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Identification of a Nonfimbrial Adhesive Factor of an Enterotoxigenic *Escherichia coli* Strain

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An enterotoxigenic *Escherichia coli* strain (strain 2230), isolated from a patient with acute infantile diarrhea, was found to adhere only to the brush border of human intestinal epithelial cells. This strain does not hemagglutinate human, bovine, chicken, or guinea pig erythrocytes. The adhesion of *E. coli* 2230 appears to be mediated by a nonfimbrial bacterial surface protein of 16,000 daltons which can be extracted by heating the bacteria at 60°C for 20 min. This surface protein is implicated as an adhesive factor because pretreatment of enterocytes with this protein extract completely inhibits the adhesion of *E. coli* 2230. This adhesive factor is serologically distinct from other adhesive factors found in enterotoxigenic *E. coli* strains. A plasmid DNA of 66 megadaltons is involved in the synthesis of this nonfimbrial adhesive factor.

Enterotoxigenic *Escherichia coli* (ETEC) causes acute diarrhea in both humans and animals by colonizing the small intestine and producing enterotoxins. In human ETEC strains, the adhesion to the intestinal epithelium has been found to be mediated by specific fimbrial antigens such as colonization factor antigen I (CFA/I) (6, 7), CFA/II (5), AgE8775 (23), 260-1 pili (9), and CFA/III (3).

The presence of pili on the bacterial surface may also be correlated with the capacity of bacteria to agglutinate erythrocytes from different species. This hemagglutination (HA) occurs even in presence of D-mannose, and has been termed mannose-resistant HA (MRHA). Of these adhesins, CFA/II is a system of CFAs. These have been called CS1, CS2, and CS3 (2, 21). These adhesins possess a fimbrial morphology, except CS3, which has recently been shown to be composed of fine fibrils (14). The existence of nonfimbrial adhesins that allow bacteria to adhere to intestinal cells is not surprising. Indeed, even though most of the adhesins involved in adhesion of *E. coli* to human uroepithelial cells are piliated (17, 25), a nonfimbrial adhesin has also been described, AFA-1 (11). Furthermore, mutagenesis experiments showed that pilus fiber formation and adhesion to human cells are two independent phenomena (16).

We examined many ETEC strains isolated from humans with diarrhea in Senegal (Africa) for their ability to adhere to the human intestinal epithelial cells. Among adhesive strains, we found one of them (strain 2230) to possess a cell surface nonfimbrial protein which mediates adherence of bacteria to the brush border of human enterocytes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *E. coli* 2230 strain studied in this paper was isolated in the Dakar area from a hospitalized infant with diarrhea. In addition, strain 2230p is a spontaneous adhesion negative variant of strain 2230, obtained after subculture on CFA-agar in the laboratory.

The following *E. coli* strains were used for comparative studies: H10407 with the colonization factor antigen CFA/I (7), Pb176 with CFA/II (5), and their negative variants that lacked CFA, H10407p and Pb176p.

Unless otherwise stated, all tests were performed with 18-h cultures of *E. coli* grown at 37°C on CFA-agar (6) containing 1% Casamino Acids (Difco Laboratories), 0.15% yeast extract, 0.005% magnesium sulfate, and 0.0005% manganese chloride in 2% agar.

In vitro enterocyte adhesion assay. The in vitro adhesion assay was conducted as previously described (13). Briefly, human duodenal enterocytes were mixed with 10^8 *E. coli* cells in phosphate-buffered saline (PBS). This mixture was allowed to incubate at room temperature for 20 min with slow shaking. Bacterial adhesion was quantified by examination under phase-contrast microscopy at a magnification of ×1,000. The number of *E. coli* cells adhering to each of 20 enterocytes was counted in duplicate. The adhesion index was expressed as the number of bacteria attached to the brush border of one enterocyte.

HA tests. HA tests were performed by slide agglutination by the method of Evans et al. (8). Fresh human (group A, rhesus positive), bovine, chicken, and guinea-pig erythrocytes were used. Fresh blood was diluted 1/4 with PBS (pH 7.2), to test for HA and 1/4 with PBS containing 1% (wt/vol) D-mannose to test for MRHA. HA was observed at room temperature and at 4°C.

Adhesion inhibition tests. To test for adhesion inhibition by D-mannose, a final concentration of 1% D-mannose (wt/vol) was allowed to incubate with *E. coli* cells and enterocytes.

To test for adhesion inhibition by 2230 antiserum, a suitable dilution of antiserum was added to 10^8 *E. coli* cells in PBS, and the mixture was allowed to incubate at room temperature for 20 min. Then enterocytes were added, and the adhesion test was performed as described above.

To test for adhesion inhibition by competing protein, 2230 purified protein obtained by chromatography on a Sepharose CL-6B column in the presence of 6 M guanidine was used. A suitable concentration of protein was added to the human enterocyte suspension, the mixture was incubated at room temperature for 20 min, and then 10^8 *E. coli* cells were added.

Assays for enterotoxin activity. Heat-stable enterotoxin activity was determined by the suckling mouse assay of Dean et al. (4).

Extraction and purification of adhesion antigens. The extraction and purification of adhesion antigens were performed essentially as described by Stirm et al. (22). Overnight culture on 10 Roux flasks with CFA-agar was harvested in 0.1 M phosphate buffer (pH 7.2). The adhesion

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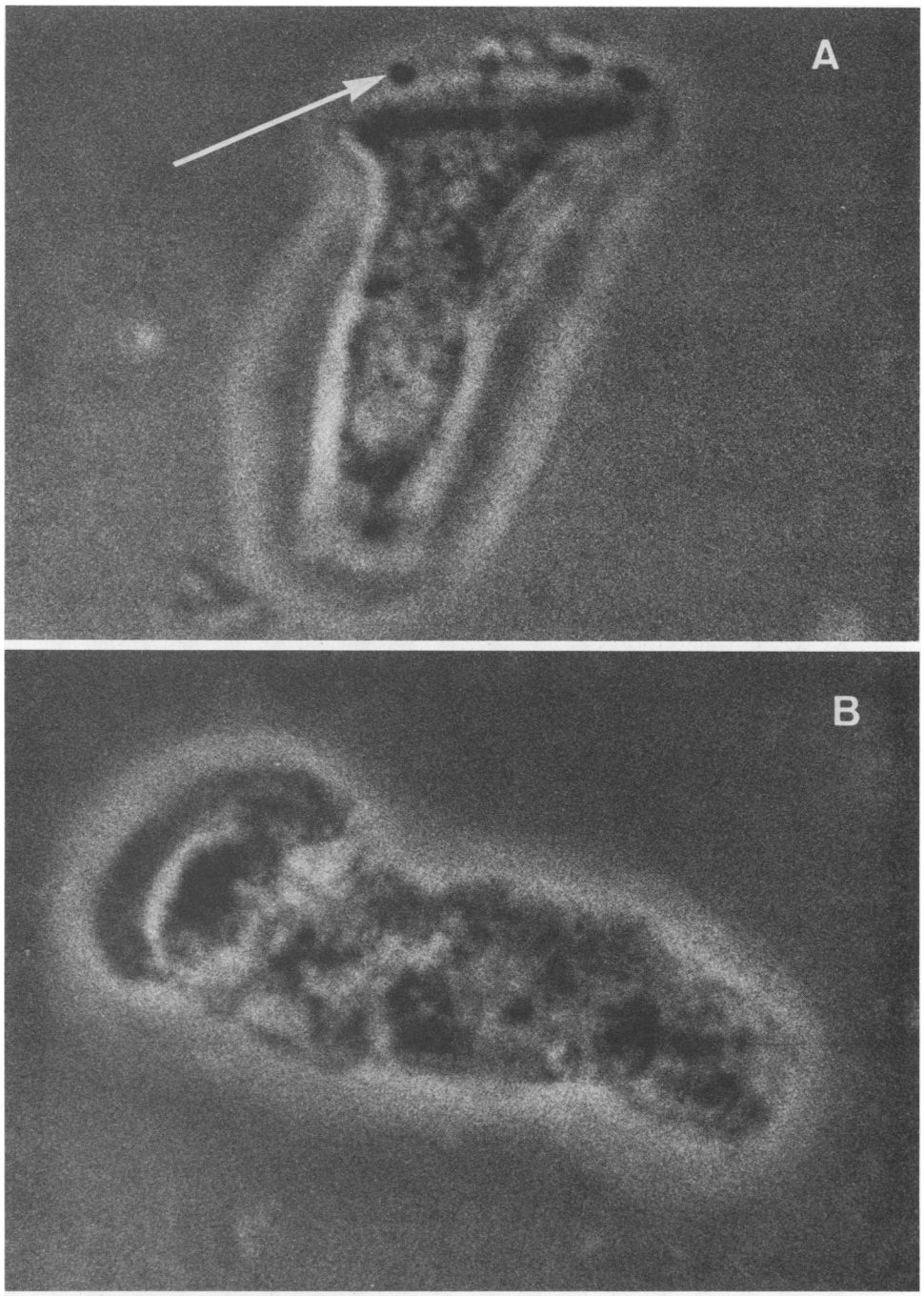

FIG. 1. Phase-contrast micrographs showing isolated human duodenal enterocytes. ETEC strain 2230 adhered only to the enterocyte brush border (A), whereas strain 2230p did not adhere (B). The arrow indicates adhering bacteria.

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 at 10,000 × *g* for 10 min. The supernatant was brought to pH 4.0 by the slow addition of orthophosphoric acid 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 phosphate buffer (pH 7.2). Partial purification was performed by two ultracentrifugations; the sediment of the first centrifugation at 115,000 × *g* for 10 min was discarded, and the supernatant was submitted to a second centrifugation at 115,000 × *g* for 200 min. The sediment suspended in 0.1 M phosphate buffer (pH 7.2) represented the crude adhesion antigen extract.

Purified 2230 protein was obtained by chromatography on a Sepharose CL-6B column equilibrated in 100 mM sodium phosphate with 6 M guanidine hydrochloride as described by Levine et al. (14).

Electrophoretic study. Molecular weight determinations of CFA/I, CFA/II, and 2230 adhesion antigen subunits were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (12). SDS-polyacrylamide gel electrophoresis was performed in

vertical slab gels (Bio-Rad Laboratories apparatus). For the separation gel, the acrylamide concentration was 12% (wt/vol) with 0.8% (wt/vol) bisacrylamide. The electrophoresis buffer was composed of 0.025 M Tris, 0.28 M glycin, and 0.1 (wt/vol) SDS (pH 8.6). The molecular weight standards were phosphorylase B (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). 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 3 min at 100°C, just before being loaded onto the gel.

Preparation of antisera. Rabbit antisera were prepared with purified antigens CFA/I, CFA/II, and 2230 as described previously (26). Rabbits were immunized intravenously four times at 4- to 5-day intervals with 175, 350, 700, and 700 μg of purified protein, respectively. The rabbits were bled on day 7 after the last injection. To prepare a CFA-specific antiserum, this crude antiserum was adsorbed with a negative variant of each CFA-producing strain.

Double immunodiffusion tests. Purified 2230 protein was tested against each CFA antiserum by the Ouchterlony gel immunodiffusion technique (18). Similar purified extracts or partially purified CFA from strains H10407 and Pb176 was also tested against the adsorbed antiserum specific for 2230. Furthermore the anti-CS3 serum, kindly provided by M. Levine, was tested against 2230 protein.

Immunoblotting. Western blotting was performed by the technique of Towbin et al. (24), with minor modifications. Strain 2230 protein extracts were first subjected in duplicate to electrophoresis in the presence of SDS (12). One part of the gel was stained with Coomassie blue, and the other part was electroblotted onto nitrocellulose paper. The adsorbed 2230 antiserum was applied to the nitrocellulose filter, and the filter was incubated overnight at room temperature. After thorough washing the filter was incubated with peroxidase-labeled sheep anti-rabbit immunoglobulin G at 1:3,000 dilution (Institut Pasteur Production). The blot was soaked in a solution composed of 0.5 mg of 3-3'-diaminobenzidine (Sigma Chemical Co.) per ml–0.1% H_2O_2–0.05 M Tris hydrochloride (pH 7.6) and dried between filter papers.

Electron microscopy. Bacterial cells of strains 2230 and 2230p were grown at 37°C on CFA-agar, harvested, and observed with a transmission electron microscope (Hitachi) after staining with 1% phosphotungstic acid (pH 6.8). Purified antigen preparation was also examined by this negative staining.

Immune electron microscopy. A gold immunolabeling technique was also realized, as described by Levine et al. (14). A washed bacterial suspension of strain 2230 or 2230p 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 adsorbed 2230 antiserum for 15 min. After many washings, the grid was placed on a drop of gold-labeled goat anti-rabbit serum (Janssen Pharmaceutical) for 15 min. After a further thorough washing, the grid was negatively stained with 1% ammonium molybdate. To prevent nonspecific labeling, 1% bovine serum albumin and 1% Tween 20 were added to the wash solutions.

Plasmid curing. Plasmid curing was performed by the method of Bouanchaud et al. (1). Briefly, portion (5 ml) of Trypticase soy broth (pH 7.2; Institut Pasteur Production) supplemented with trypsin (1 mg/ml) and ethidium bromide (100 μg/ml) was inoculated with approximately 1,000 cells of the bacterial strain to be cured. Trypsin was added to inactivate colicin produced by colicinogenic strains. Cultures were incubated overnight at 37°C without agitation and then plated out for well-separated colonies that were subsequently tested for loss of plasmid-determined characters.

TABLE 1. Adhesion to the brush border of human enterocytes of strain 2230 and its derivatives

Strain	Adhesion index (no. of bacteria/brush border) in:			
	PBS alone	PBS plus 1% D-mannose	PBS plus 2230 antiserum	PBS plus 2230 protein[a]
2230	2.4	2.2	0	0
2230p	0	ND[b]	ND	ND
2230a[c]	0	ND	ND	ND
2230b[c]	0	ND	ND	ND
2230c[c]	2.2	2.4	0	0
2230 (18°C)[d]	0	ND	ND	ND

[a] Purified strain 2230 protein.
[b] ND, Not determined.
[c] Strain 2230 derivatives obtained by ethidium bromide curing (see Table 3).
[d] Adhesion test with strain 2230 after growth at 18°C.

TABLE 2. Adhesion capacity of ETEC strains after pretreatment of enterocytes with different protein extracts

Protein extract	Adhesion index (no. of bacteria/brush border) of ETEC strains		
	2230	H10407 (CFA/I)	Pb176 (CFA/II)
Control[a]	2.4	1.6	1.8
2230	0	1.8	1.9
CFA/I	2.3	0	1.6
CFA/II	2.1	1.5	0

[a] Without any protein extract.

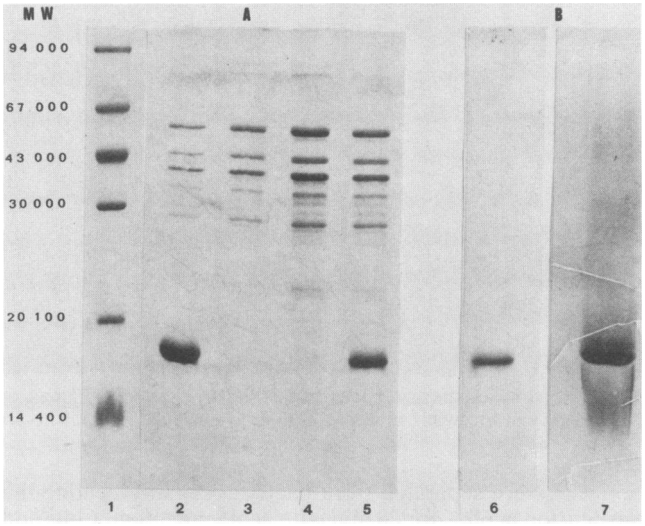

FIG. 2. (A) SDS-PAGE of crude bacterial surface protein extracts from the wild 2230 strain (lane 2) and its cured derivatives stained with Coomassie blue. Other lanes: 3, 2230a (derivative without the 66- and 56-Mdal plasmids); 4, 2230b (derivative without the 66-Mdal plasmid); 5, 2230c (derivative without the 56-Mdal plasmid). (B) SDS-PAGE of purified 2230 protein obtained by chromatography column of Sepharose CL-6B. Lanes: 6, Coomassie blue staining; 7, silver staining. Samples of 10 μl from each extract were analyzed in 12% polyacrylamide gel. Lane 1 contains the molecular weight standards.

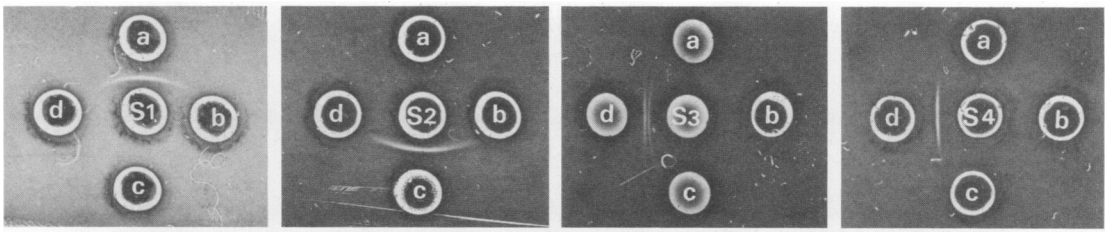

FIG. 3. Immunodiffusion of antigen extracts. Wells: a, antigen 2230; b, 2230p extract; c, antigen H10407 (CFA/I); d, antigen Pb176 (CFA/II). Other wells contain specific antisera prepared against purified antigens 2230 (S1), H10407 (S2) Pb176 (S3), and CS3 (S4).

Preparation of plasmid DNA. Plasmid DNA was extracted from wild-type strains and from different plasmid-cured derivatives by the rapid procedure of Kado and Liu (10). The method utilized the molecular characterization of covalently closed circular DNA that is released from cells under conditions that denature chromosomal DNA by using alkaline SDS (pH 12.6) at elevated temperature (65°C); proteins and cell debris were removed by extraction with phenol-chloroform. The clarified extract was used directly for electrophoretic analysis.

Agarose gel electrophoresis. Plasmid DNA extracts were analyzed on horizontal 0.7% (wt/vol) agarose gel at 12 V/cm for 2 h in a low-salt buffer (pH 7.9) composed of 40 mM Tris, 5 mM sodium acetate, and 2 mM EDTA. The molecular weights of the plasmids were measured by comparing the mobility of the plasmid DNA with the mobilities of plasmids of known molecular weights (range, 100×10^6 to 2×10^6) run on the same gel.

RESULTS

Characterization of strain 2230. Strain 2230 was isolated from stool samples of an infant suffering from diarrhea. This strain belonged to the ETEC group because it produced the heat-stable enterotoxin (determined by using the infant mouse test). Its serotype, kindly determined by Ida and Frits Orskov, was O25:K?:H16.

Since adhesive ETEC caused MRHA of different kinds of erythrocytes, we tested the ability of strain 2230 to agglutinate human, bovine, chicken, and guinea pig erythrocytes. The HA pattern of *E. coli* 2230 differed from those of the other adhesive ETEC strains: *E. coli* 2230 did not agglutinate any of the four kinds of erythrocytes.

Strain 2230 adhered to the brush border of human intestinal enterocytes (Fig. 1A). Its adhesion index was superior to that of two bacterial cells adhering to the brush border of one enterocyte (Table 1). The adhesion of strain 2230 was not mediated by type 1 pili, because strains possessing only type 1 pili did not adhere to the brush border but to the basolateral surface of the enterocytes. Furthermore the adhesion of strain 2230 was not inhibited in the presence of 1% D-mannose. The spontaneous variant (strain 2230p) did not adhere to the brush border (adhesion index, 0) (Fig. 1B). After growth at 18°C, strain 2230 could no longer adhere to enterocyte brush borders.

The adhesion of strain 2230 did not occur after pretreatment of the *E. coli* cells by the adsorbed 2230 antiserum and after pretreatment of enterocytes by a preparation of purified 2230 antigen (Table 1).

Inhibition of adhesion by 2230 purified protein extract seems to be specific; this protein extract did not block adhesion of strains H10407 (CFA/I) and Pb176 (CFA/II) (Table 2). The same specificity was observed with H10407 and Pb176 protein extracts.

Analysis of the strain 2230 adhesive factor. Bacterial surface components were extracted by heating a bacterial suspension at 60°C for 20 min by the method of Stirm et al. (22). After analysis of the components by SDS-slab gel electrophoresis, the preparation was shown to contain a major protein (Fig. 2A). The molecular weight of the 2230 protein subunit was 16,000. The adhesion-negative variant 2230p never produced such a protein.

The purity of 2230 protein obtained by chromatography on Sepharose CL-6B in 6 M guanidine hydrochloride was analyzed by SDS-polyacrylamide gel electrophoresis with Coomassie blue and silver staining. The 2230 extract contained only the 2230 protein subunit of 16,000 daltons (Da) (Fig. 2B). We investigated the presence of carbohydrate by silver staining; only the 2230 protein subunit was stained.

Antigenicity of *E. coli* 2230 antigen. The 2230 antiserum, adsorbed against the negative variant 2230p, did agglutinate 2230 antigen-positive derivatives, but did not agglutinate 2230 antigen-negative derivatives. With the Ouchterlony double-gel diffusion test (Fig. 3), the 2230 antigen gave a single precipitation line against its homologous antiserum and no precipitation line against anti-CFA/I, anti-CFA/II, or anti-CS3. The negative variant 2230p gave no precipitation line against 2230 antiserum.

Immunoblot analysis. Western immunoblotting studies showed that only the 16,000-Da subunit reacted specifically with adsorbed 2230 antiserum. No protein extracted from

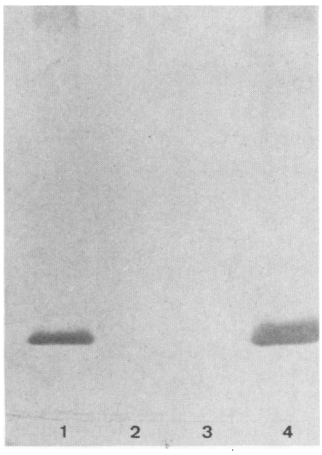

FIG. 4. Immunoblot analysis with adsorbed 2230 antiserum of crude bacterial surface protein extracts from 2230 wild strain (lane 1) and its cured derivatives. Other lanes: 2, 2230a; 3, 2230b; 4, 2230c. The positive reactions observed on the top of the gel would correspond to polymerized protein subunits that did not run into the gel.

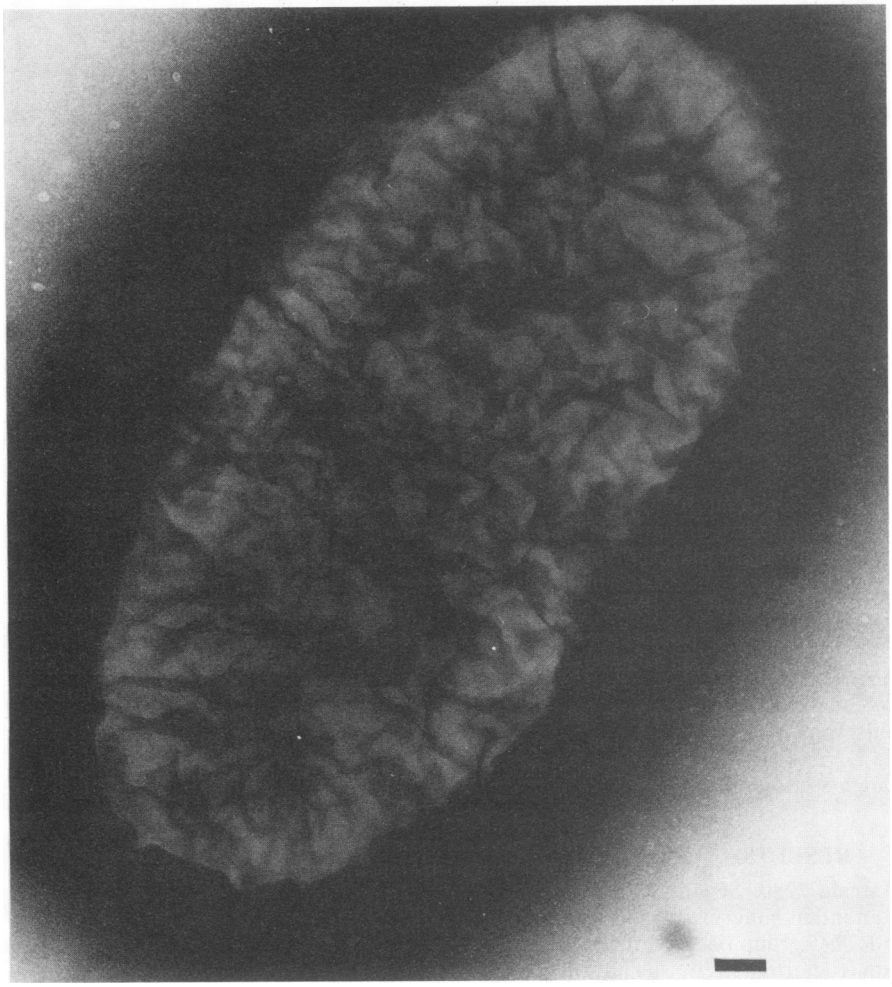

FIG. 5. Electron micrograph of a negatively stained preparation of *E. coli* 2230; ×72,000; bar, 0.1 μm.

derivatives 2230a or 2230b reacted with 2230 antiserum (Fig. 4).

Electron microscopy. An electron micrograph of a negatively stained preparation of *E. coli* 2230 did not show any filamentous structure on the bacterial surface (Fig. 5). No difference was observed between the adhesion-positive strain (strain 2230) and its negative variant.

Gold immunolabeling microscopy with the adsorbed 2230 antiserum showed that the positive 2230 strain was coated with antibodies. This antigen did not show any filamentous structure and was apparently easily shed from the bacterial cells, because a lot of 2230 antigen was detached from bacteria (Fig. 6A). No labeling occurred when 2230 antiserum was reacted with derivatives from strains 2230a and 2230b, which have lost the adhesive capacity (Fig. 6B).

Analysis of plasmid DNA. Wild strain 2230 carried four plasmids of 66, 56, 3, and 2 MDa. The negative variant (strain 2230p) lacked the 66-MDa plasmid, although it did possess the 56-, 3-, and 2-MDa plasmids. After growth of bacteria in the presence of ethidium bromide and nonselective plating for single colonies, a large number of derivatives were tested for their DNA plasmid content, their adhesive capacity, and their production of the adhesion-related surface protein (subunit of 16,000 Da) (Table 3). We obtained the following DNA plasmid patterns: cured derivative 2230a without the 66- and 56-MDa plasmids, derivative 2230b without the 66-MDa plasmid, and derivative 2230c without the 56-MDa plasmid (Fig. 7). We never observed the loss of the 3- and 2-MDa plasmids. The cured derivatives 2230a and 2230b, which had lost the 66-MDa plasmid, did not adhere to the human intestinal epithelial cells. The loss of this 66-MDa plasmid was also correlated with the loss of production of the 16,000-Da subunit surface protein as shown in Fig. 2.

DISCUSSION

The adhesive factor (antigen 2230) produced by the ETEC 2230 strain of serotype O25:H16 may represent a new type of adhesive factor antigen. This adhesive factor shares some properties with CFA/I and CFA/II (5, 7), namely (i) lack of production when bacteria are grown at 18°C, (ii) lack of bacterial adhesion to HepII cells (unpublished data), and chiefly (iii) bacterial adhesion to human enterocyte brush borders. Indeed, the adhesion of *E. coli* strains to the intestinal brush border is always correlated with the presence of specific adhesive factor only possessed by human ETEC strains. On the other hand, the adhesion of *E. coli* 2230 strain cannot be mediated by type 1 pili, because strains possessing only type 1 pili do not adhere at all to the brush border, but adhere to the basolateral surface of the intestinal enterocyte. Thus, we can consider the adhesive factor 2230 as an adhesive factor similar to the well-known CFA/I or CFA/II.

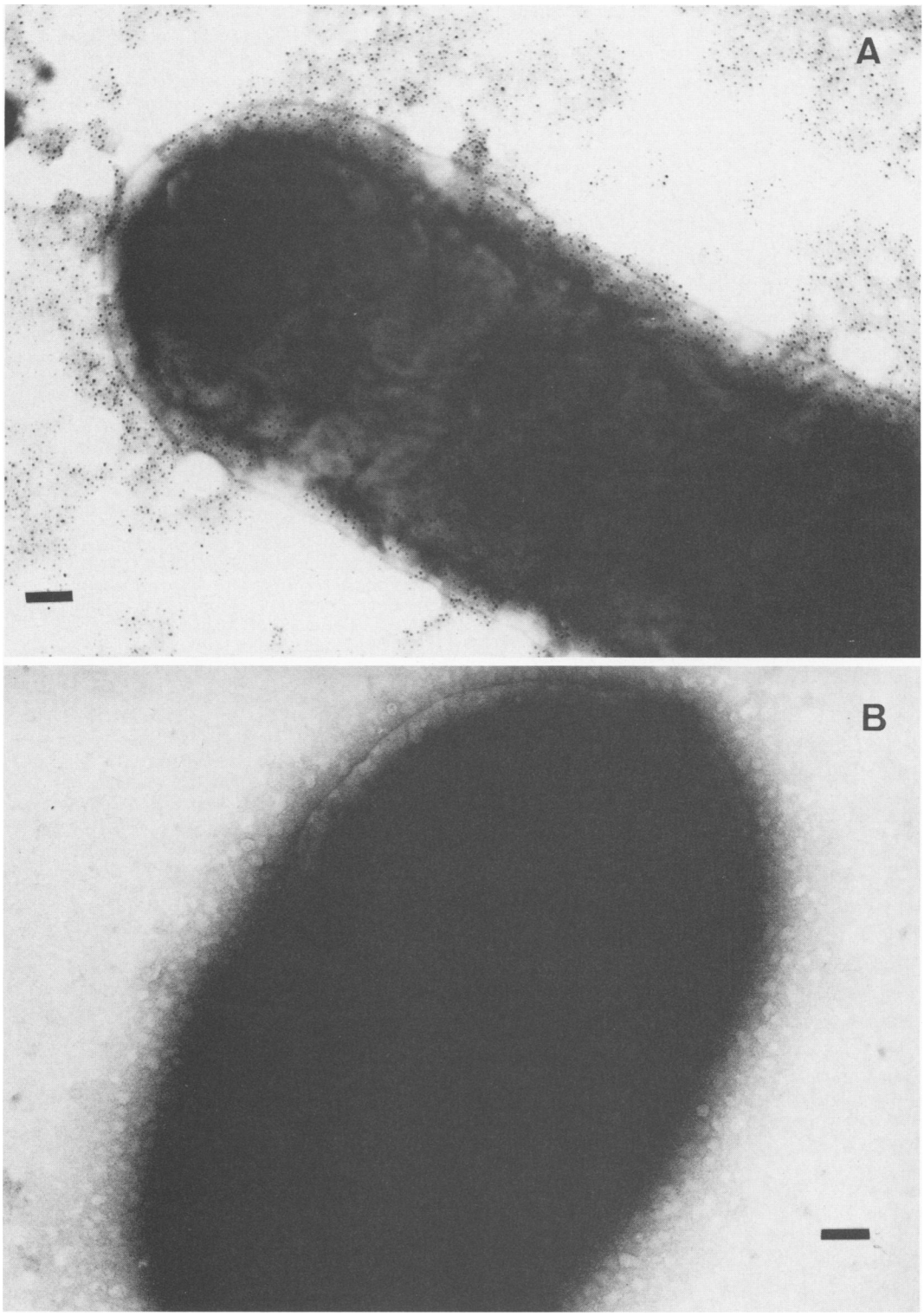

FIG. 6. Electron micrographs after colloidal gold immunolabeling of *E. coli* 2230 (A) and *E. coli* 2230p (B); ×72,000; bar, 0.1 μm.

However the adhesive factor produced by strain 2230 differs from CFA/I or CFA/II by (i) the antigenic specificity, (ii) the absence of MRHA with human, bovine, or chicken erythrocytes, and (iii) its morphology. The presence of CFA on the surface of ETEC strains is always correlated with a MRHA of these strains with human, bovine, or chicken erythrocytes (8). On the other hand, bacteria possessing CFAs are always heavily piliated (5, 7), and CFA extracts appear in electron microscope as fimbriae. Wild-type 2230 bacterial cells are not piliated.

However, the procedure of Stirm et al. (22) for extraction of K88 antigen allows us to obtain a bacterial surface protein

TABLE 3. Plasmid contents of *E. coli* 2230 and its cured derivatives; correlation between adhesive capacity, synthesis of specific 2230 nonfimbrial protein, and the 66-MDa plasmid

Strain	Adhesion capacity[a]	2230 nonfimbrial protein[b]	Presence of the following plasmids (MDa):			
			66	56	3	2
2230	+	+	+	+	+	+
2230p	−	−	−	+	+	+
2230a	−	−	−	−	+	+
2230b	−	−	−	+	+	+
2230c	+	+	+	−	+	+

[a] Capacity of bacterial strain to adhere to the brush border of human intestinal enterocytes.

[b] Presence of 2230 nonfimbrial protein was determined by SDS-slab gel electrophoresis of bacterial surface components and by agglutination with the specific adsorbed anti-2230 serum.

of 16,000 Da which does not appear as fimbriae in electron microscopy even after gold immunolabeling. This protein is found on the surface of adhesive bacteria (strain 2230 and derivative 2230c), but is not present on nonadhesive bacteria (strain 2230p and derivatives 2230a and 2230b). Thus, there is a correlation between the adhesion capacity and the presence of this nonfimbrial surface protein. Furthermore, the pretreatment of enterocytes with the purified nonfimbrial surface protein competitively inhibits the adhesion of strain 2230 to brush borders, and for that reason this protein may represent the adhesive factor.

The ability to produce CFA/I or CFA/II may be lost or transferred from strains possessing these factors, since the factors are plasmid mediated (7, 15, 19, 20). Spontaneous variants of strain 2230 have been found; they had lost the ability to adhere to the brush border and to produce the bacterial surface nonfimbrial adhesin, suggesting that the ability to adhere and the synthesis of the nonfimbrial protein are plasmid coded. Analysis of DNA plasmid contents of the different cured derivatives shows that a 66-MDa plasmid is involved in the synthesis of this nonfimbrial adhesive factor.

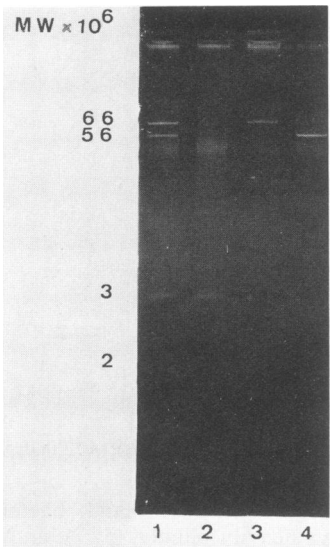

FIG. 7. Agarose gel electrophoresis of plasmid DNAs from 2230 wild strain (lane 1) and its cured derivatives. Other lanes: 2, 2230a without 56- and 66-Mdal plasmids); 3, 2230c (without 56-Mdal plasmid); 4, 2230b (without 66-Mdal plasmid).

Other adhesive factors have been identified, such as antigen 260-1 (9), antigen E8775 (23), and CFA/III (3), but they appear to be pili. Among adhesive factors, such a nonfimbrial adhesive factor has been identified; it is the CS3 antigen, a component of the initial CFA/II (14). Strain 2230 antigen did not react with anti-Pb176 serum, which must contain anti-CS1 and anti-CS3 antibodies according to the biotype of Pb176 strain. Moreover the anti CS3 serum was also tested; no cross-reaction was observed between 2230 antigen and CS3. Another nonfimbrial protein has been described by Williams et al. (27), but this surface protein is described as a mannose-resistant hemagglutinin and seems to be involved in adhesion of *E. coli* to HeLa and HepII cultured cells. The adhesive factor described in this paper is different because it is not involved in MRHA and in adhesion to cultured cells (unpublished data). Furthermore, in vivo studies are necessary to determine whether this 2230 adhesive factor is implicated in colonization of intestinal epithelium like CFA/I and CFA/II.

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