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New Adhesive Factor (Antigen 8786) on a Human Enterotoxigenic *Escherichia coli* O117:H4 Strain Isolated in Africa

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An enterotoxigenic *Escherichia coli* strain, *E. coli* 8786, of serotype O117:H4 produced only heat-stable enterotoxin and gave mannose-resistant hemagglutination with human and bovine erythrocytes. The strain adhered to the brush border of human enterocytes and to enterocytelike cell line Caco-2. Adhesion inhibition assays using Caco-2 cells with different adhesive factor extracts showed that the adhesive factor of *E. coli* 8786 is different from colonization factor antigen I (CFA/I), CFA/II, CFA/III of Darfeuille et al. (A. Darfeuille, B. Lafeuille, B. Joly, and R. Cluzel, Ann. Microbiol. Inst. Pasteur 134A:53–64, 1983), CS6, and antigen 2230. A bacterial surface protein, designated antigen 8786, with a molecular mass of 16,300 Da was responsible for the adhesion to intestinal cells. It was immunologically different from previously described adhesive factors as determined by immunoblotting. Antigen 8786 was detected on the bacterial cell surface and appeared to be nonfimbrial. NH_2-terminal analysis of antigen 8786 showed no homology with the previously described adhesive factors. Nevertheless, antigen 8786 is closely related to the NH_2-terminal sequence of *Salmonella enteritidis* fimbrin. A hybridization experiment using a synthetic oligonucleotide probe based on the NH_2-terminal amino acid sequence of antigen 8786 revealed that the coding region was located on a 70-MDa plasmid.

Enterotoxigenic *Escherichia coli* (ETEC) strains are a major cause of infant and traveller's diarrhea in developing countries (1, 10, 34). Their pathogenicity is due to the ability to adhere to and colonize the small intestine and produce enterotoxins, such as heat-labile or heat-stable enterotoxin or both.

Adhesion to the intestinal epithelium is mediated by colonization factor antigens (CFAs). Several CFAs or putative colonization factors (PCFs) have been described in human ETEC strains: CFA/I (12), CFA/II (11), CFA/III (6, 19), CFA/IV or PCF8775 (38), PCF0159:H4 (37), antigen 2230 (8), PCF0166 (29), CS17 (31), and PCF09 (17). Of these, CFA/II and CFA/IV are heterogeneous and are composed of three coli surface (CS) antigens called CS1, CS2, and CS3 for CFA/II and CS4, CS5, and CS6 for CFA/IV (35, 39). CFAs are either fimbrial or fibrillar. Antigen 2230 has been described as a nonfimbrial antigen (8). Although these CFAs have been described as antigenically unrelated, immunological cross-reactions between CFA/I, CS1, CS2, CS4, and CS17 were detected by immunoblotting with polyclonal antibodies (30). Moreover, the NH_2-terminal amino acid sequences of CFA/I, CS1, CS2, and CS4 are very similar (16, 22, 24, 43).

In vitro adhesion assays using human intestinal biopsies or the human enterocytelike cell line Caco-2 showed that binding of ETEC occurs only on the enterocyte brush border (7, 26). In addition, adhesion inhibition assays using purified adhesive factors suggested that a different cell receptor was involved for each adhesive factor (7).

The structural genes of colonization factors are located on high-molecular-weight plasmids, except CS1 and CS2, which are chromosomal. Expression of CS1, CS2, CFA/I, CS4, and antigen 2230 requires the presence of a regulatory gene always located on a high-molecular-weight plasmid (4, 5, 42; unpublished data).

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The strain described in this report, 8786 (O117:H4), has been previously studied by Forestier et al. (14). It was isolated from the diarrheal stool of an infant and adhered to the brush border of human enterocytes (14). We considered this an ETEC strain because we detected thermostable enterotoxin genes by using a specific toxin probe (unpublished data). In this study, we identified the adhesive factor of *E. coli* 8786 and compared it with reference ETEC adhesive factors by immunoblotting with polyclonal antibodies and NH_2-terminal amino acid analysis. We also localized the structural gene of the adhesive factor.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* 8786 (O117:H4) was isolated in Burundi (Africa) from a child with diarrhea. This strain produced the thermostable enterotoxin (unpublished data) and adhered to the brush border of human enterocytes (14).

The following enterotoxigenic *E. coli* strains were used: H10407 producing CFA/I (12); Pb176 producing CFA/II (CS1 and CS3) (11); C91f producing CS2 (3); 1373 producing CFA/III (6); 2230 producing antigen 2230 (8); E8775A producing CS4 and CS6 (39); E17018A producing CS5 and CS6 (39); and M17 producing CS6 (43). All strains were grown at 37°C on Mueller-Hinton agar (Institut Pasteur Production, Marnes la Coquette, France).

Adhesion to human colon carcinoma cell line Caco-2 in culture. The cell adhesion test was performed as described previously (7). Briefly, monolayers of differentiated Caco-2 cells were grown in Dulbecco modified Eagle medium (Flow Laboratories, Les Ulis, France) containing 20% (vol/vol) fetal bovine serum, 1% nonessential amino acids, 200 U of penicillin per liter, 50 mg of streptomycin per liter, and 2 mg of amphotericin B per liter in six-well Falcon tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.) at 37°C in 10% CO_2. Cultures were used at postconfluence after 15 days of culture. Before the adhesion test, cells were washed

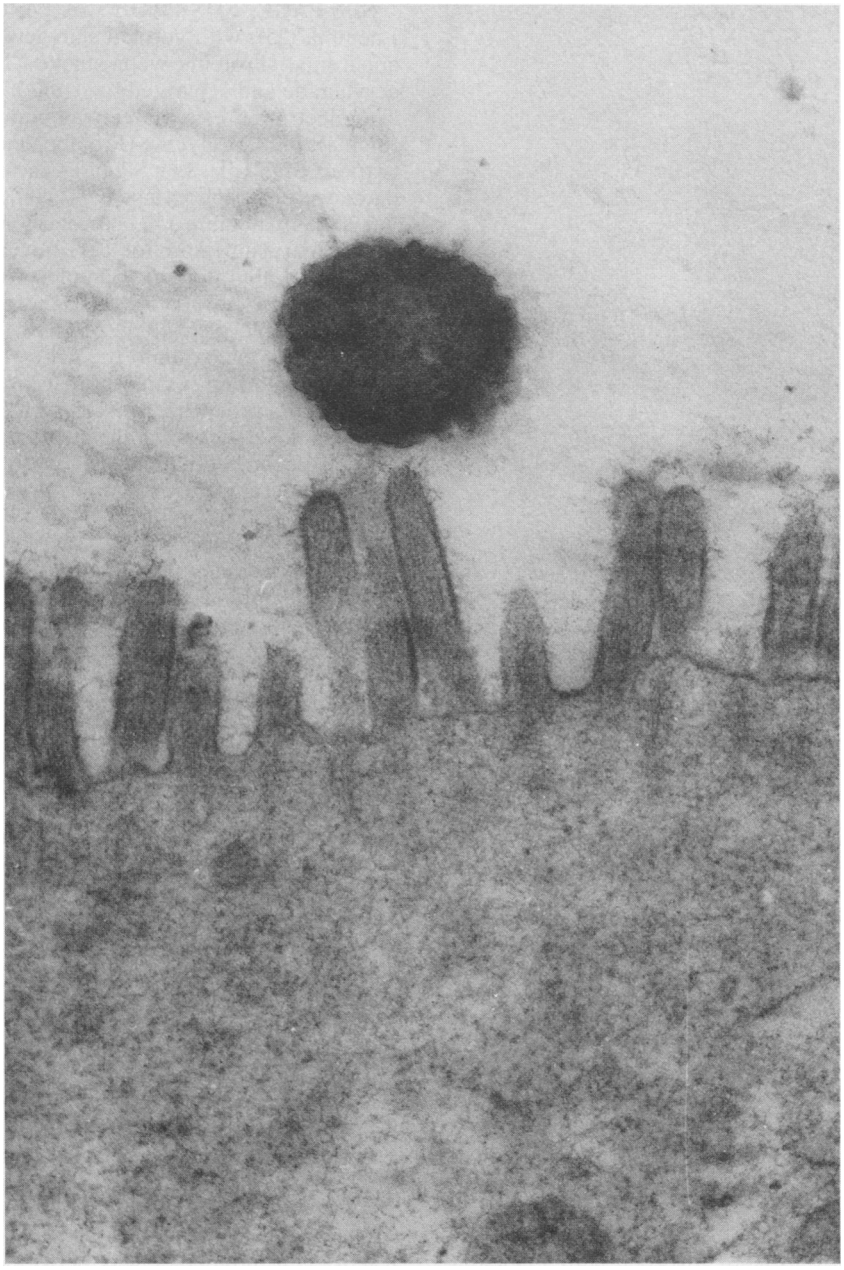

FIG. 1. Transmission electron micrograph showing the brush borderlike microvilli of the Caco-2 cell line with *E. coli* 8786. Magnification, ×43,000.

with phosphate-buffered saline (PBS; pH 7.2). A suspension of approximately 10^8 bacteria per ml in Dulbecco modified Eagle medium (without antibiotic) containing 0.5% D-mannose was added to the tissue culture and incubated for 3 h at 37°C. After three washes, cells were fixed in methanol, stained with 20% Giemsa stain, and examined under a microscope (magnification, ×1,000). An adhesion index representing the average number of bacteria per cell was determined by examining 100 cells.

Adhesion inhibition tests. CFAs of the different strains were extracted and purified as described below. Overnight cultures grown in 10 Roux flasks with Mueller-Hinton agar were harvested in 0.1 M PBS (pH 7.2). The surface antigens were separated from bacterial cells by heating the suspension at 60°C for 20 min with gentle agitation. Cells and bacterial debris were sedimented by centrifugation at 10,000 × *g* for 10 min. The supernatant was brought to pH 4.0 by slow addition of P_i and stored overnight at 4°C. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 30 min and suspended in 0.1 M PBS (pH 7.2). The suspension was mixed with cesium chloride (final density, 0.38 g/cm^3) in PBS (pH 7.2) and centrifuged in an L8-50 M/E ultracentrifuge (Beckman instruments, Inc., Palo Alto, Calif.) for 44 h at 180,000 × *g*. Protein bands were collected by tube puncture and dialyzed against water for 2 days. A suitable concentration of purified protein was added to

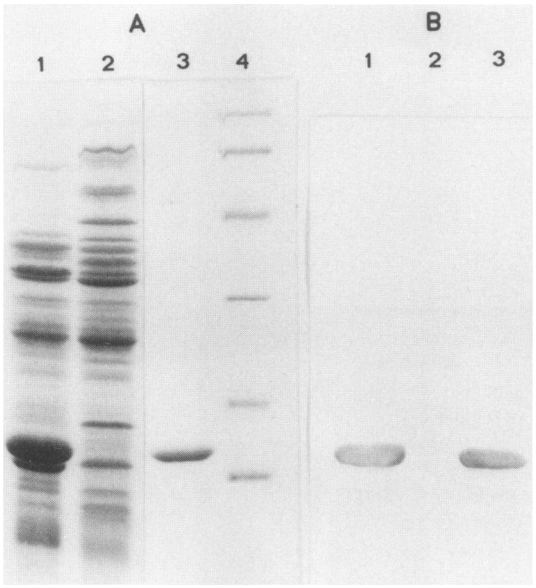

FIG. 2. SDS-PAGE (A) and Western immunoblot (B) of crude bacterial surface components of strain 8786 and purified antigen 8786. Lanes: 1, strain 8786 grown on Mueller-Hinton agar at 37°C; 2, strain 8786 grown at 18°C; 3, purified antigen 8786; 4, molecular mass markers (from the top, 97,400, 66,200, 45,000, 31,000, 21,500, and 14,400 Da). Antiserum against antigen 8786 was used at a 1:200 dilution.

Caco-2 cells and incubated at room temperature for 20 min. Then 10^8 *E. coli* cells were added, and the adhesion test was performed as described above.

For adhesion inhibition by antibodies, antisera raised against CFA/I, CFA/II (CS1 and CS3), CFA/III, antigen 2230, CS6, and antigen 8786 were obtained as follows. Crude preparations of surface antigens were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The portions of the gel containing the different surface antigens were excised and washed in PBS at 4°C to eliminate the SDS. The band was crushed and suspended in Freund complete adjuvant (Sigma Chemical Co., St. Louis, Mo.). An adult female rabbit weighing 2 kg was inoculated intradermally at multiple sites along the back. Booster injections were given 1 and 2 weeks later. The rabbit was bled 7 days after the last booster injection. Sera were stored at −20°C. All of the antisera were adsorbed with a negative variant of the corresponding CFA-producing strain, except the antiserum raised against antigen 8786, which was adsorbed with strain 8786 grown at 18°C. Each adsorbed antiserum was first incubated with 10^8 *E. coli* cells at room temperature for 20 min. The mixture was then added to Caco-2 cells, and the adhesion test was performed as described above.

Statistical analysis. The results were expressed as means ± standard deviations. Each adhesion index represents the results of four separate experiments. Statistical significance was determined by analysis of variance using the F distribution of Fisher.

Hemagglutination. Mannose-sensitive and mannose-resistant hemagglutinations of human group A and bovine erythrocytes were determined at room temperature on rocked glass slides. Erythrocytes were diluted 1:4 in PBS with (mannose-resistant hemagglutination) or without (mannose-sensitive hemagglutination) 1% (wt/vol) D-mannose.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (25), with vertical slab gels (Bio-Rad Laboratories apparatus). Proteins were stacked by using 4.5% (wt/vol) acrylamide and separated by using 15% (wt/vol) acrylamide. The electrophoresis buffer was composed of 0.025 M Tris (Sigma), 0.28 M glycine (Prolabo, Paris, France), and 0.1% (wt/vol) SDS (pH 8.6; Sigma). The molecular weight standards were phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400) (Bio-Rad Laboratories). Samples were denatured in 1.5% (wt/vol) SDS–1.5% (vol/vol) β-mercaptoethanol in 0.50 M Tris hydrochloride buffer (pH 6.8) for 5 min at 100°C just before being loaded onto the gel.

Immunoblotting. Crude preparations of bacterial surface components separated by SDS-PAGE were transferred onto nitrocellulose sheets by the method of Towbin et al. (40). The nitrocellulose membranes were incubated overnight at room temperature with an appropriate dilution of polyclonal antiserum in Tris-buffered saline (10 mM Tris base, 0.9% NaCl, pH 7.5) and 2% (wt/vol) bovine albumin (Sigma). After three washes with Tris-buffered saline–0.05% Tween 20, the membranes were incubated with peroxidase-labeled goat immunoglobulin G at a dilution of 1:1,500 (Nordic Immunological laboratories, Tilbeug, The Netherlands), washed as described above, and developed with a solution of 0.6 mg of 4-chloro-1-naphthol (Sigma) per ml–0.1% H_2O_2–16.5% methanol in Tris-buffered saline. Antisera raised against CS3 and CS4-CS6 were kindly provided by M. Levine and A. M. Svennerholm, respectively.

Electron microscopy. *E. coli* strains were grown at 37°C on Mueller-Hinton agar (Institut Pasteur Production), and bacterial cells were stained negatively with 1% (wt/vol) phosphotungstic acid (pH 6.8). Bacterial cells were observed with a transmission electron microscope (HU 12A; Hitachi). Immunolabeling with colloidal-gold-labeled goat anti-rabbit serum was performed as described by Levine et al. (27). A washed bacterial suspension was placed on carbon-coated grids. Excess liquid was removed, and the grid was placed face down on a drop of a suitable dilution of antiserum for 15 min. After several washings, the grid was placed on a drop of gold-labeled goat anti-rabbit serum (Janssen Life Sciences products, Olen, Belgium) for 15 min. After washing, the grid was negatively stained with 1% ammonium molybdate. To prevent nonspecific labeling, 10% bovine serum albumin and 1% Tween 20 were added to the wash solutions.

Transmission electron microscopy of cross-sections of the Caco-2 cell monolayer was performed as described before (7).

Amino acid analysis. Amino acid analysis was performed on cesium chloride-purified protein on a Kontron amino acid analyzer (Chromakon 500). Samples were hydrolyzed in a nitrogen atmosphere for 24 h at 110°C in 6 N HCl.

NH_2-terminal amino acid analysis. NH_2-terminal analysis was carried out on purified undenatured protein (2 nmol) by automatic Edman degradation with a 470A sequencer (Applied Biosystems, Foster City, Calif.). Phenylthiohydantoins were analyzed on line by an Applied Biosystems 120A phenylthiohydantoin analyzer. All reagents and solvents were from Applied Biosystems.

Research of protein similarity. The NH_2-terminal sequence of antigen 8786 was compared with those of other proteins by computer analysis using the FAST program and the National Biomedical Research Foundation Protein Bank.

DNA preparation and analysis. *E. coli* 8786 plasmids were isolated by the method of Kado and Liu or a modification of

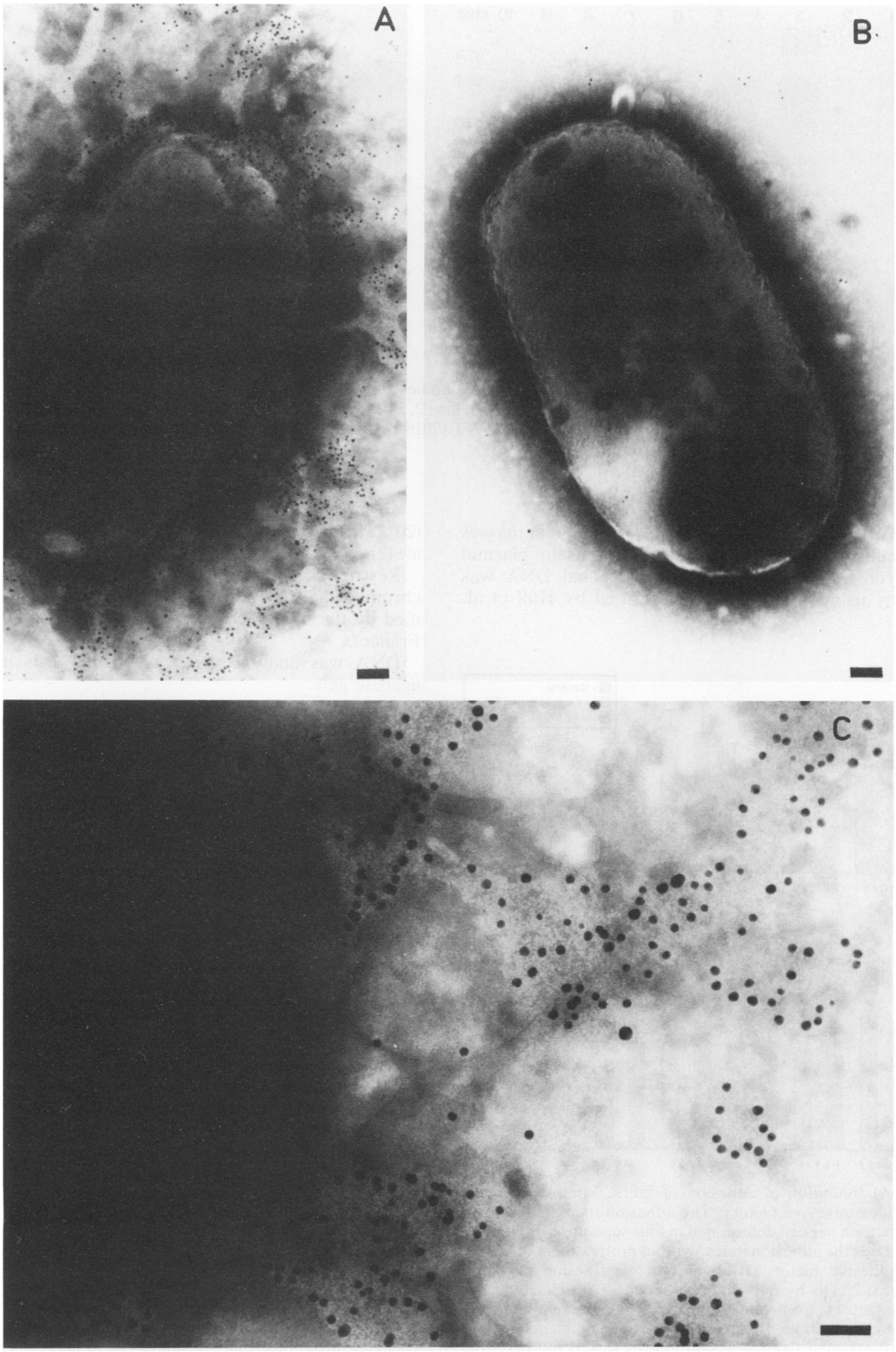

FIG. 3. Electron micrographs after colloidal-gold immunolabeling with antibodies raised against antigen 8786. Panels: A and C, *E. coli* 8786 grown at 37°C; B, *E. coli* 8786 grown at 18°C. Magnifications: A and B, ×50,000 (bar, 10 μm); C, ×150,000 (bar, 5 μm).

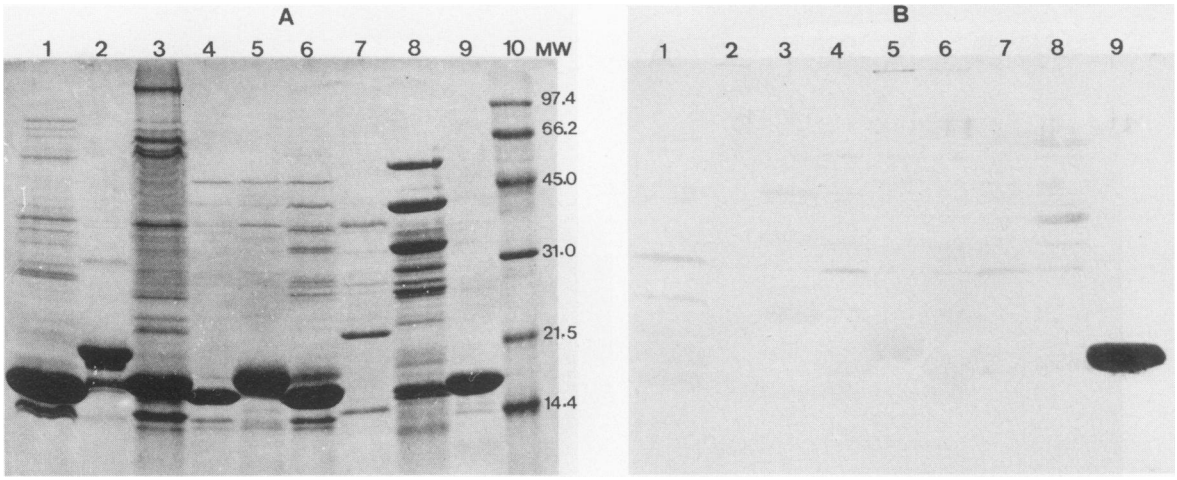

FIG. 4. SDS-PAGE (A) and Western immunoblot (B), with antibodies raised against antigen 8786, of crude bacterial surface protein extracts from the following *E. coli* strains: lane 1, H10407 (CFA/I); lane 2, Pb176 (CS1 and CS3); lane 3, C91f (CS2); lane 4, 1373 (CFA/III); lane 5, 2230 (antigen 2230); lane 6, E8775A (CS4 and CS6); lane 7, E17018A (CS5 and CS6); lane 8, M17 (CS6); lane 9, 8786 (antigen 8786); lane 10, molecular mass standards (in kilodaltons).

the method of Birnboim and Doly (21, 28). Molecular masses of the plasmids were determined relative to those of plasmid standards run on the same gel. Chromosomal DNA was extracted by using the technique described by Hull et al. (20). Plasmids were cured by the method of Bouanchaud et al. (2).

Restriction endonuclease (*Eco*RI) digests of plasmid and chromosomal DNAs were performed under conditions specified by the manufacturer (Boehringer Mannheim, Meylan, France).

DNA was analyzed after electrophoresis in 0.7 or 1% agarose gels.

Oligonucleotide probe and Southern hybridization. A mixed-sequence oligonucleotide (24-mer) based on the NH_2-terminal amino acid sequence from residues 19 to 26 were synthesized with an Applied Biosystems 380B DNA synthesizer. The sequence was as follows:

5′-CCA AGT AGC ATT AAT AGT ATT TTG-3′
G T G G T G C
C T

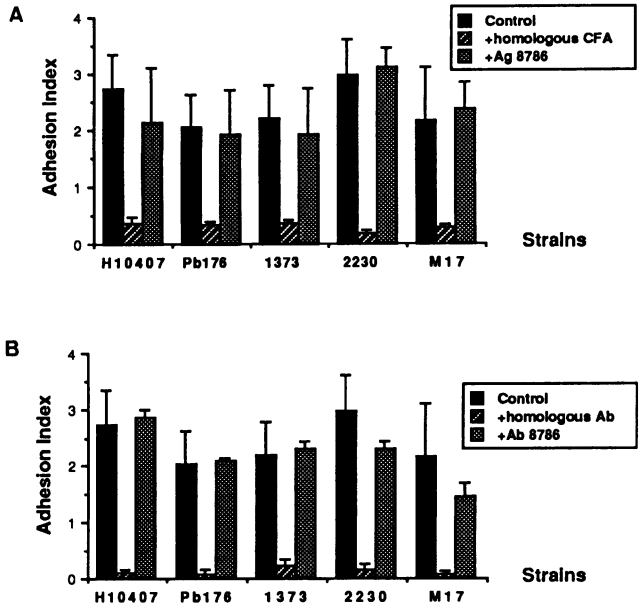

FIG. 5. (A) Inhibition of adhesion of ETEC strains to Caco-2 cells by purified adhesive factors. The adhesion index is the mean number of bacteria per cell determined in four separate experiments. Control indicates the adhesion index without pretreatment of Caco-2 cells with adhesive factor. (B) Inhibition of adhesion of ETEC strains to Caco-2 cells by polyclonal antibodies (Ab) raised against the adhesive factors. Control indicates the adhesion index without pretreatment of bacteria with Ab. The bars indicate standard deviations. Statistical analysis of adhesion indices was as follows: after pretreatment with homologous adhesive factors or Ab, $P < 0.001$; after pretreatment with antigen 8786 or antibodies raised against antigen (Ag) 8786, $P > 0.05$.

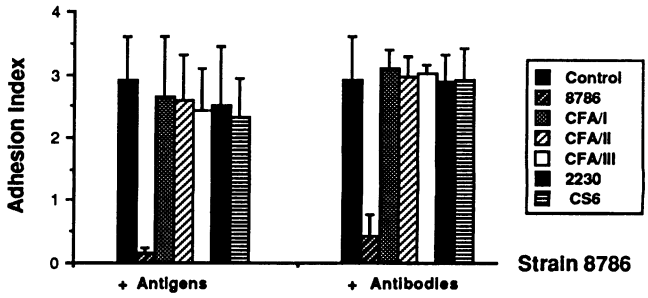

FIG. 6. Inhibition of adhesion of strain 8786 to Caco-2 cells by purified adhesive factors and antibodies raised against the adhesive factors. The adhesion index is the mean number of bacteria per cell determined in four separate experiments. Control indicates the adhesion index without pretreatment of Caco-2 cells with purified adhesive factor or bacterial cells with antibodies. The bars indicate standard deviations. Statistical analysis of adhesion indices was as follows: after pretreatment with nonhomologous adhesive factors or antibodies, $P > 0.05$; after pretreatment with homologous antigen or antibodies, $P < 0.001$.

TABLE 1. Amino acid compositions of ETEC adhesive factors and antigen 8786

Amino acid	No. (%) of residues/mol[a]					
	CFA/I	CS1	CS2	CS3	Antigen 2230	Antigen 8786
Alanine	19	14	16	13	14	16 (10.58)
Arginine	1	1	4	1	0	8 (5.26)
Asparate or asparagine	12	23	19	23	20	9 (5.91)
Glutamate or glutamine	11	9	17	6	10	19 (12.19)
Glycine	10	11	13	8	15	17 (11.21)
Cysteine	0	0	0	0	2	0
Histidine	1	3	2	2	2	4 (2.22)
Isoleucine	5	9	10	9	4	9 (5.75)
Leucine	12	17	13	15	10	11 (7.25)
Lysine	8	9	10	6	6	9 (5.55)
Methionine	3	1	3	0	4	1 (0.26)
Phenylalanine	2	3	4	3	3	7 (4.31)
Proline	7	9	7	5	5	10 (6.53)
Serine	17	20	13	16	12	10 (6.63)
Threonine	15	19	18	27	19	11 (6.93)
Tryptophan	1	ND[b]	ND	1	ND	1[c] (0.74)
Tyrosine	4	3	3	1	6	4 (2.22)
Valine	19	20	12	9	17	11 (7.19)
Total	147	171	164	145	149	157
Relative molecular mass (kDa)	15	16.8	17.4	15.3	16	16.3
Hydrophobicity (%)[d]	48.2	44.6	41.2	37.9	42.3	44.1

[a] Data are from references 15, 16, and 23.
[b] ND, Not determined.
[c] This tryptophan residue was found by analysis of the NH_2-terminal amino acid sequence.
[d] Percentages of Pro, Ala, Val, Met, Ile, Leu, Tyr, and Phe.

The oligonucleotide was end labeled with [γ-^{32}P]ATP by using polynucleotide kinase (Boehringer Mannheim).

Transfer of DNA to nitrocellulose membranes was done by the method of Southern as described by Maniatis et al. (28). Southern hybridizations were done in 5× SSPE (20× SSPE is 3.6 M NaCl, 200 mM NaH_2PO_4 [pH 7.4], and 20 mM EDTA [pH 7.4]) at 37°C for 2 days. Washing was performed twice, in 2× SSC (1× SSC is 0.15% NaCl plus 15 mM sodium citrate)–0.1% SDS for 10 min and then in 0.5× SSC–0.1% SDS for 10 min. Autoradiography was performed at −80°C for 1 to 2 days.

RESULTS

Hemagglutination ability. Since most ETEC strains caused mannose-resistant hemagglutination of different kinds of erythrocytes, we tested the ability of strain 8786 to agglutinate human and bovine erythrocytes in the presence of D-mannose. Like ETEC strains expressing CFA/I, CFA/III of strain 1373, CS4, and CS5, it was able to agglutinate both kinds of erythrocytes.

Adhesion to human colon carcinoma cell line Caco-2 in culture. It is known that strain 8786 adheres to human enterocytes (14), but to compare its adhesion with that of reference ETEC strains, we studied its ability to adhere to Caco-2 cells. Strain 8786 adhered to Caco-2 cells with an adhesion index of 2.93 ± 0.76, which was very close to those of ETEC reference strains (1.81 to 2.39) observed by us in a previous study (7). Moreover, when the strain was grown at 18°C instead of 37°C, the bacteria no longer adhered to the Caco-2 cell line and the adhesion index obtained was 0.26 ± 0.23 bacteria per cell. Transmission electron microscopy of cross-sections of a Caco-2 cell monolayer showed that strain 8786 adhered closely to the microvilli but in no way altered them (Fig. 1).

Analysis of bacterial surface components. The bacterial surface components of strain 8786 were extracted by heating a bacterial suspension at 60°C for 20 min by the method of Stirm et al. (36). When the bacterial extract was run on SDS-PAGE, a major protein with an estimated molecular mass of 16.3 kDa was observed and designated antigen 8786 (Fig. 2A). Protein patterns showed that the 16.3-kDa protein was expressed at 37°C but not at 18°C. Other proteins from the extract also presented temperature-dependent expression. Purification of the 16.3-kDa protein was performed by cesium chloride gradient centrifugation of the crude extract. After purification by CsCl gradient, only one band with a molecular mass of 16.3 kDa was observed on SDS-PAGE after Coomassie blue staining (Fig. 2A).

Electron microscopy. Immunogold labeling with absorbed anti-antigen 8786 serum showed that gold particles uniformly covered the bacterial surface when strain 8786 was grown at 37°C (Fig. 3A). No labeling occurred for the strain grown at 18°C (Fig. 3B). Antigen 8786 was apparently easily shed from the bacterial surface because gold particles were detected far from the bacteria. At a magnification of ×150,000, we observed an arrangement of gold particles which suggests a fibrillar structure (Fig. 3C). However, no structures were observed after negative staining, even at a magnification of ×216,000 (data not shown). Since fibrillar structures have already been observed after negative staining at a magnification of ×150,000 (27), this last result suggests that the structures had a diameter of less than 2 nm and were beyond the limits of resolution by electron microscopy.

Immunological studies. Western immunoblot analysis showed that the antiserum raised against the SDS-denatured form of antigen 8786 reacted with antigen 8786 (Fig. 2B). A nitrocellulose replicate with surface proteins of CFA/I-, CFA/II-, CFA/III-, antigen 2230-, and CFA/IV-producing strains was tested with anti-8786 serum. There was a weak

TABLE 2. N-terminal amino acid sequence of antigen 8786[a]

Surface antigen	Sequence[b]
CFA/I	VEKNI TVTAS VDPVI DLLQA
CS1	VEKTE SVTAS VDPTV DLLQ
CS2	AEKNI TVTAS VDPVI DLLQA
CS3	AAGPT LTKEL ALNVL SPAAL DAT
CS4	VELNI TVCAS VDPTI CIKQA
CS5	AV TNGQL TFNWQ GVVPS APVTQ SSQPF VNG
2230	GNVLSG GNGTQ TVTMP VNAAT XTVSM PTD
PCF09	D SQQDS AFNGN IELGG TLSPE VKKLP RELR
S. enteritidis fimbrin	AGFV GNKA VVQAA VTIAA QNTTS ANWSQ DPGFT GPAVAA
	: : :. : : : . .: ::: : :.: : . :
8786	ATAV GDVA TVRAP LVFSA QNTIN ATWGQ DSSVX G

[a] The published sequences of ETEC fimbriae (15–18, 22, 24, 43) and *S. enteritidis* fimbrin (13) are given for comparison.
[b] Double dots, Identical amino acid between antigen 8786 and *S. enteritidis* fimbrin; single dot, conservative amino acid change; X, unidentified residue.

cross-reaction with antigen 2230 (Fig. 4). The reactions observed with the other proteins were analogous to those obtained with the preimmune serum (data not shown). The antigenic difference from nonfimbrial structures like CS3, CS6, and antigen 2230 was confirmed by Western immunoblot analysis with anti-CS3, anti-CS4-CS6, and anti-antigen 2230 sera, since each antiserum reacted only with its homologous protein.

Adhesion inhibition assays. The results of adhesion inhibition assays are illustrated in Fig. 5 and 6. Adhesion of reference ETEC strains to Caco-2 cells did not occur when the cells were preincubated with the corresponding purified adhesive factors (Fig. 5A) and when the bacteria were preincubated with the corresponding antibodies raised against adhesive factors (Fig. 5B). The adhesion indices obtained after treatment of Caco-2 cells or bacteria varied from 0.09 to 0.38, whereas the adhesion indices of ETEC strains without any treatment varied from 2.04 to 2.98. The adhesion of the different reference ETEC strains was not affected by pretreatment of Caco-2 cells with purified antigen 8786 (Fig. 5A) or pretreatment of bacteria with antibodies raised against antigen 8786 (Fig. 5B). These results were confirmed by adhesion inhibition of strain 8786 with the different purified reference adhesive factors or their antibodies. As shown in figure 6, adhesion of strain 8786 to Caco-2 cells was affected only by pretreatment of the cells with purified antigen 8786 or pretreatment of the bacteria with antibodies raised against antigen 8786. Slight decreases in the adhesion indices of strain 8786 were observed when Caco-2 cells were treated with the different adhesive factors, but these differences were not significant ($P > 0.05$).

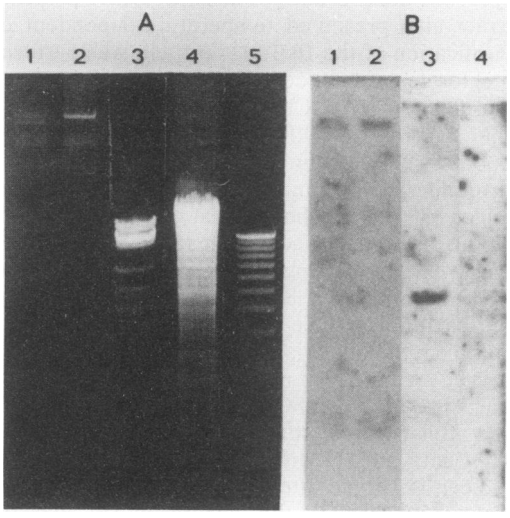

FIG. 7. Hybridization profile of plasmid and chromosomal DNAs of *E. coli* 8786 with the oligonucleotide probe based on the NH_2-terminal amino acid sequence of antigen 8786. Panels: A, agarose gel electrophoresis; B, Southern blot. Lanes: 1, *E. coli* 8786 with 70- and 60-Mda plasmids; 2, *E. coli* 8786 without the 60-Mda plasmid; 3, restriction endonuclease *Eco*RI digest of the 70-Mda plasmid; 4, restriction endonuclease *Eco*RI digest of chromosomal DNA of strain 8786; 5, molecular weight standards (1-kb ladder; Gibco BRL SARL, Cergy Pontoise, France.)

Characterization of antigen 8786: amino acid analysis and NH_2-terminal sequence. The amino acid composition of antigen 8786 was calculated from the average and/or extrapolated values and on the basis of the molecular mass of 16.3 kDa. A comparison of its amino acid composition with those of reference ETEC adhesive factors is given in Table 1. The proportion of basic amino acids (lysine, arginine, and histidine) was greater in antigen 8786 (13%) than in CFA/I (6.8%), CS1 (7.7%), CS2 (7.7%), CS3 (6.2%), and antigen 2230 (5.5%) because of the high content of arginine residues. The aromatic amino acid content (7.3%) was high and more similar to that of antigen 2230 (6.2%) than to those of CS1, CS2, and CS3 (3.5%) and CFA/I (4.7%). Nevertheless, the 53% nonpolar amino acid content was comparable to those of CFA/I, CS1, CS2, CS3, and antigen 2230, which were 52, 49, 47, 43, and 48%, respectively. Moreover, antigen 8786 had 44% hydrophobic amino acids (proline, alanine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine) and contained no cysteine. These results are similar to those obtained for CFA/I, CS1, and CS2 (Table 1). The apparent molecular mass of the antigen 8786 subunit as determined by SDS electrophoresis was 16.3 kDa. On the basis of the amino acid composition, the calculated molecular mass was 16.8 kDa. The NH_2-terminal amino acid sequence of antigen 8786 was determined by automatic Edman degradation. A comparison of this sequence with the NH_2-terminal sequences of CFA/I, CS1, CS2, CS3, CS4, CS5, antigen 2230, and PCF09 is shown in Table 2. The NH_2-terminal sequence of antigen 8786 showed no homology with the NH_2-terminal sequences of ETEC adhesive factors. In contrast, computer analysis using the FAST program and the National Biomedical Research Foundation Protein Bank indicated close homology between the NH_2-

terminal sequence of antigen 8786 and that of a *Salmonella enteritidis* fimbrin. Of the first 34 amino acids, 44% were identical. Moreover, five conservative amino acid changes (15%) were localized at positions 6, 14, 17, 27, and 31 (Table 2).

Analysis of plasmid DNA. The plasmid content of wild strain *E. coli* 8786 included two plasmids of 70 and 60 MDa (Fig. 7A). After growth of bacteria in the presence of ethidium bromide and nonselective plating for single colonies, approximately 500 derivatives were tested for DNA plasmid content. Only one type of derivative, without the 60-MDa plasmid, was obtained. These cured derivatives still adhered to the brush border of Caco-2 cells and produced antigen 8786.

Localization of antigen 8786 structural gene. Hybridization experiments using as a probe a 24-mer synthetic oligonucleotide based on the NH_2-terminal sequence revealed that the coding region of antigen 8786 was located on the 70-MDa plasmid (Fig. 7B). After digestion of this plasmid with restriction endonuclease *Eco*RI, a very strong signal was obtained with a 6-kb fragment (Fig. 7B).

DISCUSSION

Many studies have been carried out to determine the adhesive factors harbored by ETEC strains from different geographical areas. A vaccine composed of the different adhesive factors would be useful against ETEC diarrhea. However, this would require identification of all of the adhesive factors synthesized by ETEC strains in the different developing countries. Most studies of this kind are done in Asia, and only a few are done in Africa (44). We previously observed that ETEC strains isolated from infants suffering from acute diarrhea in Africa adhered to the brush border of the small intestine by means of CFA/I, CFA/III, or antigen 2230 (9). In this study, we identified another adhesin produced by an ETEC strain (strain 8786) of serogroup O117:H4 isolated in Burundi (Africa).

Surveys of ETEC strains have shown that each colonization factor is associated with particular O serogroups. Serogroup O117 has been observed among ETEC strains producing the thermolabile enterotoxin, but no colonization factor has been described (29, 31, 32).

In a previous study, we showed that the way in which ETEC strains adhere to the microvilli of Caco-2 cells is the same as for human enterocytes (7). In the present study, strain 8786 adhered closely to the microvilli of Caco-2 cells, like strain 2230, and did not induce any detectable lesion of the brush border. This close adhesion suggests that the adhesive factor is nonfimbrial. Furthermore, electron microscopic examinations of negatively stained bacterial cells did not show a fimbrial structure.

A prominent 16.3-kDa protein was observed when an extract of bacterial surface components was run on SDS-PAGE. Expression of this protein is dependent on growth temperature, a property associated with all of the adhesive factors. Inhibition of adhesion of *E. coli* 8786 to Caco-2 cells after treatment of the cells with the purified 16.3-kDa protein permitted the conclusion that this protein, designated antigen 8786, was the adhesive factor. Adhesion inhibition assays with purified ETEC adhesive factors and specific antibodies suggest that the Caco-2 cell receptor for antigen 8786 is different from those of CFA/I, CFA/II, CFA/III, antigen 2230, and CS6 and that antigen 8786 is different from any ETEC adhesive factor yet described. This difference was confirmed by immunological studies. The antiserum raised against the denatured form of purified antigen 8786 reacted only with its homologous protein in immunoblots.

Another nonfimbrial protein with a mannose-resistant hemagglutination property has also been described (41), but this protein is different from antigen 8786 because it is involved in adhesion to HeLa and HEp-2 cultured cells, whereas our strain, *E. coli* 8786, did not adhere to these cell lines.

The amino acid composition of antigen 8786 differs from those of other adhesive factors. Nevertheless, like others, this antigen is relatively hydrophobic, with approximately 44% hydrophobic residues, and lacks cysteine, which suggests that noncovalent forces are responsible for the structural integrity of the native protein. The amino-terminal sequence of antigen 8786 showed no homology with the amino-terminal sequences of other ETEC adhesive factors. Protein comparison by computer analysis indicated that antigen 8786 is closely related to the *S. enteritidis* fimbrin (44% homology and 15% conservative changes). This homology suggests that *S. enteritidis* fimbrin and antigen 8786 have a common ancestral gene. It would be interesting to study the role of this *S. enteritidis* fimbria and to determine whether such fimbriae are involved in the adhesion of *S. enteritidis* strains to the human intestinal epithelium.

The association of genes coding for an adhesin and enterotoxins on the same high-molecular-weight plasmid is common among ETEC strains (33, 37). In strain 8786, structural genes of antigen 8786 are located on the 70-MDa plasmid, which also carried the gene encoding heat-stable enterotoxin.

To facilitate epidemiological studies on the incidence of adhesive factor 8786 among ETEC strains involved in infant diarrhea in Africa, we intend to develop a specific DNA probe, and thus we propose to clone the genes encoding antigen 8786.

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