INFECTION AND IMMUNITY, Mar. 1990, p. 695–702
0019-9567/90/030695-08$02.00/0

Vol. 58, No. 3

Purification and Characterization of the Dr Hemagglutinins Expressed by Two Uropathogenic *Escherichia coli* Strains

MARION L. KIST,[1] IRVING E. SALIT,[2,3] AND THEO HOFMANN[1]*

Departments of Biochemistry[1] and Microbiology,[2] University of Toronto, and Division of Infectious Diseases, Toronto General Hospital,[3] Toronto, Ontario M5S 1A8, Canada

Received 15 September 1989/Accepted 5 December 1989

The fibrillar Dr hemagglutinins expressed by two uropathogenic *Escherichia coli* isolates were mechanically sheared from whole cells and subsequently purified by using anion-exchange high-pressure liquid chromatography. The isolated hemagglutinins were proteins with apparent subunit molecular masses of 14,500 daltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric points of 5.4 in denaturing isoelectric focusing gels. The two proteins were serologically related to each other but distinct from P fimbriae, as assessed by bacterial agglutination and immunoblotting. The amino acid compositions of the two hemagglutinins were highly similar both to each other and to other Dr hemagglutinins. N-terminal amino acid sequencing of the major hemagglutinin subunit proteins demonstrated homology with afimbrial *E. coli* adhesins.

The adherence of pathogenic *Escherichia coli* to host uroepithelium is a prerequisite for successful colonization of the urinary tract and the subsequent establishment of infection. The adherence of uropathogenic *E. coli* to specific receptors is mediated by proteins (adhesins) which are either expressed on the bacterial cell surface in association with filamentous structures termed fimbriae (or pili) or as afimbrial adhesins. In some cases, the specific receptor substances are also present on erythrocytes; hence, some adhesins are also hemagglutinins. The adhesins expressed by uropathogenic *E. coli* have been broadly classified as either mannose-sensitive or mannose-resistant (MR) hemagglutinins depending on their ability to agglutinate erythrocytes in the presence of D-mannose-containing compounds. MR hemagglutinins have been further subdivided according to their receptor specificities: 80 to 90% of all pyelonephritogenic *E. coli* isolates express P fimbriae, which bind the α-D-Gal-(1→4)-β-D-Gal moiety of the human P blood group antigen as well as glycosphingolipids of the uroepithelium (11, 18). The binding specificities of other MR hemagglutinins have also been identified: F adhesins mediate binding to the galactose-*N*-acetyl-α(1→3)galactose-*N*-acetyl moeity of the Forssman antigen (present on sheep erythrocytes and cells of the human renal pelvis) (20, 21), S fimbriae recognize sialyl galactosides (14), G fimbriae bind terminal *N*-acetylglucosamine residues (30), and M adhesins adhere to the M blood group antigen (35). Other MR hemagglutinins constitute a heterogeneous group with unknown receptor specificities and are termed X adhesins. One group of X adhesins is frequently associated with *E. coli* strains belonging to serogroup O75 and have been called O75X adhesins. They have been shown to bind preferentially to basement membranes of human and canine kidneys, Bowman's capsule, and, to a lesser extent, to the bladder epithelium (15, 26, 38, 40). Several O75X+ *E. coli* strains have also been shown to bind the Dr blood group antigen of the human IFC blood group complex and have, accordingly, been renamed Dr hemagglutinins (26). The Dr adhesin-encoding (*dra*) operon has been identified (25, 27), and a high frequency of *dra*-related sequences has been detected among *E. coli* strains isolated from patients with cystitis, as opposed to other clinical forms of urinary tract infection (27). Dr adhesins, therefore, may be important virulence factors in lower urinary tract infection (27).

This paper describes the purification and biochemical characterization of the Dr hemagglutinins expressed by two uropathogenic *E. coli* strains and demonstrates a relationship between these hemagglutinins and *E. coli* afimbrial adhesins.

MATERIALS AND METHODS

Bacteria. *E. coli* 950 (O75:K5:H-) and 5026 (O14:K5:H-) were clinical isolates obtained from the blood of infected patients. They were subcultured once on tryptic soy agar (Difco Laboratories) and stored as suspensions in 5% (wt/vol) bovine serum albumin–5% (wt/vol) monosodium glutamate. The bacteria were thawed as required and grown on colonization factor antigen (CFA) agar (5). *E. coli* 917 (O75:K5:H1) is a P-fimbriated uropathogenic strain which has been previously characterized (8).

Agglutinations. (i) Hemagglutination. Hemagglutination assays were performed on glass microscope slides over crushed ice. Packed erythrocytes were suspended to a final concentration of 3% (vol/vol) in 10 mM phosphate-buffered saline (PBS), pH 7.4, containing 1% (wt/vol) D-methylmannoside. A drop of the erythrocyte suspension was mixed on a slide with an equal volume of purified hemagglutinin or a wire loopful of agar-grown bacteria. Hemagglutination was scored visually. Agglutination of Dr (a−) cells was kindly performed in a similar manner by S. Hull, Department of Microbiology, Baylor College of Medicine, Houston, Tex. For some experiments, human erythrocytes were suspended in PBS containing 1.0 mg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml and incubated for 3 h at 37°C. The cells were then pelleted by low-speed centrifugation and washed several times with PBS prior to testing for hemagglutination. p̄ erythrocytes were kindly provided by the Canadian Red Cross. Sheep, guinea pig, chicken, horse, goat, rat, cow, and rabbit erythrocytes were obtained from Woodlyn Laboratories, Guelph, Ontario, Canada.

(ii) P-particle agglutination assay. To test for the expression of P fimbriae, a loopful of agar-grown bacteria was mixed on a glass slide with latex beads coated with the P

* Corresponding author.

fimbria receptor substance (αD-gal-β1→4-D-gal) (Carbohydrates International, Chicago, Ill.). Agglutination of the latex beads was assessed visually.

(iii) Yeast cell agglutination. Type 1 fimbriae were detected by yeast cell agglutination assays by using *Saccharomyces cerevisiae* cells (Sigma), as described by Korhonen (13).

Electron microscopy. Samples were negatively stained by placing a drop (approximately 15 μl) of the sample suspension on the surface of a Formvar carbon-coated copper grid with a micropipette. The sample was allowed to dry onto the grid. The grid was then floated on a drop of 2.0% (wt/vol) ammonium molybdate, pH 7.0. Excess fluid was removed by touching the grid to filter paper. Specimens were viewed in a Hitachi 7000 transmission electron microscope operating at 100 kV.

Crude hemagglutinin preparation. Cells from confluent overnight growth (16 h, 37°C) on 50 CFA agar plates (15 cm in diameter) were suspended in ice-cold 0.05 M Tris hydrochloride buffer, pH 8.5, by using a bent glass rod. The cell suspension was blended on ice for 10 min in a Sorvall Omni-mixer operating at medium speed. Whole cells and large cell debris were removed by centrifugation (45 min; 20,000 × *g*) at 4°C. The supernatant was recentrifuged under identical conditions and then ultracentrifuged (200,000 × *g*) for 2 h at 4°C. The pellet was suspended in a small volume of 0.05 M Tris hydrochloride buffer, pH 8.5, and allowed to stand at 4°C for 48 h. Insoluble material was removed by centrifugation (10 min; 12,000 × *g*) in an Eppendorf centrifuge. The supernatant possessed hemagglutinating activity and constituted the crude hemagglutinin preparation.

For some experiments, 1 M $MgCl_2$ was slowly added to the crude preparation to a final concentration of 0.1 M. The preparation was incubated at 4°C for 24 h, after which time it became cloudy. $MgCl_2$-precipitated material was removed by centrifugation (12,000 × *g*) for 5 min at 4°C in an Eppendorf centrifuge. The supernatant was removed and dialyzed against distilled water at 4°C for 48 h with two changes. The dialysate was then concentrated by membrane ultrafiltration by using a YM 10 membrane (10,000 daltons, molecular mass cutoff; Amicon Corp., Danvers, Mass.). The pellet ($MgCl_2$-precipitated material) was suspended in a small volume of distilled water, dialyzed against distilled water, and concentrated as described above. The supernatant and pellet fractions were each tested for mannose-resistant hemagglutination (MRHA) of human erythrocytes and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy.

Purification of hemagglutinins by anion-exchange HPLC. Purification of hemagglutinins by anion-exchange high-pressure liquid chromatography (HPLC) was accomplished by applying 100 to 250 μg of protein to a DEAE-5-PW anion-exchange column (75 by 7.5 mm in diameter; Bio-Rad Laboratories, Richmond, Calif.) previously equilibrated with 0.02 M Tris hydrochloride, pH 8.5. The column was washed for 10 min with the same buffer. The bound proteins were eluted by using an NaCl gradient (0 to 0.5 M NaCl in 0.02 M Tris hydrochloride, pH 8.5) with a flow rate of 1.0 ml/min. Eluted protein was detected at 215 nm. All peak fractions were collected, dialyzed against distilled water to remove buffer salts, and concentrated by membrane ultrafiltration. Each peak was tested for MRHA of human erythrocytes and analyzed by SDS-PAGE.

Electrophoresis. Proteins were analyzed in 15% polyacrylamide slab gels by the method of Laemmli (17) except that SDS was omitted from the gels. Samples were diluted 2:1 in sample buffer (0.0625 M Tris, pH 6.8, containing 20% [vol/vol] glycerol, 4% [vol/vol] β-mercaptoethanol, 2% [wt/vol] SDS, and 0.005% [wt/vol] bromophenol blue) and heated at 100°C for 5 min before electrophoresis. Polypeptides were stained with Coomassie brilliant blue R250. The relative molecular masses of the hemagglutinin subunit proteins were determined by comparing their migration in slab gels with those of calibration proteins (Bio-Rad). Lipopolysaccharide was detected by silver staining SDS-PAGE gels, as described by Tsai and Frasch (33).

Protein estimations. Protein concentrations were determined either by the dye-binding method of Bradford (2) by using a commercially available reagent (Bio-Rad) with bovine serum albumin (ICN Immunobiologicals; Lisle, Ill.) as a standard or by quantitative amino acid analysis by using norleucine as an internal standard.

Amino acid analysis. Purified hemagglutinins were hydrolyzed in 6 N HCl at 107°C for 22 h in evacuated, sealed tubes. Their amino acid compositions were determined by using a Beckman Model 121M single-column analyzer. Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, following performic acid oxidation. Tryptophan was not determined.

N-terminal amino acid sequencing. Proteins were sequenced by using model 470A gas phase sequenator (Applied Biosystems, Foster City, Calif.) with an in-line Applied Biosystems model 120 PTH amino acid analyzer. The computer program ALIGN (Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C.; 24) was used to compare the N-terminal amino acid sequences of the Dr hemagglutinins with those of other *E. coli* adhesins. Alignment scores, which reflect similarities between sequences, were calculated by using a mutation data scoring matrix (3, 31) and a gap penalty of 6.

Isoelectric focusing. The isoelectric points of the hemagglutinins were determined in denaturing isoelectric focusing tube gels (130 by 4.5 mm in diameter) by the method of O'Farrell (28). Protein (40 μg) was applied to each gel. Focusing was performed for 18 h at 300 V and then for an additional 1.5 h at 400 V. Polypeptides were simultaneously fixed and stained by immersing the gels in 0.115% (wt/vol) Coomassie brilliant blue R250–5% (wt/vol) trichloroacetic acid–5% (wt/vol) sulfosalicylic acid–30% (vol/vol) methanol and then destained by using 25% (vol/vol) methanol–7% (vol/vol) acetic acid. Duplicate unstained gels were cut into 5 mm sections, placed in individual vials containing 2.0 ml of distilled water, and incubated at 22°C with shaking. The pH of each solution was determined after 2 h. The pIs of the hemagglutinins were determined by comparing the relative positions of the Coomassie blue-stained polypeptides with the pH gradient.

Preparation of antiserum. Rabbit hyperimmune antiserum specific for the native Dr hemagglutinin isolated from strain 950 was available from a previous study (8). Crude antiserum was repeatedly adsorbed with *E. coli* 950 cells grown at 22°C to remove antibodies directed against nonfimbrial surface components.

Bacterial agglutination. To test for bacterial agglutination, CFA agar-grown bacteria were suspended in PBS, and a drop of the solution was mixed on a glass microscope slide with an equal volume of antiserum appropriately diluted with PBS. Agglutination was scored visually after 30 s.

Immunoblotting. Crude bacterial hemagglutinin preparations were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose, essentially as described by Towbin et al. (32). Additional binding sites were blocked by

TABLE 1. Agglutination properties of *E. coli* strains grown on agar

Cell type and source	Agglutination by strain[a]: 950 (Dr+)	5026 (Dr+)	917 (P+)
Human erythrocytes			
P1	+	+	+
p̄	+	+	+
Trypsinized P1	−	−	+
P1 + 10 mM chloramphenicol	−	−	+
Dr (a−)	−	−	+
Erythrocytes from other species			
Sheep	−	−	+
Guinea pig	−[b]	−[b]	−
Horse	−	−	−
Chicken	−	−	+
Goat	−	−	+
Cow	−	−	−
Rat	−	−	−
Rabbit	−	−	−
Yeast cells			
PBS	−[b]	−[b]	−
PBS + 1% D-methylmannoside	−	−	−
P-particle agglutination assay	−	−	+

[a] +, Agglutination; −, no agglutination.
[b] Agglutination was positive when bacteria were grown in liquid broth.

immersing the nitrocellulose membrane in Tris-buffered saline containing 5% (wt/vol) nonfat milk for 1 h. The membrane was then incubated with antiserum (diluted 1:1,000 in Tris-buffered saline containing 1% nonfat milk) for 2 h at room temperature. Bound antiserum was detected by using horseradish peroxidase-conjugated goat anti-rabbit antiserum (Bio/Can Scientific, Inc., Mississauga, Ontario, Canada) (diluted 1:1,000), with subsequent color developed by using 4-chloro-1-naphthol (Bio-Rad).

RESULTS

Agglutination properties of whole cells. Strains 950 and 5026 grown to stationary phase in liquid broth demonstrated mannose-sensitive agglutination of yeast cells, indicating the expression of type 1 fimbriae (13) (Table 1); their expression was inhibited when the bacteria were grown on solid media (4). These strains agglutinated both human P1 and p̄ erythrocytes in the presence of 1% D-methylpyrannoside and failed to agglutinate digalactoside-coated latex beads (Table 1). Both strains, therefore, expressed MR, but P blood group-independent, adhesins. Because strain 950 had been serotyped as O75 (serotyping kindly performed by F. Ørskov and I. Ørskov, Copenhagen, Denmark), there was the possibility that the adhesin expressed by this strain was related to the O75X adhesins previously described by Väisänen-Rhen (36). Several O75X adhesins have been shown to bind the Dr blood group antigen of the human IFC blood group complex and have been renamed Dr hemagglutinins (26). Strains 950 and 5026 both failed to agglutinate Dr (a−) erythrocytes, which lack the Dr blood group antigen and, therefore, express Dr hemagglutinins (26). Furthermore, hemagglutination was inhibited by the addition of chloramphenicol (26). Pretreatment of erythrocytes with trypsin also abolished hemagglutination by both strains. Strain 950 and 5026 agglutinated human erythrocytes but not the erythrocytes from eight other animals (Table 1).

Morphological observations. Negative staining-electron microscopy of whole cells and hemagglutinating preparations showed that *E. coli* 950 and 5026 were capable of expressing a variety of surface structures (Fig. 1). Typical rigid type 1 fimbrial filaments (approximately 7 nm in diameter) were detected when the bacteria were grown in CFA broth (Fig. 1A), which supported the observed mannose-sensitive agglutination of yeast cells by these bacteria (Table 1). Cells grown on solid media did not express rigid filaments; however, there were fine threadlike fibers (approximately 2 to 2.5 nm in diameter) which projected from the surface of these cells when grown at 37°C (Fig. 1B). The fibers tended to aggregate; individual strands intertwined to form fibrillar, ropelike structures (Fig. 1B). Tangled masses of flexible fimbrialike structures were also found surrounding most agar-grown cells, either associated with the outer membrane (usually at one pole) or lying separate from the cell body (Fig. 1D). Individual filaments of this tangled mass (approximately 3 to 3.5 nm in diameter) frequently aggregated as parallel strands. These filaments, although fimbrialike in appearance, were not believed to play a role in hemagglutination, because cells grown at 22°C expressed identical structures but did not agglutinate human erythrocytes.

The surface structures were detached from whole cells by mechanical shearing and separated from unbroken cells and large debris by differential centrifugation. Some of the contaminating membrane vesicles aggregated upon incubation of the preparation at 4°C and were subsequently removed by low-speed centrifugation. The supernatant had hemagglutinating activity, and this constituted the crude hemagglutinin preparation. Negative staining-electron microscopy showed that this preparation consisted of the 2- to 2.5-nm fibers and a small proportion of what appeared to be more typical fimbrial structures (approximately 6.5 nm in diameter). Similar 6.5-nm filaments were never detected on whole cells, even when the latter were fixed with glutaraldehyde prior to negative staining (data not shown). These structures may be readily detected only when concentrated in a fimbrial preparation. The filaments may represent type 1 fimbriae, although concentrated fimbrial preparations did not agglutinate yeast cells (13). Alternatively, these 6.5-nm filaments may represent aggregates of the 3- to 3.5-nm fimbrialike structures, since we could detect a fine, densely staining region along these filaments which appeared to separate these structures into two filaments of equal diameter (Fig. 1D, inset). The structures were always blunt ended, suggesting that aggregation occurred prior to mechanical shearing of whole cells. The 3- to 3.5-nm fimbrialike filaments were also expressed by cells grown at room temperature; furthermore, similar types of aggregated filaments (Fig. 1D) were found when cells grown at room temperature were mechanically sheared and the liberated surface structures were collected by differential centrifugation (data not shown).

The two morphologically different fimbria types could be effectively separated by $MgCl_2$ precipitation of the crude hemagglutinin preparation. The 6.5-nm filaments were precipitated by the addition of $MgCl_2$ and could be removed by centrifugation (Fig. 1E), leaving the 2- to 2.5-nm fibers in the supernatant fraction (Fig. 1F). MRHA activity was found to be associated only with the latter fraction.

Purification of the hemagglutinin. SDS-PAGE of the crude hemagglutinin preparations showed a major polypeptide with an apparent relative molecular mass of 14,500 and a number of polypeptides of higher molecular masses (Fig. 2, lanes B and C). The 14.5K proteins were in the molecular

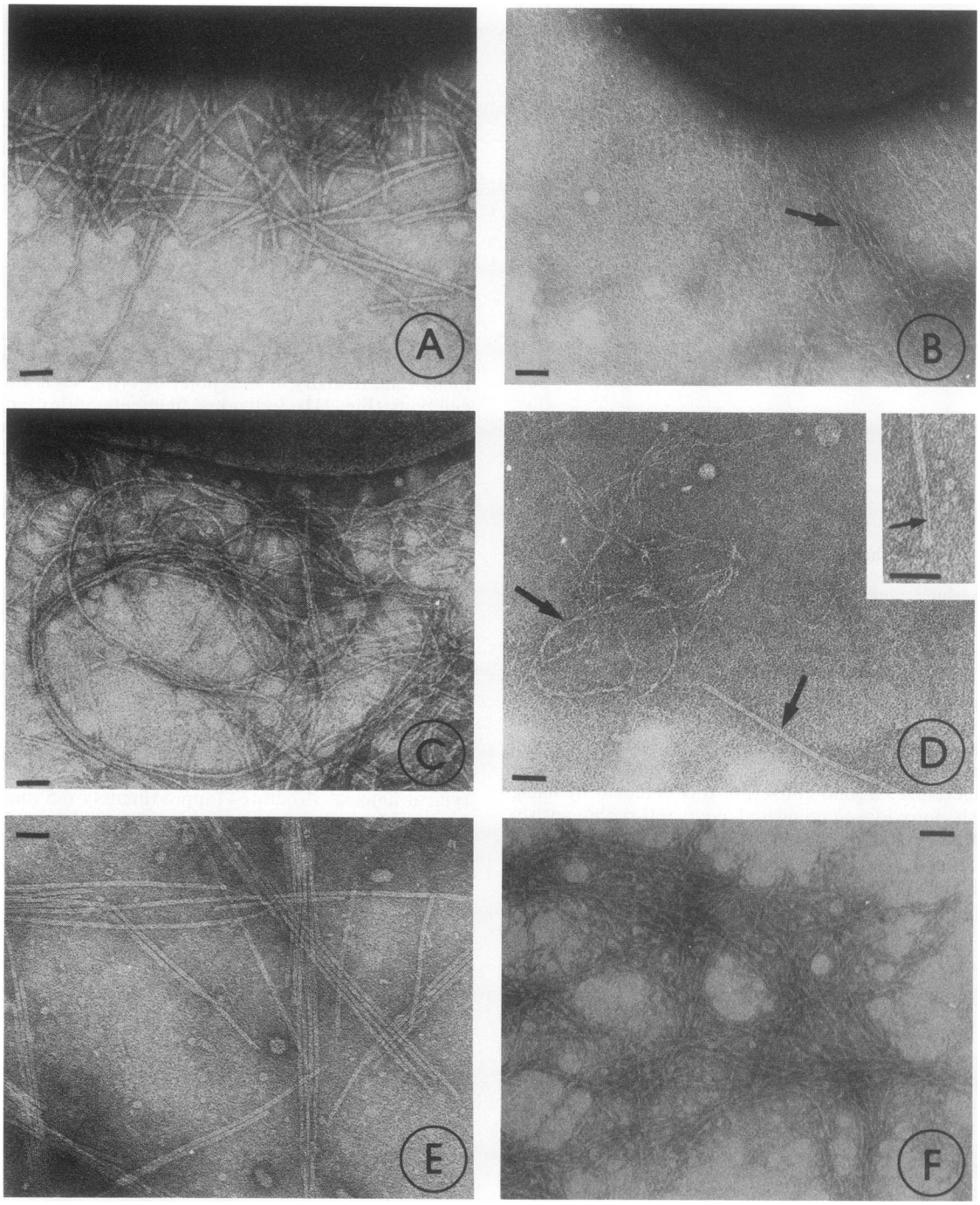

FIG. 1. Electron micrographs of *E. coli* 950 cells and crude hemagglutinin preparations negatively stained with ammonium molybdate. Cells grown in CFA broth expressed type 1 fimbriae (A), whereas cells grown on solid media expressed both 2- to 2.5-nm fibrils (B) and flexible 3- to 3.5-nm fimbrialike filaments (C). The crude hemagglutinin preparation (D) contained both fibrils and filaments (arrows indicate the two fimbrial types), which were separated by $MgCl_2$ precipitation of the crude preparation followed by low-speed centrifugation (inset, arrow indicates the densely staining midregion of a single filament). (E) $MgCl_2$-precipitated filaments; (F) nonprecipitated material containing 2- to 2.5-nm fibrillar structures. Bar = 50 nm.

mass range characteristic of fimbrial subunit proteins and, therefore, were probably the hemagglutinins. Hemagglutinating activity was found to be associated with the 2- to 2.5-nm fibers, which remained in the supernatant fraction following $MgCl_2$ precipitation of the crude hemagglutinin preparation and subsequent centrifugation. Moreover, SDS-PAGE showed that the 14.5K protein was associated with this fraction (Fig. 2, lane E).

The 14.5K proteins were purified by using anion-exchange HPLC. The crude hemagglutinin preparations from strains 950 and 5026 showed almost identical separation patterns. Each protein peak was analyzed by SDS-PAGE and tested

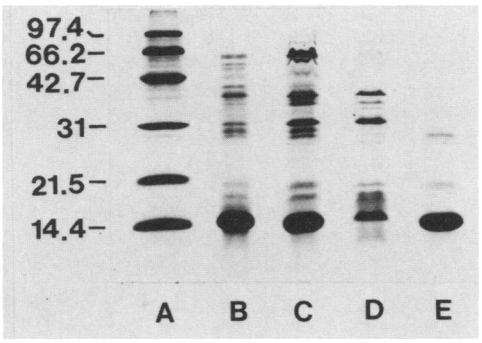

FIG. 2. SDS-PAGE of the crude hemagglutinin preparations from strains 5026 (lane B) and 950 (lane C). Lane D, Pellet following $MgCl_2$ precipitation of the crude preparation. Lane E, nonprecipitated protein. Lane A, Molecular mass markers (kilodaltons): phosphorylase *b*, 97.4; bovine serum albumin, 66.2; ovalbumin, 45.7; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5; and lysozyme, 14.4.

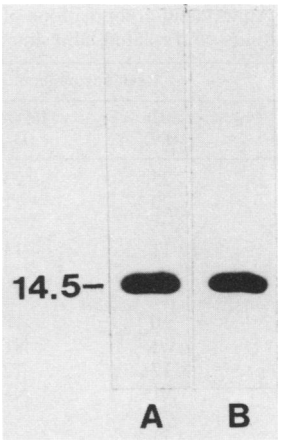

FIG. 4. SDS-PAGE of the Dr hemagglutinins purified from strains 950 (lane A) and 5026 (lane B). The molecular mass of the subunit proteins is shown in kilodaltons.

for MRHA activity by using a slide agglutination assay. In each instance, only peak 4 (Fig. 3) agglutinated human erythrocytes. SDS-PAGE showed that this peak consisted of the 14.5K polypeptide. The peak 4 fractions from several separations were pooled, dialyzed against distilled water to remove the buffer salts, concentrated by membrane ultrafiltration, and reapplied to the ion-exchange resin to separate any tightly associated contaminating proteins. The eluted 14.5K proteins were judged to be homogeneous by SDS-PAGE (Fig. 4) and subsequent repeat chromatography. Negative staining-electron microscopy showed that this fraction consisted of fibrillar structures similar to the 2- to 2.5-nm fibers previously detected on the surface of whole cells (Fig. 5).

The 6.5-nm filaments (detected in crude hemagglutinin preparations) eluted as a small shoulder immediately after the hemagglutinins (Fig. 3, peak 5). Negative staining-electron microscopy showed that this peak also contained numerous membrane vesicles and other debris (data not shown). This fraction did not possess hemagglutinating activity, and these filaments were not subjected to further purification.

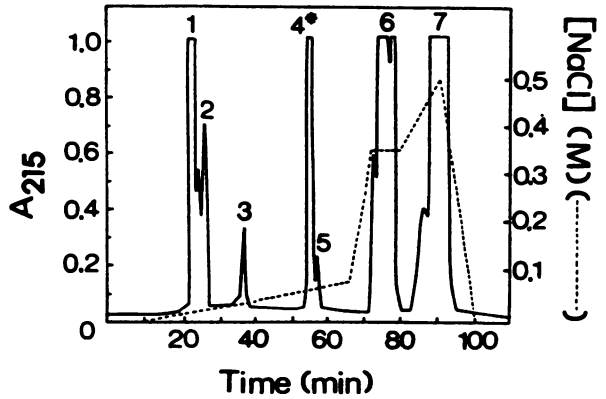

FIG. 3. Purification of Dr hemagglutinins by anion-exchange HPLC. Crude hemagglutinin preparations were injected onto the column at time zero, and the column was washed for 10 min with buffer. Protein was eluted by using an increasing NaCl gradient (– – –) and monitored at A_{215}. *, Peak with hemagglutinating activity.

Biochemical properties. The amino acid compositions of the Dr hemagglutinins expressed by *E. coli* 950 and 5026 were very similar both to each other and to the published amino acid composition of the *E. coli* O75X hemagglutinin studied by Väisänen-Rhen (Table 2, IH11128) (36), which has since been shown to possess Dr specificity (26). N-terminal amino acid sequencing of the Dr hemagglutinins expressed by *E. coli* 950 and 5026 showed that the two were identical for the first 25 residues (Table 3). Residue 19 of either protein could not be identified but is probably a cysteine, since (i) cysteine is not routinely identified by automated Edman degradation sequencing procedures without prior derivitization and (ii) cysteine is found in position 19 of the AFA-1 adhesin, which shares 50% identity with the Dr hemagglutinins (Table 3) (39). The N-terminal amino acid sequences of the Dr hemagglutinins also shared 25% identity with an afimbrial adhesin isolated from enterotoxigenic *E. coli* 2230 (6). No significant homology to other fimbrial or afimbrial adhesin types could be detected.

The pIs of the hemagglutinin subunit proteins from both strains (950 and 5026) were 5.4 (Table 2).

Serological properties. The two Dr hemagglutinins proved to be serologically related when assayed by bacterial agglu-

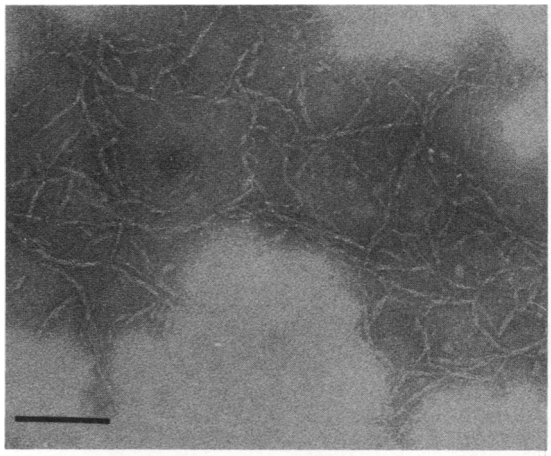

FIG. 5. Electron micrograph of HPLC-purified Dr hemagglutinin negatively stained with ammonium molybdate. Bar = 100 nm.

TABLE 2. Amino acid compositions of purified Dr hemagglutinins and related adhesins in *E. coli*

Amino acid	Residues/molecule in[a]:			
	950 (Dr)	5026 (Dr)	IH11128 (Dr)[b]	AFA-1[c]
Asx	25	25	23	15
Thr	19	20	21	15
Ser	5	5	5	11
Glx	11	11	13	10
Pro	6	6	7	2
Gly	18	17	22	19
Ala	10	10	12	6
Cys	1	1	ND	2.5
Val	12	12	12	7
Met	ND	ND	ND	3
Ile	5	5	5	4
Leu	11	11	11	8
Tyr	5	5	7	ND
Phe	6	5	6	2
His	ND	ND	ND	4
Lys	8	8	8	3
Arg	6	6	Tr	6

[a] Tr, Trace; ND, not detected. pIs for *E. coli* 950 and 5026 were 5.4. pIs were not reported for IH11128 and AFA-1.
[b] Composition, as described by Vaisanen-Rhen (36).
[c] Composition, as described by Walz et al. (39).

tination and immunoblotting. Antiserum specific for the native hemagglutinin isolated from strain 950 agglutinated both strains 950 and 5026 and also reacted by immunoblotting with the denatured 14.5K hemagglutinin subunit proteins of both strains (Fig. 6, lanes D and E). Both fimbrial proteins also bound antibodies present in serum obtained from the patient originally infected with strain 5026 (immunoblot not shown). Neither serum agglutinated P-fimbriated *E. coli* 917 or recognized the major 17K P fimbria subunit protein isolated from that strain (Fig. 6, lane F).

DISCUSSION

We have described the purification and characterization of the Dr hemagglutinins expressed by two uropathogenic *E. coli* strains. Väisänen-Rhen (36) originally described a MR P blood group-independent hemagglutinin (M_r, 15,000) which was expressed by a number of uropathogenic *E. coli* strains belonging to serogroup O75; accordingly, this adhesin was named O75X. The O75X adhesin-encoding operon has been cloned (25), and the fibrillar structures expressed by recombinant *E. coli* strains closely resemble those expressed by *E. coli* 950 and 5026 in this study (Fig. 1).

TABLE 3. N-terminal amino acid sequences of the purified Dr hemagglutinins and other *E. coli* adhesins

Source of adhesin[a]	Residue[b]					
	1	5	10	15	20	25
950	GFTPSGTTGTTKLTVTEEXQVYVGD					
5026	**********					
AFA-1	N**S***N*KVD**I***CR*T*ES					
2230	*NVL**GN**QTV*MPVNAATXTVS					
Pap A	AP*IPQGQ*KVTFNG*VVDAPCSIS					
Type 1	AA*TVNGGTVH*KGEVVNAACAVDA					

[a] N-terminal amino acid sequences of the AFA-1 (39), 2230 (6), Pap A (1), and type 1 (12) fimbrial and afimbrial subunit proteins are included for comparison.
[b] Amino acids are shown in standard single letter code. X, Unidentified residue; *, residue identical to that in the 950 Dr hemagglutinin sequence.

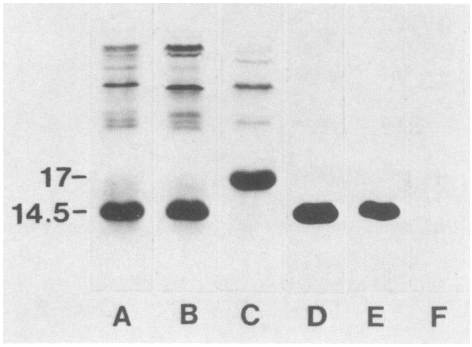

FIG. 6. Immunoblot analysis of crude Dr hemagglutinin and P fimbria preparations. Crude Dr hemagglutinin preparations from strains 950 (lane A) and 5026 (lane B) and P fimbriae from strain 917 (lane C) were electrophoresed on a 15% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with antiserum specific for the strain 950 Dr hemagglutinin. Lanes A, B, and C, are from a Coomassie blue-stained polyacrylamide gel. Lanes D, E, and F are from an immunoblot of hemagglutinin preparations from strains 950, 5026, and 917, respectively, probed with anti-950 Dr hemagglutinin antiserum. Molecular masses are shown in kilodaltons.

The binding of O75X *E. coli* strains to human and canine kidney structures has been examined (15, 26, 38, 40), and recently, the Dr blood group antigen of the human IFC blood group complex has been identified as the erythrocyte receptor (26). The adhesins expressed by *E. coli* 950 and 5026 were identified as Dr hemagglutinins because both strains failed to agglutinate Dr (a−) erythrocytes which lack the Dr blood group antigen (26). The chemical nature of the erythrocyte receptor is not known; however, trypsin treatment of erythrocytes abolished their agglutination by strains 950 and 5026, which supports the suggestion that the erythrocyte receptor is a protein (26). It is not known if all O75X adhesins possess Dr antigen-binding specificity, but it is known that O75X adhesins are not restricted to *E. coli* O75 strains. Väisänen-Rhen (36) reported the expression of such an adhesin by an *E. coli* strain belonging to serogroup O2 as well as by one nontypeable and two rough strains. More recently, Nowicki et al. (27) constructed a DNA probe specific for certain sequences within the Dr adhesin-encoding (*dra*) operon and detected *dra*-related sequences in a number of clinical urinary tract infection isolates belonging to a variety of O serotypes including O2, O4, O6, O11, O12, and others. Similarly, strain 5026 in this study has been serotyped as O14 (rough) (serotyping kindly performed by F. Ørskov and I. Ørskov). X adhesins from *E. coli* O75 strains also differ morphologically and biochemically (29). The term O75X adhesin is, therefore, inappropriate.

The limited biochemical characterization of Dr hemagglutinins to date has shown them to be chemically similar to typical *E. coli* fimbriae in that they are primarily polymers of a major subunit protein which consists of a high proportion of nonpolar amino acid residues (36). Prior to this study, amino acid sequence data have not been available and, therefore, it has not been possible to assess the extent of primary structure homology.

The Dr hemagglutinins expressed by *E. coli* 950 and 5026 were each purified from a crude mixture of proteins by using anion-exchange HPLC. Gander and Thomas (7) had previously used this method successfully to separate multiple pilus types expressed by a single uropathogenic *E. coli*

isolate. One of the advantages of ion-exchange chromatography is the use of relatively mild separation conditions, which allows the isolation of biologically active proteins. The adhesins purified in this study retained biological (i.e., receptor-binding) activity, as shown by the hemagglutination assay.

Several genetic studies of other fimbrial adhesins have demonstrated that the adhesin function and filament expression are encoded by separate genes (22, 34). Intact fimbriae consist of approximately 10^3 copies of the major subunit protein and only a few copies of minor accessory proteins, including the adhesin protein which actually mediates receptor binding (20). The minor adhesin protein has been identified for P, S, and type 1 fimbriae (9, 20, 23) and, in one case, has been localized to the tip of the fimbrial filament (19). Moch et al. (23) physically separated the minor adhesin protein from 70°C-treated S fimbriae by using anion-exchange HPLC. Relatively harsh conditions (6 M guanidine HCl) were required, however, to obtain this separation, since the two were found to be tightly associated. Similarly, Hoschützky et al. (10) separated the P-specific adhesin from P fimbria-adhesin complexes by heating the latter in detergent (Zwittergent 3-16; Calbiochem-Behring) at 80°C followed by anion-exchange chromatography in the presence of 4 M urea and detergent. The Dr hemagglutinins isolated from *E. coli* 950 and 5026 in this study demonstrated hemagglutinating activity and appeared to be composed of a single protein species. We cannot rule out the possibility that a separate adhesin protein may have copurified with the 14.5K fimbrial structural subunit protein, although silver staining of SDS-polyacrylamide gels failed to detect additional minor proteins (data not shown). The Dr adhesin-encoding (*dra*) operon consists of at least five genes, of which four (*draA*, *draC*, *draD*, and *draE*) are required for full expression of the MRHA phenotype (27). Mutations in *draA* (which encodes the 15K hemagglutinin structural subunit protein) abolish MRHA, which suggests that the adhesive properties of the Dr hemagglutinin reside within the structural subunit protein and not in a separate adhesin protein (27).

The amino acid compositions of the 14.5K proteins were very similar both to each other and to the published amino acid composition of an O75X adhesin (36), which has since been shown to possess Dr specificity (26). The N-terminal amino acid sequence of the latter protein was not determined and so is not available for comparison with those presented in this study. The N-terminal amino acid sequences of the two proteins are identical and share 50% identity with an afimbrial adhesin (AFA-1) isolated from a uropathogenic *E. coli* strain (39) and 25% identity with an afimbrial adhesin isolated from an enterotoxigenic *E. coli* strain (6). Labigne-Rousell and Falkow (16) previously showed that a DNA probe specific for the AFA-1 operon hybridized to whole DNA isolated from several *E. coli* O75X strains. A DNA probe specific for the structural gene of the AFA-1 operon, however, did not recognize restricted DNA from the same O75X strains, indicating that, although the latter possessed AFA-1 related operons, the structural subunit proteins were not identical to the AFA-1 adhesin. Antisera specific for denatured, but not native, AFA-1 adhesin bound denatured O75X adhesins, as shown by immunoblotting. The authors (16) concluded that the O75X fimbrialike adhesin represented a serological variant of the AFA-1 adhesin. Our results indicate that AFA-1 adhesins are indeed structurally related to Dr (O75X) hemagglutinins. An alignment score of 8.53 standard deviations above random was calculated when the program ALIGN was used to compare the N-terminal amino acid sequences of the AFA-1 and 950 and 5026 Dr hemagglutinins. When the Dr hemagglutin and *E. coli* 2230 adhesin sequences were compared, an alignment score of 2.17 was obtained, indicating a more distant relationship. The amino acid compositions of the Dr hemagglutinins isolated in this study showed no obvious similarities with the published amino acid composition of the AFA-1 hemagglutinin (Table 2) (39). This suggests that, although the N-terminal regions of the proteins are somewhat conserved, major differences likely occur later in the sequence.

The Dr hemagglutinins isolated from strains 950 and 5026 are very similar biochemically: they have identical isoelectric points, N-terminal amino acid sequences, and almost identical amino acid compositions. The complete amino acid sequences of these proteins are required, however, to determine the extent of this primary structure homology. The N- and C-terminal regions of the major subunit protein of P fimbriae, for example, are highly conserved, while intervening regions contain variable and hypervariable sequences (37). These sequences encode immunodominant type-specific antigenic determinants and are thought to contribute to the observed serological diversity among P fimbriae (37). This diversity has hindered the use of purified P fimbriae as vaccine molecules, because the antibody response is primarily directed at the nonconserved determinants. Immunization with purified P fimbriae fails to elicit broadly cross-reacting antibodies (8) and therefore would likely provide only limited protection against infection by heterologous strains. Dr hemagglutinins may prove to be less serologically diverse. Väisänen-Rhen (36) found that the O75X adhesins expressed by eight *E. coli* strains were serologically related. Antiserum raised against the native O75X adhesin isolated from one strain recognized the native adhesins expressed by seven other strains in agglutination, immunoprecipitation, and colony immunoblotting experiments (36). Similarly, antiserum raised against the native Dr hemagglutinin isolated from strain 950 in this study reacted with the native (and denatured) hemagglutinin isolated from strain 5026.

In this study, we have described a strategy for the purification of *E. coli* Dr hemagglutinins and have demonstrated a relationship between these hemagglutinins and AFA-1 adhesins. The extent of the observed primary structure homology between the two Dr hemagglutinins is currently being examined.

ACKNOWLEDGMENTS

We are grateful to F. and I. Ørskov (Statens Seruminstitut, Copenhagen, Denmark) for serotyping *E. coli* strains, S. Hull for performing hemagglutination assays with Dr (a−) erythrocytes, and M. Blum and C. Yu (Protein Analysis Service, Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada) for amino acid composition and sequence analyses.

This research was supported by a Medical Research Council of Canada grant (MT 1982) to T.H. and a Physicians Services Incorporated grant and Kidney Foundation of Canada grant to I.E.S.

LITERATURE CITED

1. **Båga, M., S. Normark, J. Hardy, P. O'Hanley, D. Lark, O. Olsson, G. Schoolnik, and S. Falkow.** 1984. Nucleotide sequence of the *papA* gene encoding the pap pilus subunit of human uropathogenic *Escherichia coli*. J. Bacteriol. **157**:330–333.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. **72**:248–254.
3. **Dayhoff, M. O., R. M. Schwartz, and B. C. Orcutt.** 1979. A model of evolutionary change in proteins, p. 345–362. *In* M. O. Dayhoff (ed.), Atlas of protein sequence and structure, vol. 5,

suppl. 3. National Biomedical Research Foundation, Washington, D.C.
4. **Evans, D. G., D. J. Evans, Jr., and H. L. DuPont.** 1977. Virulence factors of enterotoxigenic *Escherichia coli*. J. Infect. Dis. **136**:S118–S123.
5. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
6. **Forestier, C., K. G. Welinder, A. Darfeuille-Michaud, and P. Klemm.** 1987. Afimbrial adhesin from *Escherichia coli* strain 2230: purification, characterization, and partial covalent structure. FEMS Microbiol. Lett. **40**:47–50.
7. **Gander, R. M., and V. L. Thomas.** 1986. Utilization of anion-exchange chromatography and monoclonal antibodies to characterize multiple pilus types on a uropathogenic *Escherichia coli* O6 isolate. Infect. Immun. **51**:385–393.
8. **Hanley, J., I. E. Salit, and T. Hofmann.** 1985. Immunochemical characterization of P pili from invasive *Escherichia coli*. Infect Immun. **49**:581–586.
9. **Hanson, M. S., J. Hempel, and C. C. Brinton, Jr.** 1988. Purification of the *Escherichia coli* type 1 pilin and minor pilus proteins and partial characterization of the adhesin protein. J. Bacteriol. **170**:3350–3358.
10. **Hoschützky, H., F. Lottspeich, and K. Jann.** 1989. Isolation and characterization of the α-galactosyl-1,4-β-galactosyl-specific adhesin (P adhesin) from fimbriated *Escherichia coli*. Infect. Immun. **57**:76–81.
11. **Kallenius, G., R. Mollby, S. B. Svenson, J. Winberg, A. Lundblad, S. Svensson, and B. Cedergren.** 1980. The P^k antigen as receptor for the haemagglutinin of pyelonephritic *Escherichia coli*. FEMS Microbiol. Lett. **7**:297–302.
12. **Klemm, P.** 1984. The *fimA* gene encoding the type 1 fimbrial subunit of *Escherichia coli*: nucleotide sequence and primary structure of the protein. Eur. J. Biochem. **143**:395–399.
13. **Korhonen, T. K.** 1979. Yeast cell agglutination by purified enterobacterial pili. FEMS Microbiol. Lett. **6**:421–425.
14. **Korhonen, T. K., V. Väisänen-Rhen, M. Rhen, A. Pere, J. Parkkinen, and J. Finne.** 1984. *Escherichia coli* fimbriae recognizing sialyl galactosides. J. Bacteriol. **159**:762–766.
15. **Korhonen, T. K., R. Virkola, V. Väisänen-Rhen, and H. Holthöfer.** 1986. Binding of purified *Escherichia coli* O75X adhesin to frozen sections of human kidney. FEMS Microbiol. Lett. **35**: 313–318.
16. **Labigne-Roussel, A., and S. Falkow.** 1988. Distribution and degree of heterogeneity of the afimbrial-adhesin-encoding operon (*afa*) among uropathogenic *Escherichia coli* isolates. Infect. Immun. **56**:640–648.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Leffler, H., and C. Svanborg-Edén.** 1980. Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. FEMS Microbiol. Lett. **8**:127–134.
19. **Lindberg, F., B. Lund, L. Johansson, and S. Normark.** 1987. Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. Nature (London) **328**:84–87.
20. **Lund, B., F. Lindberg, B.-I. Marklund, and S. Normark.** 1987. The Pap G protein is the α-D-galactopyranosyl-(1→4)-β-D-galactopyranose-binding adhesin for uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**:5898–5902.
21. **Lund, B., B.-I. Marklund, N. Strömberg, F. Lindberg, K.-A. Karlsson, and S. Normark.** 1988. Uropathogenic *Escherichia coli* can express serologically identical pili of different receptor binding specificities. Mol. Microbiol. **2**:255–263.
22. **Minion, F. C., S. N. Abraham, E. H. Beachey, and J. D. Goguen.** 1986. The genetic determinant of adhesive function in type 1 fimbriae of *Escherichia coli* is distinct from the gene encoding the fimbrial subunit. J. Bacteriol. **165**:1033–1036.
23. **Moch, T., H. Hoschützky, J. Hacker, K.-D. Kröncke, and K. Jann.** 1987. Isolation and characterization of the α-sialyl-β-2,3-galactosyl-specific adhesin from fimbriated *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**:3462–3466.
24. **Needleman, S. B., and C. D. Wunsch.** 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. **48**:443–453.
25. **Nowicki, B., J. P. Barrish, T. Korhonen, R. A. Hull, and S. Hull.** 1987. Molecular cloning of the *Escherichia coli* O75X adhesin. Infect. Immun. **55**:3168–3173.
26. **Nowicki, B., J. Moulds, R. Hull, and S. Hull.** 1988. A hemagglutinin of uropathogenic *Escherichia coli* recognizes the Dr blood group antigen. Infect. Immun. **56**:1057–1060.
27. **Nowicki, B., C. Svanborg-Edén, R. Hull, and S. Hull.** 1989. Molecular analysis and epidemiology of the Dr hemagglutinin of uropathogenic *Escherichia coli*. Infect. Immun. **57**:446–451.
28. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
29. **Pawelzik, M., J. Heesemann, J. Hacker, and W. Opferkuch.** 1988. Cloning and characterization of a new type of fimbria (S/F1C-related fimbria) expressed by an *Escherichia coli* O75: K1:H7 blood culture isolate. Infect. Immun. **56**:2918–2924.
30. **Rhen, M., P. Klemm, and T. K. Korhonen.** 1986. Identification of two new hemagglutinins of *Escherichia coli*, *N*-acetyl-D-glucosamine-specific fimbriae and a blood group M-specific agglutinin, by cloning the corresponding genes in *Escherichia coli* K-12. J. Bacteriol. **168**:1234–1242.
31. **Schwartz, R. M., and M. O. Dayhoff.** 1979. Matrices for detecting distant relationships in proteins, p. 353–358. *In* M. O. Dayhoff (ed.), Atlas of protein sequence and structure, vol. 5, suppl. 3. National Biomedical Research Foundation, Washington, D.C.
32. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
33. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
34. **Uhlin, B. E., M. Norgren, M. Båga, and S. Normark.** 1985. Adhesion to human cells by *Escherichia coli* lacking the major subunit of a digalactoside-specific pilus-adhesin. Proc. Natl. Acad. Sci. USA **82**:1800–1804.
35. **Väisänen, V., T. K. Korhonen, M. Jokinen, C. G. Gahmerg, and C. Ehnholm.** 1982. Blood group M specific hemagglutinin in pyelonephritogenic *Escherichia coli*. Lancet **i**:1192.
36. **Väisänen-Rhen, V.** 1984. Fimbria-like hemagglutinin of *Escherichia coli* O75 strains. Infect. Immun. **46**:401–407.
37. **van Die, I., W. Hoekstra, and H. Bergmans.** 1987. Analysis of the primary structure of P-fimbrillins of uropathogenic *Escherichia coli*. Microb. Pathog. **3**:149–154.
38. **Virkola, R., B. Westerlund, H. Holthöfer, J. Parkkinen, M. Kekomäki, and T. K. Korhonen.** 1988. Binding characteristics of *Escherichia coli* adhesins in human urinary bladder. Infect. Immun. **56**:2615–2622.
39. **Walz, W., M. A. Schmidt, A. F. Labigne-Rousell, S. Falkow, and G. Schoolnik.** 1985. AFA-1, a cloned afimbrial X-type adhesin from a human pyelonephritic *Escherichia coli* strain. Eur. J. Biochem. **152**:315–321.
40. **Westerlund, B., J. Merenmies, H. Rauvala, A. Miettinen, A.-K. Järvinen, R. Virkola, H. Holthöfer, and T. K. Korhonen.** 1987. The O75X adhesin of uropathogenic *Escherichia coli*: receptor-active domains in the canine urinary tract and interaction with laminin. Microb. Pathog. **3**:117–127.