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Identification and Characterization of CS20, a New Putative Colonization Factor of Enterotoxigenic *Escherichia coli*

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An enterotoxigenic *Escherichia coli* (ETEC) strain producing a previously undescribed putative colonization factor was isolated from a child with diarrhea in India. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of bacterial heat extracts revealed a polypeptide band of 20.8 kDa when the bacteria were grown at 37°C which was absent after growth at 22°C. A specific rabbit antiserum raised against the purified 20.8-kDa protein bound specifically to the fimbriae, as shown by immunoelectron microscopy, and inhibited bacterial adhesion to tissue-cultured Caco-2 cells. Transformation with a recombinant plasmid harboring the *cfaD* gene, which encodes a positive regulator for several ETEC fimbriae, induced hyperexpression of the 20.8-kDa fimbrial subunit and a substantial increase in the proportion of bacterial cells that were fimbriated. The N-terminal amino acid sequence of the polypeptide showed 65 and 60% identity to the PCFO20 and 987P fimbriae of human and porcine ETEC, respectively. We propose the term CS20 for this new putative colonization factor of human ETEC.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhea in children in developing countries. ETEC colonization factor antigens (CFAs) and putative colonization factors (PCFs), jointly referred to as colonization factors (CFs), mediate the attachment of bacteria to the intestinal mucosa (32, 47, 48), whereas enterotoxins induce a net secretion of electrolytes and water into the gut lumen (4, 31, 46). The toxins involved in human ETEC diarrhea are the heat-labile enterotoxin (LT) LTh, which is genetically and functionally similar to cholera toxin (55), and the heat-stable enterotoxins (ST) STaII (STh) and STaI (STp).

CFA/I is a single fimbrial structure (13), whereas CFA/II is composed of three distinct coli surface antigens (CSs) in various permutations. Thus, CFA/II is composed of CS3 alone or CS3 in combination with CS1 or CS2 fimbriae (8, 42). Likewise, CFA/IV strains express CS6 alone or together with CS4 or CS5 fimbriae (36). During recent years, several new PCFs, including PCFO159:H4, PCFO166, CS7, CS17, CFA/III, PCFO20, and CS19, have been discovered (18, 21, 23, 33, 49, 51). The ability of several of these PCFs to mediate intestinal colonization and induce an immune response has been shown in animal models (47). The antibacterial immunity induced by ETEC is to a large extent CF specific (1, 7, 32, 48). Accordingly, detailed information on the distribution of ETEC carrying the various CFs in different geographic regions is a prerequisite for the design of effective ETEC vaccines (31, 32, 46). A considerable proportion of ETEC strains do not possess identifiable CFs (3, 35, 51). These strains, for which the term 0-ETEC has been suggested (45), may either have lost their ability to express known CFs or produce hitherto undescribed CFs. The identification and characterization of such new CFs are important for the construction of a vaccine with a broad antigenic coverage.

Bacterial heat extracts of ETEC contain fimbrial proteins which, when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), migrate corresponding to proteins of 14 to 31 kDa (5). The fimbrial proteins are efficiently expressed when the bacteria are grown at 37°C but not after growth at room temperature (22°C). CfaD, a positive regulator belonging to the AraC protein family (25), is capable of inducing the expression of several different ETEC CFs (15, 18, 22, 41, 54). In a recent study using up-to-date hybridization or immunodetection tests for CFA/I, CS1 to CS7, CFA/III, CS17, CS19, PCFO20, PCFO159:H4, and PCFO166, we found that 27 of 111 ETEC isolates obtained from North Indian children were CF negative (45). Bacterial heat extracts of these 0-ETEC isolates were examined by SDS-PAGE to identify temperature-dependent protein bands within the specified molecular weight range. A selection of such strains were then transformed with the cloned *cfaD* gene to induce hyperexpression of potential fimbrial proteins. Adhesion of bacteria to Caco-2 cells and N-terminal amino acid sequencing of hyperexpressed fimbriae were also performed. By these methods, a previously undescribed fimbria was identified on the STaI$^+$LT$^+$ O17:K23:H45 0-ETEC isolate H721Ab.

MATERIALS AND METHODS

Bacterial strains. The characteristics of the human ETEC strains used in the study are shown in Table 1. The toxin profiles were determined by colony hybridization (43). Strain H721A was isolated in 1988 from a child with diarrhea in New Delhi.

Preparation of bacterial heat extracts. ETEC strains were grown at 37°C and at room temperature on CFA agar (12) and CFA agar containing 1.5 g of Bacto Bile Salts no. 3 (Difco, Detroit, Mich.) per liter (CFA-BS agar). Overnight bacterial cultures were suspended in 350 μl of phosphate-buffered saline (PBS; pH 7.2) to a final volume of 500 μl. The tubes containing the bacterial suspensions were placed in a water bath at 60°C for 30 min and then centrifuged at 14,000 × *g* for 20 min. The supernatants were used as crude protein extracts for SDS-PAGE, immunoblotting, and purification of fimbrial polypeptides.

SDS-PAGE and immunoblotting. Crude protein extracts and purified fimbrial

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TABLE 1. Characteristics of the human ETEC strains used in the study

Strain	CF designation	Enterotoxin gene profile		Origin[a] or reference
		ST	LT	
H10407	CFA/I$^+$	+	+	13
247425	CS1$^+$ CS3$^+$	+	+	A
416C	CS2$^+$ CS3$^+$	+	+	B
E34420	CFA/III$^+$ CS6$^+$	−	+	C
128A	CS5$^+$ CS6$^+$	+	+	B
H334A	CS7$^+$	−	+	B
F222A	CS17$^+$	−	+	B
F595	CS19$^+$	+	+	B
350C1	PCFO159:H4$^+$	+	+	49
H574B	PCFO166$^+$	+	−	44
ARG-2	PCFO20$^+$	+	+	51
ARG2/1	PCFO20$^-$	+	+	51
950425	987P$^+$	+	+	40
I36	987P$^-$	+	+	24
H721Ab	CS20$^+$	+	+	B
H721Aa	CS20$^-$	+	+	B

[a] Origins: A, Dhaka, Bangladesh, provided by A.-M. Svennerholm, Göteborg, Sweden; B, New Delhi, India; C, provided by M. M. McConnell, London, United Kingdom.

polypeptides were electrophoresed through 15% SDS-PAGE gels (30). The gels were stained with Coomassie brilliant blue R250 or electroblotted onto nitrocellulose membranes (Trans-Blot transfer medium; Bio-Rad Laboratories, Richmond, Calif.) with the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories) as specified by the manufacturer. Protein transfer was carried out at 90 V for 1.5 h. Absorbed rabbit antiserum was diluted 1:500 in PBS–0.05% Tween 20 (Sigma, St. Louis, Mo.)–0.1% bovine serum albumin (BSA; Sigma) and incubated with the nitrocellulose membranes overnight at room temperature. The membranes were washed in a PBS solution containing 0.05% Tween and gently agitated for 90 min at room temperature in goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.) diluted 1:2,000 in PBS–0.05% Tween 20–0.1% BSA. The membranes were again thoroughly washed and developed with hydrogen peroxide substrate and 4-chloronaphthol chromogen (Bio-Rad Laboratories).

Transformation with plasmids harboring regulatory genes. Bacteria were transformed with pIVB3-100, a pUC18 derivative containing the *cfaD* gene (41), or with pPK198 (27) containing the *fapR* gene, which encodes the positive regulator for the 987P fimbriae of ETEC infecting neonatal piglets (11), in pACYC184 as the cloning vector. Transformation was performed by electroporation (56) with the Gene Pulser apparatus (Bio-Rad Laboratories), and transformants were selected after overnight growth at 37°C on CFA agar containing 100 mg of ampicillin per ml or 20 mg of chloramphenicol per ml, as appropriate. The transformants were henceforth grown in the presence of the adequate antibiotic to ensure the presence of the recombinant plasmids.

Purification of fimbrial proteins. Crude protein extract from the *cfaD* transformant H721Ab(pIVB3-100) was subjected to SDS-PAGE with 1.5-mm-thick 15% polyacrylamide gels without wells as described by Laemmli (30). Immobilon P^{SQ} (Millipore Corp., Bedford, Mass.) polyvinylidene difluoride membranes corresponding to the size of the gels were soaked for 5 min in methanol, rinsed three times in distilled water, and then immersed in 1 M 3-cyclohexylamino-1-propanesulfonic acid (Sigma)–10% methanol (pH 11) (CAPS transfer buffer). Upon completion of electrophoresis, protein transfer was carried out in CAPS transfer buffer at 70 V for 2 h. The Immobilon P^{SQ} membrane was then stained for 1 min with 1% Ponceau S (Sigma) and washed with distilled water until the protein pattern became visible. The band of interest was excised and placed in an Eppendorf tube containing 0.5 ml of eluant (0.5 mM Tris HCl, 2% SDS, 1% Triton X-100 [pH 9.0]) per cm^2 of Immobilon strip. The samples were centrifuged at room temperature for 15 min at 14,000 × *g* to release bound proteins from the membrane, and the liquid was subsequently centrifuged in an Eppendorf centrifuge for 5 min at 14,000 × *g* to remove any insoluble material. Four volumes of acetone were added to the protein solution, which was left to precipitate at −80°C for 60 min, followed by a 20-min centrifugation in an Eppendorf centrifuge (14,000 × *g* at 4°C). The acetone precipitate was solubilized with 800 μl of 100 mM Tris HCl–0.1% Triton X-100 (pH 7.7).

Antisera. Rabbit immune serum was obtained following four intramuscular injections with 100 μg of purified protein at 2-week intervals. The initial immunization was given in Freund's complete adjuvant, and the three subsequent injections were given in Freund's incomplete adjuvant. The final bleeding was performed 10 days after the last immunization. Specific ϕ78 antiserum was prepared by multiple absorptions with nonfimbriate bacteria from a mutant of strain H721A termed H721Aa (Table 1), which had spontaneously lost its ability to express the protein of interest. Specific rabbit antiserum against PCFO20 (R1868) was obtained from G. Viboud (51).

Slide agglutination assays. H721Ab and its transformants were grown on CFA agar at 37°C. The overnight bacterial cultures were tested by slide agglutination with polyclonal antiserum ϕ78 and, when indicated, with the anti-PCFO20 polyclonal antiserum R1868. In addition, one strain each of types CFA/I$^+$, CS1$^+$ CS3$^+$, CS2$^+$ CS3$^+$, CFA/III$^+$ CS6$^+$, CS5$^+$ CS6$^+$, CS17$^+$, CS19$^+$, PCFO159:H4$^+$, PCFO166$^+$, PCFO20$^+$, and 987P$^+$ were examined in slide agglutination with ϕ78 after overnight growth at 37°C on CFA or CFA-BS agar, as appropriate.

N-terminal amino acid sequencing. Crude protein extracts from the *cfaD* transformant H721Ab(pIVB3-100) were separated by SDS-PAGE and electroblotted to an Immobilon P^{SQ} membrane as described above. The protein band of interest was excised and subjected to N-terminal amino acid sequence analyses by a model 477A protein sequencer with an on-line model 120A phenylthiohydantoin analyzer. The sequence was compared with that of other proteins in the Experimental Geninfo Blast Network Service Blaster database by computer analysis.

Electron microscopy. After overnight culture on CFA agar at 37°C, bacterial cells were harvested, suspended in 200 μl of distilled water, and applied to carbon-coated nickel grids, as described previously (44). For transmission electron microscopy, the bacteria were negatively stained with 1% ammonium molybdate (pH 7). Fifty bacteria from each of the cultures were examined by transmission electron microscopy to quantify the proportion of cells exhibiting fimbrial structures, the ratio being termed the fimbriated-cell percentage (FCP). For immunoelectron microscopy, the grids were placed for 15 min on drops of the absorbed antifimbrial antiserum ϕ78 diluted 1:100 in PBS–1% BSA–1% Tween 20 (PBS-BSA-Tween). Following five washings for 5 min each in PBS-BSA-Tween, the grids were placed for 15 min on drops with gold-labeled protein A (British BioCell International, Cardiff, United Kingdom) diluted 1:10 in PBS-BSA-Tween. Then, each grid was washed three times in PBS-BSA-Tween and then three times in distilled water. This was followed by negative staining in 1% ammonium molybdate. The grids were examined in a JEOL JEM 100 CX transmission electron microscope at 80 kV.

Adhesion to the cultured human colon carcinoma cell line Caco-2. The Caco-2 cell adhesion assay (9) was used to assess the ability of strain H721Ab and its *cfaD* transformant to adhere to intestinal mucosa. Caco-2 cells were maintained at 37°C in a 7% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Bio Whittaker, Verviers, Belgium) containing fetal calf serum (Advanced Protein Products Ltd., Brockmoor, United Kingdom) and 2 mM glutamine (Bio Whittaker) on 12-mm circular Assistant plastic coverslips in 24-well plates (Nunc Inc., Naperville, Ill.). The cultures were used at postconfluence after 15 days of culture (2, 10). Prior to the adhesion tests, the cells were washed in PBS (pH 7). A suspension of 5 × 10^8 bacteria per ml in culture medium containing 1% D-mannose was added to the Caco-2 cells. Three hours after infection, the samples were washed three times with PBS, fixed in methanol, stained with modified Giemsa stain (Sigma), and examined by light microscopy to assess bacterial adherence. The adhesion index was determined as the percentage of epithelial cells with adhering bacteria (51, 53). Furthermore, we recorded the average number of bacteria bound per cell by examining 100 representative Caco-2 cells.

Adhesion inhibition tests. To test for adhesion inhibition by the rabbit antiserum ϕ78, 100 μl of undiluted serum was added to 900 μl of a suspension of 5 × 10^8 bacteria per ml. The mixture was allowed to incubate at room temperature for 20 min and added to the wells with the Caco-2 cells. The adhesion test was then performed as described above. The absorbed rabbit antiserum ϕ79, raised against the CS19 fimbrial protein (18), was used as control serum.

RESULTS

SDS-PAGE and immunoblotting. Heat extracts of strain H721Ab yielded a prominent 20.8-kDa protein band when the bacteria were grown on CFA agar at 37°C, whereas this polypeptide was absent after growth at room temperature (Fig. 1A, lanes 2 and 3; Fig. 2, lanes 3 and 4). The presence of bile salts in the growth medium did not enhance the expression of the 20.8-kDa polypeptide (Fig. 1A, lane 2). Direct comparison in SDS-PAGE showed differences in migration between the 20.8-kDa protein of strain H721Ab and the CS5 and CS7 subunits (Fig. 1B, lane 2 to 5).

Transformation with a plasmid harboring regulatory genes. To test whether the CfaD protein can induce the expression of the 20.8-kDa polypeptide, the *cfaD* gene was introduced into strain H721Ab. The expression of the polypeptide was substan-

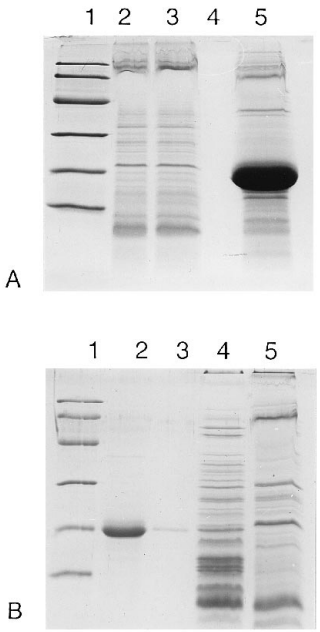

FIG. 1. SDS-PAGE analysis of crude heat extracts from ETEC strains grown on CFA agar at 37°C and of purified fimbrial subunit protein. (A) Lanes: 1, prestained low-molecular-mass standard proteins (Bio-Rad laboratories), lysozyme (20.5 kDa), soybean trypsin inhibitor (28.7 kDa), carbonic anhydrase (34.9 kDa), ovalbumin (53.2 kDa), BSA (84 kDa), and phosphorylase *b* (112 kDa); 2, heat extract of strain H721Ab grown on CFA agar at 37°C; 3, heat extract of strain H721Ab grown on CFA-BS agar; 5, heat extract of the *cfaD* transformant H721Ab(pIVB3-100) grown on CFA agar containing ampicillin. Lane 4 is empty. (B) Lanes: 1, low-molecular-mass standards identical to those in panel A; 2, diluted (1:20) heat extract of H721Ab(pPIVB3-100); 3, purified fimbrial protein from the *cfaD* transformant H721Ab(pIVB-100); 4, heat extract of a CS5-positive strain; 5, heat extract of a CS7-positive strain.

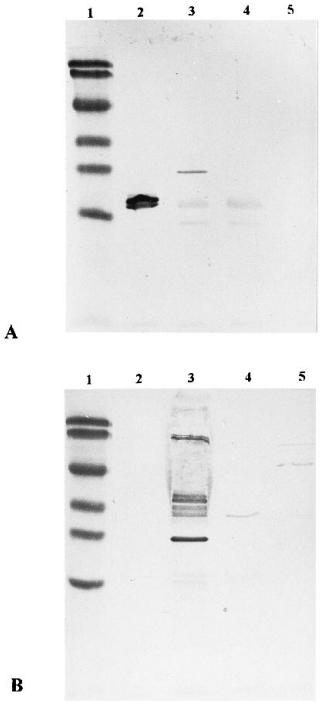

FIG. 3. Immunoblot analysis. Lanes: 1, low-molecular-mass standards identical to those in Fig. 1; 2, heat extract diluted 1:20 from the *cfaD* transformant H721Ab(pIVB3-100) grown on CFA agar containing ampicillin at 37°C; 3, heat extract of the PCFO20$^+$ strain ARG-2 grown on CFA agar at 37°C; 4, heat extract of the 987P$^+$ strain 950425 grown on blood agar at 37°C; 5, heat extract of the CFA/I$^+$ strain H10407 grown on CFA agar at 37°C. (A) The absorbed rabbit antiserum ϕ78 raised against purified CS20 protein was used as the antibody. (B) The specific antiserum R1868 raised against PCFO20 was used as the antibody.

tially enhanced in the transformant H721Ab(pIVB3-100) (Fig. 1A, lane 5). On the basis of the observed N-terminal amino acid sequence similarity between 987P and the 20.8-kDa polypeptide (see below), the 987P regulatory gene *fapR* was introduced into strain H721Ab. Expression of the 20.8-kDa polypeptide was enhanced, albeit to a lesser extent than after transformation with pIVB3-100 (Fig. 2, lane 7).

Purification of fimbrial proteins. As shown in Fig. 1A, lane 5, the 20.8-kDa polypeptide was the dominating protein of the crude heat extracts from the transformant H721Ab(pIVB3-100). After purification, a single band was observed on SDS-PAGE; its migration was indistinguishable from that of the temperature-dependent protein band of strain H721Ab (Fig. 1B, lane 3; Fig. 2, lanes 2 to 4). The concentration of the purified protein solution was determined by Bradford dye staining (6) to be 1 mg/ml.

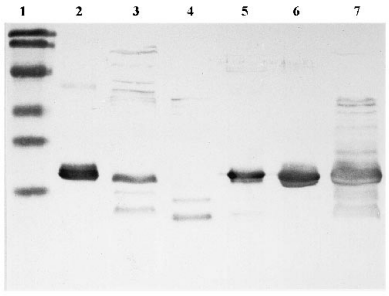

FIG. 2. Immunoblot analysis. Lanes: 1, low-molecular-mass standards identical to those in Fig. 1; 2, purified fimbrial protein from the *cfaD* transformant H721Ab(pIVB-100); 3, heat extract of strain H721Ab grown on CFA agar at 37°C; 4, heat extract of strain H721Ab grown on CFA agar at 22°C; 5, heat extract diluted 1:20 from the *cfaD* transformant H721Ab(pIVB3-100) grown on CFA agar containing ampicillin at 37°C; 6, heat extract diluted 1:20 from the *cfaD* transformant H721(pIVB3-100) grown on CFA agar containing ampicillin at 22°C; 7, heat extract from the *fapR* transformant H721(pPK198) grown on CFA agar containing chloramphenicol at 37°C. The specific antiserum ϕ78 raised against purified CS20 fimbrial protein was used as antibody.

Immunoblotting and slide agglutination. When crude protein extracts of strain H721Ab were examined with the absorbed antiserum ϕ78 in immunoblotting, the 20.8-kDa polypeptide band was detected after growth of the bacteria at 37°C (Fig. 2, lane 3). The antiserum also bound to the purified 20.8-kDa polypeptide (lane 2). After growth of the wild-type strain H721Ab at room temperature, no polypeptides with this

TABLE 2. N-terminal amino acid sequence of CS20, PCFO20, and 987P fimbrial polypeptides[a]

Fimbrial subunit	Sequence (amino acids 1–20)	Identity (%)
CS20	**APAAN DSSQA TLNFS GGVTS**	100
PCFO20	**ALPAN DSSKA TLDFT GNVTA**	65
987P	**APAEN NTSQA NLDFT GKVTA**	60

[a] For sequence details, see references 51 and 52 for PCFO20 and reference 28 for 987P.

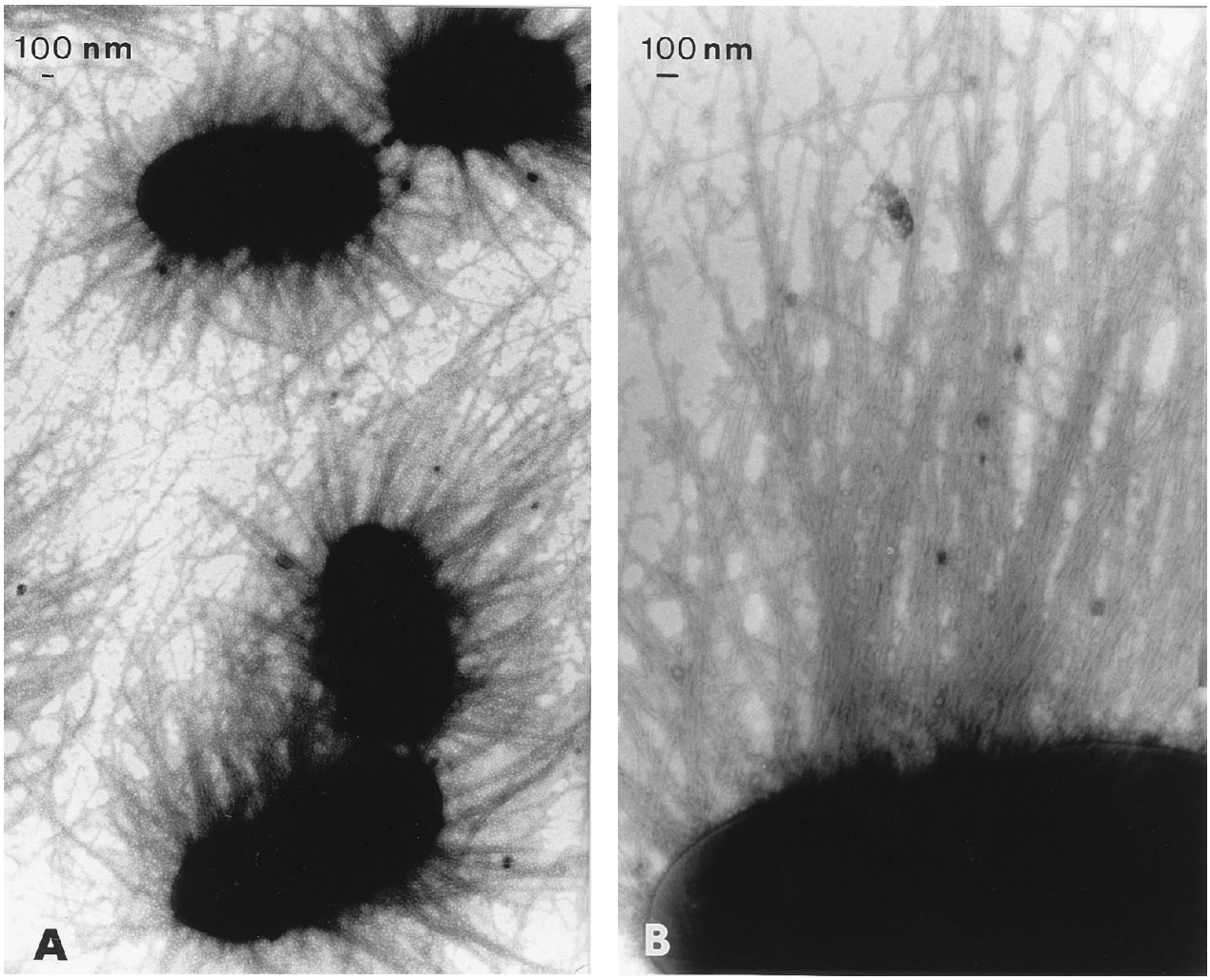

FIG. 4. (A) Transmission electron micrograph of the *cfaD* transformant H721Ab(pIVB3-100) negatively stained with 1% ammonium molybdate. (B) Transmission electron micrograph of the same preparation as in panel A but at a higher magnification, showing up to 1-μm-long fimbriae with a diameter of 7 nm. (C) Transmission electron micrograph showing the *cfaD* transformant H721Ab(pPIVB3-100) immunogold labeled with antiserum against CS20.

molecular mass reacted with the antiserum (lane 4). The 20.8-kDa polypeptide was detected by ϕ78 antiserum in heat extracts from H721Ab(pIVB3-100) independent of whether the bacteria were grown at 37 or 22°C (lanes 5 and 6). The ϕ78 antiserum did not react with the 987P or CFA/I subunits (Fig. 3A, lanes 4 and 5). However, a weak reaction to the 25-kDa PCFO20 polypeptide was observed (lane 3). The antiserum R1868 raised against the PCFO20 fimbriae did not cross-react with the 20.8-kDa subunits of strain H721Ab(pIVB3-100) (Fig. 3B, lane 2). Only the transformants H721Ab(pIVB3-100) and H721Ab(pPK198) were agglutinated with the ϕ78 antiserum, whereas the untransformed strain H721Ab, as well as CFA/I$^+$, CS1$^+$ CS3$^+$, CS2$^+$ CS3$^+$, CFA/III$^+$ CS6$^+$, CS5$^+$ CS6$^+$, CS17$^+$, CS19$^+$, PCFO159:H4$^+$, PCFO166$^+$, PCFO20$^+$, and 987P$^+$ strains were all negative. Keeping in mind the sequence similarities between the 20.8-kDa polypeptide and the PCF O20 subunit protein, the transformant H721Ab(pIVB3-100) and the PCFO20$^+$ strains were also tested for agglutination with R1868. Only the PCFO20$^+$ strain ARG-2 was agglutinated.

N-terminal amino acid sequence of the *cfaD*-induced polypeptide of strain H721Ab. The N-terminal amino acid sequence of the *cfaD*-induced 20.8-kDa polypeptide differed from those of known colonization factors. It showed a 60 and 65% identity with the corresponding sequences of 987P and PCFO20, respectively (Table 2).

Electron microscopy. Strains H721Ab and H721Ab(pIVB3-100) produced rod-like fimbriae, up to 1 μm long and with a diameter of 7 nm when the bacteria were grown at 37°C. Of the 50 wild-type bacteria examined, 6 were fimbriated, yielding an FCP of 12%. Following transformation, all the bacterial cells were fimbriated; i.e., the FCP was 100%, irrespective of culture conditions and growth temperature (Fig. 4A and B). Immunoelectron microscopy showed that antiserum ϕ78 bound to the fimbrial structures (Fig. 4C).

Adhesion to the human colon carcinoma cell line Caco-2 in culture. The wild-type strain H721Ab and its corresponding *cfaD* transformant H721Ab(pIVB3-100) adhered to the Caco-2 cells with indices of 15 and 100%, while the average number of bacteria bound per cell was 2 and 9.5, respectively (Fig. 5A and B). H721Ab(pIVB3-100) bacteria incubated with ϕ78 or ϕ79 yielded adhesion indices of 5 and 100%, whereas the average number of bacteria per cell was 0.8 and 9, respectively (Fig. 5C and D).

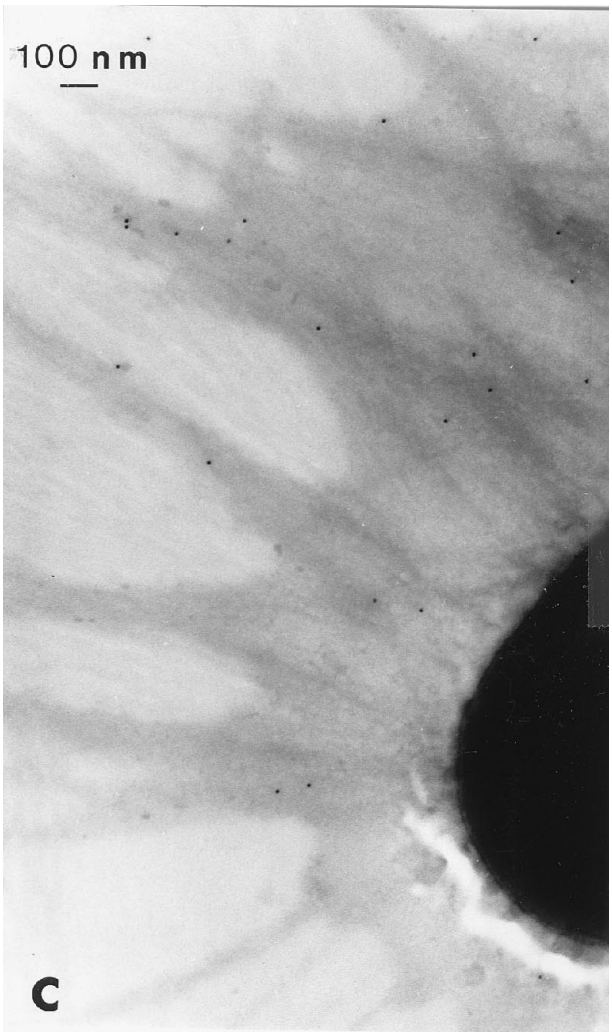

FIG. 4—*Continued.*

DISCUSSION

Several studies have been carried out to determine the distribution of CFs on human ETEC strains from different geographical areas. However, there are still many ETEC strains, 0-ETEC, which do not possess any of the hitherto identified CFs. Since mucosal colonization is a prerequisite for virulence, it is assumed that most clinical ETEC isolates express one or more adhesins. Secretory immunoglobulin A directed against ETEC CFs confers CF-specific protection against bacterial colonization of the small intestine (32, 48). Thus, the identification and molecular characterization of new adhesins is important, not only for extending the knowledge of these organelles but also for vaccine development.

In the present study, a 20.8-kDa temperature-dependent protein was a major component of the heat extract of surface components from strain H721Ab; temperature dependence is a characteristic of ETEC fimbriae (34, 37). A positive association between the presence of bile salts in the growth medium and fimbrial production has been shown for CS5, CS7, CS17, CS19, and PCFO166 (18, 21, 33, 34). The expression of the 20.8-kDa protein on strain H721Ab was found to be independent of bile salts.

The 20.8-kDa molecular mass of the protein subunit is distinct from those of most other protein subunits of human ETEC CFs. Although the reported molecular masses of CS5 (21 kDa) and CS7 (21.5 kDa) (21) are similar to that of the *cfaD*-induced protein of strain H721Ab, direct comparison of the bands in SDS-PAGE showed differences in migration. Specific antiserum raised against the purified 20.8-kDa polypeptide agglutinated H721Ab(pIVB3-100) bacteria but not ETEC expressing CS5 or CS7. Furthermore, the N-terminal amino acid sequence of the 20.8-kDa polypeptide was distinct from those of any of the hitherto described ETEC fimbrial types. In addition, strain H721Ab was negative in assays for CFA/I, CFA/II, CFA/III, CFA/IV, CS7, CS17, PCFO20, PCFO159:H4, and PCFO166 (16, 17, 43, 44).

Strain H721Ab was not examined for the adhesins PCFO9, antigen 8786, antigen 2230, PCFO148, or longus. Antigens 2230 and 8786 are nonfimbrial (2); PCFO148 ETEC produces curly fibrils, 3 nm in diameter (29); whereas strain H721Ab produces distinct fimbriae with a diameter of 7 nm, as visualized by electron microscopy. The fimbrial subunits of the reference PCFO9 strain are approximately 27 kDa (20), in contrast to the 20.8-kDa fimbrial protein of strain H721Ab. Furthermore, the N-terminal amino acid sequence of the fimbrial protein from H721Ab(pIVB3-100) is distinct from the corresponding sequences of antigen 8786, antigen 2230, PCFO9, and PCFO148 (2, 10, 20, 29). Recently, a bundle-forming type of pilus, named longus, was found in ETEC (14). Longus is composed of a repeating subunit of 22 kDa. In contrast to strain H721Ab, the strain expressing this pilus produces ST only and shows no adhesion to Caco-2 cells, and the pilus protein subunit has no N-terminal amino acid sequence similarity to the 20.8-kDa polypeptide of the transformant H721Ab (pIVB3-100) (14).

The fimbria of H721Ab did not react with antibodies directed against CFA/I, CS1, CS2, CS4, CS5, CS6, CFA/III, CS7, CS17, CS19, PCFO159:H4, PCFO166, 987P, and PCFO20, even after CfaD-induced hyperexpression. The specific antiserum raised against the 20.8-kDa polypeptide showed a weak cross-reaction in immunoblotting with heat-extracted PCFO20 proteins. This indicates that PCFO20 and the 20.8-kDa polypeptide share internal epitopes, which is in line with the observed N-terminal amino acid sequence similarity between the two fimbrial polypeptides. Nevertheless, antiserum against PCFO20 showed no binding to the 20.8-kDa polypeptide from the transformant H721Ab(pIVB3-100) in immunoblotting, and the specific antisera against the 20.8-kDa polypeptide did not agglutinate any of the ETEC carrying known CFs, including PCFO20. Interestingly, the N-terminal amino acid sequence of the 20.8-kDa protein subunit showed a 65% identity to the 25-kDa PCFO20 subunit (51, 52) of human ETEC and a 60% identity to the 987P fimbrial protein of porcine ETEC isolates (28). The N-terminal amino acid sequence of the PCFO20 fimbrial protein is more similar to the corresponding sequence of the 987P protein than to that of strain H721Ab. Although complete nucleotide sequence information is required for assessment of genetic relatedness, these observations suggest that these three fimbria may have evolved from a common ancestral gene.

The Caco-2 cell adhesion assay is probably the best available test to study the adhesion of human enteropathogens to the intestinal epithelium (9, 53). The cell line exhibits structural and functional differentiation patterns characteristic of mature small intestinal enterocytes. The adhesion observed for several ETEC strains, including H721Ab, is not mediated by the mannose-sensitive type 1 pili, since the cell-binding tests were performed in the presence of mannose. In the present study, the ϕ78 antiserum specifically inhibited the binding of the trans-

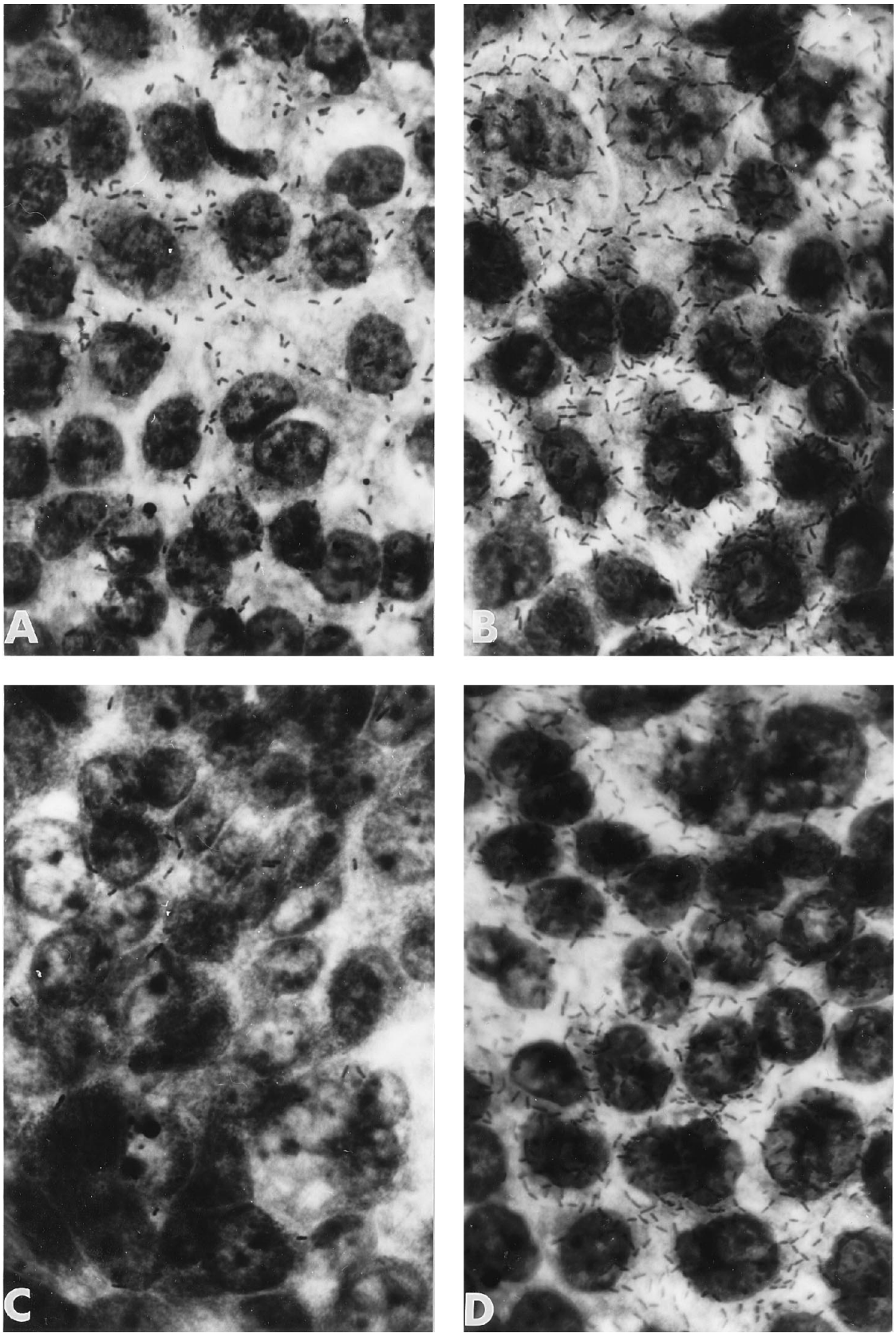

FIG. 5. Micrographs showing adhesion and adhesion inhibition of H721Ab and H721Ab(pPIVB3-100) bacteria to tissue cultured Caco-2 cells. (A) Strain H721Ab. (B) The *cfaD* transformant H721Ab(pPIVB3-100). (C) Adhesion inhibition test with H721(pPIVB3-100) to Caco-2 cells, using the specific antiserum ϕ78 raised against purified CS20 fimbrial proteins. (D) Adhesion inhibition test with H721Ab(pPIVB3-100) to Caco-2 cells, using the rabbit antiserum ϕ79 raised against purified CS19.

formant H721(pIVB3-100) to the Caco-2 cells, since the polyclonal antiserum ϕ79 raised against CS19 did not significantly affect the adhesion index or the number of bacteria per cell. We conclude that the 20.8-kDa polypeptide represents the subunit of a previously undescribed CF, for which we suggest the name CS20.

On the basis of extensive studies of ETEC adhesion to Caco-2 cells, it has been suggested that the uneven distribution of cells with bound bacteria may be due to disparities in the degree of differentiation of the individual Caco-2 cells with corresponding variations in receptor availability (9, 53). Our observations that the CS20-hyperexpressing transformant bound with an adhesion index of 100% whereas the wild-type bacteria, having a considerably lower FCP, bound to only 15% of the Caco-2 cells questions this hypothesis. Instead, our observations raise the possibility that the variation in binding of wild-type ETEC is associated with disparities in the proportion of fimbriated bacteria (15, 44).

Hibberd et al. have shown that transformation of CS5-, CS6-, CS7-, CS17-, PCFO9-, PCFO159:H4-, and PCFO166-positive strains with pNTP513, a recombinant plasmid containing the CFA/I fimbrial subunit operon which lacks a functional *cfaD* sequence, resulted in CFA/I production (22). Positive regulation of CFA/I, CS4, CS19, PCFO159:H4, and PCFO166 fimbrial expression by *cfaD* transformation has been described elsewhere (15, 18, 44, 50, 54). Similarly, in the present study, *cfaD* transformation of strain H721Ab increased the number of fimbriated bacterial cells from 12 to 100%. This observation were corroborated by SDS-PAGE, which showed that the transformants produced large amounts of the 20.8-kDa fimbrial protein, irrespective of the incubation temperature. In addition, it was demonstrated that transformation with a plasmid harboring the gene encoding the FapR protein, a positive regulator of 987P fimbria in neonatal piglet ETEC (27), also increased the expression of the 20.8-kDa polypeptide. This observation supports our hypothesis that CS20, like PCFO20, is closely related to 987P fimbriae.

The commercial fimbrial vaccines against animal ETEC infections have been used extensively, seemingly without the emergence of new or previously low-prevalence fimbrial antigen type ETEC (39). However, as there has been no active direct surveillance, it is possible that such emergence has taken place unnoticed (38). Therefore, after ETEC immunization in Third World children is initiated, a continuous and active monitoring system involving up-to-date CF identification methods (17–19, 26, 51) should be established to reveal ETEC expressing previously uncommon or novel CFs, such as CS20, which are immunologically sufficiently distinct to fill the ecological niche left by the antigen types targeted by the vaccine. The inclusion of such CFs may prevent immune evasion from eroding on long-term vaccine effectiveness.

Previous surveys of human ETEC strains have revealed wide variations in the proportions and types of CFs found in different geographical areas (3, 35, 51). A considerable proportion of ETEC do not possess identifiable CFs. It is possible that CS20 will account for a certain proportion of these hitherto unknown colonization factors. Actually, a recent study of North Indian ETEC isolates indicates that CS20 may be as common as PCFO166 (45, 50), suggesting that CS20 should be considered as a component of a future ETEC vaccine.

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