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Isolation and Characterization of F12 Adhesive Fimbrial Antigen from Uropathogenic *Escherichia coli* Strains

PER KLEMM,[1]* IDA ØRSKOV,[2] AND FRITS ØRSKOV[2]

Department of Microbiology, The Technical University of Denmark, DK-2800, Lyngby-Copenhagen, Denmark,[1] and Collaborative Centre for Reference and Research on Escherichia coli *(World Health Organization), Statens Seruminstitut, DK-2300, Copenhagen S, Denmark[2]*

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The adhesive fimbrial antigen F12 from a strain of uropathogenic *Escherichia coli* has been isolated and characterized. The antigen was purified by ammonium sulfate precipitation and gel chromatography. The protein subunit of the F12 fimbria has a molecular weight of 18,200; the N-terminal amino acid sequence of the subunit shows close resemblance to that of the subunits of other F fimbriae and the type 1 fimbriae. We identified in these proteins a pattern of alternating conserved and variable amino acid residues which could indicate a special structural and functional feature.

Bacterial infection of the urinary tract in humans often results in cystitis or pyelonephritis. The latter must be given particularly serious concern, as kidney infections can be difficult to eradicate and tend to become chronic. However, the human urinary tract is efficiently constructed for preventing bacterial invasion of the bladder and the kidneys. Apart from the physical impediments provided by the physiology of the urinary tract, other factors play important roles in preventing infections. Thus, the flow of urine helps cleanse the system by flushing out microorganisms (4). Furthermore, normal urine contains antimicrobial substances in the form of antibodies (17) and urine mucus (15, 17), a large percentage of which consists of Tamm-Horsfall glycoprotein (17, 19). The natural function of both secretory immunoglobulins and Tamm-Horsfall glycoprotein seems to be the entrapment and removal of bacteria (7, 14, 19).

Escherichia coli strains account for about 50% of urinary tract infections. The invading strains have been shown, by serological studies, to originate in almost all cases from the intestinal flora of the patients (17). Many strains of uropathogenic *E. coli* possess specific attachment fimbriae that enable them to colonize the urinary tract epithelium (6, 15), thus circumventing the defenses of the host. Fimbriae are thin thread-like surface structures found on most *E. coli* strains. A single fimbria consists of several hundred identical protein subunits. A diversity of such fimbrial antigens exists on uropathogenic *E. coli* strains exhibiting both mannose-sensitive and mannose-resistant modes of adhesion (11, 12, 15, 17, 18). Six antigenically different fimbrial antigens (termed F7 to F12) that confer mannose-resistant adhesion to human erythrocytes and urinary epithelial cells have been described (15, 15a, 16).

Here we describe the isolation and characterization of the F12 fimbrial antigen found on *E. coli* strains, particularly those of serotype O16:K1:H6. Furthermore, a fimbrial antigen related to F12 was found to be present in several O4:K12:H5 strains (14a).

MATERIALS AND METHODS

Bacterial strain and culture. *E. coli* C1979, serotype O16:K1:H6, isolated from a case of urinary tract infection in Sweden and earlier chosen as the test strain for fimbrial antigen F12, was used for the isolation of this antigen. Cells were grown confluently overnight on 50 large (14-cm) ox heart infusion agar plates, prepared as previously described (15).

Crude extract of fimbriae. The bacteria were harvested and suspended in 150 ml of 0.1 M sodium phosphate, pH 7.0. The suspension was heated to 60°C for 20 min and then sheared two times, 1 min each time, in an Ultra Turrax dispension apparatus (Janke and Kunkel, Staufen, Federal Republic of Germany). Cells and large debris were removed by centrifugation at 27,000 × *g* for 15 min, and the supernatant was filtered through a 0.80-μm pore size filter (Millipore Corp., Bedford, Mass.).

Ammonium sulfate precipitation. To precipitate the F12 antigen, we added saturated ammonium sulfate to the filtered supernatant to 50% saturation. This was carried out under vigorous stirring at 4°C. The solution was left overnight in the cold, and the flocculent precipitate was collected by centrifugation. The pellet was suspended in 0.1 M ammonium hydrogen carbonate and filtered to remove a small amount of insoluble material, presumably of capsular origin.

Gel filtration. Gel chromatography of the crude extract was performed on a Sepharose 2B column (5

by 82 cm), and gel filtration of the F12 protein in 6 M guanidinium chloride was carried out on a Sepharose CL 6B column (1.5 by 90 cm). Gel filtration media were obtained from Pharmacia (Uppsala, Sweden).

Polyacrylamide gel electrophoresis. The purity and molecular weight of the F12 fimbrial subunit were assessed by electrophoresis in polyacrylamide slab gels, in the presence of 0.1% sodium dodecyl sulfate (SDS) (1). Samples were boiled for 5 min before electrophoresis. Protein bands were located by staining the gels with Coomassie brilliant blue G in 4.5% perchloric acid.

Amino acid analysis. Amino acid analysis was performed on a Durrum D-500 amino acid analyzer. Duplicate samples were hydrolyzed for 24, 48, and 72 h at 110°C in 6 M HCl containing 0.1% phenol. Samples of performic acid-oxidized protein were hydrolyzed for 24 h to determine cysteine as cysteic acid. For serine and threonine, values extrapolated to zero time were used, whereas the values for 72-h hydrolysis were used for valine and isoleucine. The tryptophan content was determined after hydrolysis in methane sulfonic acid containing 0.2% 3-(2-aminoethyl)indole.

N-terminal sequence analysis. The primary structure of the N-terminal part of the F12 subunit was determined by manual Edman degradation in the presence of 1% SDS. The sequencing and the identification of the phenylthiohydantoin derivatives and the parent amino acids were performed as previously described (8, 10).

Secondary structure prediction. For predicting the secondary structure of the N-terminal region of the F12 sequence, the method of Chou and Fasman was employed (3). The calculations were carried out with an SPC/1 microcomputer.

Crossed immunoelectrophoresis (CIE). The method of Weeke (20, 21) was used as reported previously (15). Glass plates (5 by 5 cm) were covered by a 1.5-mm layer of agarose (Litex HSA) in Tris-barbital buffer (pH 8.6; ionic strength, 0.05). Extract of strain C1979 (15 μl), prepared as described previously (15), was placed in the well in the lower gel. The first-dimension electrophoresis was run at 5 V/cm for 60 min. Then a 1:2 dilution of the antiserum prepared against whole-cell culture of C1979 and absorbed with culture grown at 18°C was incorporated in the top gel (30 μl/cm^2). To monitor the purification of the antigen and to examine the relatedness of the various preparations, samples (1 mg/ml) originating from the different purification steps were incorporated in the lower gel wells (tandem CIE [13]) or in the intermediate gel (32 μl/cm^2) (absorption in situ [2]). Application of antigen extract in the intermediate gel will cause elevation of the line formed by a related antigen present in the extract in the lower gel. The second-dimension electrophoresis was run at 2 V/cm overnight. The dried gels were stained with Coomassie brilliant blue in acetic acid-ethanol-water (2:9:9 by volume).

RESULTS

Isolation of F12 antigen. To suppress any potential capacity for producing type 1 fimbriae, we grew the C1979 strain used for the isolation of the F12 antigen on solid medium (15). After harvesting the cells, the F12 fimbriae were liberated by heat shocking and shearing. Further purification included ammonium sulfate precipitation, followed by gel filtration on Sepharose 2B. The latter method took advantage of the large sizes of intact fimbriae or large fragments thereof. Accordingly, the F12 protein eluted in the void volume and was thereby isolated to approximately 80% purity. As a final purification step, the fimbriae were disrupted in 6 M guanidinium chloride and the liberated subunits were purified by gel chromatography on a Sepharose CL 6B column (Fig. 1). The purification was monitored by SDS gel electrophoresis (Fig. 2). Preparations of the F12 antigen at the three stages of purity are referred to as F12.1, F12.2, and F12.3, respectively.

Like most other uropathogenic strains, C1979, the strain used for the purification and characterization of the F12 antigen in this study, produces excessive amounts of capsular material that hampers the purification. Furthermore, the output of fimbriae appears to be considerably smaller than that of *E. coli* strains expressing plasmid-specified fimbriae, from which large amounts of these proteins can be obtained (8, 9). This seemingly low output had been encountered earlier in other uropathogenic *E. coli* (11).

Immunology. Samples of equal concentrations from the different purification steps of the F12 antigen were compared by CIE with each other and with the C1979 crude extract, using antiserum absorbed with culture grown at 18°C; by this procedure, antibodies against the F12 antigen(s) are left. Figure 3A shows that the F12 antigen(s) precipitates in two lines positioned very close to each other. The antigens forming these lines have not yet been defined as two specific, different antigens, since until now it has only been possible to visualize them as separate antigens by application of antiserum and not antigen in the intermediate gel (14a). The presence of two lines in the CIE gel indicates that the F12 antigen preparations used in this work in fact contain two highly homologous fimbrial proteins having identical sizes and N-terminal amino acid sequences (see below). Figure 3B shows that the F12 precipitation lines are hardly elevated in the presence of the F12.1 preparation in the intermediate gel, whereas they are significantly elevated in the presence of the F12.2 preparation (Fig. 3C) and markedly elevated by F12.3 (Fig. 3D). Furthermore, with the F12.3 preparation, a further separation of the two F12 lines was obtained. An excess of F12 antigen in the F12.3 preparation would cause an excessive elevation of the two F12 lines, leading to their disappearance from the gel; therefore, we diluted the F12.3 preparation 20 times before adding it to the intermediate gel (Fig. 3D). Tandem CIE (Fig. 3E through H) shows antigenical identity

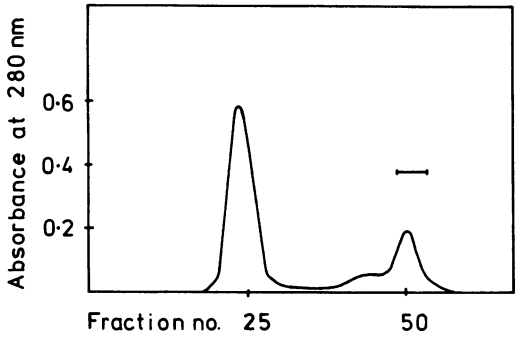

FIG. 1. Fractionation of partially purified F12 fimbrial protein on a Sepharose CL 6B column (1.5 by 90 cm) by elution with 6 M guanidinium chloride in 0.2 M ammonium hydrogen carbonate. Fractions of 2 ml were collected. Bar indicates pooled fractions containing F12 protein.

between the F12 fimbrial lines formed by the F12.2 and F12.3 preparations and between the lines formed by the F12.3 preparation and the C1979 crude extract (Fig. 3G and H). The greater amount of F12 antigenic determinants in the F12.3 preparation compared with F12.2 is reflected in the much higher peak formed by that preparation (Fig. 3G).

Characterization of F12 antigen. The apparent molecular weight of the F12 subunit as determined by SDS gel electrophoresis was 18,000 (Fig. 2). In good agreement with this result is the molecular weight of 18,200 calculated on the basis of the amino acid composition of the subunit (Table 1). The subunit has a content of 32% hydrophobic amino acid residues. The amino acid composition of F12 resembles that of the type 1 fimbrial proteins of other uropathogenic *E. coli* (11).

The amino acid sequence of the 18 N-terminal residues of the F12 fimbrial subunit was determined (Fig. 4). The sequencing efficiency was severely hampered by the presence of two proline residues at positions 2 and 5 and by an asparaginyl-glycyl bond at residues 14 and 15. Other N-terminal sequences of fimbrial proteins from various uropathogenic *E. coli* strains (11, 17a) as well as the sequence of the fimbrial protein of *Klebsiella pneumoniae* (5) are presented for comparison. The F12 N-terminal sequence is highly homologous to that of the F7 fimbrial protein. Except for residues 2, 6, 10, 12, and 14, the two N-terminal sequences are identical, the observed changes being the substitutions Ala → Pro, Gln → Glu, Glu → Lys, Ala → Thr, and Lys → Asn. A comparison of the F12 sequence with those of the type 1 fimbriae also reveals a homology, although they seem more distantly related, with only the residues in positions 9, 11, 13, 15, 17, and 18 being identical.

A secondary structure prediction (3) based on the F12 N-terminal sequence allocates a very high potential for β-sheet structure to the segment comprising residues 9 to 18. When this segment is compared with the corresponding segments in the other fimbriae, it becomes evident that every other residue (i.e., numbers 9, 11, 13, 15, and 17) is identical in all of the depicted sequences and has thus been conserved. However, the intervening positions are occupied by variable residues, e.g., Lys, Glu, or Thr in position 10. It is therefore tempting to infer that the sequence segment represented by residues 9 to 18 in this family of homologous proteins exists in β-sheet conformation, with the common residues 9, 11, 13, 15, and 17, i.e. Gly, Val, Phe, Gly, and Val, constituting the inside of the sheet and the intervening residues forming the outward-facing part of the sheet. This conclusion is based on the fact that the residues which would form the inside of the sheet and would therefore be buried in the molecule are predominantly of hydrophobic character, whereas the residues on the outside of the sheet, facing the surrounding aqueous milieu, are predominantly hydrophilic.

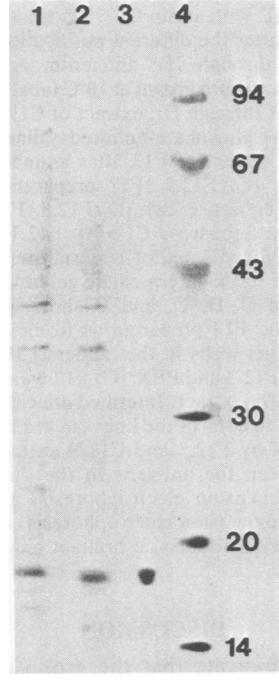

FIG. 2. SDS-polyacrylamide gel electrophoresis of F12 preparations. Lane 1, crude extract after ammonium sulfate precipitation (F12.1); lane 2, F12 preparation after gel filtration on Sepharose 2B (F12.2); lane 3, F12 after final purification on Sepharose CL 6B (F12.3); lane 4, marker proteins.

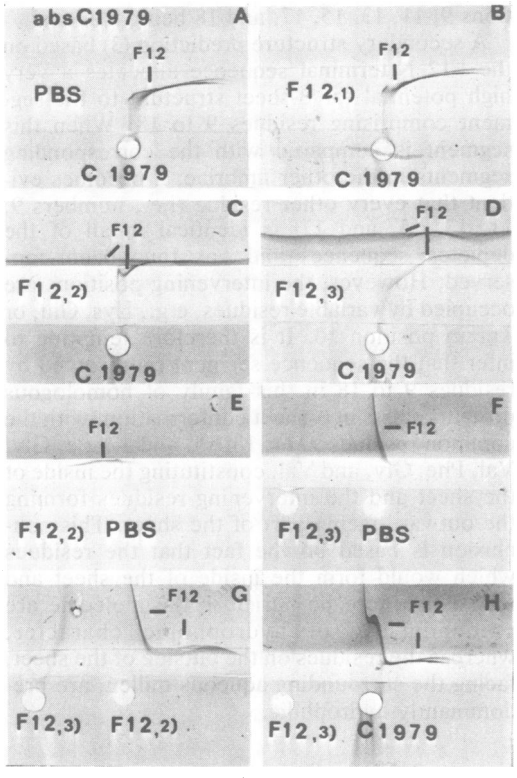

FIG. 3. CIE with strain C1979 extract and the F12 preparations after the different purification steps. Upper gels (A through H); antiserum against C1979 absorbed with culture grown at 18°C (absC1979). Lower gel wells (A through D); extract of C1979. Intermediate gels: (A) phosphate-buffered saline (PBS); (B) F12.1 (crude extract of F12 after ammonium sulfate precipitation); (C) F12.2 (F12 preparation after gel filtration on Sepharose 2B); (D) F12.3 (F12 after final purification on Sepharose CL 6B). F12.1, F12.2, and F12.3 all contained 1 mg of material per ml, but the amount added to the intermediate gel in D was 1/20 of that in B and C. B, C, and D show an increasing capacity of the F12 preparations to elevate the F12 lines. Lower gel wells in the tandem CIE: (E) F12.2 and PBS; (F) F12.3 and PBS; (G) F12.3 and F12.2; (H) F12.3 and C1979 extract. Intermediate gels (E through H); PBS. The fusions of the lines caused by F12.3 and F12.2 (G) and by F12.3 and C1979 extract (H) show identity between the antigens in the three preparations. First-dimension electrophoresis, anode to the right; second-dimension electrophoresis, anode at the top. Stained with Coomassie brilliant blue.

DISCUSSION

Our data indicate that the group of fimbriae comprising the F12, F9, and F7 antigens, as well as the type 1 proteins, constitute a group of evolutionarily related proteins. One can therefore envisage that the F12, F9, and F7 proteins have all evolved from the type 1 fimbriae. The type 1 fimbriae are present in 50 to 70% of all wild-type *E. coli* strains (16), whereas the F antigens are found only in relatively few serogroups, notably those capable of invading the urinary tract (14a), and therefore may represent specialized versions.

Fimbrial proteins of uropathogenic *E. coli* can be considered as important virulence factors; they certainly interact with the immune system of a host; i.e., fimbriae are excellent immunogens (17). One would therefore expect these surface proteins to evolve in ways that would result in maximum immunological heterogeneity. At the same time, they would have to conserve those parts of the peptide chain necessary for fulfilling the structural constraints imposed on a fimbrial protein, i.e., (i) to provide the required structural integrity of the fimbrial superstructure and (ii) to provide a binding site for a host cell receptor. Positions at the surface of the proteins not subject to too many functional constraints can therefore be expected to absorb a substantial number of amino acid substitutions.

Regarding the structure-function aspects of the present study, it is possible to envisage that the part of the peptide chain segment in the F12 protein that forms the outward-facing side of the potential β-sheet, i.e., Lys, Thr, Asn, and Thr (residues 10, 12, 14, and 16), constitutes an antigenic determinant, or at least part of one. Similarly, it would be expected that the corresponding residues in the homologous proteins make up surface features that can interfere with

TABLE 1. Amino acid composition of the F12 fimbrial subunit

Amino acid	No. of residues per subunit[a]
Asx	24 (24.1)
Thr	16 (16.1)
Ser	12 (12.3)
Glx	14 (14.1)
Pro	7 (6.8)
Gly	18 (18.0)
Ala	19 (18.8)
½ Cys	2 (1.6)
Val	13 (12.9)
Met	2 (2.0)
Ile	10 (9.7)
Leu	12 (12.2)
Tyr	3 (2.9)
Phe	9 (9.1)
His	2 (2.0)
Lys	12 (12.1)
Arg	0 (0.1)
Trp	0 (0.1)
Total amino acid residues per subunit	175

[a] Values in parentheses represent the experimentally obtained values.

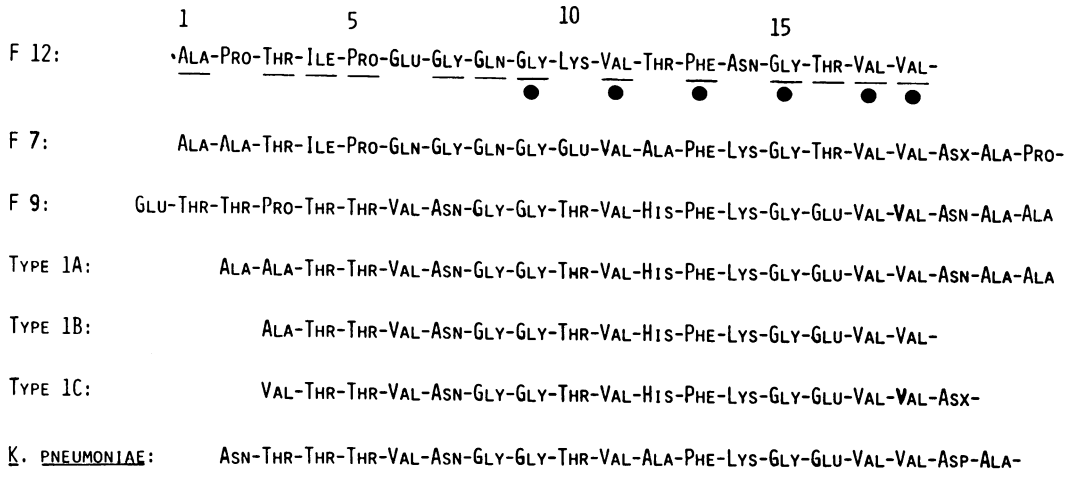

FIG. 4. N-terminal amino acid sequence of the F12 fimbrial subunit of *E. coli* C1979. The N-terminal sequences of other fimbrial proteins from *E. coli* (i.e., F7, types 1A, 1B, and 1C [11], and F9 from strain 3669 [17a]) and from *K. pneumoniae* are given for comparison. Residues underlined and indicated with dots indicate homology with the F7 and the type 1A sequences, respectively.

the immune system of the host, e.g., Glu, Ala, Lys, and Thr in the case of the F7 antigen. This part of the proteins can thus be viewed as an example of a molecular structure that in evolution maintained residues in positions that are important for the structural integrity of the molecule (namely, positions 9, 11, 13, 15, and 17) while at the same time at other, less-restricted positions exhibiting a large array of different antigenic potential, presumably to avoid and foil the immune defenses of the host.

Other and possibly more extensive sequence differences are expected to exist among these F fimbriae in parts of the molecules not yet analyzed. The identified differences among the structures can alone hardly account for their immunological differences (14a). Another aspect which is noteworthy is that the F-type fimbriae confer mannose-resistant adhesion to epithelial cells (11, 15, 17), whereas the mode of adhesion of the type 1 fimbriae is mannose sensitive. The F7 to F12 fimbriae have thus evolved not only in ways that have altered their antigenic determinants but also in ways that have resulted in their acquisition of receptor specificities different from the type 1 fimbriae.

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