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Production, Purification, and Characterization of the Fimbrial Adhesive Antigen F41 Isolated from Calf Enteropathogenic *Escherichia coli* Strain B41M

FRITS K. DE GRAAF* AND INGRID ROORDA
Department of Microbiology, Biological Laboratory, Vrije Universiteit, Amsterdam, The Netherlands

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Enterotoxigenic *Escherichia coli* strains of serogroups O9 and O101 produce an adhesive antigen, provisionally designated as F41. Production of the F41 antigen was shown to be dependent on the composition of the growth medium. A minimal salts medium or Minca medium was the most suitable medium to obtain a high production. The biosynthesis of the F41 antigen was repressed at 18°C or in the presence of L-alanine. The F41 antigen was isolated from the bacteria by mechanical detachment, concentrated by precipitation with ammonium sulfate, and purified by gel filtration on Sepharose CL-4B and treatment with deoxycholate. The purified F41 was composed of protein subunits with an apparent molecular weight of 29,500 on sodium dodecyl sulfate-polyacrylamide gels. The isoelectric point of the antigen was 4.6. The N-terminal amino acid sequence was determined. The F41 antigen had strong hemagglutinating activity with guinea pig and human group A erythrocytes and weaker hemagglutinating activity with horse and sheep erythrocytes. In immunoelectrophoresis at pH 8.4 the purified antigen migrated to the anode. Purified F41 antigen has a filamentous structure with an average diameter of 3.2 nm.

The majority of noninvasive enteropathogenic *Escherichia coli* strains that cause diarrhea in calves, lambs, and occasionally in piglets possess the filamentous surface antigen K99 (18, 24, 26). The K99 antigen possesses hemagglutinating properties and enables the bacterium to adhere to the intestinal mucosa (1, 12, 18, 28). K99 fimbriae are composed of protein subunits with a molecular weight of 18,500 (3). Upon immunoelectrophoresis the K99 antigen migrates to the cathode (7). Production of the K99 antigen is encoded by a 52-megadalton conjugative plasmid (26, 29).

Recently, Morris et al. (22) have demonstrated that certain K99-producing strains possess an additional surface antigen which, like the K99 antigen, exhibits hemagglutinating and adhesive properties but, in contrast to the K99 antigen, migrates to the anode in immunoelectrophoresis. This adhesin, provisionally designated as F41, is associated with K99-positive enteropathogenic *E. coli* strains belonging to O-serogroups 9 and 101 but is not detected so far on K99-positive strains belonging to O-serogroups 8, 20, and 64. For many years the presence of two different adhesins on K99-positive strains had not been discerned. Inconsistent data with respect to the hemagglutinating properties and the behavior of isolated adhesin preparations in immunoelectrophoresis have been reported by different laboratories (10, 11, 19–21).

In this paper we describe the purification and characterization of the F41 antigen. Purification was facilitated by the isolation of a spontaneous K99-negative mutant derived from the K99 reference strain B41 (O101:K99). This mutant did not react with OK antisera to an *E. coli* K-12 K99-positive exconjugant or with OK antisera to K99-positive organisms of serogroups O8, O20, or O64, but it did react with OK antisera to K99-positive strains of the O9 and O101 serogroups. The mutant showed mannose-resistant hemagglutination of sheep erythrocytes and in vitro adhesion to calf intestinal brush borders. These properties were associated with the presence of fimbriae which were detected on whole cells by electron microscopy (J. A. Morris et al., submitted for publication).

MATERIALS AND METHODS

Bacterial strains and media. The F41 antigen was isolated from the K99-negative *E. coli* strain B41M. Strain B41M is a spontaneous mutant from the K99 reference strain *E. coli* B41 [O101:K(A)?:NM] and was isolated by Morris et al. (submitted for publication). *E. coli* strain H926 (O8:K85:K99) was used as a control strain producing only the K99 antigen.

Minca medium, nutrient broth (Oxoid), Trypticase soy broth (BBL Microbiology Systems), and a minimal

medium were used for cultivation of the bacteria. Minca medium (pH 7.5) contained: KH_2PO_4, 1.36 g; $Na_2HPO_4 \cdot 2H_2O$, 10.1 g; glucose, 1.0 g; Casamino Acids (Difco Laboratories), 1 g; and trace salts solution, 1 ml, as described by Guinée et al. (7). It was supplemented with 1 g of yeast extract (Oxoid) per liter. Minimal medium contained: KH_2PO_4, 3 g; $Na_2HPO_4 \cdot 2H_2O$, 7.5 g; NaCl, 0.5 g; NH_4Cl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.12 g; $CaCl_2 \cdot 2H_2O$, 11 mg; $FeSO_4 \cdot 7H_2O$, 5 mg; and glucose, 5 g/liter.

Buffers. The buffers used were phosphate buffer (50 mM, pH 7.2), phosphate-urea buffer (phosphate buffer containing 2 M urea [E. Merck AG]), and phosphate-deoxycholate (DOC) buffer (phosphate buffer, pH 7.5, containing 0.5% [wt/vol] sodium DOC [BDH Chemicals]). KTM buffer is 0.01 M Tris, pH 7.4, containing: NaCl, 7.5 g; KCl, 0.383 g; $MgSO_4 \cdot 7H_2O$, 0.318 g; $CaCl_2 \cdot 2H_2O$, 0.404 g; and mannose, 5 g/liter. PBSM is 10 mM phosphate-buffered saline (pH 7.4) containing 5 g of mannose per liter.

Antisera. Absorbed antiserum to F41 was prepared by absorbing antiserum to the B41M strain with live B41M cells grown at 18°C and was kindly supplied by J. A. Morris. To obtain antiserum against purified F41 antigen, a solution of purified F41 in phosphate-buffered saline (2 mg/ml) was mixed with an equal volume of complete Freund adjuvant (Difco), sonicated for 5 min, and then injected subcutaneously at four different sites into two rabbits (1 mg of purified antigen per animal). After 3 weeks a similar amount of antigen but mixed with incomplete Freund adjuvant (Difco) was injected intravenously. The rabbits were bled 2 weeks after the booster injection. Antiserum against K99 was prepared by immunization of rabbits with purified K99 antigen prepared by preparative electrophoresis of an ultrasonic extract of *E. coli* strain H416 [O101:K(A)?:K99:NM] and was supplied by P.A.M. Guinée (7).

Immunological procedures. The method of Scheidegger (25) as described by Ørskov et al. (23) was used for immunoelectrophoresis. The amount of F41 antigen in various samples was estimated by double-diffusion tests. Double diffusion was performed in petri dishes (9-cm diameter) overlaid with 10 ml of a 1.3% solution of Noble agar (Difco) in saline. Diffusion proceeded overnight at room temperature. Sepharose-protein A-immunoglobulin G was prepared by incubating Sepharose-protein A (Pharmacia Fine Chemicals, Inc.) with specific antiserum against purified F41 antigen in phosphate buffer for 3 h at room temperature. Subsequently, the Sepharose was washed extensively with phosphate buffer containing 0.1 M NaCl.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 13.2% acrylamide slab gels and preparation of samples were done as described by Lugtenberg et al. (16).

Electrofocusing. The isoelectric point of the F41 antigen was determined by analytical electrofocusing of a sample of purified F41 in a 0.5-mm thin-layer agarose gel (LKB Instruments), using LKB Ampholine carrier ampholytes (pH 3.5 to 9.5), an isoelectric focusing calibration kit (Pharmacia), and the LKB Multiphor system.

Hemagglutination. Direct hemagglutination tests, using a suspension of 1% (vol/vol) washed erythrocytes in KTM buffer, were done by mixing 0.2 ml of erythrocyte suspension with an equal volume of an antigen suspension. The titer was determined in serial twofold dilutions of the antigen. After 3 h of incubation at 4°C the trays were examined. Blood samples were kindly provided by W. H. Jansen.

Amino acid analysis. Amino acid analysis was performed on a Kontron amino acid analyzer. Samples were hydrolyzed for 24, 48, and 72 h at 100°C in 6 N HCl containing 0.1% phenol. For serine and threonine, values extrapolated to zero time were used, whereas the values for 72-h hydrolysis were used in the case of valine and isoleucine.

Determination of the N-terminal amino acid sequence. The primary structure of the N-terminal part of the F41 antigen was determined by automatic Edman degradation on a Beckman 890C sequenator. The repetitive yield was 94%. PTH amino acid derivatives were identified by high-performance liquid chromatography as described by Frank and Strubert (5).

Chemical analysis. Protein was determined by the method of Lowry et al. (15), using bovine serum albumin as the standard. Carbohydrate was determined by the phenol method (8) with glucose as the standard.

Electron microscopy. A lyophilized preparation of purified F41 antigen was dissolved in 0.1 M phosphate buffer (pH 7.3) to a concentration of 1 mg/ml. This solution was then diluted 10 times, and $MgCl_2$ was added to a final concentration of 10 mM. The preparation was sprayed on carbon-coated grids and stained with uranyl acetate (1%, wt/vol). The micrographs were taken with a JEM-100B electron microscope operating at 80 kV.

Adhesion experiments. Calf intestinal epithelial cells were prepared as described by Middeldorp and Witholt (17). For adhesion experiments the bacterial strains were grown overnight in Minca medium, harvested, and resuspended in PBSM (10^9 cells per ml). A 50-μl sample of a bacterial suspension was then mixed with 50 μl of epithelial cells also suspended in PBSM (10^6 cells per ml), incubated for 30 min at 37°C in polyvinyl chloride tubes on a rotary shaker, and examined for adhesion. For adhesion inhibition experiments 50 μl of the epithelial cell suspension was preincubated with a solution of a particular antigen for 30 min at 37°C before the addition of 50 μl of a bacterial suspension. The concentrations of the bacterial and epithelial cell suspensions were determined by microscopic evaluation.

RESULTS

Production of the F41 antigen. In previous papers we described that production of the K99 antigen is strongly dependent on the composition of the medium. Minimal salts medium and Minca medium are the most suitable media for obtaining high production of the K99 antigen (4). In complex media, K99 production is strongly reduced, probably because of the presence of alanine, which was shown to repress the biosynthesis of the K99 antigen (2).

To determine whether F41 production is also dependent on medium composition, strain B41M was cultivated on various liquid as well as solid media. The extent of F41 production was mea-

TABLE 1. Relative production of F41 fimbriae by *E. coli* B41M grown in different media[a]

Medium	Production[b] in:	
	Liquid medium	Solid medium
Minca	8	8
Nutrient broth	ND	2
Trypticase soy broth	ND	ND
Minimal	16	16
Minimal + 10 mM L-alanine	ND	ND

[a] *E. coli* B41M was cultivated on either liquid or solid medium, harvested, and resuspended in phosphate buffer to an OD of 20 at 660 nm. Extracts were prepared by ultrasonication of the concentrated cell suspension for 5 min at 75 W and 4°C (Branson Sonifier, type B12, setting 4.5).

[b] Reciprocal value of the highest dilution to yield a positive reaction in the Ouchterlony assay, using undiluted absorbed antiserum against the F41 antigen. ND, Not detectable.

sured in ultrasonic extracts, using the double-diffusion test as a semiquantitative assay (Table 1). High production of the F41 antigen was detected on cells grown in either minimal or Minca medium, but no F41 could be detected in ultrasonic extracts from cells grown in more complex media such as nutrient broth or Trypticase soy broth. No difference was observed between cells grown on liquid or solid media. L-Alanine strongly repressed F41 production to a level no longer detectable with the Ouchterlony assay. The results indicate that the biosynthesis of the F41 antigen is regulated by a mechanism comparable to that for the K99 antigen. Likewise, neither antigen is produced at 18°C (not shown).

Isolation and purification. Cells of *E. coli* strain B41M were grown in 10 liters of minimal medium at 37°C in a Microferm fermentor (New Brunswick Scientific Co.) under constant aeration. The culture was harvested by centrifugation in the late logarithmic growth phase (optical density [OD] of 2.0 at 660 nm). The cell pellet was then resuspended in phosphate-urea buffer to an OD of 100 measured at 660 nm and homogenized for 20 min in a Sorvall Omni-Mixer (maximal setting). After homogenization, the cells were removed by centrifugation of the suspension for 15 min at 30,000 × *g*. The F41 antigen was precipitated from the supernatant fluid by adding ammonium sulfate (60% saturation) and stirring for 2 h at 4°C. The precipitate was collected by centrifugation, resuspended in 10 ml of phosphate-urea buffer, and dialyzed against successive portions of the same buffer at 4°C. Repeated homogenization did not improve the final yield.

For purification, the concentrated F41 preparation was fractionated on a Sepharose CL-4B column equilibrated in phosphate-urea buffer. The extract eluted from the column in two fractions (Fig. 1). Only the first fraction (tubes 6 to 15) reacted positively in the Ouchterlony test. Analysis of this fraction on a sodium dodecyl

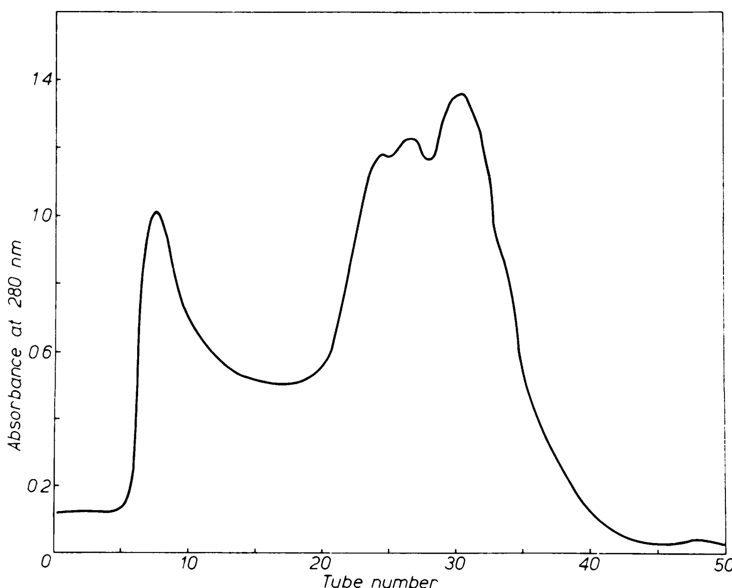

FIG. 1. Fractionation of a crude F41 preparation on a Sepharose CL-4B column (1.5 by 90 cm) equilibrated in phosphate-urea buffer. After application of a concentrated sample (5 ml), the column was eluted with the same buffer (v = 10 ml/h).

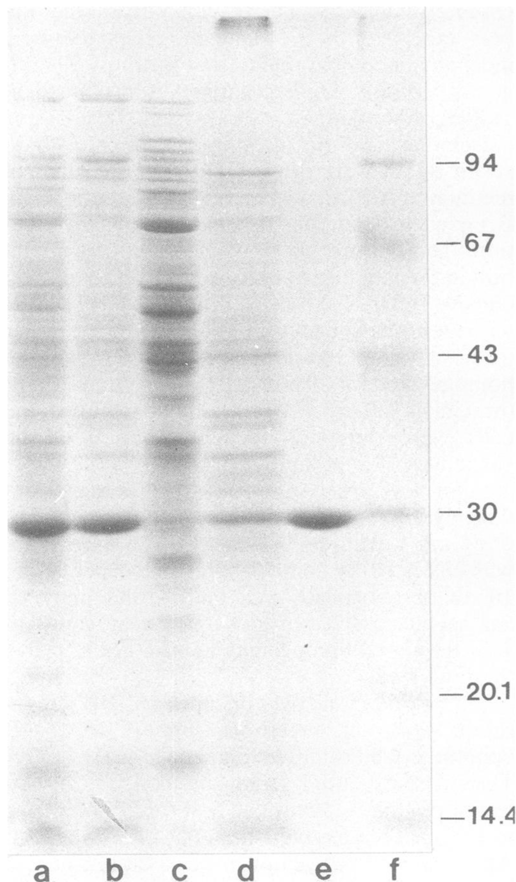

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of F41 preparations. Lanes: a, ammonium sulfate precipitate; b, first fraction eluted from the Sepharose CL-4B column; c, second fraction eluted from this column; d, DOC-insoluble material; e, DOC-soluble material (purified F41 antigen); f, marker proteins. The apparent molecular weights of the marker proteins ($\times 10^3$) are indicated on the right.

sulfate-polyacrylamide gel (Fig. 2b) showed a prominent protein band with an apparent molecular weight of 29,500 and minor quantities of various other polypeptides. The second fraction (tubes 15 to 40) was heterogeneous with respect to polypeptide composition and without the preponderance of a particular polypeptide as observed in the first fraction (Fig. 2c). Final purification of the F41 antigen was achieved by treatment with DOC according to the procedure described by Korhonen et al. (14). For this purpose, the first fraction eluted from the Sepharose CL-4B column was dialyzed against phosphate buffer (pH 7.5) and then supplemented with DOC to a final concentration of 0.5% (wt/vol). This suspension was dialyzed for 72 h against phosphate-DOC buffer, and the DOC-insoluble material was then removed by centrifugation (15 min at 30,000 × *g*). The supernatant fluid was first dialyzed for 48 h against successive portions of phosphate buffer followed by extensive dialysis against distilled water and then lyophilized. Analysis of the DOC-soluble and -insoluble material on a sodium dodecyl sulfate-polyacrylamide gel showed that the DOC-soluble fraction was homogeneous and contained only one polypeptide whereas the DOC-insoluble material contained various contaminating polypeptides in addition to the 29,500-dalton protein (Fig. 2d and e). The final yield of F41 antigen isolated from the 10-liter culture was about 15 mg.

Chemical characterization. The purified F41 antigen migrated as a single protein band upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with an apparent molecular weight of 29,500 (Fig. 2e). The pI of the purified antigen was 4.6 as determined by electrofocusing in a thin-layer agarose gel.

Chemical analysis of three different samples of lyophilized F41 antigen dissolved in phosphate buffer (1.0 mg/ml) showed that the purified material contained 95% protein and about 5% carbohydrates, with a variation of 2%. Whether or not any carbohydrate is linked to the protein is not known.

The amino acid composition of the purified antigen is given in Table 2. The number of residues of each amino acid was calculated assuming a molecular weight of 29,500. The N-terminal sequence of the first 22 residues of the F41 protein subunit is shown in Fig. 3 and compared with the N-terminal sequences of other fimbrial adhesive antigens isolated from enterotoxigenic *E. coli* strains. At five different positions within the N-terminal sequence, the F41 and K99 protein subunits contained an identical amino acid residue.

Specificity of absorbed F41 antiserum and immunoelectrophoresis. To demonstrate that the absorbed antiserum against F41 was specific for the isolated protein, an ultrasonic extract of a concentrated cell suspension of *E. coli* B41M (OD of 40 measured at 660 nm) was mixed with Sepharose-protein A to which antibodies raised against the purified F41 antigen were coupled. After incubation at 4°C the mixture was centrifuged to remove the Sepharose, and the supernatant was tested in a double-diffusion experiment with the absorbed antiserum as well as with antiserum raised against the purified antigen. Precipitation lines were not observed with either of these antisera. This observation indicated that (i) the Sepharose-protein A coupled with antibodies against the purified antigen had removed all of this antigen from the ultrasonic extract and (ii) the absorbed antiserum against

TABLE 2. Amino acid composition of F41 and K99 protein subunits

Amino acid	No. of residues[a]	
	F41	K99[b]
Asp	27.4 (27)	23.2 (23)
Thr	17.6 (18)	18.9 (19)
Ser	33.0 (33)	14.6 (15)
Glu	24.3 (24)	9.9 (10)
Pro	10.8 (11)	4.7 (5)
Gly	39.4 (39)	18.9 (19)
Ala	16.4 (16)	18.0 (18)
Cys	ND	3.4 (4)
Val	16.5 (17)	8.0 (8)
Met	2.9 (3)	3.1 (3)
Ile	10.4 (10)	8.7 (9)
Leu	13.8 (14)	9.4 (9)
Tyr	13.2 (13)	7.3 (7)
Phe	9.3 (9)	8.6 (9)
Lys	11.9 (12)	8.9 (9)
Hydroxy Lys	10.5 (10)	
His	6.5 (6)	3.2 (3)
Arg	4.2 (4)	5.7 (6)
Trp	ND	ND

[a] The number of residues was calculated assuming molecular weights of 29,500 and 18,500 for the F41 and K99 protein subunits. Nearest integers are in parentheses. ND, Not determined.

[b] Values reported by de Graaf et al. (3).

the F41 antigen is specific for the isolated material. In immunoelectrophoresis experiments (not shown) the purified F41 antigen gave a precipitation line towards the anode with F41 antiserum and no line with K99 antiserum. Purified K99 antigen gave an extended precipitation line towards the cathode and showed no reaction with F41 antiserum.

Hemagglutination. The ability of the purified F41 antigen to hemagglutinate various types of erythrocytes in the absence and in the presence of 0.5% mannose was determined and compared with the hemagglutinating activity of purified K99 antigen (Table 3). The F41 antigen exhibited a strong mannose-resistant hemagglutinating activity with guinea pig and human group A erythrocytes in contrast to the K99 antigen, which did not hemagglutinate these cells. Both antigens had weaker hemagglutinating activity with sheep erythrocytes. The K99 antigen had the strongest reaction with horse erythrocytes; F41 showed a weaker reaction with this type of erythrocytes.

TABLE 3. Mannose-resistant hemagglutination of erythrocytes by K99 and F41 antigens

Erythrocytes	Hemagglutination[a]	
	F41[b]	K99[b]
Guinea pig	4,096	4
Human (group A)	8,192	4
Sheep	512	256
Horse	512	4,096
Rabbit	64	2

[a] Reciprocal value of the highest dilution that showed a positive hemagglutination reaction.

[b] Standard solutions containing 1.0 mg of purified antigen per ml of KTM buffer were used. The K99 antigen was purified as described previously (3). Omission of mannose from the buffer did not affect the results.

Electron microscopy. Purified F41 antigen was negatively stained and examined in the electron microscope (Fig. 4). Filamentous structures with a strong tendency to curl were observed. The structures had an average diameter of 3.2 nm; the length could not be estimated. Variation in pH of the buffer used to prepare the sample in the range of 5.8 to 8.8 did not significantly affect the result.

Adhesion. In vitro, strain B41M and its parent B41 adhere to calf intestinal epithelial cells, but the number of mutant cells that adhered was much lower than the number of cells of strain B41, which possesses both the K99 and the F41 antigens (Table 4). *E. coli* strain H926 (O8:K85), which possesses only the K99 adhesive antigen, showed an intermediate adherence. All strains failed to adhere when grown at 18°C.

The attachment of strain B41M could be inhibited by preincubation of the epithelial cells with the F41 antigen. Likewise, attachment of strain H926 was completely inhibited by purified K99 antigen. The adherence of strain B41 was only partially inhibited by either F41 or K99 antigen.

All adhesion experiments were performed in

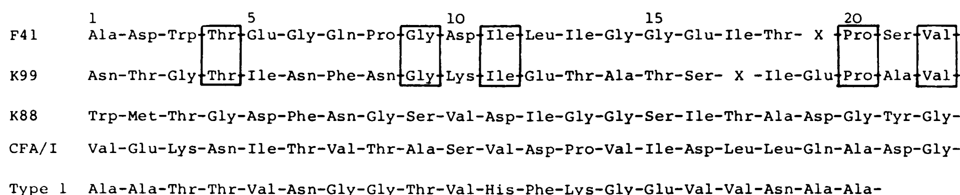

FIG. 3. N-terminal amino acid sequence of the F41 protein subunit. The N-terminal sequences of other *E. coli* fimbrial proteins are given for comparison (3, 6, 9, 13). Homology between the F41 and K99 protein subunits is indicated by boxes.

TABLE 4. In vitro adhesion of various enterotoxigenic *E. coli* strains to epithelial cells and the effect of purified adhesins on adhesion

Strain	Adhesin added[a]	No. of adherent bacteria per cell[b]	
		Mean	Range
B41	None	33	25–40
	F41	29	25–35
	K99	8.5	5–15
B41M	None	7.6	5–10
	F41	0.8	0–2
	K99	7.4	5–10
H926	None	22	15–30
	F41	23	15–30
	K99	6.8	5–10

[a] The concentration of F41 and K99 was 1 mg/ml. The K99 antigen was purified as described previously (3).

[b] All data are based on three experiments, in each of which 20 epithelial cells were examined.

the presence of 0.5% mannose. Omission of mannose from the incubation mixtures did not affect adhesion.

DISCUSSION

The K99 reference strain *E. coli* B41 produces two mannose-resistant hemagglutinins which are not expressed at 18°C (21, 22). One of these hemagglutinins is the K99 adhesin composed of protein subunits with a molecular weight of 18,500 and with a pI of 9.7 (3). The second hemagglutinin, previously characterized as an anionic component (21), is described in this paper and provisionally referred to as F41.

The F41 antigen was isolated from a K99-negative mutant of reference strain B41 by mechanical detachment and purified by chromatography on Sepharose CL-4B. The purified antigen was shown to be composed of protein subunits with a molecular weight of 29,500 and had a pI of 4.6. An electron micrograph of the purified material showed that the F41 antigen had a filamentous structure. The rather curled appearance of the filaments might be an artifact of the preparation procedure (i.e., treatment with DOC). Whole cells of *E. coli* B41M possess very thin fimbriae which are not produced at 18°C (Morris et al., submitted for publication). Antiserum against *E. coli* B41M cells grown at 37°C and absorbed with live cells of B41M grown at 18°C was shown to be specific for the purified F41 antigen, indicating that the purified antigen was identical to the fimbriae detected on whole cells.

The N-terminal amino acid sequence of the F41 protein subunit showed a limited homology with the K99 protein subunit. The significance of this homology, however, is questionable. No cross-reactivity of both antigens was observed upon immunoelectrophoresis.

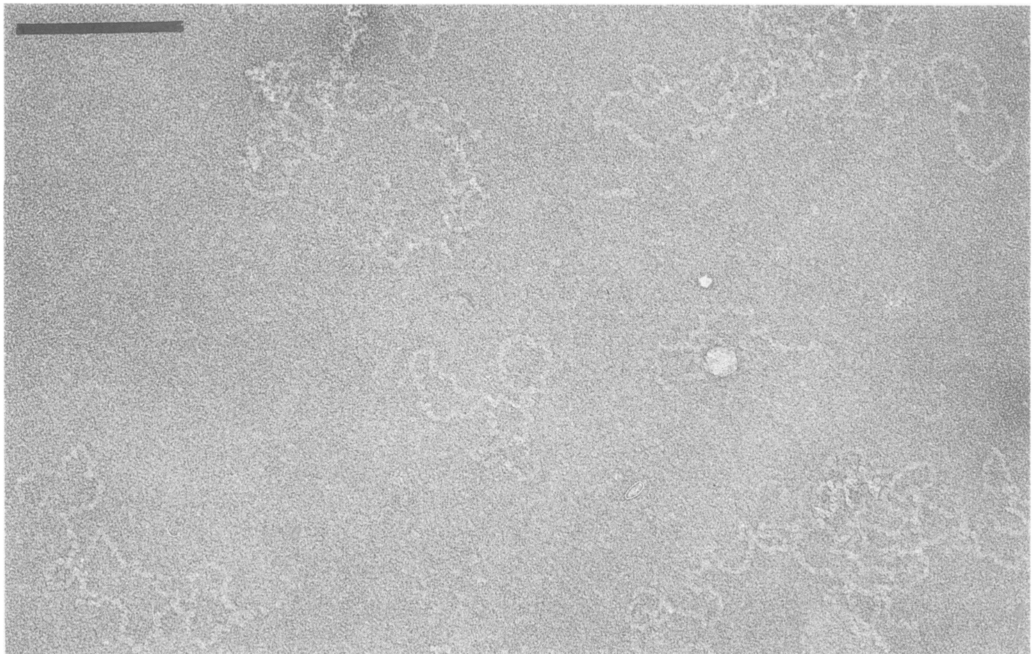

FIG. 4. Electron micrograph of purified F41 antigen. Bar = 100 nm.

The hemagglutinating properties of the F41 antigen with various types of erythrocytes were compared with that of the K99 antigen. In agreement with the data published by Tixier and Gouet (28) and by Burrows et al. (1), the K99 antigen hemagglutinated horse and sheep erythrocytes, respectively, but failed to react with guinea pig erythrocytes, as reported by Isaacson (10). In contrast to these observations, Morris et al. (20) previously described that cell-free K99 antigen hemagglutinated sheep as well as guinea pig erythrocytes and possessed a pI of 4.2. This contradiction can now be explained since these authors used the *E. coli* B41 reference strain to isolate cell-free K99 antigen by acid precipitation. This strain produces both K99 and F41 fimbriae, and acid precipitation obviously results in a preferential isolation of the anionic F41 antigen possessing a strong hemagglutinating activity with guinea pig erythrocytes (11; this paper).

Production of the F41 antigen appeared to depend on the composition of the medium in the same way as observed for K99 production (3). Likewise, F41 production was repressed by alanine, indicating that the regulation of K99 and F41 prouction might have a similar molecular basis. At the moment, however, no explanation for the inhibiting activity of alanine can be provided. Another intriguing question is whether the genetic determinants of both antigens are located on the same plasmid. Morris et al. (22) reported that antibodies to F41 were not detected in the antiserum to an *E. coli* K-12 K99-positive exconjugant of strain B41, and the mutant strain B41M did not react with this antiserum. Isaacson, however, described that a concentrated suspension of *E. coli* K-12 K99$^+$ was able to hemagglutinate guinea pig erythrocytes (10). In a preliminary experiment, we examined the presence of the F41 antigen in an ultrasonic extract of a concentrated cell suspension (OD = 20) prepared from a K99-positive *E. coli* K-12 strain. No antigen could be detected with antiserum prepared to purified F41 fimbriae. Since the production of F41 might be repressed in *E. coli* K-12, the K99 plasmid carried by this strain was transferred to an *E. coli* O9:K$^-$ strain by conjugation. Also on this exconjugant, no F41 antigen could be detected. Although this experiment indicated that the genetic determinant for F41 production is not located on the K99 plasmid, the question of whether the F41 determinant is located on the chromosome or on another plasmid carried by *E. coli* B41 remains to be answered. A good candidate for such a plasmid might be the 65-megadalton large plasmid detected in *E. coli* B41 by So et al. (27) and encoding for heat-stable enterotoxin production.

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