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Identification and Characterization of a Uroepithelial Cell Adhesin from a Uropathogenic Isolate of *Proteus mirabilis*

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***Proteus mirabilis* is a frequent cause of urinary tract infections in rehabilitation hospitals and among persons with structural abnormalities of the urinary tract. Adherence to uroepithelial tissues may be an important virulence determinant in these infections because most *Proteus* strains adhere to desquamated uroepithelial cells. To identify the adherence factor responsible for this phenomenon, we sheared outer membrane material from $^{35}SO_4$-radiolabeled bacteria and allowed it to bind to uroepithelial cells. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the major adherence element was a protein with an apparent molecular weight of 17,500 and was provisionally designated as the uroepithelial cell adhesin. This adhesin was purified by heat shock and gel filtration on Sepharose CL-4B. After purification, the adhesin was seen assembled as long, flexible rods by electron microscopy. The N-terminal amino acid sequence of the subunit had limited homology with that of the K99 fimbriae of *Escherichia coli*.**

Urinary tract infections are the most commonly acquired nosocomial infections (26). *Proteus mirabilis* is a frequent cause of these infections, especially in rehabilitation hospitals and in persons with structural abnormalities of the urinary tract (5, 20, 27). These bacteria generally produce a more severe disease than does *Escherichia coli*, owing to the production of urease, which in turn leads to struvite stone formation (10). Several bacterial determinants are associated with uropathogenic strains of *P. mirabilis*, including hemolytic activity, invasiveness in tissue cultures, motility, and certain proticine types (22–24, 27).

The capacity to adhere to uroepithelium is believed to be another bacterial determinant related to virulence in *Proteus* strains. In other genera studied to date, this adherence is mediated by structures present on the outer surfaces of the cells (glycolayxes, capsules, and fimbriae) (6). For example, uropathogenic *E. coli* uses P fimbriae to attach to the P blood group antigen on human tubule cells of the kidney (12). A similar phenomenon probably occurs in *Proteus* strains, as demonstrated by electron micrographs which show organisms attached to rat renal epithelium by fimbriae of the type IV category (7 nm in diameter) (28). In human studies, *Proteus* strains have been shown to adhere to desquamated uroepithelium; however, attempts to correlate virulence with adherence were unsuccessful, owing to the high frequency of adherent strains among stool isolates used as controls (29). Certainly, the contribution of adherence factors to the pathogenicity of *Proteus* isolates is not well understood.

In this study, we identified a protein from a uropathogenic isolate of *P. mirabilis* which adheres to desquamated human uroepithelial cells. This protein was purified and found to be organized as flexible rods (fimbriae) by electron microscopy. In addition, these fimbriae were characterized as to N-terminal amino acid sequence and amino acid composition. We suggest that these fimbriae are the adherence element responsible for binding uropathogenic *Proteus* strains to uroepithelium.

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MATERIALS AND METHODS

Strains, media, buffers, and reagents. The uropathogenic strains of *P. mirabilis* were obtained from the Ben Taub County Hospital, Houston, Tex.

Minimal medium or L medium was used for the cultivation of bacteria. L medium consisted of 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. Minimal medium consisted of 60 mM potassium phosphate (pH 7.4); 2 mM disodium citrate; 0.8 mM $MgCl_2$; 15 mM NH_4Cl; 0.08 mM $(NH_4)_2SO_4$; 0.02 mg each of glycine, serine, arginine, threonine, histidine, isoleucine, valine, lysine, leucine, and tryptophan per ml; 0.4% glycerol; and 0.5 mg of yeast extract per ml.

Buffered saline with gelatin (BSG) contained 0.15 M NaCl, 0.004 M potassium phosphate (pH 6.0), and 0.1% gelatin.

Carrier-free $H_2{}^{35}SO_4$ was obtained from ICN Radiochemicals, Irvine, Calif.

Bacterial cell adherence assay. Uroepithelial cells were isolated from the urine of three healthy female volunteers by centrifugation at 400 × *g* for 10 min. The cells were washed in BSG, and pellets containing 2×10^5 cells were suspended in 1 ml of BSG containing 5×10^9 bacteria. Suspensions were rotated for 1 h at 37°C. The cells were washed four times in BSG and examined under a light microscope. The number of bacteria adhering per cell was counted.

Hemagglutination. Bacteria were grown in L broth, in minimal broth, or on L agar for 16 h, washed, and suspended in BSG to an A_{600} of 1. Then, 0.2 ml of bacterial suspension was mixed with a equal volume of a 2% suspension of fresh or tanned erythrocytes in BSG. The mixtures were rocked for 5 min at room temperature and examined for agglutination.

Preparation of radiolabeled outer membrane material. Bacteria (10^{10}) were harvested after 16 h of growth at 37°C from 10 ml of minimal broth containing 50 μCi of $^{35}SO_4$ per ml and washed with BSG. Bacteria were suspended in 1.0 ml of BSG, and outer membrane material was sheared from the bacterial surface by passage through a 26-gauge needle. Whole bacteria were removed by centrifugation for 10 min at 10,000 × *g*.

TABLE 1. Adherence of *P. mirabilis* to desquamated uroepithelial cells[a]

Strain	Mean no. of bacteria/uroepithelial cell		
	L agar	L broth	Minimal broth
HU1069	15.5 ± 1.6[b]	25.5 ± 5.1[b]	29.2 ± 4.5[b]
HU1070	5.6[c]	ND[d]	ND
HU1071	6.5[c]	ND	ND

[a] A total of 64 uroepithelial cells were examined by light microscopy, and the number of adherent bacteria was determined.
[b] Four experiments.
[c] Two experiments.
[d] ND, Not determined.

Adsorption of bacterial outer membrane material by uroepithelial cells. Uroepithelial cells were isolated as described above. Pellets containing 2×10^5 cells were suspended in BSG containing radiolabeled outer membrane material. Reaction volumes were 1.0 ml. After incubation for 1 h at 37°C, the reaction mix was centrifuged, and both the cell pellet and the supernatant were retained. The cells were then washed extensively with BSG, and a sample was counted in a Beckman scintillation counter. The number of counts in the entire sample was calculated, and an equal number of counts was removed from the supernatant fraction for further processing. The cells with adhered bacterial components and the counts from the supernatant fraction containing unattached bacterial components were dialyzed against distilled H_2O and lyophilized. Samples were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the procedure of Laemmli (18). Gels were dried and autoradiographed.

Purification of UCA. Bacteria were grown in minimal broth and harvested after 18 h. Cells were washed in 0.05 M Tris (pH 8.0). Bacteria were suspended in 50 mM sodium phosphate (pH 7.2) with 2 M urea and incubated at 65°C for 20 min. Whole cells were removed by centrifugation at 7,500 × *g* for 10 min. An equal volume of saturated ammonium sulfate was added to the supernatant, and the solution was stirred for 2 h at 4°C. The precipitate was collected by centrifugation at 12,000 × *g* for 20 min. The pellet was suspended in 0.05 M Tris (pH 7.8) and dialyzed against the same buffer for 16 h. Insoluble material was removed by centrifugation at 25,000 × *g* for 20 min. Sodium deoxycholate was added to a concentration of 0.5%, and the sample was dialyzed against this buffer for 48 h. The deoxycholate-insoluble material was removed by centrifugation at 12,000 × *g* for 20 min, and the supernatant was dialyzed against 0.05 M Tris (pH 7.8) for 72 h. The protein was then dialyzed against water and lyophilized. Samples were suspended in 50 mM sodium phosphate (pH 7.2) with 6 M urea and chromatographed in the same buffer over a Sepharose CL-4B column. The flow rate was 5 ml/h. The void volume contained the uroepithelial cell adhesin (UCA). The yield from 3 liters of minimal broth was approximately 0.5 mg of UCA.

N-terminal amino acid sequencing. Approximately 20 μg of protein was further purified by SDS-PAGE and electroeluted as described previously (11). The protein was lyophilized and sequenced by using the Applied Biosystems model 470A protein sequencer and the 02RPTH program with trifluoroacetic acid conversion to phenylthiohydantoin derivatives (7). Phenylthiohydantoin amino acids were identified by high-performance liquid chromatography on a Waters Nova Pac C18 column by using a Waters high-performance liquid chromatography system. Solvent A was 84% sodium acetate (33 mM, pH 5.0)–16% acetonitrile; solvent B was 60% isopropanol–40% water. Phenylthiohydantoin amino acids were eluted at a flow rate of 1 ml/min by using the following gradient: 0 to 0.5 min, 0% solvent B; 0.5 to 3.5 min, convex gradient to 35% solvent B; 3.5 to 12 min, isocratic elution at 35% solvent B. The amino-terminal sequence was determined three times.

Amino acid analysis. The amino acid composition was determined by using the Waters Pico-Tag system after acid hydrolysis of SDS-PAGE-purified *P. mirabilis* UCA (3).

Electron microscopy. Bacteria (*P. mirabilis* HU1069) grown overnight in minimal broth, in L broth, or on L agar were washed and suspended in BSG. One drop of the bacterial suspension or one drop of a 1:100 dilution of *P. mirabilis* UCA in phosphate-buffered saline was placed on a cube of 2% agarose and stained with 0.5% uranyl acetate by a pseudoreplicating technique (19). Specimens were examined with a JEOL 100CX electron microscope. Bacteria were also fixed in 3% glutaraldehyde prior to being stained. Fixed and unfixed bacteria appeared identical when examined.

RESULTS

Adherence of strain HU1069 to uroepithelium. Three uropathogenic isolates of *P. mirabilis* were grown on L agar for 16 h and tested for their capacity to bind desquamated human uroepithelial cells as described in Materials and Methods. The strains tested bound uroepithelium to various degrees (Table 1). Strain HU1069 attached to the uroepithelial cells in the greatest numbers (15 bacteria per cell) and was chosen for subsequent experiments.

Strain HU1069 was then tested for its capacity to adhere to uroepithelium after growth in various types of media. Minimal broth cultures yielded bacteria which adhered better than bacteria grown on L agar or in L broth.

Bacteria grown under these three conditions were also tested for their capacity to agglutinate fresh erythrocytes from humans, guinea pigs, and sheep and tanned human erythrocytes. No agglutination was detected.

Identification of strain HU1069 UCA. Because adherence is commonly mediated by proteinaceous outer membrane appendages among members of the family *Enterobacteriaceae*, a similar phenomenon was expected to occur in *P. mirabilis*. To identify this uroepithelial cell adhesin (UCA), we labeled bacterial cells by growth in minimal broth (conditions optimal for adherence) containing $^{35}SO_4$ and removed their outer membrane appendages by passing the suspension through a 26-gauge needle. The whole cells were removed by centrifugation, and the supernatant was incubated with desquamated human uroepithelial cells in BSG. Following incubation, the cells and attached bacterial components were subjected to SDS-PAGE followed by autoradiography (Fig. 1). The radiolabeled bacterial components which were removed by shearing of the bacteria are shown in Fig. 1, lane A. The profile is similar to that reported by Driver and Lambert (9). The mixture was heterogenous; however, the label was concentrated in two proteins of M_r 17,500 and M_r 39,000. The radiolabeled bacterial components which remained in the supernatant following adsorption by uroepithelial cells were electrophoresed in Fig. 1, lane B. The profile is identical to that in Fig. 1, lane A. Figure 1, lane C, contains the bacterial components adsorbed by the uroepithelial cells. The major protein which adhered to uroepithelium (i.e., the UCA) was the M_r-17,500 protein. The other radiolabeled proteins that were represented in the supernatant, including the M_r-39,000 protein, were also

present among the attached bacterial components, although in a reduced quantity, suggesting they have a low affinity for uroepithelium.

Purification of the M_r-17,500 protein from strain HU1069. The M_r-17,500 major adherence protein (UCA) was believed to mediate the attachment of bacteria to uroepithelial cells because of its capacity to bind uroepithelium in vitro. A protein having an identical migration in SDS-PAGE was purified from strain HU1069 by using fimbria isolation techniques (17). Bacteria were grown in minimal broth, washed, suspended in 50 mM PO_4 with 2 M urea, and incubated at 65°C for 20 min. The whole bacteria were removed by centrifugation. This procedure yielded a complex mixture of proteins (Fig. 2, lane C). This mixture was concentrated by ammonium sulfate precipitation and dialyzed against 50 mM Tris (pH 7.8). Insoluble material was removed by centrifugation, the supernatant was treated with 0.5% deoxycholate, and insoluble material was again removed. The deoxycholate-soluble material was chromatographed over a Sepharose CL-4B column. The void volume from this column contained the M_r-17,500 protein and a contaminant of M_r 37,000 (Fig. 2, lane D). An alternative isolation procedure, in which strain HU1069 was grown on complex medium (L agar) and outer membranes appendages were blended off in a Waring blender, yielded principally the M_r-39,000 protein (Fig. 2, lane B). Significant amounts of the M_r-17,500 protein could not be isolated by this method.

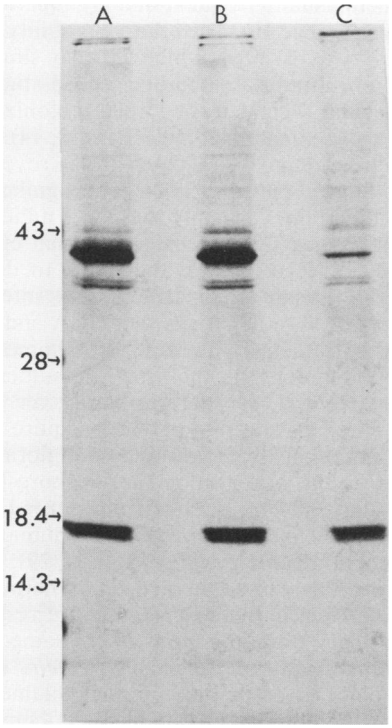

FIG. 1. Adherence of *P. mirabilis* outer membrane material to human uroepithelial cells. Radiolabeled outer membrane material was sheared from strain HU1069 after growth in minimal medium containing 50 μCi of $^{35}SO_4$ per ml and incubated with 2×10^5 desquamated uroepithelial cells in BSG. Uroepithelial cells and adsorbed material were removed by centrifugation and washed. Samples were lyophilized and electrophoresed on SDS gels. Gels were dried and autoradiographed. Lanes: A, sheared material from HU1069; B, outer membrane material remaining in the supernatant after adsorption to uroepithelial cells; C, outer membrane material adsorbed to uroepithelial cells. The positions of molecular weight markers (in thousands) are indicated on the left.

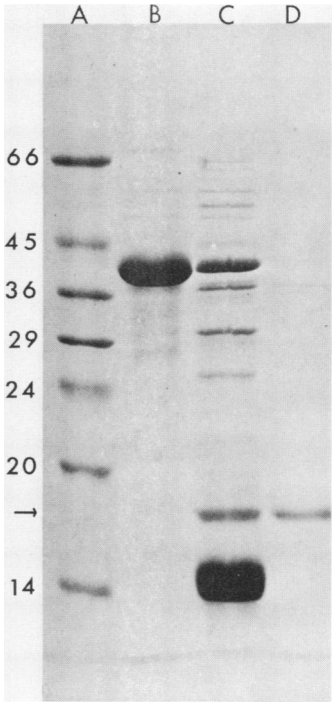

FIG. 2. Purification of the M_r-17,500 protein from *P. mirabilis* HU1069. Lanes: A, standard marker proteins; B, sheared outer membrane material from HU1069 grown on complex medium; C, material derived from heat shock of HU1069 grown in minimal medium; D, purified M_r-17,500 protein. Numbers on the left are the apparent molecular weights (in thousands) of the marker proteins; the arrow indicates an M_r of 17,500.

Binding of purified UCA to uroepithelial cells. To determine if the purified M_r-17,500 protein possessed the same functional characteristics as the UCA (as identified by adsorption studies), we purified the M_r-17,500 protein from bacterial cells grown in minimal broth containing 30 μCi of $^{35}SO_4$ per ml by using the technique described above (i.e., Sepharose CL-4B column chromatography). The radiolabeled M_r-17,500 protein (82,154 cpm) was incubated with 10^5 uroepithelial cells. After washing, 7,335 cpm remained attached to the cells (approximately 9% binding). For comparison, an equal number of counts contained in bacterial proteins which were retained on the Sepharose CL-4B column (nonfimbrial proteins) were also incubated with uroepithelial cells. These proteins attached less well to uroepithelial cells (1.5% binding). To determine if the increased binding (9%) by fimbrial proteins was due to the adherence of the M_r-17,500 protein, we subjected the cells in this assay to SDS-PAGE (Fig. 3). The majority of the counts were due to the attachment of the M_r-17,500 protein to uroepithelium (Fig. 3, lane B). Thus, the purified M_r-17,500 protein possessed the adhesive capacity characteristic of the UCA in addition to having an identical migration in SDS-PAGE. Hence, we assumed that the purified M_r-17,500 protein was the UCA.

Organization of UCA as fimbriae. The column-purified UCA was stained with uranyl acetate by a pseudoreplicating technique and viewed with a JEOL 100CX electron microscope. The UCA was seen organized as long, flexible rods (Fig. 4A). The rods in the preparation varied in diameter. A

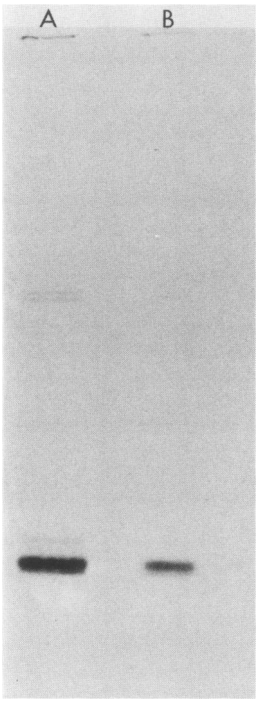

FIG. 3. Binding of the purified M_r-17,500 protein to uroepithelial cells. Lanes: A, radiolabeled proteins contained in the void volume after Sepharose CL-4B chromatography; B, radiolabeled proteins from the column void volume which attached to uroepithelial cells.

thin filament of 4 nm predominated, although a thicker filament of 6 nm was also present (approximately 10% of the filaments). Similar structures were seen radiating from intact organisms. These fimbrial structures were expressed most frequently on cells grown in minimal broth (Fig. 4B), although the majority of the cells were heavily flagellated and not fimbriated. Organisms grown in L broth expressed fimbriae less frequently than those grown in minimal broth, and L agar-grown organisms rarely expressed fimbriae (data not shown).

Characterization of purified UCA. The UCA was purified to homogeneity by electroelution from a Coomassie blue-stained 12.5% SDS-polyacrylamide gel. The protein was lyophilized, ethanol precipitated, and subjected to N-terminal amino acid sequence analysis (Fig. 5). The *P. mirabilis* UCA has significant homology to the K99 pilus associated with diarrhea-causing *E. coli* of calves. Among the first 15 residues, there is 46% homology (7 residues are identical). Among the eight remaining positions, one has a very conservative isoleucine-to-valine change, four other changes can also be attributed to 1-base-pair differences in the DNA, and only three positions represent more radical changes in the DNA (aspartic acid to threonine, valine to threonine, and glutamine to alanine). Homology to other fimbriae of *E. coli* is very limited, ranging from 26% for colonization factor antigen to 13% for P fimbriae in the first 15 residues.

Amino acid composition analysis was performed on SDS-PAGE-purified UCA (Table 2). The moles percent were calculated from the peak area following high-performance liquid chromatography and, based on a molecular weight of 17,600, the number of residues of each amino acid was calculated. Threonine and asparagine or aspartic acid were the most frequent amino acids. Arginine, cysteine, and methionine were detected only in small amounts (one residue per molecule). The concentration of tryptophan was not determined.

DISCUSSION

These studies identified a protein from a uropathogenic strain of *P. mirabilis* which may be responsible for the attachment of the bacteria to human uroepithelium. This main characteristic of the UCA was its capacity to adhere to uroepithelial cells. Furthermore, the UCA was seen organized as rod-shaped structures or fimbriae following purification. Similar structures were seen radiating from intact organisms. The expression of the fimbriae on the organisms was greatest under conditions allowing maximal adherence of the bacteria to uroepithelium. These data suggest that the UCA is organized on the bacterial cell surface as fimbriae which mediate the attachment of the bacteria to uroepithelium.

The UCA was purified to homogeneity by a combination of deoxycholate treatment, gel filtration, and SDS-PAGE from bacteria grown in minimal broth cultures. Following purification, the UCA was physically characterized as to amino acid composition, subunit size, and N-terminal amino acid sequence. The UCA has an amino acid composition similar to that reported for the fimbriae of *E. coli*, i.e., threonine was unusually frequent and arginine was rare (for a review, see reference 16). The relative mobility of the UCA in SDS-PAGE was 17,500, which is also similar to that reported for other fimbrial subunits. Those studied to date range in molecular weight from 15,000 (colonization factor antigen 1) to approximately 29,500 (F41) (8, 14). N-terminal amino acid sequencing of the UCA fimbriae revealed limited homology to the K99 pilus of *E. coli*. The significance of this homology is uncertain. Certainly these two adhesins bind to very different tissues: K99 to the intestional epithelium of calves and sheep erythrocytes and UCA to desquamated epithelium from human urinary tracts. It is interesting that there is much less homology between UCA and the urinary tract adhesins of *E. coli* (P. fimbriae, 20% homology; type 1 fimbriae, 25% homology) in the first 20 residues. This may indicate that UCA is, in fact, primarily an adhesin for intestional epithelium and that patients acquire *P. mirabilis* urinary tract infections from natural bowel flora. In support of this theory is the observation of Svanborg-Eden et al., who showed that *Proteus* isolates from normal stools have the same adhesive capacity for exfoliated human uroepithelium as do uropathogenic *Proteus* isolates (29). Prospective studies are in progress to investigate whether intestinal *Proteus* strains from individual patients are responsible for subsequent urinary tract infections in the same patients.

Previous ultrastructural studies of *Proteus* strains have identified two types of fimbria, a thick filament approximately 7 nm in diameter and a thin filament 4 nm in diameter (4, 28). These are, in addition to the numerous flagella (12 nm), present on the surface. Silverblatt has reported that the thicker pili appear to be related to infection, i.e., cells expressing 7-nm-thick fimbriae were more virulent in a rat model of pyelonephritis (28). Other workers have attempted to associate patterns of hemagglutination with expression of these fimbriae (1, 21). Old and Adegbola (21) reported that the thin filaments were responsible for *Klebsiella*-like hemagglutination (agglutination of tanned ox erythrocytes) and that the thicker filaments may be associated with *Proteus*-like hemagglutination (agglutination of fresh fowl, guinea

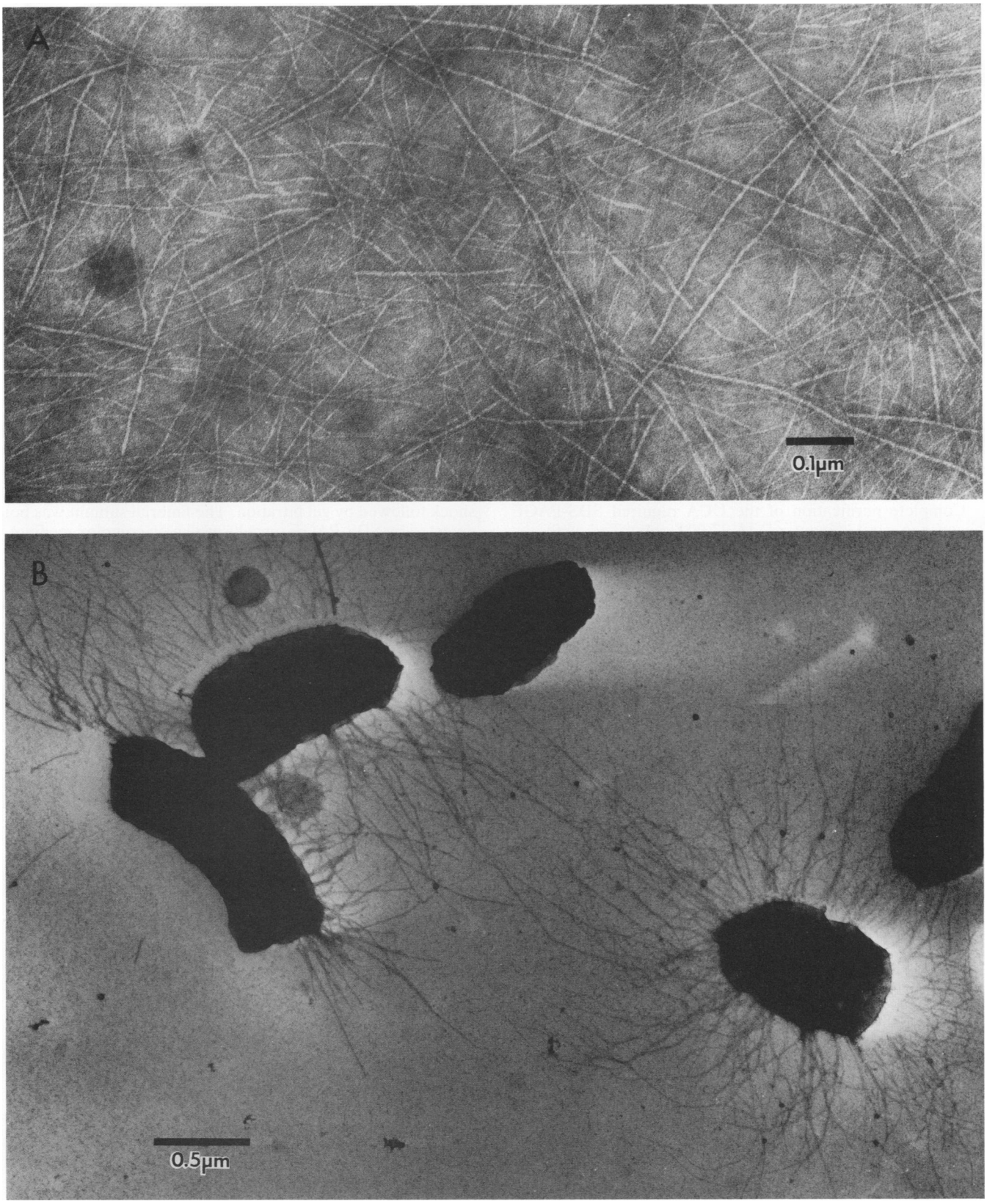

FIG. 4. (A) Electron micrograph of purified UCA. (B) Electron micrograph of *P. mirabilis* HU1069 grown for 16 h in minimal broth.

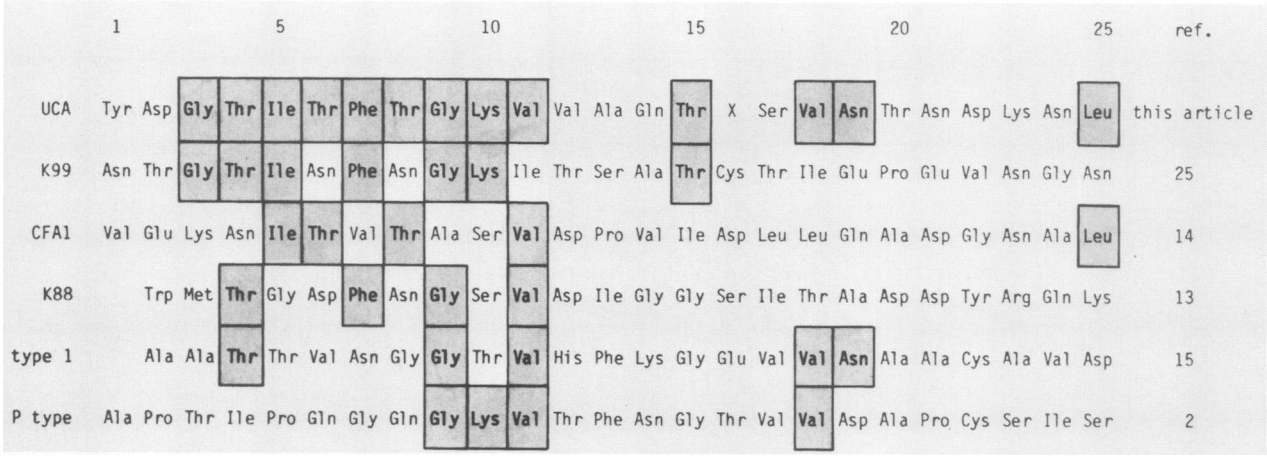

FIG. 5. N-terminal amino acid sequence of UCA from *P. mirabilis*. The N-terminal amino acid sequence of UCA is compared with those of various fimbriae isolated from *E. coli*. Residues that are common between UCA and other fimbriae are shaded. Numbering corresponds to the UCA sequence. CFA1, Colonization factor antigen 1.

pig, horse, human, and sheep erythrocytes). Unfortunately, these experiments were hampered by the inability to isolate strains expressing only one type of fimbria. In the present study, these two types of fimbria were both excluded on the Sepharose CL-4B column and could be observed in the electron micrographs.

Complete purification of the UCA required SDS-PAGE because a contaminant of M_r-37,000 copurified with the UCA during gel filtration. This larger protein was not a dimer of the UCA subunit, as its N-terminal sequence was different from that of the UCA (data not shown). The second protein may be the subunit of the second type of fimbria seen in the micrographs, although the molecular weight of the protein appears to be large for a fimbrial subunit. Alternatively, this contaminant may be a dimer of flagellin. Driver and Lambert (9) reported that the dimer of flagellin migrates in SDS-PAGE with an apparent molecular weight of 40,000, which is similar to the molecular weight of the contaminant. This possibility seems unlikely because flagella were not seen in the fimbria preparations.

P. mirabilis HU1069 was seen to be heavily flagellated. Even after growth in minimal medium, only a minority of the cells expressed fimbriae. Despite this relatively low level of expression, the UCA fimbriae could be isolated by heat shock followed by gel filtration. The fimbrial subunit was not detected by SDS-PAGE on cells grown on complex medium, due to reduced expression under these conditions. This relatively low level of expression of fimbriae in this strain may explain the inability to detect any agglutination of sheep, guinea pig, or human erythrocytes. Alternatively, Adegbola et al. (1) reported that a small percentage of *Proteus* isolates agglutinate only fowl erythrocytes, which were not tested here; strain HU1069 may be similar to these strains. We believe that this is a more plausible explanation because strain HU1069 adhered well to uroepithelium, suggesting that sufficient fimbriae were present for interaction with mammalian tissues if the appropriate receptors were present.

In summary, we have detected a protein from a uropathogenic isolate of *P. mirabilis* which is related to the K99 fimbriae of enterotoxigenic *E. coli*. The functional and physical characteristics of this protein suggest that it may be responsible for the attachment of *P. mirabilis* to human uroepithelium during urinary tract infections.

TABLE 2. Amino acid composition of *P. mirabilis* UCA[a]

Amino acid	Mol %	No. of residues
Thr	0.158	25
Asx	0.146	23
Ala	0.112	18
Glx	0.086	14
Val	0.086	14
Gly	0.074	12
Leu	0.067	11
Ser	0.057	9
Lys	0.036	6
Ile	0.036	6
Pro	0.039	6
Tyr	0.034	6
Phe	0.025	4
His	0.020	3
Met	0.007	1
Arg	0.005	1
Cys	0.005	1

[a] SDS-PAGE-purified UCA was hydrolyzed and analyzed by using the Waters Pico-Tag system. The number of residues was calculated by assuming a molecular weight of 17,600 for the UCA and an average molecular weight of 110 per amino acid. The number of residues was rounded to the nearest integer.

ACKNOWLEDGMENTS

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