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A New Fimbrial Putative Colonization Factor, PCFO20, in Human Enterotoxigenic *Escherichia coli*

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The ability to colonize the small intestine is essential for enterotoxigenic *Escherichia coli* (ETEC) to cause diarrhea. Several colonization factor antigens (CFAs) and putative colonization factors (PCFs) have been described for ETEC. However, there are still many ETEC strains isolated from patients with diarrhea which do not possess any of these antigens. To identify CFAs in ETEC lacking the above-mentioned antigens, we exploited the ability of ETEC to adhere to tissue-cultured cells from an enterocyte-like cell line, Caco-2. An ETEC strain producing heat-labile toxin and heat-stable toxin of serotype O20:K27:H− (ARG-2) that was isolated from a child with diarrhea in Argentina and bound to Caco-2 cells was studied in further detail. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of this strain revealed a band of 25 kDa when bacteria were grown at 37°C that was missing when the same strain was cultured at 20°C. Furthermore, electron microscopy examination revealed the presence of fimbriae on the surfaces of cells of this strain when cells were grown at 37°C but not at 20°C. Rabbit antiserum raised against purified fimbriae reacted with the 25-kDa protein in immunoblotting and bound specifically to the fimbriae, as shown by immunoelectron microscopy. The presence of fimbriae, adhesion to Caco-2 cells, and the 25-kDa band seen in the SDS-PAGE were all simultaneously lost by single-insertion mutations. The N-terminal amino acid sequence of the protein subunit of the fimbriae showed no relation with those of the known colonization factors of ETEC. Furthermore, the fimbriae of the ARG-2 strain did not cross-react immunologically with any of the previously described adhesive factors in human ETEC when specific antisera against colonization factor antigens and putative colonization factors were used. Moreover, a specific antiserum raised against the fimbriae in ARG-2 did not react with ETEC carrying known colonization factors. We propose to name these new fimbriae PCFO20.

Enterotoxigenic *Escherichia coli* (ETEC) causes diarrhea in humans through intestinal colonization and production of either a heat-labile toxin or a heat-stable toxin or both. Several colonization factor antigens (CFAs) in ETEC have been described. Among these, CFA/I, CFA/II, and CFA/IV are the most-studied factors. Whereas CFA/I is a single antigen (7, 9), both CFA/II and CFA/IV consist of three antigens each, i.e., the coli surface (CS) antigens CS1, CS2, and CS3, and CS4, CS5, and CS6, respectively (6, 23, 24, 30). In addition, a number of so-called putative colonization factors (PCFs), e.g., CFA/III, CS7, CS17, and PCFO159 (5, 11, 12, 19, 28), have recently been shown to promote colonization in experimental animals (25). Although PCFO166 (18) did not colonize the rabbit intestine (25), in vitro studies have shown that this factor is responsible for adhesion of ETEC to human enterocytes and Caco-2 cells (18, 32). Other less-characterized possible colonization factors, such as PCFO9, PCFO148, and antigens 2230 and 8786, have also been described elsewhere (1, 4, 10, 14).

The different CFAs and PCFs may be fimbrial, nonfimbrial, or fibrillar in nature, and most of them mediate hemagglutination. Most of them are encoded by genes in high-molecular-weight plasmids that also contain the genes for the toxins (9). The expression of the genes is thermoregulated; i.e., fimbriae are produced at 37°C but not at room temperature (12, 23).

During a recent epidemiological study in Argentina, expression of CFA/I, CFA/II, and CFA/IV were found in 52% of ETEC strains isolated from children with diarrhea (2). Among the remaining isolates which did not express any of these colonization factors, only 27% possessed CFA/III, CS7, CS17, PCFO166, or PCFO159 (31). Thus, the percentage of ETEC with identified colonization factor increased from 52 to approximately 65% in the Argentinian ETEC strains tested. On the basis of this finding of a high proportion of ETEC that did not possess any of the known CFAs or PCFs, we have initiated a search for additional adhesins in such ETEC strains. In previous studies, many of the colonization factors were identified by their ability to cause mannose-resistant hemagglutination (8, 9), whereas others were found during searching for strains which belonged to certain serotypes (28). In the present study, we have exploited the ability of a number of clinical ETEC isolates which did not produce any known CFA or PCF to adhere to the enterocyte-like cell line Caco-2 and to yield distinct bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) when they were grown at 37°C but not when they were cultured at room temperature. By these methods, we identified one ETEC strain, ARG-2, that possesses a new type of fimbriae that we have designated PCFO20.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Seventy clinical ETEC isolates which did not react with either monoclonal

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antibodies or polyclonal antisera raised against CFA/I, CS1 to CS7, CS17, CFA/III, PCFO159, or PCFO166 were studied. The ETEC strains were kept at −70°C in Trypticase soy broth (Difco, Detroit, Mich.) supplemented with 15% glycerol. All the strains were grown in Casamino Acids-yeast extract agar (CFA agar) with bile salts (18) at 37°C overnight.

Enterotoxin test. The strains were analyzed for the production of heat-labile and heat-stabile enterotoxins by means of ganglioside GM1 enzyme-linked immunosorbent assays (26, 27) with a direct culture in plate modification.

Serotyping. The ETEC strains had been serotyped by Ida Ørskov at The International Escherichia and Klebsiella Centre, Statens Seruminstitutet, Copenhagen, Denmark (2, 31).

Adhesion to human colon carcinoma cell line Caco-2 in culture. Caco-2 cells were grown in Dulbecco's modified Eagle's medium containing fetal bovine serum (20% [vol/vol]) in eight-well chamber slides (Nunc Inc., Naperville, Ill.) at 37°C in 7% CO_2. Cultures were used at postconfluence after 15 days of culture as previously described (1, 4). Before the adhesion test, cells were washed in phosphate-buffered saline (PBS), pH 7.2. A suspension of 5×10^8 bacteria per ml in culture medium containing 1% D-mannose was added to the tissue culture, and the mixture was incubated at 37°C for 3 h. After three washes, cells were fixed in methanol, stained with 10% Giemsa stain, and examined by oil immersion light microscopy to determine bacterial adherence. The percentage of epithelial cells with adherent bacteria was determined by counting 10 randomly chosen microscopic fields. Each strain was tested in duplicate, and assays were performed doubly blind.

SDS-PAGE and immunoblotting. SDS-PAGE was carried out according to the method described by Laemmli (15). Bacterial heat extracts and purified fimbrial preparations were applied on a 4 to 20% acrylamide gradient and run with the mini PROTEAN II vertical electrophoresis cell (Bio-Rad) at 200 V for 40 min. The gels were either stained with Coomassie brilliant blue R or electroblotted to nitrocellulose. Protein profiles immobilized on nitrocellulose sheets were reacted with an antifimbrial antiserum, and antibody-antigen complexes were detected with anti-rabbit immunoglobulin G antibody labelled with peroxidase. Filters were developed with hydrogen peroxide substrate and 4-chloronaphthol chromogen (Bio-Rad).

Electron microscopy. Bacterial cells were grown on CFA agar with bile salts at 37°C overnight, harvested, suspended in distilled water, and applied on Formvar-carbon-coated grids (200 mesh). The coated grids were either negatively stained with 1% ammonium molybdate or placed on drops with a rabbit antifimbrial antiserum (diluted 1/100 in 1% bovine serum albumin [BSA] in PBS-0.05% Tween for 1 min and subsequently on drops with gold-labelled protein A (Auro Probe EM; Amersham International, Amersham, United Kingdom) diluted 1/30 in 1% BSA–PBS-0.05% Tween before negative staining was performed. The grids were then examined in an electron microscope (JEOL, Ltd., Tokyo, Japan).

Fimbrial purification. Whole-cell suspension of one of the strains, i.e., ARG-2, was prepared by growing bacteria on CFA agar containing bile salts (18) at 37°C overnight. Bacteria were harvested with PBS. Homogenization was carried out with a blender for 5 min at maximal speed in an ice bath. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C. Thereafter, the supernatant was sequentially precipitated with ammonium sulfate (20 and 40%), and the precipitates were removed by centrifugation under the conditions described above. The pellet material was resuspended in 0.05 M sodium sulfate buffer and dialyzed to remove residual salts.

Preparation of antisera. Antisera were prepared in New Zealand White rabbits weighing approximately 3 kg each at the onset of the immunization. Four injections, with 100 μg of fimbrial preparation in each, were given subcutaneously at 2-week intervals. The initial immunization was given in Freund's complete adjuvant. The final bleeding was performed 1 week after the last immunization.

Transposon mutagenesis. Transposon mutagenesis was performed on ARG-2 by using Tn*phoA*. Tn*phoA* is a Tn*5*-derived transposon which encodes kanamycin resistance and the carboxy terminus of *E. coli* alkaline phosphatase (PhoA) (17). Strain SM10 containing the suicide vector plasmid pRT733 was used as a Tn*phoA* donor (29), while the ETEC strain ARG-2, which was nalidixic acid resistant, was used as the recipient. Conjugation was carried out on Luria agar plates by standard procedures. Selection of mutants was performed after 24 or 48 h by plating transconjugants on Luria agar containing nalidixic acid, kanamycin, and XP (5-bromo 4-chloro 3-indolyl phosphate), the chromogen for alkaline phosphatase. Mutants containing transpositional insertions producing fusion proteins were detected by blue colonies on agar plates containing XP. All mutants were tested for ampicillin sensitivity in Luria agar medium to exclude mutants which could have retained the pRT733 plasmid. Transformants which did not agglutinate with the antiserum raised against the purified fimbriae and which were ampicillin sensitive were streaked onto LB (Luria-Bertani) agar plates containing kanamycin and were stored at −70°C until use.

Amino-terminal amino acid analysis. Purified fimbriae were subjected to SDS-PAGE and transferred electrophoretically to Immobilon membranes (Millipore Corp., Bedford, Mass.). The transferred proteins were stained with Ponceau S (Sigma), and the bands were excised for sequence analyses by automatic Edman degradation. The amino-terminal sequence of the ARG-2 fimbrial subunit protein was compared with those of other proteins by computer analysis by using the IBI Pustell sequence analysis program.

RESULTS

Adhesion to human enterocyte-like Caco-2 cells. Seventy ETEC strains negative for CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17, CFA/III, PCFO159, and PCFO166 (2, 31) were tested for adhesion to the human enterocyte-like cell line Caco-2. The adhesion index was determined as the percentage of epithelial cells with adhering bacteria; if more than 10% of the epithelial cells had adhering bacteria, the strain was considered positive. Thirty-seven of the 70 strains tested adhered to Caco-2 cells with an index of ≥10%. The adhesion observed was not mediated by type 1 pili, since the cell-binding assay was performed in the presence of mannose. CFA-deficient ETEC strains, which were included as negative controls, gave adhesion indices of <10% (data not shown). Figure 1A and B shows the adhesion of positive and negative control strains, respectively, to Caco-2 cells.

SDS-PAGE. CFAs of ETEC are usually expressed when bacteria are grown at 37°C but not when they are grown at room temperature, in contrast to type 1 pili. When the 37 strains that adhered to the Caco-2 cells were grown at these two temperatures and the heat extracts made from these cultures were subjected to SDS-PAGE, a band in the 37°C-

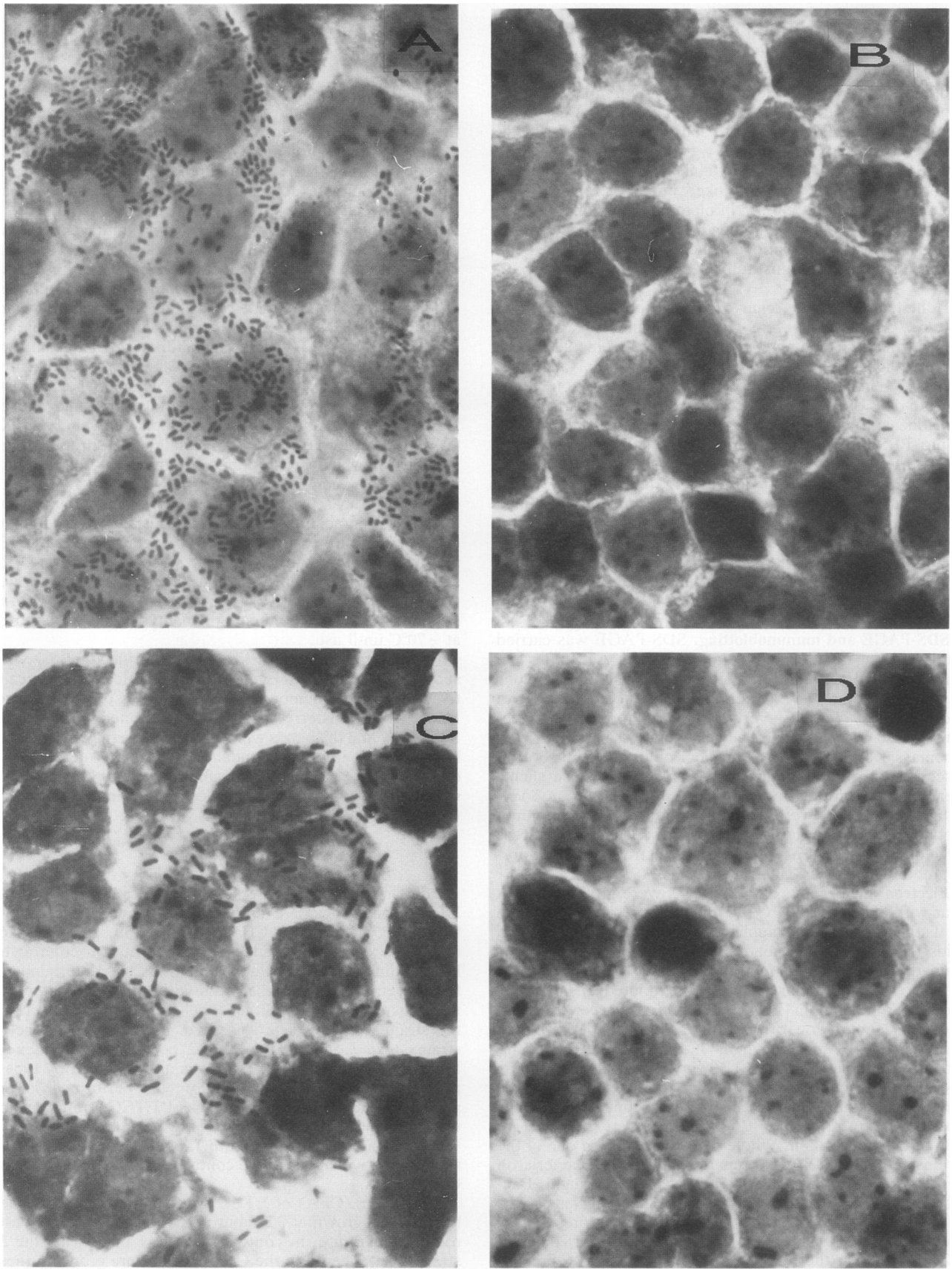

FIG. 1. Micrograph showing adhesion of different ETEC bacteria to tissue-cultured Caco-2 cells. (A) ETEC strain E11881/23 (CS4+ and CS6+); (B) nonadherent E11881/2 strain (CS4− and CS6−); (C) ETEC strain ARG-2; (D) fimbriae-negative mutant ARG-2/1 strain.

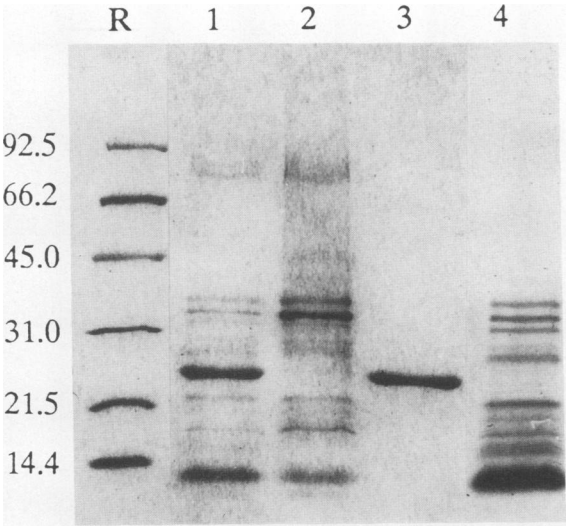

FIG. 2. SDS-PAGE analysis of crude heat extracts of strain ARG-2 and of purified fimbrial antigen. Lanes: 1, heat extract of strain ARG-2 grown on CFA agar at 37°C; 2, heat extract of strain ARG-2 grown on CFA agar at 18°C; 3, purified fimbriae from strain ARG-2; 4, heat extract of the fimbria-negative mutant strain ARG-2/1 grown on CFA agar at 37°C; R, molecular mass markers (in kilodaltons).

grown cell preparations, which was absent in the 18°C-grown cell extract, was seen in 26 of these strains. One ETEC strain producing heat-labile and heat-stable enterotoxins (ARG-2) of serotype O20:K27:H− which presented these characteristics was selected for further examination. SDS-PAGE of bacterial extract of strain ARG-2 grown at 37°C showed a distinct band of 25 kDa (Fig. 2, lane 1), which was absent in the bacterial preparation grown at room temperature (Fig. 2, lane 2). This strain adhered to Caco-2 cells (Fig. 1C) with an index of 25%. ARG-2 did not react in slide agglutination with any of the monoclonal antibodies or polyclonal antisera raised against CFA/I, CS1 to CS7, CFA/III, PCFO159, PCFO166, antigen 2230, and antigen 8786. Antiserum against PCFO9 was not available at the time of the study; however, a comparison of the molecular masses of the respective subunits in SDS-PAGE revealed that the subunit of ARG-2 (25 kDa) was smaller than that of PCFO9 (27 kDa) (data not shown).

Electron microscopy. When strain ARG-2 was grown at 37°C and examined in an electron microscope, heavily fimbriated bacteria were seen (Fig. 3A). The pili were about 6 to 8 nm in diameter and 1 to 2 μm long and were rod-like shaped (Fig. 3B). At higher magnification, the pili appeared as hollow cylindrical structures with a central hole penetrated by the negative stain (data not shown), like CFA/I fimbriae (13). Bacteria grown at room temperature lacked these fimbriae (Fig. 3C).

Purification of the fimbria and preparation of an antiserum. Serial ammonium sulfate precipitations and centrifugations of crude fimbrial preparation yielded purified fimbriae, as suggested by the single band corresponding to the protein subunit in SDS-PAGE (Fig. 2, lane 3). This band corresponded exactly to the band that was found in the crude heat extract of bacteria grown at 37°C which was absent in the room temperature-grown bacteria. Figure 3D shows an electron micrograph in which the purified fimbriae look morphologically identical to those seen on the cell surface.

A polyclonal antiserum raised against the purified fimbriae agglutinated ARG-2 bacteria strongly when they had been grown at 37°C on CFA agar but did not agglutinate the corresponding organisms grown at room temperature. Furthermore, this antiserum did not agglutinate strains expressing CFA/I, CS1 to CS7, CFA/III, CS17, PCFO9, PCFO159, and PCFO166.

A crude extract and purified fimbriae of strain ARG-2 were tested in immunoblots with the antiserum against the purified fimbriae. Only one band (25 kDa) was detected in the heat extract of bacteria grown at 37°C and in the purified fimbrial preparation (Fig. 4). The antiserum was also used to label ARG-2 by the immunogold technique. As shown in Fig. 5A, the gold particles were specifically bound along the fimbriae on bacteria grown at 37°C. In contrast, no gold particles were bound to bacteria grown at room temperature (Fig. 5B).

N-terminal amino acid analysis. The sequence of the first 25 amino acids of the N-terminal part of the protein subunit of fimbriae from strain ARG-2 was determined (Table 1). Comparison between this sequence and sequences of other fimbrial proteins by the sequence analysis program revealed a 43% homology with porcine ETEC 987P. The homology with the N-terminal sequence of other ETEC fimbriae is very low.

Tn*phoA* mutagenesis. Tn*phoA* mutagenesis was used to produce mutants of ARG-2 deficient in the fimbrial structures. After mutagenesis, 140 kanamycin-resistant colonies that were blue on XP agar were isolated and screened by slide agglutination with the polyclonal antiserum raised against the purified fimbriae. Three stable clones which did not react with the specific antiserum in immunoblots were obtained (data not shown). When the heat extracts of one of the mutants (ARG-2/1) was tested in SDS-PAGE, it showed protein bands identical to those of the parent strain, with the exception of a 25-kDa fimbrial subunit protein band that was lacking in the mutant preparation (Fig. 2A, lane 4). In addition, immunoblot analyses of the same mutant showed no reaction with the antiserum raised against purified fimbriae (Fig. 4, lane 4). Electron microscopy of the mutants revealed bacteria which did not express fimbriae on their surfaces (data not shown). Moreover, when one of the transformants (ARG-2/1) was tested in the Caco-2 cell assay, no adhesion was observed (Fig. 1D).

Prevalence of the new fimbriae. The 70 Argentinian strains included in this study were screened for expression of the new fimbriae by slide agglutination, dot blot analysis, and immunoblotting with specific absorbed serum against the purified fimbriae of strain ARG-2. Only one strain, i.e., ARG-2, reacted specifically with the antiserum.

DISCUSSION

In human ETEC, specific adhesins in about 50 to 70% of clinical isolates from different parts of the world have been identified (2, 3, 16, 20, 31). However, there are still many ETEC strains that have been isolated from patients with diarrhea which do not possess any of the hitherto identified colonization factors. Since colonizing ability by means of adhesins is probably a prerequisite for virulence, it is assumed that most clinical ETEC isolates express one or more adhesins. Many efforts have been made to search for new colonization factors in such strains. Different methods have been used for this purpose, e.g., mannose-resistant hemagglutination of erythrocytes from different species (7), aggregation of bacteria with ammonium sulfate to assay hydro-

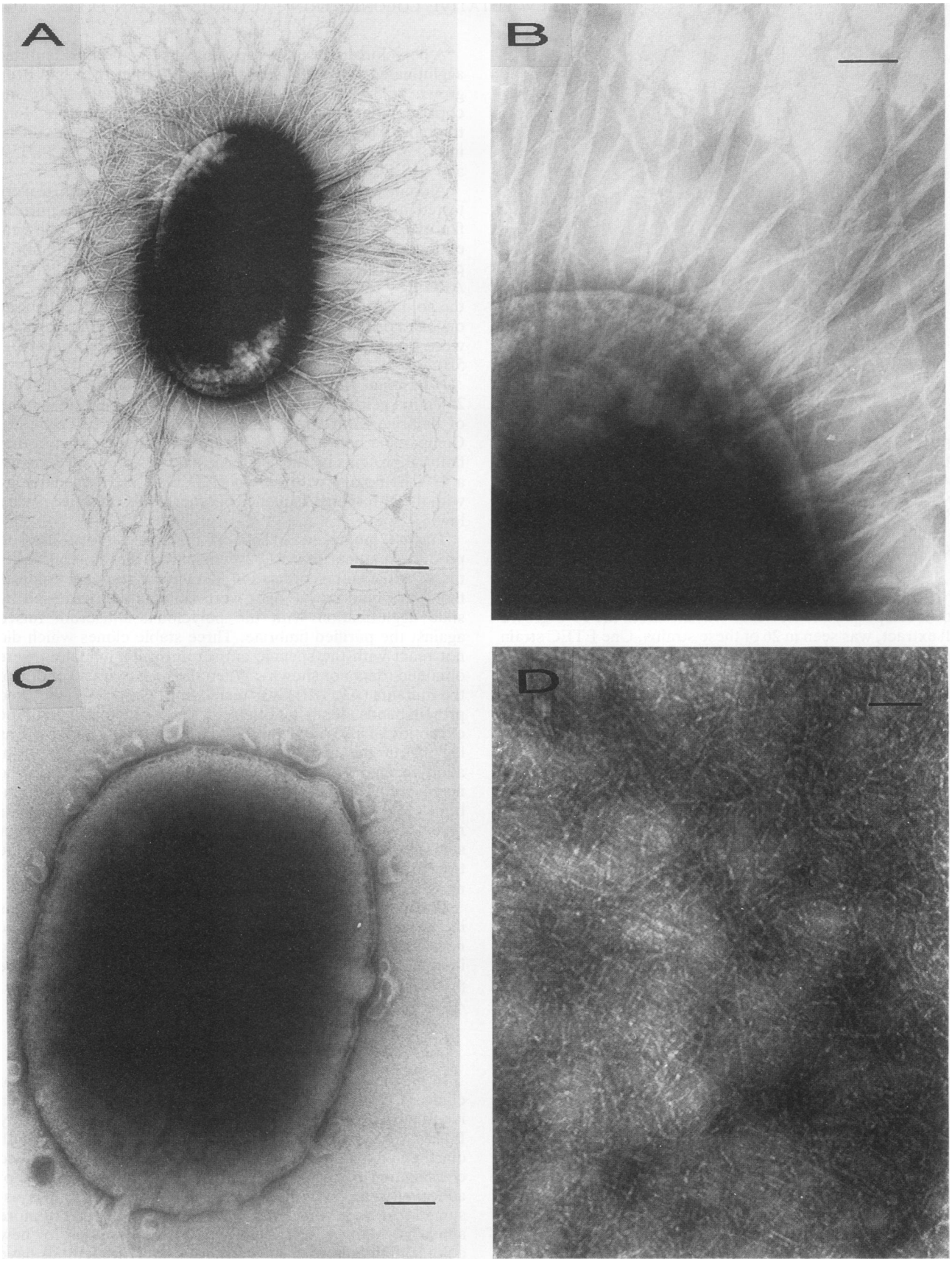

FIG. 3. (A) Electron micrographs of strain ARG-2 grown at 37°C after negative staining with 1% ammonium molybdate (bar = 500 nm); (B) the same preparation at higher magnification, showing fimbriae of about 7 nm in diameter (bar = 100 nm); (C) strain ARG-2 grown at 18°C (bar = 100 nm); (D) purified fimbrial preparation (bar = 50 nm).

FIG. 4. Immunoblot analysis. Lanes: 1, bacterial heat extract of strain ARG-2 grown on CFA agar at 37°C; 2, bacterial heat extract of strain ARG-2 grown at 18°C; 3, ARG-2 purified fimbrial antigen; 4, bacterial heat extract of strain ARG-2/1 grown on CFA agar at 37°C. A rabbit antiserum against the purified fimbriae was used as antibody.

phobicity (12), binding of ETEC to human buccal mucosal cells (5), or adhesion of ETEC to the Caco-2 cell line (1, 4). The Caco-2 cell line, which is derived from a human colonic carcinoma, differentiates into an epithelial cell layer which resembles the small intestinal epithelium (22). Even though these cells do not have properties identical to those of enterocytes from the human small intestine (32), they are probably the best-available cell line to study adhesion of human enteropathogens to intestinal epithelium. Our findings in the present study, i.e., that approximately 50% of the ETEC strains tested that were negative for the established CFAs and PCFs adhere to these cells, suggest that new previously uncharacterized adhesins may be present in these strains. The adhesion observed was not mediated by the mannose-sensitive type 1 pili, since the cell-binding test was performed in the presence of mannose. The strains that did not bind to Caco-2 cells might have lost the genes coding for the adhesin (21) or might express adhesins for which Caco-2 cells do not have receptors (32).

In the present report, we describe the identification, purification, and characterization of a new type of ETEC fimbriae in a strain (ARG-2) isolated from a child with diarrhea in Argentina, for which we propose the designation PCFO20. These fimbriae are antigenically distinct from all other hitherto identified adhesins in human ETEC.

The available data, albeit preliminary, suggest that PCFO20 is a colonization factor. Thus, PCFO20 shares characteristics with most ETEC fimbriae, i.e., temperature-dependent expression of fimbriae and adhesion to enterocyte-like culture cells. The fimbriae have a diameter of ca. 7 nm, and their morphology involves hollow cylindrical rod-

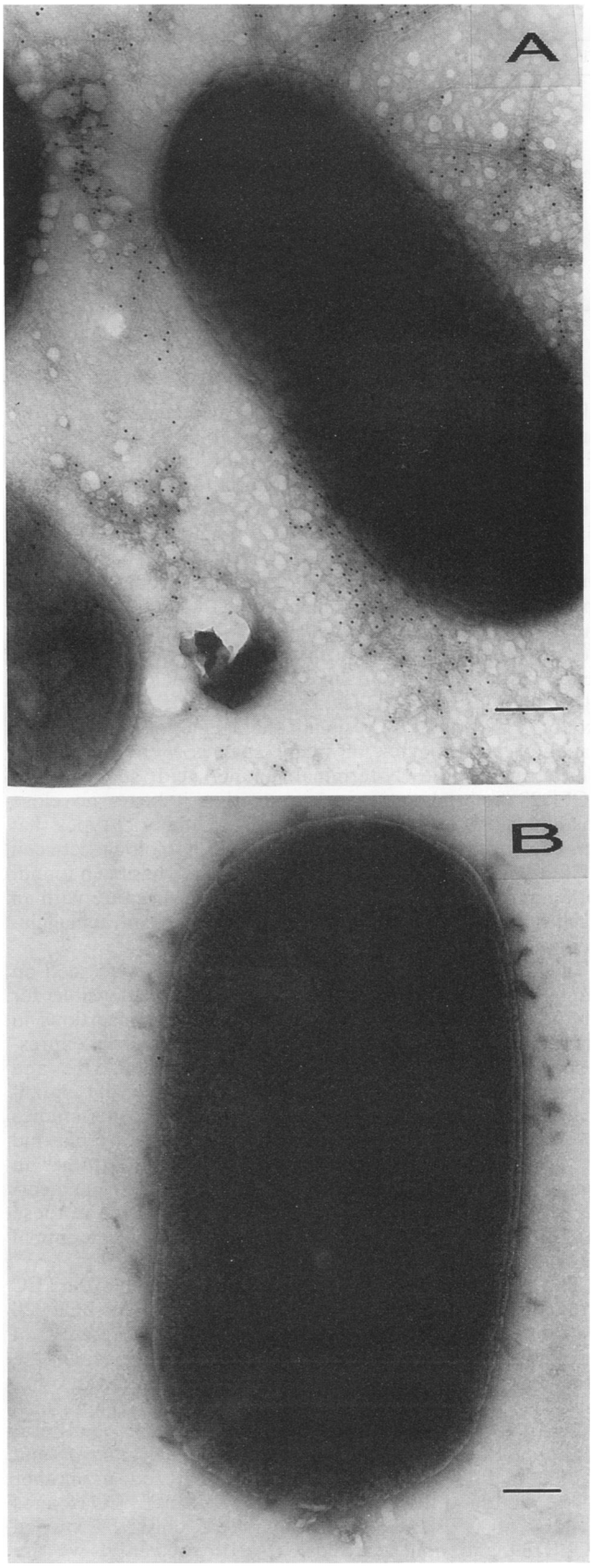

FIG. 5. Electron micrograph showing strain ARG-2 gold immunolabeled with antiserum against purified ARG-2 fimbriae after growth on CFA agar at 37°C (A) and at 18°C (B). Bars = 200 nm.

TABLE 1. Comparison of amino-terminal sequences of some fimbrial subunits

Fimbrial subunit	Sequence[a]
	1* 10* 20*
PCFO20	ALPAN DDSXX ATLDF FTGNV AXLXQ
	1*
987P	PAE NNTSQ ANLD- FTGKV T
	1* 10* 20*
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

[a] 1* indicates the position of the amino acid number 1.

like fimbriae resembling CFA/I. The molecular mass of the protein subunit of PCFO20 was found to be 25 kDa, which is slightly higher that those reported for most other protein subunits of human ETEC colonization factor. Although the protein subunit of PCFO9 has a molecular mass rather similar to that of PCFO20 (27 kDa), comparison of the two bands in SDS-PAGE showed a clear difference in size. Stronger evidence that PCFO9 is distinct from PCFO20 is the fact that PCFO9 is a fibrillar protein, whereas, as already mentioned, PCFO20 is a typical rod-like fimbrium. In addition, antiserum against purified PCFO20 antigen does not react with a strain producing PCFO9. In analogy with some other fimbriae, e.g., PCFO159 and CFA/III, ARG-2 did not exhibit any mannose-resistant hemagglutination when a panel of erythrocytes of many species were tested (2). Furthermore, the N-terminal amino acid sequence of PCFO20 fimbrial antigen suggests that it bears no close relationship with any of the other ETEC fimbrial types that have been examined. However, a 43% homology between PCFO20 fimbriae and 987P of porcine ETEC has been found. Other analogies with 987P are the fimbrial structure with an axial hole and the absence of mannose-resistant hemagglutination.

In this study, we demonstrate that the fimbriae seen on strain ARG-2 in electron microscopy are responsible for the adhesion to Caco-2 cells. Thus, Tn*phoA* insertions in strain ARG-2 that completely abolished the fimbrial expression, as seen by electron microscopy, SDS-PAGE, and immunoblot analyses, resulted in the inability of the mutant strain to adhere to intestinal epithelial cells. Further studies to determine whether the fimbriae are encoded by plasmid or chromosomal DNA are in progress. Future studies in which the fimbrial genes are inactivated by genetic engineering will allow precise evaluation of the importance of these fimbriae, independent of toxin, in the disease in an animal model.

The low frequency (1.4%) of PCFO20 among the 70 ETEC strains of this study may be because the search for PCFO20 was done retrospectively, i.e., on strains subcultured several times with the risk of loss of PCFO20-coding genes. On the other hand, this frequency is similar to that found in other retrospective epidemiological studies (20, 31). Thus, in a study of 196 strains from Burma, central Africa, and Peru, McConnell found PCFO159 in 0.5%, CFA/III in 0.5%, and PCFO9 in 1.5% of the ETEC isolates analyzed; in another study, we found that among 105 Argentinian ETEC isolates negative for CFA/I, CFA/II, and CFA/IV, only 3.8% carried PCFO159 and none carried CFA/III. Presently, we are performing a prospective study in Argentina in which the prevalence of PCFO20 in fresh clinical isolates will be evaluated.

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