removal of the minor proteins from the pili. The interactions between the 17K pilin subunits were not broken by heating in SDS; thus, pilin rods with or without the minor proteins attached eluted in the void volume. The peak void volume fractions from three such experiments are shown in Fig. 2. Temperatures of at least 80°C were required for complete removal of the three minor proteins from the rods. The SDS-treated pili were assayed for HA activity after removal of the detergent. The void volume fractions, containing about 250 μg of pili per ml, were mixed with 2 volumes of n-butanol and centrifuged briefly to aid phase separation. After three such extractions, the SDS remaining in the aqueous phase with the pili was less than 1 μg/ml, as determined by a dye-binding assay (24). It was found that removal of the minor proteins from the pili correlated with loss of HA activity (Table 1). Pili lacking the three minor proteins occasionally showed weak HA activity, but this was not mannose inhibitable.

It appeared that the three pilus-associated proteins were not merely contaminants but integral minor proteins of the pilus rods. This was supported by three lines of evidence. (i) They copurified with pili with the same relative stoichiometry from batch to batch of pili from one strain and among type 1 pili purified from different strains (not shown). (ii) They resisted removal from the main pilus rod by all purification techniques tried unless treated under the most denaturing conditions (SDS, 80 to 100°C). (iii) One or more of them were required for pilus adhesion.

In the course of attempts to remove the minor pilus proteins from the pili with SDS, it was observed that pilus rods were insoluble in higher concentrations of SDS (Fig. 3). The resistance of type 1 pili to depolymerization by SDS has been known for some time (19), but the fact that concentrations of SDS above 5 mg/ml precipitate the pili was not anticipated. Thus, the resistance of type 1 pili to SDS

### Table 1. Effect of temperature and detergent on pilus HA ability and minor protein removal

<table>
<thead>
<tr>
<th>SDS treatment</th>
<th>Minor proteins present</th>
<th>HA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>No SDS</td>
<td>Yes</td>
<td>512</td>
</tr>
<tr>
<td>40°C</td>
<td>Yes</td>
<td>128</td>
</tr>
<tr>
<td>60°C</td>
<td>Yes</td>
<td>64</td>
</tr>
<tr>
<td>80°C</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>100°C</td>
<td>No</td>
<td>0</td>
</tr>
</tbody>
</table>

The minor proteins were depolymerized and eluted from the gel filtration column (see Table 1). The 28K, 16.5K, and 14.5K proteins by silver-stained SDS-PAGE (Fig. 2).

Column-fractionated soluble pili were treated to remove these (see text), and their passive HA activities were determined. The starting pilus concentration was approximately 0.25 mg/ml.

![Absorbance vs. SDS Concentration](http://jb.asm.org/)

**FIG. 3.** Solubility of pili in SDS. Soluble pili (1 mg/ml in distilled water) were mixed with various volumes of 20% SDS and incubated overnight at room temperature, and absorbance at 400 nm was read in a Gfirdom 2600 spectrophotometer.
depolymerization allowed the fractionation of pilin from the minor proteins by differential centrifugation. The pellet was composed of pure pilin in the form of rods. SDS-PAGE and silver staining revealed no bands other than pilin at loads of 75 μg per lane (Fig. 4). It has been reported that some SDS-denatured proteins can be reactivated by removing SDS through acetone precipitation, suspension in 6 M guanidine hydrochloride, and dilution in a renaturing buffer (21). While the HA activity of non-denatured pilin was stable to this treatment, the pilin rods free of minor proteins could not be reactivated in this manner.

**Purification of the adhesin and other minor proteins.** Evidence presented elsewhere supports the idea that the 28K minor protein is the actual pilus adhesin (22). Steps were taken to fractionate this protein from the 16.5K and 14.5K polypeptides. Small amounts of insoluble pilin in the SDS supernatant containing the minor proteins were removed by filtration, and the soluble proteins were concentrated by organic solvent precipitation. (Losses due to unpurified protein were slight.) Small fragments of pilin rods that would fractionate as higher-molecular-weight species on gel filtration columns were converted to a monomeric (17K) form to avoid contaminating the fractions containing the 28K protein. Treatment of the organic solvent-precipitated protein with formic acid was found to be an effective means of accomplishing this while minimizing hydrolysis of the minor proteins. When intact soluble pilin were treated with formic acid at concentrations of 40% or higher, pilin entered the SDS running gel even without having been boiled in dilute HCl (Fig. 5, lanes 9, 11, 13, and 15). The 28K protein was found to be only slightly soluble in 40% formic acid, while pilin and the other two minor proteins were partially soluble. Sedimentation of the 28K protein at this step afforded about a 50% purification from the other soluble proteins (Fig. 4).

After removal of organic solvents, the 28K and other proteins were resuspended in SDS and fractionated by gel filtration chromatography in the same detergent (Fig. 6). Earlier experiments with Sepharose 4B showed that the minor proteins solubilized in hot SDS eluted later from such columns if DTT was included during solubilization and chromatography. Presumably, disulfide bonds play a role in inter- or intrachain interactions among the pilus proteins. Thus far, column conditions have not been found that adequately resolve the lower-molecular-weight (17K, 16.5K, and 14.5K) proteins to allow their purification. We are currently investigating the use of reverse-phase HPLC for this task. The 28K protein has been purified by the above methods to apparent homogeneity by SDS-PAGE and silver staining, with yields approaching 50% relative to amount of protein present on purified, intact pilin.

Preparative SDS-PAGE was explored as an alternative to gel filtration chromatography. The 28K protein was the most easily resolved. Both methods of electroelution proved to be effective. Protein collected by the salt trap method gave essentially the same amino acid analysis (data not shown) as did protein purified by gel filtration. Protein eluted into dialysis bags maintained better solubility than protein precipitated onto the salt trap. The 28K protein purified from preparative gels was homogeneous, as determined by silver-stained SDS-PAGE (Fig. 7).

**Stoichiometry of pilus proteins.** A preliminary estimate of the stoichiometry of the major (pilin) and minor pilus proteins was obtained from densitometry analysis of Coomassie blue-stained SDS gels. Pellets of crystalline pilin were suspended in formic acid at depolymerizing (40%) or subdepolymerizing (10%) concentrations to about 10 mg of protein per ml. It was found necessary to load at least 20 to 30 μg of protein per lane to adequately visualize the minor proteins against the background of Coomassie blue staining.
areas (not shown) were used with apparent molecular weights of 28K, 17K (pilin), 16.5K, and 14.5K proteins to obtain molar ratios of 2:(300–400):1:1.

Amino acid sequence analysis. The amino acid analyses of the 28K protein purified by gel filtration chromatography are shown in Table 2. There was good agreement among the analyses of the proteins purified, hydrolyzed, and analyzed by the different methods (data not shown). The composition showed substantial differences from all *E. coli* pilins (30) as well as other surface proteins, such as OmpA and OmpF. Most notably, the 28K protein lacked methionine, as does type 1 pilin (4, 45). Both proteins showed resistance to cleavage by cyanogen bromide (data not shown). The analysis of the 28K protein confirmed the prediction of abundant tyrosine made from its sensitivity to labeling by 125I (22). Similar predictions can be made for the 16.5K and 14.5K minor pilus proteins. Analyses of these are in progress. Amino acids with nonpolar side chains (Ile, Phe, Pro, Leu, Val, Met, Ala, and Gly) accounted for about 59% of the 28K protein composition and about 53% of the residues in type 1 pilin. The “solvent parameter” of Levitt (36) is one way of expressing amino acid hydrophobicity. Neglecting Asx and Glx, the weighted average solvent parameters for the 28K protein and pilin were −725 and −634 cal/mol, respectively. By either of these simple approximations, the 28K protein has a more hydrophobic character than the main rod subunit, pilin.

Two attempts at sequencing the 28K protein purified by elution from SDS gels were unsuccessful. This may have been due to artifactual blocking of the amino terminus during electrophoresis (27) or during electroelution into the salt-urea trap. One sequence analysis attempt suffered from incomplete removal of buffer salts. Additionally, the 28K protein was largely insoluble in the 15% acetic acid used to load the gas-phase sequenator. On the other hand, Edman degradation of the 28K protein purified by gel filtration proceeded with a 93% repetitive yield for over 30 cycles on

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>34.0</td>
</tr>
<tr>
<td>Thr</td>
<td>24.4</td>
</tr>
<tr>
<td>Ser</td>
<td>16.8</td>
</tr>
<tr>
<td>Glx</td>
<td>15.9</td>
</tr>
<tr>
<td>Pro</td>
<td>16.2</td>
</tr>
<tr>
<td>Gly</td>
<td>32.3</td>
</tr>
<tr>
<td>Ala</td>
<td>24.9</td>
</tr>
<tr>
<td>Cys</td>
<td>2.4</td>
</tr>
<tr>
<td>Val</td>
<td>32.1</td>
</tr>
<tr>
<td>Met</td>
<td>0.0</td>
</tr>
<tr>
<td>Ile</td>
<td>13.0</td>
</tr>
<tr>
<td>Leu</td>
<td>19.3</td>
</tr>
<tr>
<td>Tyr</td>
<td>14.6</td>
</tr>
<tr>
<td>Phe</td>
<td>8.9</td>
</tr>
<tr>
<td>Trp</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>His</td>
<td>1.1</td>
</tr>
<tr>
<td>Lys</td>
<td>6.0</td>
</tr>
<tr>
<td>Arg</td>
<td>6.0</td>
</tr>
<tr>
<td>Total</td>
<td>273.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from analysis of protein from G-100 column, dialyzed and then hydrolyzed in 6 N HCl-0.5% phenol except as noted. Composition based on M, 28,000.

<sup>b</sup> Amide determinations were not made.

<sup>c</sup> Data from analysis of protein from SDS-PAGE, dialyzed and then hydrolyzed in 6 N HCl-5% dimethyl sulfoxide.

<sup>d</sup> nd. Not determined.
the liquid-phase sequenator. The results through residue 30 are shown in Fig. 8.

While the 28K adhesin protein amino-terminal sequence was distinct from that of E. coli pilins and other periplasmic and outer membrane proteins (not shown), it did show several amino acid matches with type 1 pilin and two other pilins in the 28K range, K88 and F41.

**DISCUSSION**

By exploiting the unusual stability and solubility properties of type 1 pilus rods in SDS solutions, the pilin subunit assemblies and 28K adhesin proteins have been purified to apparent homogeneity. The subunit interactions between pilin monomers are resistant to disruption by all detergents, indicating that hydrophobic interactions may not be the major forces binding them together. The opposite seems to be true of the interactions among the minor proteins and of these proteins with the main rod.

The use of deoxycholate and velocity sedimentation to remove membrane contaminants from intact pili (32) was found to be less efficient than Triton-EDTA and differential centrifugation, as used here, when silver staining was used to determine purity. Additionally, our method is applicable to larger samples and to most of the clinical E. coli isolates we have encountered. Although not necessary for strain Bam, flagella from other piliated E. coli strains can be depolymerized by the addition of 1 N NaOH to pH 12 and incubation for 1 h (P. Fusco, Ph.D. thesis, University of Pittsburgh, 1983). The use of concentrated urea is also effective for flagellar depolymerization (32).

The unexpected ability of the depolymerized pilin rods to aggregate in concentrated SDS solutions allowed purification of these structures to homogeneity, devoid of minor proteins or other contaminants. These pure pilin rods retained the major chemical and antigenic properties of intact pili but failed to promote HA.

Due to the extremely low copy number of the 28K adhesin protein on type 1 pili, about three to six per rod, large quantities of pili (1 to 2 g) were necessary in order to work out the purification procedures for this novel protein. Obtaining pili in this quantity was greatly facilitated by use of the protocol described here. Note that in contradiction to some reports (30), richly piliated bacteria can be grown effectively on solid medium if careful attention is given to manipulation of pilination phases by selection of the appropriate colonial types and growth conditions (3, 4).

The amino-terminal sequences of the type 1 pilus adhesin and pilin proteins show limited homology through the first 20 residues. Similarities may be noted with F41 and K88 pilins, proteins of sizes similar to that of the type 1 pilus adhesin. Many of the nonidentical amino acids shown in the alignment could result from single-nucleotide base substitutions. At least at the amino terminus, the adhesin seemed to show some structural similarity to pilin. Similar observations have been made for the deduced amino acid sequences of the major pilin subunit and a putative minor protein of Pap pilin (2). The adhesin and the pilE gene product (39) are of similar Mr, and each has been shown to be required for HA activity. We propose that they are the same protein, the type 1 pilus adhesin.

Similarities in the amino-terminal portions of type 1 pilin and type 1 adhesin are not reflected in antigenic cross-reactivity. By ELISA and immunoblotting, no cross-reactivity is seen between antipilin serum and adhesin or between antiahesive serum and the pilin, 16.5K, or 14.5K proteins (22).

The difficulty in resolving the 16.5K and 14.5K proteins from each other and from soluble pilin has thus far precluded obtaining any sequence or serological information about their relatedness to the other two pilus proteins. They are similar to the adhesin in their solubility in SDS (Fig. 3), but once removed from the pilus were more similar to pilin in their solubility in 40% formic acid (Fig. 5). We speculate that their role in pilus structure may be as adapter proteins between the terminal pilus subunits and the 28K adhesin protein. They may also provide some shielding of this hydrophobic protein from the aqueous environment, as well as shielding the highly conserved adhesin (22) from immunological pressure. The hydrophobic nature of the tip adhe-

<table>
<thead>
<tr>
<th>Type 1 pilin</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 adhesin</td>
<td>phe</td>
<td>ala</td>
<td>x</td>
<td>lys</td>
<td>thr</td>
<td>thr</td>
<td>thr</td>
<td>val</td>
<td>asn</td>
<td>gly</td>
<td>gly</td>
<td>thr</td>
<td>val</td>
<td>his</td>
<td>phe</td>
</tr>
<tr>
<td>F41 pilin</td>
<td>ala</td>
<td>asp</td>
<td>trp</td>
<td>thr</td>
<td>glu</td>
<td>gly</td>
<td>gln</td>
<td>pro</td>
<td>gly</td>
<td>asp</td>
<td>ile</td>
<td>leu</td>
<td>ile</td>
<td>gly</td>
<td>gly</td>
</tr>
<tr>
<td>K88 pilin</td>
<td>trp</td>
<td>met</td>
<td>thr</td>
<td>gly</td>
<td>asp</td>
<td>phe</td>
<td>asn</td>
<td>gly</td>
<td>ser</td>
<td>val</td>
<td>asp</td>
<td>ile</td>
<td>gly</td>
<td>gly</td>
<td></td>
</tr>
<tr>
<td>K99 pilin</td>
<td>asn</td>
<td>thr</td>
<td>gly</td>
<td>thr</td>
<td>ile</td>
<td>asn</td>
<td>phe</td>
<td>asn</td>
<td>gly</td>
<td>lys</td>
<td>ile</td>
<td>chr</td>
<td>ser</td>
<td>ava</td>
<td>thr</td>
</tr>
</tbody>
</table>

**FIG. 8.** N-terminal amino acid sequence of the type 1 pilus 28K adhesin protein compared with those of type 1 (45), F41 (15), K88ab (28), and K99 (47) pilins. The amino-terminal sequence of strain Bam type 1 pilin (26) is homologous to that of PilA (45). In cycles 3 and 17 of the 28K sequence, no derivative was detectable (indicated by X); this result is compatible with the presence of an unmodified cysteine residue at these positions. The uncertain assignment of aspartic acid at position 30 in the 28K sequence is indicated as (asp). Alignments were made by visual inspection. Amino acids identical to those in the 28K sequence are indicated by boxes.
sin may also facilitate its interaction with the membrane receptors of eucaryotic cells.

The first evidence of tip adherence by pili was the observation that free type 1 pilus adhere endwise to polystyrene latex (PSL) particles as viewed by electron microscopy (4). This adherence is monovalent, since free pili suspensions essentially free of aggregates do not agglutinate PSL particles (S. Polen and C. C. Brinton, Jr., unpublished). Adherence of free pili to erythrocytes (RBC) is also tipwise and monovalent (22). Monovalent tip adhesion is explainable without invoking a tip adhesin, since the helical symmetry of pilus rods (4) implies that different pilin domains are exposed at the distal and proximal ends of the free pilus rod as well as at the lateral surface. Pilus families without a tip adhesin may adhere tipwise by their exposed cell-distal pilin domains, which, because they are normally buried inside the pilus rod, are expected to be more hydrophobic than their exposed pilin-lateral domains.

Our recent discovery of the type 1 tip adhesin (22) suggests that it, rather than a distal pilin domain, may be involved in PSL adhesion as well as RBC adhesion. The higher hydrophobicity of adhesin than of pilin that we report here may be responsible for endwise adhesion to PSL particles and may also strengthen the adhesive bond to RBC. Alternative explanations of monovalent endwise PSL adhesion by free pili is that the hydrophobic cell-proximal pilin domain or a hydrophobic pilus anchor protein is the PSL adhesion site.

This report represents the first purification and characterization of a pilus adhesin minor protein. Antiserum raised against the adhesin (22) has proved useful in identifying this protein as containing the mannose-binding sites of the pilus and may be useful in studies of pilus morphogenesis. We are currently applying principles described here toward the purification of other pilus adhesin proteins. These techniques may prove applicable to the production of broadly cross-reactive antidiadhesin and colonization vaccines against diseases caused by piliated bacteria.

After original submission of the manuscript of this paper, the sequence of the fimFGH gene cluster was published (31). The products of these genes are required for mannose-specific adhesion of E. coli K-12. The predicted amino acid sequence of fimH indicates a protein of similar size to the strain Bam type 1 adhesin and has nearly complete identity to the adhesin N-terminal sequence shown here. The leader peptide cleavage site in fimH, predicted by using the von Heijne rule (53), is two residues downstream of the amino terminus observed here for the mature adhesin. At least for strain Bam, cleavage occurs at the position of the second-highest "processing probability" predicted by this rule rather than at the highest.

LITERATURE CITED


