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Characterization of 20K Fimbria, a New Adhesin of Septicemic and Diarrhea-Associated *Escherichia coli* Strains, That Belongs to a Family of Adhesins with *N*-Acetyl-D-Glucosamine Recognition

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Bovine septicemic *Escherichia coli* 31A agglutinates bovine, rabbit, and human erythrocytes and adheres in vitro to the brush border of bovine or ovine intestinal epithelial cells and to the human colon carcinoma Caco-2 cell line. The adhesion and hemagglutination of *E. coli* 31A are mediated by a chromosome-encoded fimbrial adhesin serologically distinct from known fimbrial adhesins found in enterotoxigenic and septicemic bovine *E. coli* strains. By electron microscopy studies the fimbriae designated 20K were observed as fine flexible filaments (diameter, 3 nm) and the purified major fimbrial subunit appeared with an apparent molecular mass of 20,000 Da. Western blot (immunoblot) analysis, N-terminal sequence alignment, and amino acid composition revealed a high homology with the *N*-acetyl-D-glucosamine-specific G fimbria of human uropathogenic *E. coli* and with fimbriae belonging to the F17 family produced by bovine enterotoxigenic and invasive *E. coli* strains. Immunological study revealed that 20K fimbria was closely related to G fimbria and represents a serological variant of F17 fimbria. Hemagglutination and adhesion inhibition assays demonstrated that 20K, G, and F17 fimbriae bind to an *N*-acetyl-D-glucosamine-containing receptor, but each probably binds to different oligosaccharide sequences or different receptors on host tissues. 20K fimbriae were produced by a limited group of clonally related strains with the unusual *m*-inositol-positive phenotype and appeared highly associated with the plasmid-encoded CS31A surface antigen. It was expected that 20K- and CS31A-positive *E. coli* strains with the *m*-inositol-positive phenotype could represent a new example of association between bacterial clones and a plasmid-mediated virulence factor. An examination of natural occurrence of 20K fimbriae among a large collection of human and animal pathogenic *E. coli* showed that 20K fimbria is the prominent adhesin among bovine septicemic *E. coli* isolated from European countries.

Pathogenic *Escherichia coli* strains cause a wide variety of diseases in humans and animals (33). Septicemic colibacillosis is a common problem in neonatal calves, especially when the passive transfer of colostral immunoglobulins fails (39). Fimbriae from many pathogens have been extensively characterized and shown to be important for virulence by mediating adhesion to the host epithelial cells and thereby allowing colonization of the epithelium (18, 29, 30, 34). An essential step in vaccine development is the identification of fimbrial antigens that occur in most pathogenic isolates and are able to induce antibodies that block bacterial adhesion (33).

E. coli strains producing the CS31A surface antigen were isolated from 0- to 12-week-old calves with diarrhea or septicemia (6). The plasmid-encoded CS31A antigen is a capsule-like surface protein belonging to the group of K88-related fimbriae (21, 22, 24). However, this surface antigen clearly differs from typical fimbriae: immunoelectron microscopy of bacteria revealed a wide capsule-like zone around the bacteria, which probably consisted of an abundance of very fine fibrils arranged without apparent order (21). The CS31A antigen does not show hemagglutinating activity or in vitro adhesive properties on the intestinal villi of various animals. However, in a recent study (26) adhesion to the Caco-2 cell line of *E. coli* strains isolated from human diarrheal stools and belonging to the diffusely adhering *E. coli* group was found to be mediated by CS31A antigen. A CS31A-related protein termed CF29K has been described for *Klebsiella pneumoniae* strains involved in nosocomial infections (11).

The well-studied CS31A-positive reference strain 31A was isolated from feces of diarrheic calves and caused experimental septicemia in gnotobiotic calves (7). This strain does not possess K88, K99, F41, F17, or F165 fimbriae and produces neither classical enterotoxins (heat stable or heat labile) nor Shiga-like toxin or cytotoxic necrotizing factors (CNF1 or CNF2). Examination of experimentally infected gnotobiotic calves revealed massive colonization of the organs (liver, lung, and kidney), blood, and urine with a constant edema of the brain and kidney (7). *E. coli* 31A, which contains a 105-MDa plasmid conferring antibiotic resistance and production of aerobactin and CS31A antigen, adhered strongly in vitro to calf intestinal villi, showed strong mannose-resistant hemagglutination (MRHA) activity with bovine erythrocytes, and was piliated after growth at 37°C on Minca medium (13, 21). The plasmid-cured strain 31A/06 lacks CS31A production but retained piliation, hemagglutination, and adhesive properties of the parental strain 31A, suggesting that these properties are mediated by a chromosome-encoded fimbrial adhesin. Although several observations support a role for the 105-MDa plasmid in the pathogenic potential of the septicemic strain 31A (13), the

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TABLE 1. Characteristics and origins of *E. coli* strains used in this study

Strain	Description	Phenotype	Reference or source
31A	Wild strain from fecal flora of a diarrheic calf (serotype O153:K$^-$:H$^-$) carrying the p31A plasmid (Aer$^+$ Iut$^+$ R$^+$)[a]	CS31A$^+$ 20K$^+$	21
31A/06	p31A plasmid-cured variant of 31A	CS31A$^-$ 20K$^+$	21
31A/06(20K$^-$)	Spontaneous 20K$^-$ mutant from 31A/06 (inositol-negative phenotype)	CS31A$^-$ 20K$^-$	This study
HB101	*E. coli* K-12 recipient strain		3
DH5α	*E. coli* K-12 recipient strain		BRL[b]
HB101(pRR5)	Strain HB101 harboring the pRR5 plasmid carrying the G fimbria operon	G fimbria$^+$	48
06/20K$^-$(pRR5)	31A/06(20K$^-$) electroporated with the pRR5 plasmid	G fimbria$^+$	This study
DH5α(pPLHD62)	DH5α harboring the *Hin*dIII-*Sma*I fragment of the pPLHD62 plasmid containing the F17 operon	F17$^+$	38
111KH86	Isolated from fecal flora of a diarrheic calf	F111$^+$	2
H209av	Strain H209 carrying the plasmid Vir	F17b$^+$	42

[a] Aer, aerobactin; Iut, ferric-aerobactin receptor; R, multiple-drug resistance to streptomycin, kanamycin, chloramphenicol, tetracycline, and sulfamide.
[b] BRL, Bethesda Research Laboratories, Inc., Gaithersburg, Md.

plasmid-cured strain 31A/06 has been shown to initiate septicemia in experimental infection of gnotobiotic calves (4).

In this paper, we describe the purification and biochemical characterization of the fimbrial adhesin of the septicemic *E. coli* 31A. The adhesin, which is composed of 20-kDa subunits and termed 20K, is arranged in flexible fimbrial filaments (diameter, 3 nm). The 20K adhesin promotes mannose-resistant hemagglutination of bovine erythrocytes and in vitro adhesion to the brush border of intestinal calf villi and to human Caco-2 cells. On the basis of N-terminal sequence and Western blot (immunoblot) analysis, we demonstrate that this fimbrial adhesin is closely related to the *N*-acetyl-D-glucosamine-specific G hemagglutinin of human uropathogenic *E. coli* (UPEC) (48). We also demonstrate a relationship between 20K, F17 (37) (formerly called FY [22]), F111 fimbriae previously described for bovine enterotoxigenic or enteropathogenic *E. coli* (2), and F17b, recently described as F17-like fimbriae expressed by an invasive *E. coli* strain isolated from bacteremic lambs (14).

MATERIALS AND METHODS

Bacterial strains and media. The characteristics and origins of the strains are given in Table 1. The plasmid-cured strain 31A/06, which lacks the CS31A antigen but retains the piliation and the adhesive properties of the parental 31A strain (21), was chosen for purification of 20K fimbriae. The 31A/06(20K$^-$) strain was a spontaneous mutant of the 31A/06 strain, which lacks simultaneously abilities to produce 20K fimbriae and ferment *m*-inositol. The pRR5 plasmid carrying the gene cluster for G fimbriae (48) was introduced into the 31A/06(20K$^-$) strain by bacterial electroporation using the Gene Pulser apparatus, according to the manufacturer's recommendations (Bio-Rad). The production of G fimbriae by the resultant strain, 06/20K$^-$(pRR5), was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Western blotting using antibodies raised against G fimbriae.

The production of 20K fimbriae was tested on 80 strains isolated from feces of lambs with a severe tubular disease (nephrosis) (1) and on 1,040 *E. coli* strains isolated from the organs and feces of diseased calves with *E. coli* diarrhea or septicemia in Belgium, Algeria, and France (5, 6, 15, 41, 47). We also tested 20K fimbria production among a collection of 169 *E. coli* strains isolated from the diarrheal stools of child and adult patients treated in the Centre Hospitalier Regional Universitaire of Clermont-Ferrand, France (26–28).

All strains were routinely cultured on Minca agar (25) supplemented with 0.2% glucose (MG2). The Minca growth medium was developed by Guinée et al. (25) for the optimal production of fimbrial antigen of *E. coli* strains isolated from bovine intestinal contents. Luria-Bertani medium supplemented with tetracycline (20 μg ml^{-1}) or ampicillin (50 μg ml^{-1}) was used with plasmid-containing strains. All cultures were grown for 16 to 18 h at 37°C.

Extraction and purification of 20K. Overnight MG2 cultures from 10 Roux flasks were harvested in 80 ml of phosphate-buffered saline (PBS) (pH 7.2), and the suspension was blended for 2 min with a Top mix blender at maximum speed. Bacterial cells and cellular debris were removed by centrifugation at 20,000 × *g* for 10 min at 4°C. The resultant supernatant (fraction 1) was collected and subjected to 10 and 20% (of saturation) sequential ammonium sulfate precipitation. The solution was stored overnight at 4°C, the 20% precipitate was collected by centrifugation at 10,000 × *g* for 10 min at 4°C, and the pellet was dissolved in 6 ml of PBS (pH 7.2). SDS was added to a final concentration of 2%, and the suspension was incubated for 2 h at 37°C without agitation. The SDS-insoluble material was collected by ultracentrifugation at 110,000 × *g* for 200 min at 20°C and suspended in 4 ml of PBS (pH 7.2) (fraction 2). After solubilization (4°C, overnight) the suspension was incubated for 2 h at 37°C in 8.5 M guanidine hydrochloride to dissociate the 20K fimbrial polymer into individual subunits. Subsequently, 5 mM Tris hydrochloride (pH 7.8) was added to obtain 6 M guanidine hydrochloride. The preparation was allowed to stand at 20°C for equilibration and was then subjected to chromatography on a Sephacryl S300 (Pharmacia) column (2.5 by 80 cm) and eluted in 5 mM Tris hydrochloride containing 6 M guanidine hydrochloride. Fractions were collected, and the optimal density of the eluate was continuously monitored at 280 nm. The fractions containing the major peak were pooled, dialyzed at 4°C for 48 h with two changes against ammonium acetate buffer (5 mM, pH 7.6), and then lyophilized (fraction 3).

The lyophilizate dissolved in Laemmli sample buffer and boiled 10 min at 100°C was loaded on a preparative acrylamide gel (thickness, 3 mm). After electrophoresis, the band with an apparent molecular mass of 20,000 Da was cut from the unstained gel corresponding to a Coomassie blue-stained part of the gel. Purified fimbrial subunits were electroeluted at 200 V in Tris glycine buffer for 5 h, dialyzed against ammonium acetate buffer, and lyophilized (fraction 4). The purity of the fimbrial preparation in the course of the purification procedure and the apparent molecular mass of the fimbrial subunit were estimated by SDS-PAGE according to the method described by Laemmli (32). Proteins were stained by a modified Oakley silver-staining procedure as previously described (45).

The isoelectric point of 20K fimbriae was determined by denaturing electrofocusing of purified fimbriae (fraction 4) in 6 M urea. Thin-layer polyacrylamide gel and LKB ampholine carrier ampholytes (pH 3.5 to 9.5) were used according to the manufacturer's recommendations.

Preparation of antisera. Polyclonal antibodies were produced against native 20K fimbriae by injecting adult rabbits subcutaneously with 250 μg of native fimbrial preparations (fraction 2) in Freund incomplete adjuvant three times at 4-week intervals. The rabbits were bled 2 weeks after the last immunization. The crude antiserum was repeatedly adsorbed with 31A/06(20K$^-$) cells grown at 37°C to remove antibodies directed against nonfimbrial surface components.

A second antiserum was produced against the denatured 20K subunit protein. Briefly, fraction 3 was electrophoresed through an SDS–15% polyacrylamide gel. The 20,000-Da band was excised, and approximately 250 μg of protein was homogenized with complete Freund adjuvant and injected subcutaneously in five mice. Each animal was immunized at 4-week intervals with three separate injections (50 μg each) of denatured 20K fimbrial subunits. The mice were bled 2 weeks after the last injection. Polyclonal rabbit anti-native F17 fimbria serum was provided by Henri de Greve, and polyclonal anti-native G fimbria serum was provided by Timo K. Korhonen.

Immunoglobulins G (IgG) were isolated from hyperimmune rabbit serum or from nonimmune rabbit serum by protein A affinity chromatography according to the manufacturer's protocol (Pierce, Chicago, Ill.).

Immunoblotting. Proteins of the crude fimbrial preparations were electrophoresed on an SDS–10% polyacrylamide gel and transblotted to nitrocellulose membranes as described by Towbin et al. (51). The membranes were blocked for 60 min in 0.2 M PBS with 5% bovine serum albumin (BSA) and incubated overnight with appropriate dilutions of the different antisera raised against 20K subunit, F17, or G fimbria. The nitrocellulose membranes were washed in PBS with 0.05% Tween 20 and incubated in 1:2,000 conjugate (goat anti-rabbit or anti-mouse IgG–horseradish peroxidase; Nordic Immunological Laboratories).

After washings, the membranes were developed with hydrogen peroxide substrate and 4-chloro-1-naphthol chromogen.

N-terminal amino acid sequence. A 15-μg sample of denatured 20K fimbria (fraction 4) was subjected to SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) in transfer buffer (10 mM 3-cyclohexylaminol-1 propane sulfonic acid [CAPS], 10% methanol). The Immobilon-bound 20-kDa protein was stained with Coomassie brilliant blue R-250 (Merck), excised from the membrane, and sequenced directly by automatic Edman degradation with an Applied Biosystems model 477A gas phase sequencer with an in-line 120A PTH amino acid analyzer. Comparison of the N-terminal sequence of 20K fimbriae with proteins listed in the GenBank database and the National Biomedical Research Foundation protein sequence data bank was made with the FASTA program (46).

Hemagglutination and inhibition of hemagglutination. Hemagglutination tests were performed on glass slides at 4°C in the absence and presence of 0.5% D-mannose as described by Evans et al. (16). A suspension of *E. coli* strains (50 μl, 10^9 cells ml^{-1}) was incubated with an identical volume of a 3% (vol/vol) suspension of erythrocytes from human blood group A and from animals (calf, sheep, horse, goat, pig, chicken, rabbit, and mouse). The hemagglutination titers of bovine erythrocytes by *E. coli* strains were determined at 4°C in the presence of 0.5% D-mannose. Serial twofold dilutions of 50 μl of *E. coli* strains were performed in Veronal Ca-Mg buffer containing 1% D-mannose in 96-well microplates (Greiner, Nürtingen, Germany). To each well was subsequently added 50 μl of a 0.3% suspension of bovine erythrocytes, and the hemagglutination titers were determined after 6 h of incubation at 4°C. The hemagglutination titer was recorded as the reciprocal of the highest dilution of a bacterial suspension (1.2×10^9 bacteria ml^{-1}) causing complete hemagglutination, and this hemagglutination titer was assigned the value of 1 hemagglutination unit (1 HA unit).

The hemagglutination inhibition tests were performed as described by Giron et al. (23). Serial twofold dilutions of the putative inhibitors (50 μl per well) were performed as described above, and an equal volume of HA units of the different *E. coli* strains was added to the wells. An aliquot of 100 μl of a 0.3% suspension of bovine erythrocytes (containing 1% D-mannose) was then added. After 6 h of incubation at 4°C, the concentration of the substance that inhibited the hemagglutination titer of 4 HA units of each *E. coli* strain was determined. The inhibitory potency of *N*-acetylglucosamine on MRHA with 20K agglutinin was normalized at 1.0 as described by Mouricout et al. (43). Values above 1.0 indicated stronger affinity of the adhesin for the compound than for free GlcNAc. The relative inhibitory potency was expressed as concentration of GlcNAc needed for 50% MRHA inhibition/concentration of inhibitor giving 50% MRHA inhibition.

Different compounds known to be part of the receptor structure for different bacterial adhesins were tested as putative inhibitors of the MRHA shown by the studied *E. coli* strains. All compounds used were purchased from Sigma Chemical Co., St. Louis, Mo.

In vitro adhesion assay on intestinal villi. Adhesion tests on calf, lamb, and piglet intestinal villi were carried out as previously described (19) with few changes. Briefly, immediately after thawing, the villi were washed for 30 min in cold PBS containing 15 mM of 14-(2-aminoethyl)-benzensulfonyl-fluoride-hydrochloride (AEBSF) to prevent proteolysis. Just before use, the villi were washed in cold PBS (pH 7.0) and maintained on ice. Adhesion was scored by using a phase-contrast microscope at a magnification of ×1,000. A maximal attachment was usually obtained when 30 bacteria adhered to a 50-μm segment of a villus brush border (19). Inhibition of adhesion was tested in the presence of 75 mM *N*-acetyl-D-glucosamine (GlcNAc).

Adhesion to the human colon carcinoma cell line Caco-2 in culture. Adhesion tests were performed as previously described (10). Briefly, monolayers of differentiated Caco-2 cells were prepared in 24-well Falcon tissue culture plates. Cells were seeded at $4 \times 10^4/cm^2$ in Dulbecco modified Eagle medium at 37°C in a 10% CO_2–90% air atmosphere for 15 days. The medium was supplemented with 20% fetal bovine serum and 1% nonessential amino acids. The culture medium was changed every 2 days. A suspension of approximately 10^8 bacteria per ml was prepared in the cell line culture medium with 1% D-mannose, added to the tissue culture, and incubated for 3 h at 37°C. The cells were washed in sterile PBS, stained with 20% Giemsa stain, and examined microscopically under oil immersion.

Electron microscopy. Bacterial cells or crude fimbrial extracts (fraction 1) were placed on carbon-stabilized collodion-coated copper grids, negatively stained with 1% phosphotungstic acid, and observed with either a Hitachi 2A or an EM400 electron microscope (Philips Electronic Instruments) operated at 75 and 80 kV, respectively. For immunoelectron microscopy, gold immunolabeling was performed as described by Levine et al. (35) with anti-20K fimbria serum (dilution, 1:10) and 10-nm colloidal gold-labeled goat anti-rabbit IgG (Nordic). To prevent nonspecific labeling, 1% BSA and 1% Tween 20 were added to the wash solution.

Amplification procedure. DNA to be amplified was released from whole organisms by boiling. Bacteria were harvested from 1 ml of an overnight broth culture, suspended in 1 ml of sterile water, and incubated at 100°C for 10 min. Oligonucleotides used as primers were deduced from the published sequence of the F17 operon (38) and flanked a 538-bp DNA fragment corresponding to the *f17A* gene. Their sequences were 5′-GCAAAATTCAATTTATCCTTGG-3′ and 5′-CTGATAAGCGATGGTGTAATTCAG-3′. PCR was carried out in a total volume of 50 μl containing 5 μl of DNA template, each of the primers (20 mM), the four deoxynucleoside triphosphates (each at 200 μM), 5 μl of enzyme buffer, and 0.4 U of AmpliTaq DNA polymerase (Appligene-Oncor). The reaction mixture was overlaid with 50 μl of mineral oils. PCR amplification consisted of 25 cycles of denaturation at 94°C for 2 min, annealing at 59°C for 1 min, and extension at 72°C for 2 min. Ten microliters of the reaction mixture was then analyzed by electrophoresis on a 1.4% agarose gel.

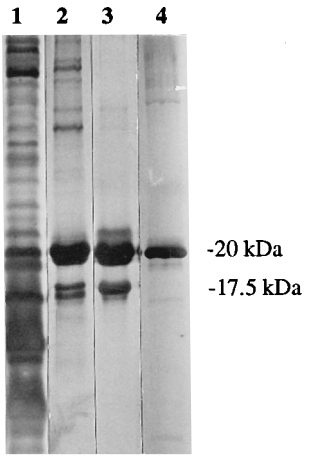

FIG. 1. SDS-PAGE of fractions obtained during the purification procedure of 20K protein. Lanes: 1, crude extract (fraction 1); 2, SDS-insoluble material (fraction 2); 3, major peak eluted from the chromatography column (fraction 3); 4, final fraction purified by preparative SDS-PAGE (fraction 4).

RESULTS

Purification and chemical characterization. The presence of filamentous fimbria-like structures on the *E. coli* plasmid-cured strain 31A/06 had been demonstrated previously by electron microscopy of whole cells (21). When bacterial cells were subjected to mechanical shearing and centrifugation, the resulting supernatant fraction contained abundant fimbrial structures. SDS-PAGE analysis of the crude extract revealed a major polypeptide band with an apparent molecular mass of 20,000 Da (20K protein) (Fig. 1). Analysis of the SDS-insoluble material (fraction 2) revealed that this fraction was enriched in the 20,000-Da major protein but contained other minor polypeptide bands (Fig. 1). Fimbriae were disrupted in 6 M guanidine hydrochloride, and the dissociated subunits were purified by gel filtration chromatography on a Sephacryl S300 column. The major peak eluted from the chromatography column (fraction 3) contained two copurified polypeptides with apparent molecular masses of 20 and 17.5 kDa. Finally, purification of the 20K fimbrial subunit (fraction 4) was obtained by preparative SDS-gel electrophoresis of this fraction. Protein analysis of the purified fimbrial subunit revealed only one polypeptide band corresponding to the 20-kDa protein (Fig. 1), and denaturing isoelectric focusing of the 20K-purified urea-treated fimbriae resulted in the presence of a single band with an acidic pI value of 4.9.

The amino acid sequence of the first 18 N-terminal residues of the 20K subunit is shown Table 2 with the amino acid sequences of previously described fimbrial proteins listed in the GenBank database and the National Biomedical Research Foundation protein data bank. A high homology (94%) was observed with the first 15 residues of the *N*-acetyl-D-glucosamine-specific G fimbria (48) and F111 (2) fimbrial subunits of human UPEC and bovine pathogenic *E. coli*, respectively. Except for the first residue, the N-terminal sequences of 20K and G fimbriae were identical. A high homology (88 and 81%) was also observed between the 20K subunit and the

TABLE 2. Comparison of the N-terminal amino acid sequence of the 20K subunit with those of other major fimbrial subunits

Fimbria	N-terminal amino acid sequence[a]	Identity (%)	Pathogenicity	Origin	Reference
20K	**YDGTINFNGKVVDQT**XX**V**	100	Septicemia	Bovine	This study
G	**TDGTINFNGKVVDQTXSV**	94	Urinary tract infection	Human	48
F111	**YDGTI**T**FNGKVVDQTCSV**	94	Diarrhea	Bovine	2
F17	**YDG**K**I**T**FNGKVVDQTCSV**	88	Diarrhea	Bovine	38
Uca	**YDGTI**T**F**T**GKVV**A**QTCSV**	83	Urinary tract infection	Human	8
F17b	**YDG**K**INF**T**GKVV**A**QTCSV**	81	Bacteremia	Bovine	14

[a] X, unidentified residue. Uncertain residues are underlined; identical residues are boldfaced.

F17-A (38) and F17b-A (14) subunits from bovine enterotoxigenic and invasive *E. coli* strains, respectively. In addition, a recently described fimbria (Uca) from a human uropathogenic *Proteus mirabilis* strain (8) was also observed with a high homology (83%) in the amino terminal part of its major fimbrial subunit.

Residue 16 of the 20K subunit could not be identified but was probably a cysteine, since a cysteine residue, which was routinely unidentified by the automated Edman degradation sequencing procedure, was found at the corresponding position in all other related proteins.

Morphological observations. Negative-staining electron microscopy of whole cells of the 20K-positive strain 31A/06 grown at 37°C on a Minca plate revealed fine and flexible fimbria-like filaments (diameter, approximately 3 to 3.5 nm) (Fig. 2a). No fimbrial structures were observed on either strain 31A/06 ($20K^-$) grown at 37°C on Minca agar (Fig. 2b) or strain 31A/06 grown at 18°C. Immunoelectron microscopy showed that the anti-20K subunit antibodies completely decorated intact native fimbriae on the 31A/06 strain (Fig. 2c), thereby confirming that the purified 20K subunit protein was the major fimbrial subunit. No immunogold labeling was observed with strain 31A/06($20K^-$) (results not shown).

Negative-staining electron microscopy of purified fractions revealed that the SDS-insoluble material (fraction 2) showing one major polypeptide band (20 kDa), as determined by SDS-PAGE, consisted of fibrillar structures (Fig. 2d) similar to the 3- to 3.5-nm fibers previously detected on the surface of whole cells (Fig. 2a). These purified filamentous structures aggregated frequently as parallel strands.

Characterization of the 20K-negative mutant strain. SDS-PAGE analysis of the 31A/06 and 31A/06($20K^-$) crude bacterial extracts revealed identical protein patterns except for the 20,000-Da polypeptide band present on the 31A/06 extract and absent on the 31A/06($20K^-$) extract (Fig. 3). PCR was used to test for the presence of an *f17A*-related gene encoding the major structural subunit. Results of primer-directed amplification show the presence of a 538-bp amplified product when DNAs from the DH5α(pPLHD62) and 31A/06 strains were used (Fig. 3). Similar results were obtained with DNAs from G-, F111-, and F17b-producing strains (results not shown). In contrast, no amplification product was detected in DNA from the 31A/06($20K^-$) strain, indicating that the gene coding for the 20K major fimbrial subunit was absent (or partly deleted) in the 20K-negative mutant.

Immunological studies. Two specific antisera to native 20K fimbriae and the denatured 20K fimbrial subunit were obtained on rabbits and mice, respectively. Antibodies raised against the denatured 20K subunit recognized native and denatured states of the 20K antigen and cross-reacted with native F17, F111, and G fimbriae and with their respective denatured subunits (Fig. 4). However, absorption of anti-20K subunit serum with either G or F17 fimbriae did not affect the reactivity of this antiserum. This suggests that cross-reacting antibodies recognized common determinants of the denatured subunits that are not accessible on the native F17 or G fimbriae. Antibodies raised against native 20K fimbriae reacted strongly with native 20K, F17, F111, and G fimbriae but did not react by Western blot after SDS-PAGE with any of the denatured fimbrial subunits.

To study the immunological cross-reactivity of these related antigens, nitrocellulose replicates of 20K, F17, F111, and G fimbriae were also tested by Western blotting with anti-F17 and anti-G sera. Antibodies raised against native G fimbriae showed high cross-reactivity with the 20K subunit but reacted more weakly with F17 and F111 subunits, suggesting a higher relatedness between 20K and G fimbrial subunits. This finding was further supported by the absence of immunological reactivity between anti-F17 fimbria serum and the 20K or G fimbrial subunit (Fig. 4). The results summarized in Table 3 show that the immunological cross-reactivity between 20K and F17 native fimbriae depends on common conformational determinants accessible at the surface of the assembled fimbrial subunits. Thus, cross-reactivity between anti-F17 fimbria serum and G or 20K fimbriae no longer occurred after the antiserum was absorbed by 20K fimbriae. Reciprocally, cross-reactivity between anti-20K fimbria serum and F17 or F111 fimbriae disappeared after the antiserum was absorbed against F17 fimbria. In addition, absorption of anti-20K-fimbria antibodies with G fimbriae abolished reactivity against 20K and G fimbriae, indicating that the two are closely related.

MRHA of 20K fimbriae and inhibition by receptor analogs; comparison with other fimbrial hemagglutinins with *N*-acetyl-D-glucosamine recognition. Strain 31A/06 showed a strong MRHA of bovine erythrocytes at 4°C and a weak MRHA of human blood group A but did not agglutinate sheep, horse, mouse, pig, chicken, or goat erythrocytes. No hemagglutination was observed with either the 20K-negative mutant 31A/06 ($20K^-$) strain grown at 37°C or the 31A/06 strain grown at 18°C. On glass slides on ice a high concentration (10 μg ml^{-1}) of purified 20K fimbriae agglutinated a 3% suspension of bovine erythrocytes mixed by constant rocking. However, as described for the G fimbriae with human erythrocytes (48), the 20K purified fraction did not agglutinate bovine erythrocytes on 96-well microplates.

To clarify the nature of the 20K agglutinin, we tested the potentials of a large number of compounds to inhibit MRHA activity. However, because of the weak agglutinating activity of the 20K and G purified fraction, an investigation of the receptor specificities of the different agglutinins was carried out with 4 HA units of intact *E. coli* cells as described by Giron et al. (23). *N*-Acetyl-β-D-glucosamine showed a strong inhibitory activity of 20K agglutinin, and activity increased twofold when GlcNAc was linked to a benzyl group. However, the inhibitory activity of the disaccharide GlcNAc–β-1-4-GlcNAc (diacetylchitobiose) decreased 30-fold. *N*-Acetylgalactosamine, *N*-ace-

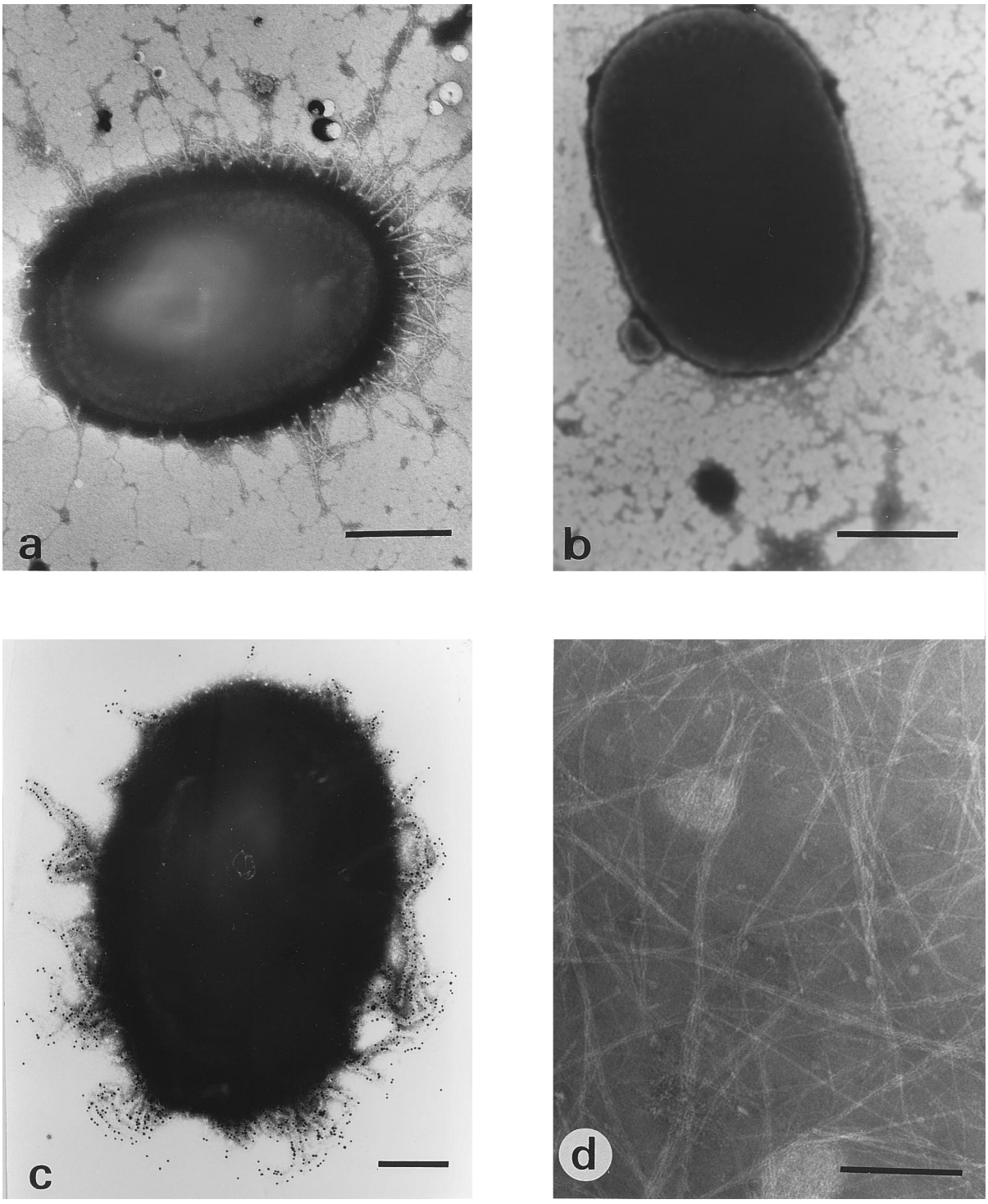

FIG. 2. Transmission electron micrograph of bacterial preparations. (a and b) *E. coli* 31A/06 and its isogenic 20K-negative mutant 31A/06(20K$^-$), respectively, negatively stained with 1% phosphotungstic acid. Bars, 0.5 μm. (c) 31A/06 bacterial cells were labeled by the immunogold technique with anti-20K subunit antibodies and negatively stained with 1% phosphotungstic acid. The 3-nm flexible fimbrial structures are clearly labeled by this technique. Bar, 0.5 μm. (d) SDS-insoluble material (fraction 2) negatively stained with 1% phosphotungstic acid showing partially purified 20K fimbriae from *E. coli* 31A/06. Bar, 200 nm.

tylneuraminic acid, and *N*-acetylneuramine lactose showed no inhibitory activity on MRHA of 20K fimbriae.

Sialylated forms of mucins of bovine submaxillary glands (BSM) and porcine gastric mucin (PGM) strongly inhibited agglutinating activity of 20K fimbria for bovine erythrocytes. Human glycophorin A and bovine glycolipids failed to inhibit the activity of the 20K agglutinin.

Given the N-terminal sequence homologies and immunological relatedness among F17, F17b, F111, and 20K fimbrial subunits, we used hemagglutination inhibition studies to deter-

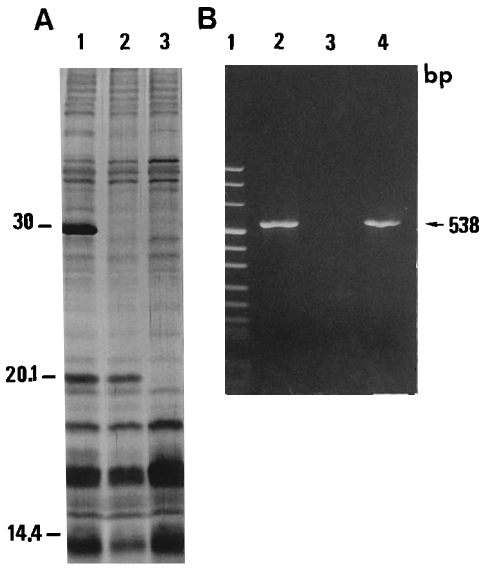

FIG. 3. SDS-PAGE and PCR amplification product analysis of bacterial strains. (A) SDS-PAGE analysis of crude bacterial extracts from *E. coli* 31A (lane 1), 31A/06 (lane 2), and 31A/06(20K$^-$) (lane 3). Protein molecular mass standards (in kilodaltons) were carbonic anhydrase (30.0), soybean trypsin inhibitor (20.1), and α-lactalbumin (14.4). (B) PCR amplification products corresponding to the *f17A* structural gene from *E. coli* 31A/06 (lane 2), 31A/06(20K$^-$) (lane 3), and HB101(pPLHD62) (lane 4). Lane 1, DNA molecular weight marker V (Boehringer Mannheim).

mine whether receptor specificities were also shared among their adhesins. The results are summarized in Table 4. An HB101 strain harboring plasmid pRR5 carrying the genetic determinants for the G fimbriae caused a strong hemagglutination of bovine erythrocytes, but this strain was also strongly autoagglutinating. With this strain, investigation of MRHA inhibition by receptor analogs was seriously hampered by the autoaggregates. To decrease bacterial surface hydrophobicity (known to be high in K-12 strains), plasmid pRR5 was electrotransferred into the nonfimbriated wild-type *E. coli* 31A/06 (20K$^-$). Plasmid pRR5 converted the nonfimbriated recipient strain into the strain 06/20K$^-$(pRR5), which produced G fimbriae and agglutinated bovine erythrocytes but did not autoagglutinate. This strain enabled us to determine and compare receptor specificities of the G fimbriae.

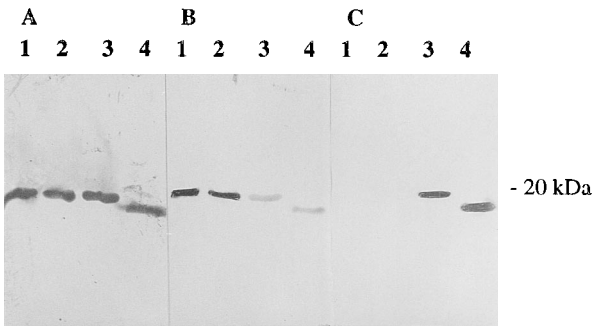

FIG. 4. Comparison of antigenic structures of 20K, G, F17, and F111 fimbrial subunits by Western blot analysis. Bacterial proteins were extracted from *E. coli* 31A/06 (lane 1), HB101(pRR5) (lane 2), DH5α(pPLHD62) (lane 3), and 111KH86 (lane 4). SDS-PAGE of crude extracts separated on a 15% polyacrylamide gel, transferred on nitrocellulose membranes, and incubated with sera raised against 20K (A), G (B), and F17 (C) fimbriae. The strain 31A/06(20K$^-$) and the two recipient strains HB101 and DH5α were used as negative controls.

TABLE 3. Immunological cross-reactivity between 20K, G, F17, and F111 fimbriae as determined by immunodot analysis

Antigen	Reactivity with antiserum[a]:					
	Anti-20K fimbria	Anti-G fimbria	Anti-F17 fimbria	Anti-20K fimbria absorbed against:		Anti-F17 absorbed against 20K fimbria
				F17 fimbria	G fimbria	
20K	+++	+++	+	++	−	−
G	+++	+++	+	++	−	−
F17	+++	+++	+++	−	−	++
F111	++	++	+++	−	−	++

[a] The antisera used were directed against native fimbriae. −, negative; +, weak; ++, moderate; +++, strong. Strains HB101, DH5α, and 31A/06(20K$^-$) were used as negative controls.

Differences in MRHA titers among strains were observed according to their fimbriae. The 111KH86 strain showed the highest hemagglutination activity (HA titer, 256), DH5α (pPLHD62) was less potent (128), and H209av, 31A/06, and 06/20K$^-$(pRR5) had the weakest HA activity (32 to 64). As shown in Table 4, substantial differences exist among the studied agglutinins displaying a GlcNAc specificity. Compared with the HA inhibitory potency of GlcNAc observed with 20K, the GlcNAc inhibitory potency decreased by 33, 8, 3, and 2 for F111, F17, G, and F17b, respectively. Addition of a benzyl group linked to GlcNAc increased the inhibitory activity of GlcNAc also for G, F17, F17b, F111, and 20K. BSM and PGM displayed the strongest inhibitory potency towards MRHA activities of the five agglutinins. However, as shown in Table 4, inhibitory potency of mucins varied considerably among the strains. The strongest inhibitory potency of PGM was observed with F111 (1,000-fold stronger than GlcNAc), and the weakest

TABLE 4. Comparative inhibitory activities of GlcNAc derivatives, oligosaccharides, glycolipids, and glycoproteins on MRHA of *E. coli* strains with *N*-acetylglucosamine-binding fimbrial agglutinins

Inhibitor	Relative inhibitory potency[a]				
	20K	F17	F17b	F111	G
N-Acetyl-β-D-glucosamine	1.0	0.13	0.5	0.03	0.34
p-Nitrophenyl-*N*-acetyl-β-D-glucosaminide	1.36	0.25	1.36	0.06	0.74
N-Acetyl-diacetylchitobiose	0.02	0.01	0.02	0.01	0.02
PGM	10	4.2	4.0	34	17
BSM	18	7	25	7	25
N-Acetyl-β-D-galactosamine	—	—	—	—	—
N-Acetylneuraminic acid (pH 7.5)	—	—	0.01	—	—
N-Acetylneuramine lactose	—	—	0.01	—	—
Ceramides from the bovine brain	—	—	—	—	—
Cerebrosides from the bovine brain	—	—	0.65	—	—
Monosialoganglioside GM1 from the bovine brain	—	—	0.55	—	0.50
Glycophorin A from human blood (Type N and type M)	—	—	—	—	0.30
	—	—	—	—	0.30

[a] The relative inhibitory potency of free GlcNAc on MRHA of strain 31A/06 was assigned the value of 1.0 (0.19 mM was required for 50% inhibition). —, no inhibition at the maximal concentration used (50 mM for oligosaccharides and 125 μg ml^{-1} for glycolipids and glycoproteins). The data are means of three separate experiments. Fimbriae and strains: 20K, 31A/06; F17, HB101 (pPLHD62); F17b, H209av; F111, 111KH86; G, 06/20K$^-$(pRR5).

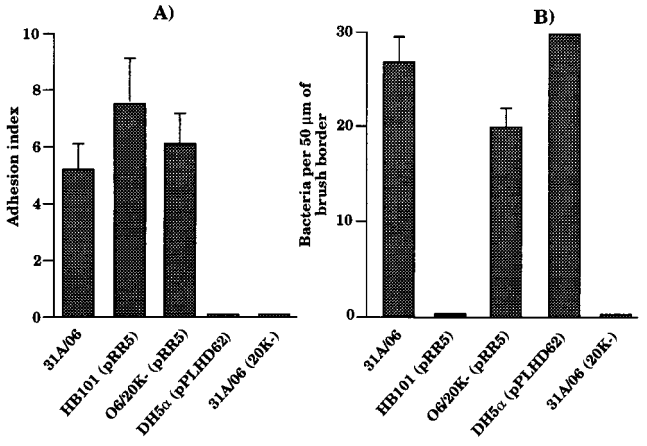

FIG. 5. Adhesion of *E. coli* strains to Caco-2 cells and to calf intestinal villi. Strains 31A/06, HB101(pRR5), and DH5α(pPLHD62) were used as 20K-, G-, and F17-positive strains, respectively. Strains 31A/06(20K$^-$) and 06/20K$^-$ (pRR5) were the 20K-negative mutant and the wild bacterial strain producing the G fimbriae, respectively. (A) The adhesion index for Caco-2 cells was the mean number of bacteria per cell determined by examining 100 cells. (B) The adhesion index to calf intestinal villi was the mean number of bacteria adhering to a 50-μm segment of the villus brush border. Standard deviations for three separate experiments are indicated.

inhibitory potency was observed with F17b (18-fold stronger than GlcNAc). BSM displayed the strongest inhibitory potency also with F111 (230-fold stronger than GlcNAc) but the weakest inhibitory activity was observed with 20K (13-fold more active than free GlcNAc). One of the main features of BSM being its very high sialic acid content (25%) and terminal *N*-acetylneuraminic acid (NeuAc) residues in major carbohydrate chains (31), the high inhibitory potency of BSM towards MRHA suggests that sialic acid may be involved in the binding of agglutinins. However, with 20K-, F17-, and F111-producing strains, no MRHA inhibition occurred with either *N*-acetylneuraminic acid or *N*-acetylneuramine lactose. In addition, monosialoganglioside GM1, ceramides, and cerebrosides from the bovine brain did not show strong inhibitory potency. For particular strains, H209av and 06/20K$^-$(pRR5), a weak MRHA inhibition could be observed with NeuAc, GM1, and human glycophorin A, indicating that F17b and G agglutinins bind with low affinity to sialic acid.

Adhesion to the human carcinoma cell line Caco-2 in culture. An adhesion index corresponding to the mean number of bacteria per cell was determined by examining 100 cells. The percentage of epithelial cells with adhering bacteria was also calculated; if more than 10% of the epithelial cells had adhering bacteria, the strain was considered positive (52). Adhesion to Caco-2 cells was measured in the presence of 1% D-mannose to prevent nonspecific type 1 pilus-mediated adhesion. Strain 31A/06 adhered to Caco-2 cells with an adhesion index of 5.19% ± 0.92%. Bacteria adhered to 91% ± 5% of Caco-2 cells (Fig. 5). Bacteria were not randomly distributed over the entire monolayer, suggesting the existence of preferential areas where the cells, according to their stage of differentiation, had more receptors for the adhesive factors. When the 20K-negative strains 31A/06(20K$^-$), HB101, DH5α grown at 37°C, and 31A/06 grown at 18°C were tested, bacteria no longer adhered to the Caco-2 cells and the adhesion index obtained was about 0.02 bacterium per cell (Fig. 5). Interestingly, the HB101(pRR5) recombinant strain harboring G fimbriae also adhered to Caco-2 cells with an adhesion index and a percentage of epithelial cells with adhering bacteria greater than those obtained with the 31A/06 strain, whereas the DH5α (pPLHD62) strain expressing the F17 fimbria no longer adhered to the Caco-2 cell line (Fig. 5).

In vitro adhesion to intestinal villi. Strains 31A/06, DH5α(pPLHD62), and HB101(pRR5), producing 20K, F17, and G fimbriae, respectively, were tested for their capacity to adhere in vitro to calf, piglet, and lamb intestinal villi. The results of the adhesion study are shown in Fig. 5 and Fig. 6. The 20K- and F17-producing strains adhered strongly in vitro to the intestinal villus brush border in calves and lambs but not in piglets. However, strong adhesion to the basolateral border of piglet enterocytes was observed. Attachment of both strains on brush borders of calf and lamb intestinal villi could be inhibited by free GlcNAc (75 mM) and BSM (50 μg ml^{-1}). No adhesion to animal intestinal villi was observed with the 20K-negative mutant 31A/06(20K$^-$) or with the G fimbria-positive strain (Fig. 6). However, a limited adhesion was observed when G fimbria was produced by the wild 20K-negative strain *E. coli* 06/20K$^-$(pRR5), indicating that the G-fimbria-mediated adhesion on calf intestinal villi was influenced by extrinsic factors (lipopolysaccharide or cell surface hydrophobicity).

To determine whether adhesion of *E. coli* 31A/06 to intestinal villi was mediated only by the 20K fimbriae, we examined the reduction of in vitro attachment by antibodies directed against 20K fimbriae. However, when total rabbit sera were used, immune and nonimmune sera had comparable inhibitory activities. Similar observations had been reported previously by Sanchez et al. (50), and plasma glycoproteins were found to be strong adhesion inhibitors for F17 fimbrial adhesin. Therefore, inhibition assays were tested with protein A-purified rabbit IgG obtained from either normal serum or anti-20K fimbria serum. When *E. coli* 31A/06 was preincubated (30 min at 20°C) with anti-20K antibodies (IgG fraction), in vitro adhesion to the calf intestinal villi was greatly decreased (80% with a 1:300 dilution of the total IgG fraction). Preincubation of bacterial strain 31A/06 with a similar concentration of nonimmune rabbit IgG had little or no effect on in vitro adhesion (<10% decrease). We also examined the role of purified 20K fimbriae in a competitive inhibition assay. Results showed that adherence of *E. coli* 31A/06 was clearly decreased (60%) by preincubation of calf intestinal villi with purified 20K fimbriae (100 μg ml^{-1}), whereas preincubation of villi with a similar concentration of BSA showed no effect on the level of binding. These findings confirmed the 20K-fimbria-mediated binding of *E. coli* 31A/06 to bovine enterocytes.

Similar results were obtained when the assay of in vitro attachment to Caco-2 cells was performed with *E. coli* 31A/06 preincubated with purified 20K fimbriae or with anti-20K fimbria sera (data not shown).

Natural occurrence of 20K fimbriae among collections of pathogenic *E. coli* strains and characteristics of 20K-positive strains. A large collection of previously described pathogenic *E. coli* strains (5, 6, 15, 27, 28, 41, 47) was reexamined in a retrospective study for the production of 20K fimbriae. As shown in Table 5, 380 (33%) of the 1,120 bovine and ovine *E. coli* strains cross-reacted in immunodot assay with anti-20K serum. Expression of 20K fimbriae was confirmed by SDS-PAGE analysis and, for part of the collection, by Western blotting. Of the bovine diarrhea and septicemia strains, 313 (46%) of the 681 strains isolated in Europe (Belgium and France) but only 43 (12%) of the 359 strains collected in Algeria were 20K positive. In contrast with bovine isolates, only 5 (3%) of the 169 strains isolated from diarrheic stools of hospitalized patients were 20K positive, suggesting that 20K fimbria does not play an important role in infantile and adult diarrhea.

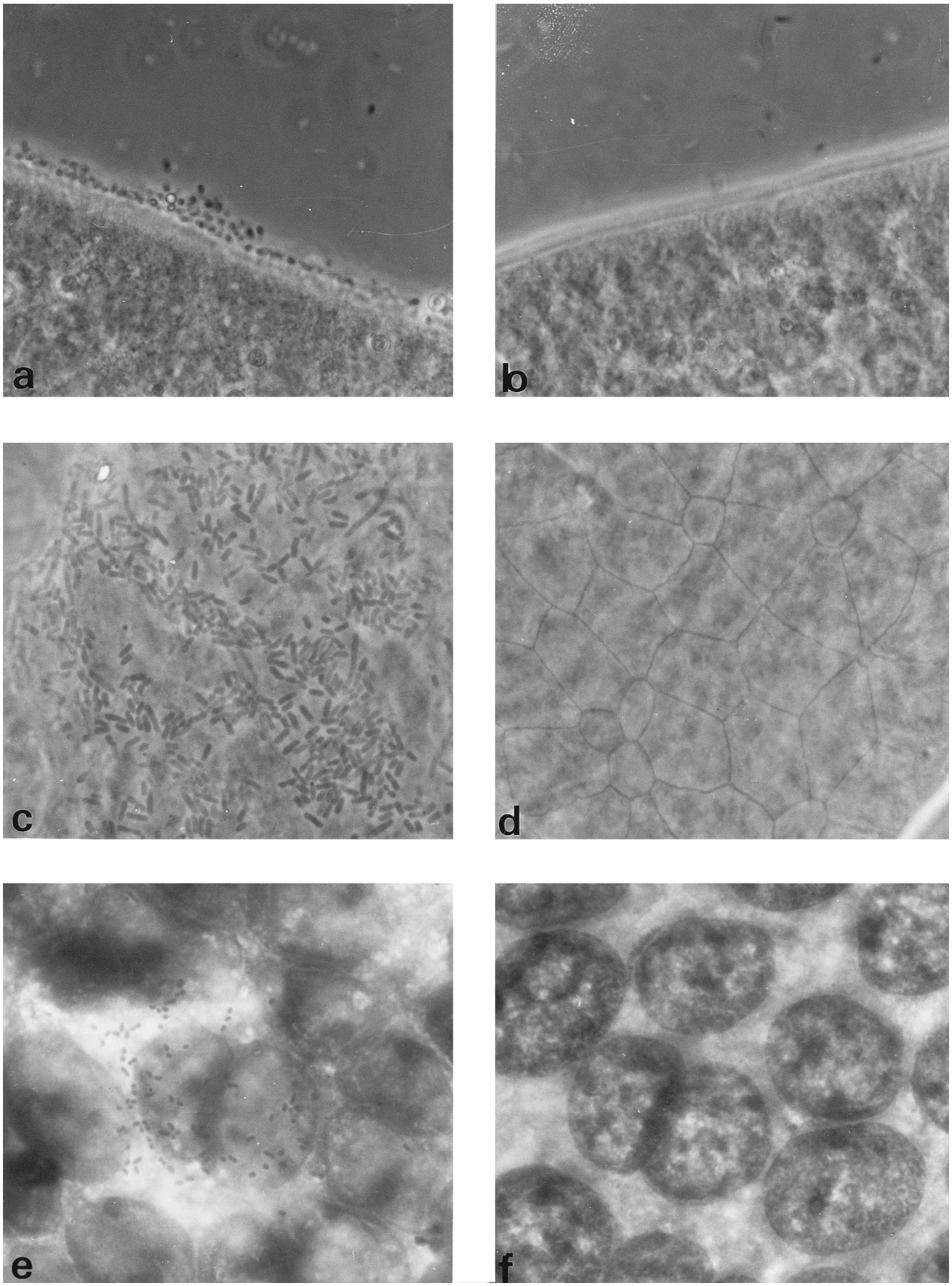

FIG. 6. Micrographs showing in vitro adhesion of *E. coli* 31A/06 and its negative variant to calf intestinal villi and Caco-2 cells. *E. coli* 31A/06 (a) adhered, but the 20K-negative variant 31A/06(20K$^-$) (b) did not adhere, on the brush border of calf intestinal villi. (c) A micrograph showing diffuse adhesion of *E. coli* 31A/06 at the surface of lamb intestinal villi. Intestinal epithelial cells were clearly observed without adhering bacteria when *E. coli* 31A/06(20K$^-$) was tested (d). Diffuse adhesion on Caco-2 cells was observed in the presence of 0.5% D-mannose with *E. coli* 31A/06 (e) but not with the 20K-negative variant 31A/06(20K$^-$) (f). Magnification, ×1,360.

TABLE 5. Prevalence and characteristics of 20K-positive strains among *E. coli* strains

Country (yr)	Reference(s)	Source	No. of strains		% 20K-positive strains associated with:	
			Total	20K positive (%)	CS31A	Inositol-positive phenotype
Belgium (87–91)	47	Diarrheic calves	166	46 (28)	85	82
France (86–89)	15	Calves with diarrheic syndrome with ataxia	473	246 (52)	96	ND[a]
Great Britain (93)	5	Lambs with nephrosis	80	23 (29)	0	80
France (77–85)	6	Septicemic calves	42	21 (52)	100	80
France (94)	27, 28	Diarrheic stools of hospitalized humans	169	4 (3)	0	ND
Algeria (93)	41	Diarrheic calves	359	43 (12)	12	ND

[a] ND, not determined.

In a previous report (6) we showed that of 22 20K-positive strains 17 (80%) fermented *m*-inositol within 24 h. In this study we assessed the significance of this phenotype in relation to 20K production. Of 45 20K-positive strains isolated in Belgium from diarrheic calves, 37 (82%) were inositol positive. In addition, 20K-positive strains isolated from lambs were also observed with a high prevalence (80%) of the inositol positive-phenotype. In contrast, of 208 strains tested for the inositol phenotype, only 6 (3%) 20K-negative strains fermented inositol. These results indicate that the inositol-positive phenotype was highly prominent among the 20K-positive strains, whereas biotyping of *E. coli* strains selected randomly from the National Collection of Type Cultures revealed an unusual prevalence (0.5%) of the inositol-positive phenotype among *E. coli* species (9).

Fimbrial adhesins and surface antigens F41, K99, F17, F165, and CS31A (12, 17, 21, 25, 37) were previously identified in our pathogenic *E. coli* collections. The association of 20K fimbriae with other adhesins was further investigated in the 20K-positive strains. From the bovine isolates collected in Europe, 296 (94%) of the 313 20K-positive strains were also positive for CS31A antigen expression, whereas only 5 (12%) of the 43 20K-positive strains isolated from diarrheic calves in Algeria were positive for CS31A antigen. None of the 20K-positive strains isolated from humans or lambs was CS31A positive. No F41, K88, K99, or F17 fimbriae were identified among the 20K-positive *E. coli* strains.

DISCUSSION

In this report, we describe identification and characterization of fimbriae termed 20K fimbriae and demonstrate that they play a role in the ability of *E. coli* 31A to adhere to intestinal epithelial cells. The 20K fimbria was purified from the plasmid-cured variant 31A/06 strain by a procedure involving differential ultracentrifugation in the presence of SDS and gel filtration of the dissociated fimbrial subunits. The 20K fimbriae consisted of flexible 3.5-nm fimbria-like structures.

The N-terminal amino acid sequence of 20K fimbriae was closely related (94%) to the major subunit of G fimbriae identified on UPEC isolated from urine of patients with acute pyelonephritis (48) and more distantly related to the F111, F17-A, and F17b-A subunits, which belong to the F17 fimbriae family found on bovine enterotoxigenic *E. coli* and septicemic *E. coli* (2, 14, 38). The results indicate a close immunological relatedness between 20K and G fimbriae but suggest that 20K and F17 fimbriae are immunologically more distant. This finding was further supported by the loss of cross-reactivity between 20K fimbriae and specific anti-F17 serum absorbed by 20K fimbriae (and vice versa). We concluded that 20K and G fimbriae are immunologically identical or closely related and constitute a serological variant of F17 fimbriae.

Fimbrial adhesins consist of numerous copies of the major subunit protein and only a few copies of minor proteins, including the adhesin protein which mediates receptor binding. The interaction between F17 fimbriae and calf intestinal mucosal cells required the F17G subunit, a minor fimbrial component responsible for binding to *N*-acetyl-D-glucosamine-containing receptors (36). On the basis of morphological, serological, and biochemical relatedness with F17 fimbriae, it was reasonable to expect that the determinant of the adhesive function of 20K fimbriae was distinct from the major fimbrial subunit. Fimbrial adhesins can be subdivided on the basis of their receptor specificity. The G fimbriae of human UPEC bind to the terminal *N*-acetyl-D-glucosamine residues of glycophorin A exposed after treatment with endo-β-galactosidase (48), and F17 fimbriae of bovine enterotoxigenic *E. coli* bind to GlcNAc residues present on the surface of intestinal cells (22, 37). Recently, Mouricout et al. (43) demonstrated that the minimal GlcNAcβ1-3Galβ1 sequence, whether in a terminal or internal position in carbohydrate moieties of bovine glycophorins and intestinal mucins, strongly bound to F17 lectin. Our MRHA inhibition experiments showed that GlcNAc bound to agglutinins with different affinities in the preferential order 20K > F17b > G > F17 > F111. Unlike GlcNAc-binding plant agglutinins which bind also to sialic acid (43, 48), our results, which agree closely with recent results obtained with F17 (43), indicated that F17, 20K, and F111 did not bind to sialic acid. However, the high inhibitory potency of BSM (which contains 25% sialic acid) and influence of free NeuAc, cerebrosides, GM1, and glycophorin A on binding of F17b and G fimbrial lectins to receptors of bovine erythrocytes suggest that sialic acid may be involved in the binding of both lectins. PGM, which contains only a very small amount of NeuAc (0.9%), also induces strong inhibition of MRHA with 20K, G, and F111. This inhibitory potency may be related to the presence of terminal GlcNAc residues in the main carbohydrate chains (31), suggesting, in agreement with earlier results obtained with G agglutinin (48), that 20K, G, and F111 bind with high affinity to the terminal GlcNAc residue. Although these results did not provide direct information on binding sites of bovine erythrocytes, the difference in the inhibitory potencies of BSM and PGM probably reflects various receptor specificities of the GlcNAc-binding fimbrial lectins. This was further investigated by in vitro adhesion experiments on animal intestinal villi and on the human colon carcinoma cell line Caco-2. We observed that bacterial strains producing 20K and F17 fimbriae bound in vitro to calf and lamb intestinal villus brush borders and that this adhesion could be inhibited by *N*-acetyl-D-glucosamine. Although both strains HB101(pRR5) and 06/20K$^-$(pRR5)

produced similar amounts of G fimbriae, only strain 06/20K$^-$ (pRR5) exhibited adhesive properties with intestinal villi. This discrepancy may have resulted from a difference in the hydrophobic patterns of the two strains and revealed that the recipient strains could influence the G-fimbria-mediated adhesion. 20K-fimbria-mediated binding of *E. coli* 31A/06 to human and bovine intestinal epithelial cells was confirmed by inhibition of binding with both anti-20K IgG and purified 20K fimbriae. The failure of 20K and F17 adhesins to recognize receptor structures in piglet brush borders may be due to the absence of a characteristic oligosaccharide sequence found only on glycoproteins or glycolipids of bovine enterocytes.

The Caco-2 cell line, derived from a human colon carcinoma, has been described as the best available cell line to study the adhesion of human enteropathogens to intestinal epithelium (52). Interestingly, the 20K and G fimbria-producing strains adhered to Caco-2 cells, whereas F17-positive strains did not, indicating that 20K and G adhesins, but not F17, recognize a receptor on human Caco-2 cells. Our results show conclusively that (i) F17 adhesin of bovine enterotoxigenic *E. coli* binds to specific receptors of calf and lamb brush border enterocytes but not human receptors on human Caco-2 cells; (ii) G fimbria of human UPEC recognizes receptors on human Caco-2 cells, but recognition of animal enterocyte receptors appears influenced by cell surface hydrophobicity; and (iii) 20K adhesin recognizes receptors on both human Caco-2 cells and bovine enterocyte brush borders. This study therefore provides evidence that 20K, F17, F17b, F111, and G fimbriae recognize an *N*-acetyl-D-glucosamine-containing receptor but that each recognizes different oligosaccharide sequences. In recent years, similar results have been obtained with AFA-I, AFA-III, Dr hemagglutinin, and F1845 fimbriae recognizing the Dr blood group antigen. However, each probably represents a single adhesin belonging to a family of Dr receptor-recognizing hemagglutinins (44). Dr hemagglutinin, AFA-I, and AFA-III adhesins were expressed by human UPEC strains, and F1845 fimbriae were expressed by enteropathogenic *E. coli*. Our study suggests that an analogous situation exists among adhesins recognizing GlcNAc-containing receptors, since F17 and 20K adhesins were isolated from bovine diarrheic *E. coli* strains and G fimbriae were isolated from human UPEC strains.

20K fimbria was first described in a limited survey of bovine pathogenic CS31A-positive *E. coli* strains belonging to serogroups O8, O11, O21, O28, O78, and O86. Outer membrane protein patterns and esterase polymorphism analysis demonstrated that these strains were clustered in a limited group of clonally related strains (6). In this study, we detected 20K fimbria in 43% of the 691 bovine pathogenic isolates. This shows that 20K fimbria was the prominent adhesin of the bovine septicemic and diarrhea-associated *E. coli* strains isolated in European countries. Unlike most *E. coli* strains, 80% of the 20K-positive strains ferment *m*-inositol. This unusual biotype suggested that the strains expressing 20K fimbriae have a clonally derived origin. The abilities to ferment *m*-inositol and to produce 20K fimbriae were thus simultaneously lost by the 20K-negative mutant in which the 20K fimbrial subunit-encoding gene was probably a target for deletion or genetic recombination as indicated by PCR experiments.

Most (94%) of the 20K-positive strains also express the capsule-like antigen CS31A, described as an adhesive factor on the human Caco-2 cell line (11). The genes encoding CS31A were located on a large conjugative R plasmid (26) reported to carry additional virulence factors such as the aerobactin iron-scavenging system (13) that contribute to the pathogenic process of *E. coli* (40). Thus, strains producing 20K fimbria and CS31A antigen and having the *m*-inositol-positive phenotype may represent a new example of association between bacterial clones and plasmid-mediated virulence factors. In developed countries, the intensive use of antibiotics in animal care could explain the high occurrence of 20K-positive strains associated with self-transmissible plasmids coding for virulence factors (CS31A and aerobactin system) and for antibiotic resistance. This finding is supported by the much lower prevalence of 20K- and CS31A-positive bovine pathogenic isolates originating from Algeria (12% versus 94% in Europe). Among the human isolates, 20K-positive strains were rare (4%) and not found in association with CS31A.

In a recent study, the amino acid sequence of the major subunit of the uroepithelial cell adherence fimbriae (Uca) isolated from *P. mirabilis* responsible for urinary tract infection was found to be closely related to those of F17 and F17b fimbrial subunits (8). The Uca-positive strains adhere to desquamated uroepithelium but did not agglutinate with bovine and human erythrocytes. Moreover, the G adhesin termed GafD has been purified, and the gene encoding GafD has been sequenced (49). The purified GafD protein was able to bind *N*-acetyl-D-glucosamine–Sepharose resin, confirming its receptor specificity. Further analysis of the genetic organization of 20K fimbriae would enable us to identify the 20K fimbrial component that acts as an adhesin protein.

In conclusion, we showed that 20K, G, F17, F17b, F111, and probably Uca belong to the family of fimbrial adhesins with *N* acetyl-D-glucosamine recognition but that each probably recognizes different receptors on host tissues. The apparent linkage between 20K fimbriae and inositol-positive phenotype, in agreement with earlier findings, suggests that 20K positive-strains are clonally related. The association between adhesin and clonally related strains may account for the different clinical and host origins of strains with the different GlcNAc adhesins, but this hypothesis remains to be tested.

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