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The Structural Gene Encoding Human Enterotoxigenic *Escherichia coli* PCFO20 Is Homologous to That for Porcine 987P

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Putative colonization factor PCFO20 was recently identified in an enterotoxigenic *Escherichia coli* (ETEC) strain of serogroup O20 isolated from a child with diarrhea in Argentina. The gene encoding the structural subunit of PCFO20 fimbriae, *fotA*, was cloned from strain ARG-2 in the expression phage vector lambda ZAP Express. One positive clone, pGV29, that carried a 3.3-kb fragment was identified on the basis of fimbrillin production by using a monospecific rabbit anti-PCFO20 serum. Nucleotide sequencing of a 1.3-kb *Sau*3A-*Cla*I fragment of the subclone pGV292 containing the region coding for PCFO20 fimbrillin revealed two open reading frames of which one was complete. A Western blot (immunoblot) showed that the cloned protein, FotA, migrated like the PCFO20 fimbrial subunit protein did. Fimbriae were not detected on the surface of *E. coli* host bacteria containing pGV292 or pGV29, suggesting that the genes needed for assembly of PCFO20 fimbriae are lacking in both clones. The *fotA* gene encodes a 20,574-Da prefimbrillin protein which contains a 21-amino-acid signal sequence; the mature protein has a size of 18.1 kDa. The subunit protein FotA was found to be more homologous to the subunit of porcine 987P than to any fimbrial subunit produced by human ETEC. Alignments of the amino acid sequences of the two proteins indicate that they are partly identical, with an overall similarity of 82%. FotA fimbrillin was shown to be transported and assembled by the fimbria assembly machinery in porcine ETEC strain 987. PCFO20 and 987P may have evolved from a common ancestral gene. They are immunologically related but have affinity for different host cell receptors, since PCFO20-producing bacteria do not bind to neonatal piglet enterocytes.

Fimbriae are bacterial adhesins of great importance in specific binding of gram-negative bacteria to eukaryotic cell receptors and subsequent colonization of the host tissue (11, 44). They are rod-like structures composed of hundreds of copies of identical polypeptides designated major fimbrial subunits or fimbrillins. These fimbrillins are produced as precursors having an N-terminal signal sequence which is subsequently removed during export across the inner membrane to the periplasm. Translocation across the inner membrane is dependent on the general secretory pathway of *Escherichia coli* (35). However, for most fimbriae, further export across the outer membrane to the cell exterior and assembly at the cell surface are dependent on the fimbria-specific export and assembly system, consisting of a periplasmic chaperone molecule and a so-called usher protein located in the outer membrane (18, 26). The genes encoding these different proteins are organized in gene clusters often constituting one or two units of transcription.

Fimbriae produced by enterotoxigenic *E. coli* (ETEC) enable bacteria to colonize the small intestine and cause diarrhea (5). Several colonization factor antigens and putative colonization factors have been described for ETEC (11, 28). Most of them are fimbriae with a diameter of approximately 7 nm or flexible, thin fibrillae with a diameter of 2 to 5 nm; they can also be nonfimbrial (11). Recently, a bundle-forming type of pilus, named Longus, was also reported for ETEC (15). Recent epidemiological studies of ETEC isolated from children with diarrhea in Argentina (4, 21, 45) revealed that there is still a high proportion (ca. 35%) of ETEC isolates in which no colonization factor antigen or putative colonization factor was detected. Since colonizing ability by means of adhesins is probably a prerequisite for virulence, it is assumed that most clinical ETEC isolates produce one or more colonization factors. Thus, the identification and molecular characterization of new adhesins are important not only for extending the knowledge on these organelles but also for developing vaccines and for modifying already existing vaccines.

We have recently identified a "new" putative colonization factor in an ETEC strain of serogroup O20 that was named PCFO20 (46). This strain, ARG-2, which produces both heat-labile toxin (LT) and heat-stable toxin (ST), was isolated from a child with diarrhea in Argentina. ARG-2 has been shown to bind to Caco-2 cells and to be heavily fimbriated (46). The rigid fimbriae are composed of subunits with an apparent molecular size of 25 kDa determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fimbriation, adhesion to Caco-2 cells, and production of the 25-kDa protein were all simultaneously lost by a single insertion mutation, suggesting that the fimbriae seen in electron microscopy are responsible for the adhesion to intestinal epithelial cells. The N-terminal amino acid sequence of the PCFO20 subunit protein is not related to corresponding sequences of any of the known colonization factors of human ETEC. However, a considerable degree of homology was found between the N-terminal sequence of PCFO20 and that of the subunit of 987P fimbriae of porcine ETEC. Eight genes have been shown to be required for expression of 987P fimbriae and adhesin, i.e., the genes coding for the major fimbrial subunit, the adhesin, and the proteins involved in transport and assembly and the regulator gene (22, 23). Bacteria producing 987P adhere to neonatal piglet enterocytes but not intestinal epithelial cells from weaned pigs (10).

To further characterize PCFO20, we have isolated the genetic determinant of its fimbrial subunit protein from the

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ETEC strain ARG-2. We hereby describe the molecular cloning and the complete open reading frame (ORF) encoding the structural subunit of PCFO20 and analyses of the homology between PCFO20 and 987P porcine fimbriae. We propose to name the described genes *fotA* and *fotB* and the fimbrial subunit FotA (for "PCFOtwenty").

MATERIALS AND METHODS

Bacterial strains, plasmid, bacteriophage, and media. The ETEC strain ARG-2 (LT/ST, serotype O20:K27:H−) (46) was used as a source of DNA. ARG-2/1, a PCFO20-negative Tn*phoA* insertion mutant, has been described earlier (46). *E. coli* XL1-Blue MRF′ (Stratagene, La Jolla, Calif.) was used for growing and screening lambda ZAP Express clones, and *E. coli* XLOLR was used for subcloning and sequencing. The phage lambda ZAP Express (Stratagene) was used as a vector for cloning fragments of genomic DNA, and subclones were constructed in plasmid pBK-CMV (Stratagene). The strains were grown in Luria-Bertani broth or on NZY agar when recommended. Casamino Acids-yeast extract agar (CFA agar) (13) was used to maximize expression of the fimbriae. Antibiotics were used at the following concentrations when necessary: tetracycline, 12.5 μg ml^{-1}; kanamycin, 50 μg ml^{-1}.

DNA preparation and manipulation. Genomic DNA from ARG-2 was prepared by digestion with proteinase K and precipitation with cetyl-trimethylammonium bromide (CTAB) as described previously (1), and plasmid DNA was isolated with the Wizard Miniprep system (Promega Corp., Madison, Wis.).

Agarose gel electrophoresis, Southern blotting, use of restriction endonucleases and other enzymes, and transformation of *E. coli* were performed essentially by standard techniques (37). Wizard DNA Clean Up (Promega) was used to purify DNA from enzymes and buffer salts. Nonradioactive labeling of DNA and hybridization analyses were done as described in the instructions supplied with the digoxigenin labeling and detection kit used (Boehringer GmbH, Mannheim, Germany).

Construction of a lambda ZAP Express phage library. DNA fragments resulting from partial *Sau*3A restriction endonuclease treatment of ARG-2 bacterial genomic DNA were separated by agarose gel electrophoresis, and 1.6- to 12-kb fragments were isolated by electroelution, purified with Wizard DNA Clean Up, and ligated into *Bam*HI-predigested lambda ZAP Express vector (Stratagene). The resulting recombinant phages were packaged by use of a lambda in vitro packaging system (Stratagene) and plated with XL1-Blue MRF′ host cells. Recombinant ZAP Express phages were screened by testing nitrocellulose plaque replicas (after an additional 16-h incubation on fresh NZY agar) for production of PCFO20 fimbrial protein with and without isopropyl-β-D-thiogalactopyranoside (IPTG), the inducer of the *lac* promoter in the vector. A rabbit antiserum against purified PCFO20 fimbriae (46) absorbed with XL1-Blue MRF′ was used as the primary antibody and a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody in immune detection. Positive plaques were rescreened and purified. The cloned DNA fragments in PCFO20-producing recombinant phages were rescued in pBK-CMV plasmid by in vivo excision from the ZAP Express vector.

DNA sequencing. DNA sequencing was performed by the dideoxy chain terminator (38) method. Double-stranded plasmids were used as template DNA. Oligonucleotides corresponding to the T3 and T7 promoter regions flanking the cloned DNA, and nucleotides subsequently designed from the obtained sequences, were used as primers. Nucleotide or protein database searches were performed with FASTSCAN or Blast Network Service. Sequence alignments were performed with NALIGN, PALIGN, or CLUSTAL programs by using the Software PCGENE (Intelligenetics, Inc., Mountain View, Calif.). Promoter consensus sequences were also searched by using the Mac Vector 4.1 program (Eastman Chemical Co.).

In vitro transcription and translation. In vitro transcription and translation experiments were performed with a prokaryotic DNA-directed translation kit (Amersham) based on Zubay's method as modified by Collins (8). The [^{35}S]methionine-labeled polypeptides synthesized with circular plasmid DNA as the template were separated by electrophoresis through an SDS–16% polyacrylamide gel and autoradiographed as recommended by the manufacturers.

Detection of PCFO20. The production of fimbrillin protein was determined by a dot blot test and Western blotting (immunoblotting) of whole bacteria with PCFO20-specific antiserum (46). Surface localization of PCFO20 was analyzed by an inhibition enzyme-linked immunosorbent assay (ELISA) (27) and by immunoelectron microscopy (IEM) (46) with the same antiserum. Binding of bacteria to Caco-2 cells was assayed as described previously (46, 47).

Adhesion to neonatal piglet enterocytes. Microscopic adhesion assays were performed as described previously (9, 10, 40) with brush borders isolated from <1-day-old hysterectomy-derived, colostrum-deprived pigs and stored in 20% glycerol at −80°C. Bacteria were grown for 18 h in Trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.), with shaking at 200 rpm, in a 37°C water bath. Adherence mixtures contained 1% methyl-α-D-mannopyranoside to inhibit mannose-sensitive binding. The 987P-positive *E. coli* strain 987 (33) and the 987P-negative *E. coli* strain I36 (19) were included as adhesive and nonadhesive controls, respectively. All cultures were tested for expression of 987P fimbriae by slide agglutination with a rabbit antiserum against 987P (34).

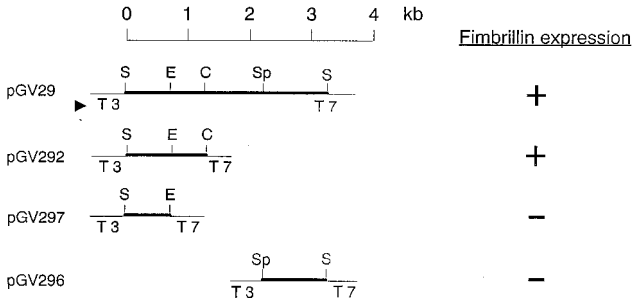

FIG. 1. Restriction map of the inserts in pGV29 and derivative plasmids. The relevant restriction sites (abbreviations: S, *Sau*3A; E, *Eco*RI; C, *Cla*I; Sp, *Spe*I) as well as the expression of PCFO20 fimbrillin protein analyzed by Western blot in XLOLR containing the different plasmids are shown. The orientation of the *lac* promoter is indicated with an arrowhead.

DNA probe and colony hybridization. The 987P DNA subunit probe, a 360-bp *Bgl*II-*Hpa*II fragment derived from recombinant plasmid pPM200 (31), was prepared as described by Nagy et al. (32). Probe DNA was labeled with [α-^{32}P]deoxycytidine with the random primer labeling kit which employs Klenow polymerase. High- and low-stringency colony hybridization analyses were performed as described previously (30).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the GenBank database under accession number U31413 (*fotA*) and U31414 (*fotB*).

RESULTS

Cloning of the PCFO20 fimbrillin gene. A lambda ZAP Express library containing 1.6- to 12-kb DNA fragments of genomic DNA from the PCFO20-positive ARG-2 strain was constructed. Approximately 15,000 plaques were tested for production of PCFO20 protein with the specific anti-PCFO20 antiserum. Five clones were strongly positive in the immune reaction after purification of the plaques. The cloned DNA from these plaques was excised directly from the lambda ZAP Express vector in pBK-CMV double-stranded phagemid and plated with the *E. coli* strain XLOLR to allow replication in this host. After screening of the resulting colonies with PCFO20 antiserum, only one clone, XLOLR(pGV29), was positive. This clone expressed PCFO20 protein in colony blots in the presence and absence of IPTG, the inducer of the *lac* promoter.

By utilizing *Cla*I, *Eco*RI, and *Spe*I restriction sites, present both in the polylinker of the vector and in the 3.3-kb insert, the subclones pGV292, pGV297, and pGV296 were created after digestion and religation (Fig. 1).

Detection of fimbrillin by immunoassays. Dot blot and Western blot assays were performed to determine in which clones immunoreactive PCFO20 fimbrillin was produced. Antiserum against PCFO20 reacted in the dot blot analysis with bacteria containing pGV29 and more weakly with those containing pGV292 but not with XLOLR harboring pGV297 or pGV296. Western blot analyses showed that both XLOLR(pGV29) and XLOLR(pGV292) produced the mature fimbrillin protein FotA, which was absent in XLOLR(pGV297) and the host strain (Fig. 2). The clone harboring plasmid pGV292 revealed an additional band, about 2 kDa larger than the fimbrial subunit, that may correspond to the unprocessed prefimbrillin protein pre-FotA (Fig. 2). The results suggest that more than 0.7 kb and possibly less than 1.3 kb of the cloned DNA downstream from the 5′ end *Sau*3A restriction site is needed for production of the PCFO20 fimbrial subunit (FotA).

In vitro transcription-translation analysis. To confirm the existence of a precursor for the PCFO20 fimbrillin, pGV29 was

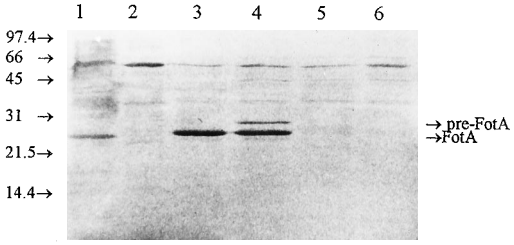

FIG. 2. Western blot analysis of wild-type ETEC ARG-2, ARG-2/1 (PCFO20-negative Tn*phoA* insertion mutant), and the clones containing the recombinant plasmids. Lanes: 1, ARG-2; 2, ARG-2/1 mutant; 3, XLOLR (pGV29); 4, XLOLR(pGV292); 5, XLOLR(pGV297); 6, XLOLR host strain. The numbers on the left indicate molecular sizes in kilodaltons.

analyzed via a DNA-dependent coupled transcription-translation in vitro system. Since the vector itself can direct synthesis of a protein (neomycin phosphotransferase) with a size close to that of the predicted PCFO20, the pGV48 clone, which failed to react with the antiserum, was included as a negative control. The autoradiogram of the radiolabeled synthesized products shows that pGV29 produced one prominent protein band with an apparent molecular mass similar to that of pre-FotA (Fig. 3). This protein was not produced by the negative control (pGV48).

Adhesion assay using Caco-2 intestinal epithelial cells. To determine whether the PCFO20 fimbrillin produced by the clones conferred adhesive properties to the host organisms, bacteria harboring the different plasmids were tested for adhesion to Caco-2 cells. None of the clones, including XLOLR (pGV29) and XLOLR(pGV292), which were shown to produce fimbrillin in a Western blot, adhered to Caco-2 cells. As expected, the parent strain, ARG-2, adhered well to this cell type.

Localization of the fimbrillin protein. To evaluate whether the fimbrial protein in the clones was surface located, a whole-cell inhibition ELISA was performed with anti-PCFO20 serum. Only the wild-type ARG-2 strain was able to inhibit the binding of anti-PCFO20 to solid-phase-bound PCFO20 fimbrial antigen, suggesting that none of the clones expresses fimbriae on the surface. This was confirmed by IEM. However, antibodies bound only weakly to the surface of XLOLR (pGV29), and no fimbrial structure was visible.

The nucleotide sequence of the fimbrillin gene. The nucleotide sequences of both strands of the 1.3-kb *Sau*3A-*Cla*I fragment of pGV292 containing the region encoding PCFO20 fimbrillin were determined (Fig. 4). Two ORFs encoded by the same strand were found. A complete 614-bp ORF has two potential in-frame ATG initiation codons at positions 246 and 261 and a stop codon at position 861. The presence of two lysine residues following the first methionine suggests that the first ATG could be the start point of translation, since the resulting product would contain a typical prokaryotic signal peptide (48). The predicted cleavage site of the prefimbrillin protein is between amino acids 21 and 22. In addition, the deduced N-terminal amino acid sequence of the mature protein is similar to that previously reported (46). A strong potential ribosome binding site (41) was found at an optimal distance from the first ATG. Two promoter consensus sequences (16) corresponding to the −35 region and the −10

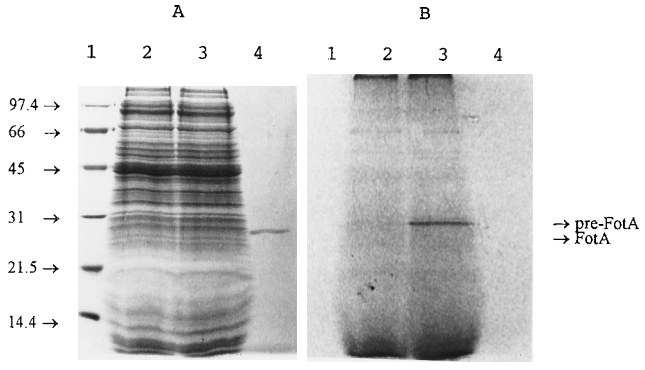

FIG. 3. In vitro transcription-translation products analyzed by SDS-PAGE (A) and autoradiography (B). [^{35}S]methionine-labeled polypeptides synthesized by pGV29 and pGV48. Lanes: 1, molecular mass reference; 2, products from pGV48; 3, products from pGV29; 4, purified PCFO20 fimbriae. The numbers on the left indicate molecular sizes in kilodaltons.

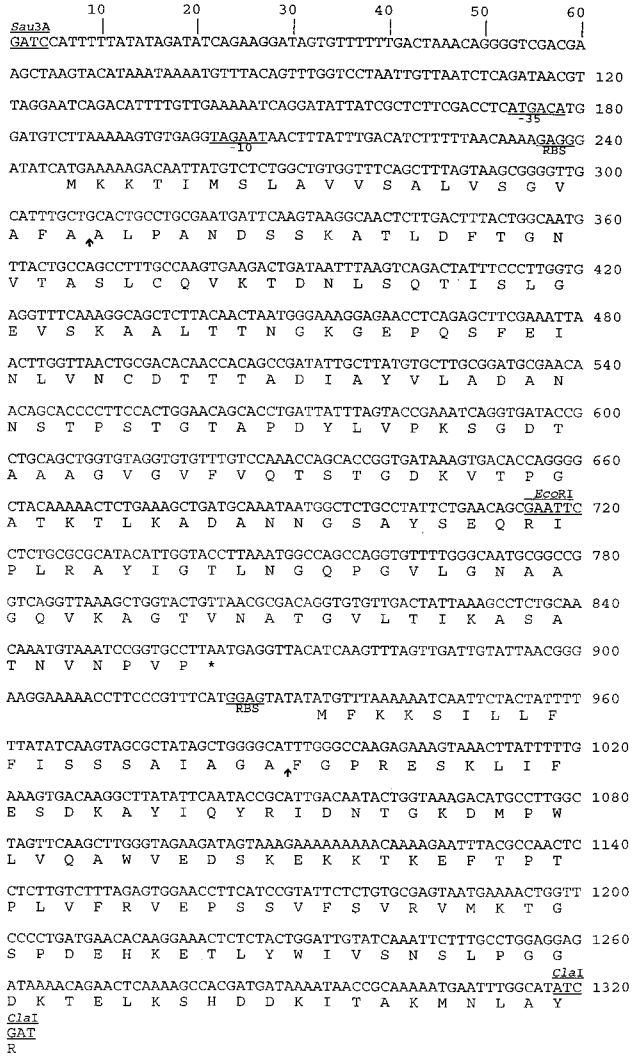

FIG. 4. Nucleotide sequence of the gene encoding PCFO20 fimbrial subunit and its flanking regions. The nucleotide sequence of a 1,323-bp *Sau*3A-*Cla*I fragment has been numbered starting from the *Sau*3A site. The deduced amino acid sequences are given below the nucleotide sequence. The restriction sites *Sau*3A, *Eco*RI, and *Cla*I, the promoter consensus sequences −10 and −35, and the putative ribosome binding sites (RBS) are underlined and labeled. The cleavage sites for signal peptidase are indicated with arrows. The stop codon is indicated by an asterisk.

FIG. 5. Comparison of the deduced primary structure of PCFO20 fimbrillin with the amino acid sequence of the 987P fimbrial subunit. Identical amino acids are boxed (96 of 171), and well-conserved residues are marked with an asterisk. Three and six gaps have been introduced in the sequences of PCFO20 and 987P fimbrillins, respectively, to obtain maximal sequence homology.

region were found at positions 175 and 204, respectively. This ORF, *fotA*, starting at the first initiation codon, would encode a polypeptide of 205 amino acids with a molecular mass of 20,574 Da. The predicted molecular mass of the mature FotA fimbrillin is 18,100 Da, a size considerably smaller than that suggested by SDS-PAGE analysis. The predicted isoelectric point of the protein is 6.53. The deduced amino acid sequence contained two cysteine residues in the N-terminal part of the protein that are likely to form a disulfide bridge.

Analysis of the second ORF, designated *fotB*, revealed two methionine codons at positions 933 and 1071. If transcription starts at the first methionine, the deduced amino acid sequence presents a signal sequence with a probable cleavage site between amino acids 19 and 20. A potential ribosome binding site was found five nucleotides upstream from the first methionine. No stop codon was found, suggesting that the gene extends beyond the cloned fragment. Analyses of sequence homology of the translation product of the incomplete *fotB* indicated that it is highly homologous to several fimbrial chaperones, e.g., FinC of type 1 fimbriae of *Salmonella typhi* (36), FimC (43) and LpfB of *Salmonella typhimurium* (2), PapD of P fimbriae (20), and ClpE of CS31A fimbriae (3). Notably, all but one of the conserved (invariant) amino acid residues within this region of proteins in the chaperone family (17) are found in FotB. The exception is a lysin replacing the consensus arginine at position 7 of the predicted mature FotB.

Genetic and antigenic relationship between PCFO20 and 987P. Homology searches in EMBL, DDBJ, and GenBank with the FASTSCAN program showed that the sequence with the highest score of similarity with *fotA* was that of the porcine ETEC 987P fimbrial subunit gene. Alignment of the two nucleotide sequences by the NALIGN program revealed that 341 bases of a 585-nucleotide overlap were identical (58.3% nucleotide homology). However, this homology was not enough to allow hybridization of ARG-2 DNA with a 987P probe. Similarly, 987P DNA was not hybridized when a *Sau*3A-*Cla*I ARG-2 DNA fragment labeled with digoxigenin was used as a PCFO20 probe in analyses performed at high (68°C) or low (45°C) stringency (data not shown). Comparison of the deduced amino acid sequence of FotA with that of the fimbrial subunit of 987P (Fig. 5) showed that they are partly identical (56%), with an overall similarity of 82%. In addition, comparison of the FotA sequence with amino acid sequences of various fimbrial subunits showed that PCFO20 and 987P fimbrillins are considerably related to other *E. coli* fimbrial types, i.e., type 1C, K99, and P fimbriae. Alignment of these proteins by the CLUSTAL program showed that the fimbrillins in this group have many conserved residues, including the two cysteines in the amino-terminal half of the subunit that are responsible for the formation of disulfide bonds. However, very poor homology was found when PCFO20 was compared with other fimbriae of human ETEC (data not shown).

To evaluate whether PCFO20 and 987P share some immunoreactive epitopes, ARG-2 and 987 bacteria were tested for cross-reactivity in a dot blot using anti-987P and anti-PCFO20 sera, respectively. No cross-reactivity was found; i.e., strains ARG-2 and 987 reacted with the corresponding homologous antisera but not with the heterologous sera (data not shown). Since common epitopes could be masked, we performed a Western blot analysis to evaluate the cross-reactivity between denatured PCFO20 and 987P fimbrial proteins. A weak cross-reactivity was revealed when purified PCFO20 and 987P fimbriae were tested against anti-987P and anti-PCFO20 sera, respectively (data not shown).

Expression of FotA in porcine ETEC 987. Given the strong homology between FotA and 987P fimbrillins, we wanted to investigate whether the fimbrial assembly systems of 987P and PCFO20 were functionally related. Wild-type ETEC strain 987 (987P positive) was transformed with plasmid pGV29 or pGV292 to let FotA fimbrillin compete with 987P subunits in the fimbrial assembly. The resulting Kn^r 987 transformants, carrying either pGV29 or pGV292, were found to be heavily fimbriated (Fig. 6A). Both PCFO20 and 987P subunits were present in these fimbriae as demonstrated by IEM with immunogold labeling using anti-PCFO20 and anti-987P antisera, respectively (Fig. 6B to D). The control wild-type strain 987 did not react with anti-PCFO20 antiserum (Fig. 6F), which on the other hand reacted with the homologous ARG-2 bacteria (Fig. 6E).

Since pGV292 has only one complete ORF, *fotA*, these results suggest an ability of the fimbrial assembly components of porcine ETEC 987 to replace the corresponding export machinery of the PCFO20 strain.

Adherence assay using neonatal piglet enterocytes. Considering the strong structural homology between PCFO20 and 987P, we explored the possibility that these two types of fimbriae might share the same binding motif. Since 987P-positive strains have been shown to adhere to neonatal piglet enterocytes (10, 19), we assessed the capacity of PCFO20 to mediate bacterial adhesion to these intestinal epithelial cells. Adherence assays showed that neither PCFO20 reference strain ARG-2 nor the negative mutant ARG-2/1 adhered to neonatal porcine brush borders. Therefore, it is not likely that PCFO20-positive strains bind to the same receptor as 987P-expressing bacteria.

DISCUSSION

In this study, we have isolated the PCFO20 fimbrillin structural gene, *fotA*, from genomic DNA of ETEC strain ARG-2 in the expression vector lambda ZAP Express, with a specific antiserum against PCFO20. In addition, we have extended our earlier observation on the similarity between human ETEC PCFO20 and porcine 987P by showing that the subunits of these fimbriae have extensive homology throughout the whole primary amino acid sequence.

The deduced amino acid sequence of the mature fimbrial subunit FotA constitutes a protein of 18.1 kDa. This value is

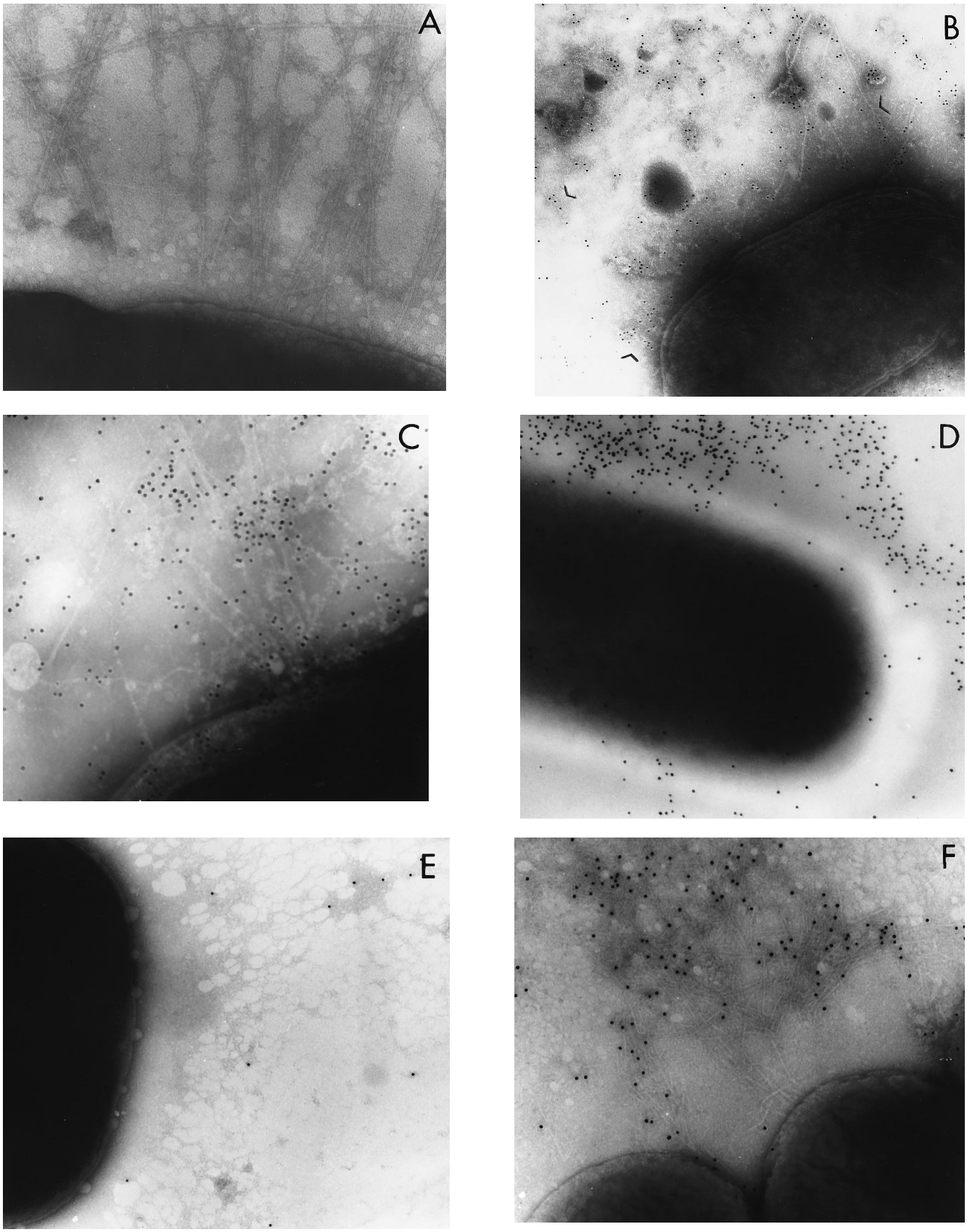

FIG. 6. (A) Electron micrograph of negatively stained ETEC strain 987 transformed with pGV292 plasmid; (B and C) immunogold-labeled 987(pGV292) bacteria reacting with anti-PCFO20 serum; (D) immunogold-labeled 987(pGV292) bacteria reacting with anti-987P serum; (E) 987 bacteria incubated with anti-PCFO20 serum; (F) ARG-2 bacteria reacting with anti-PCFO20 serum.

considerably smaller than that previously estimated by SDS-PAGE, i.e., 25 kDa (46). However, the abnormal migration in SDS-PAGE appears to be a common feature for other fimbrial subunits, such as the 17.5-kDa subunit of 987P migrating at 21.5 kDa (12) and CS31A (14). It has been proposed that the conformation adopted by the dissociated subunit may be one explanation for the abnormal migration of some fimbrial protein in SDS-PAGE (12).

The second ORF, *fotB*, is very likely to be part of the same operon as *fotA*, since both genes are situated in the same reading frame, very close to each other. In addition, no obvious promoter sequence was found upstream from *fotB*, suggesting that both genes constitute the same unit of transcription. Although *fotB* is not complete, it can be inferred that it codes for a protein that is transported through the cytoplasmic membrane since the first 19 amino acids in the sequence resemble a typical signal peptide (48). The high homology found between FotB and some chaperone proteins of other fimbrial types suggests that it might be involved in the transport of the fimbrillins across the periplasm to the outer membrane.

Immunoelectron microscopy and inhibition ELISA indicated that no PCFO20 fimbriae were exposed on intact cells carrying pGV29 or pGV292, suggesting that proteins in addition to those encoded by these plasmids are needed for the biogenesis of the fimbrial structure. However, products of the genes present in pGV29 might help in the transport of the fimbrillins to the bacterial surface, although fimbrial subunits are not assembled, since XLOLR(pGV29) bacteria are positive in a dot blot and are weakly labeled in IEM. The unexpected positive reaction obtained in a dot blot with pGV292, which has only one complete ORF, *fotA*, is probably due to leakage of PCFO20 fimbrillins from the periplasm. Furthermore, the fimbrillin FotA produced by clone pGV292 is not totally cleaved, and it is very likely that the unprocessed proteins remain stuck in the inner membrane. The existence of unprocessed FotA in pGV292 but not in pGV29 cells further suggests that in the absence of a chaperone, the turnover in the production of mature fimbrillin is slowed. It has been proposed that pili associated with most human ETEC strains constitute an evolutionarily related family (25). The amino acid sequences of the CFA/I and CS1 structural subunits are 92% similar and have 55% identical residues (25). The N-terminal amino acid sequence of a number of other fimbrial subunits from human ETEC, i.e., CS2, CS4, PCFO166, and CS17, is very similar to those of CFA/I and CS1 (25, 42). Moreover, all of these fimbriae can be positively regulated by a similar protein (6), and all lack a protein with the features of the known chaperone proteins of *E. coli* fimbrial operons, suggesting that a close relationship exists among all of these fimbrial operons. However, PCFO20 is unrelated to this type of fimbria, being more related to 987P, a porcine ETEC fimbria. Another fimbria of human ETEC, CS13 (previously referred to as PCFO9), has been shown to be more related to other animal ETEC fimbriae than to human ETEC fimbriae (7). Alignment scores indicate significant structural similarity between PCFO20 and 987P fimbrial proteins. Highly conserved regions, containing many identical amino acid residues, were found at the same position within the two sequences. Despite this homology, the two fimbriae are only weakly related immunologically; cross-reactive epitopes were detected only when fimbrial antigens were denatured. Most likely, dominating antigenic domains correspond to regions of variability between PCFO20 and 987P that might have evolved as a result of selective pressure of the host. On the other hand, constant regions are probably less well exposed and may be of importance in the biogenesis of the fimbriae.

It has been proposed that phylogenetic relationships between fimbrial gene clusters can probably be best inferred from comparison of the intracellular accessory proteins needed for export of the fimbriae to the exterior since they are less subject to selective pressures caused by the host factors, such as immune responses and variation in receptors (29). The PCFO20-gold-labeled bacteria observed by IEM after transformation of a 987P-positive strain with plasmids containing the *fotA* gene are an indication, yet not a proof, that the biosynthesis machineries of the two fimbriae are functionally similar. We cannot rule out the possibility that the PCFO20 fimbrial subunit could be assembled by an additional unknown adhesin export system present in *E. coli* 987. However, this seems unlikely since numerous studies performed with ETEC strain 987 have never suggested the existence of an extra adhesin. Experiments in which pGV29 is introduced into an *E. coli* strain containing plasmids with all of the genes for production of functional 987P fimbriae, except for the fimbrial subunit gene, would give stronger evidence of the homology between the two export machineries. Furthermore, the inability of the accessory system of an unrelated fimbria to mediate expression of PCFO20 fimbriae would further support this theory. Nevertheless, previous studies (24) have demonstrated that functional expression of heterologous fimbrial subunits can be mediated by accessory genes that are closely related genetically but not by those of an unrelated export system.

The major fimbrial subunit of CFA/I and that of K88 fimbriae mediate receptor binding (11). However, a protein different from the major fimbrial subunit mediates adhesion in 987P (22), type I, and P fimbriae (25). Whether FotA or other minor fimbrial proteins are responsible for the adhesion of PCFO20-producing ETEC to epithelial cells remains to be elucidated. Besides, our results suggest that PCFO20 and 987P have affinity to different binding structures, since only strain 987 adheres to these piglet enterocytes. It is tempting to speculate that PCFO20 and 987P adhesins stem from a common ancestor, which during evolution has adapted itself to occupy different host receptors.

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