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Gene Cluster for Assembly of Pilus Colonization Factor Antigen III of Enterotoxigenic *Escherichia coli*

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The assembly of pilus colonization factor antigen III (CFA/III) of enterotoxigenic *Escherichia coli* (ETEC) requires the processing of CFA/III major pilin (CofA) by a prepilin peptidase (CofP), similar to other type IV pilus formation systems. CofA is produced initially as a 26.5-kDa preform pilin (prepilin) and then processed to a 20.5-kDa mature pilin by CofP which is predicted to be localized in the inner membrane. In the present experiment, we determined the nucleotide sequence of the whole region for CFA/III formation and identified a cluster of 14 genes, including *cofA* and *cofP*. Several proteins encoded by *cof* genes were similar to previously described proteins, such as the toxin-coregulated pili of *Vibrio cholerae* and the bundle-forming pili of enteropathogenic *E. coli*. The G+C content of the *cof* gene cluster was 37%, which was significantly lower than the average for the *E. coli* genome (50%). The introduction of a recombinant plasmid containing the *cof* gene cluster into the *E. coli* K-12 strain conferred CFA/III biogenesis and the ability of adhesion to the human colon carcinoma cell line Caco-2. This is the first report of a complete nucleotide sequence of the type IV pili found in human ETEC, and our results provide a useful model for studying the molecular mechanism of CFA/III biogenesis and the role of CFA/III in ETEC infection.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhea in children and travelers in developing countries. The ability of ETEC to adhere to and colonize the intestinal epithelium is an essential step for pathogenicity in addition to the ability to produce heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST) (23). The colonizing ability of human ETEC depends on the presence of colonization factors (CFs) on the surface of the cells, which usually form pili (or fimbriae). Several types of colonization factor antigens (CFAs) and putative colonization factors (PCFs) have been identified on the basis of antigenic specificity and/or N-terminal amino acid sequence of the major subunit (pilin), e.g., CFA/I, CFA/II, CFA/III, CFA/IV, PCFO148, PCFO159, PCFO166, antigen 2230, and antigen 8786 (7, 23). Among these, CFA/II and CFA/IV are heterogeneous and consist of a complex of different antigens named coli surface (CS) antigens. CFA/II is composed of CS1, CS2, and CS3, which are present in different permutations. Similarly, CFA/IV is composed of CS4, CS5, and CS6. Epidemiologic studies indicated that CFA/I- or CFA/II-carrying ETEC strains seem to be the most prevalent and a wide variation in CFs was found in different parts of the world (24, 27, 44). According to our survey, 8% of ETEC strains isolated from patients with travelers' diarrhea in Japan were found to carry CFA/III (12, 13).

The best-characterized pilus genes which usually consist of operons are K88 and K99 of ETEC in animals and pap pili and type 1 pili of uropathogenic *E. coli* (22, 33). These operons contain 8 to 11 genes encoding the proteins involved in regulation of expression, major pilin, minor pilin (adhesin), periplasmic transportor, outer membrane channel, and so on. Up to now, the operons for the biosynthesis of CFA/I, CS1, CS2, CS3, and CS6 of ETEC in humans have been sequenced and characterized (6, 14, 16, 30, 48).

We have previously isolated a 55-kb plasmid controlling the expression of CFA/III from *E. coli* 260-1 after it was marked with ampicillin-resistant transposon Tn*3*, and a 17.4-kb region of the Tn*3*-marked plasmid was found to be responsible for CFA/III formation (32). We also reported the nucleotide sequences of *cofA* and *cofP* encoding major pilin and prepilin peptidase, respectively, and the evidence that CofA is produced initially as a 26.5-kDa preform pilin (prepilin) and then processed to a 20.5-kDa mature pilin by cleavage between Gly-30 and Met-31 residues by CofP which is predicted to be localized in the inner membrane (39–41). The N-terminal 30-amino-acid sequence of the mature CofA is highly hydrophobic and has homology (about 70 to 75% identity) with the type IV class B pilin family such as TcpA for toxin-coregulated pili (TCP) of *Vibrio cholerae*, BfpA for bundle-forming pili (BFP) of enteropathogenic *E. coli* (EPEC), and LngA for long pilus (longus) of ETEC (9, 10, 39, 40). They are produced as precursors which are processed at a highly conserved consensus cleavage site (QXG ↓ F[M]T[S]LXE) located close to their N termini.

We report here the entire nucleotide sequence of the region encoding the genes for CFA/III formation and evidence that the *cof* gene cluster is similar to the *tcp* gene cluster for TCP of

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TABLE 1. Bacterial strains, plasmids, and bacteriophages used in this study

Strain, plasmid, or bacteriophage	Description[a]	Reference or source
E. coli		
260-1	LT CFA/III	12
31-10	LT CFA/III	13
31-10P	CFA/III-negative spontaneous derivative of strain 31-10; LT	This study
HB101	Host for recombinant plasmids	Laboratory stock
JM109	Host for recombinant M13 bacteriophages	Laboratory stock
Plasmid		
pSH1134	32.5-kb plasmid containing CFA/III gene cluster and Tn*3* insertion; Ap^r	32
pTT201	pMW119 (*Acc*I and *Bam*HI sites) containing 8.6-kb *Cla*I_1-*Bam*HI_1 fragment from pTT202; Ap^r	40
pTT202	pMW119 (*Acc*I site) containing 11.5-kb *Cla*I_1-*Cla*I_2 fragment from pSH1134; Ap^r	32, 40
pTT206	pACYC184 (*Bam*HI site) containing 12.4-kb *Bam*HI_1-*Bam*HI_2 fragment from pSH1134; Cm^r	32, 41
pTT222	pACYC184 (*Eco*RI site) containing 5.2-kb *Eco*RI_2-*Eco*RI_3 fragment from pTT206; Tc^r	41
pTT224	pACYC184 (*Sal*I and *Bam*HI sites) containing 7.6-kb *Sal*I_4-*Bam*HI_2 fragment from pTT206; Tc^r	41
pTT237	pMW119 (*Kpn*I and *Acc*I sites) containing 9.0-kb *Kpn*I-*Cla*I_2 fragment from pTT202; Ap^r	This study
pMW119	Cloning vector; Ap^r *lac*Z	Nippon Gene
pACYC184	Cloning vector; Cm^r Tc^r	Nippon Gene
Bacteriophage		
M13mp18	Cloning vector; *lac*Z	Laboratory stock
M13mp19	Cloning vector; *lac*Z	Laboratory stock

[a] Ap^r, Cm^r, and Tc^r, ampicillin, chloramphenicol, and tetracycline resistance, respectively.

V. cholerae and *bfp* gene cluster for BFP of EPEC and demonstrate CFA/III biogenesis in the *E. coli* K-12 strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. *E. coli* strains, plasmids, and bacteriophages used in this study are listed in Table 1. *E. coli* 260-1 was used for the analysis and cloning of the CFA/III genes. A 55-kb plasmid controlling the expression of CFA/III was isolated by marking with the ampicillin-resistant transposon Tn*3*, resulting in pSH1001 (32). After construction of enzyme (*Cla*I)-deleted derivative plasmids, the 17.4-kb region in pSH1134 was found to be responsible for CFA/III formation on *E. coli* HB101 (32). pTT202 and pTT206 carry *cofA* (major pilin gene) and *cofP* (prepilin peptidase gene), respectively (40, 41). Cloning vectors pMW119 and pACYC184 belong to different compatibility groups, and they can multiply simultaneously in the same host.

Bacterial culture conditions. *E. coli* strains were routinely grown in Luria-Bertani medium, supplemented with appropriate antibiotics (31). For the optimal expression of CFA/III, *E. coli* strains were grown on CFA agar plates at 37°C for 20 h (5). 2xYT medium was used for *E. coli* JM109 to propagate phages (31). Antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 15 μg/ml.

Enzymes and chemicals. Restriction endonucleases, exonuclease III, bacterial alkaline phosphatase, T4 DNA polymerase, and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). [α-^{32}P]dCTP was obtained from Amersham Japan Co., Ltd. (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

General cloning techniques. Plasmid DNA was extracted from *E. coli* strains by the alkaline lysis method (31). Digestion of DNA with restriction enzymes, gel electrophoresis, ligation, and transformation were performed using standard procedures (31).

DNA sequencing. Suitable restriction fragments were subcloned into M13mp18 and M13mp19 and then digested by exonuclease III to generate a series of nested deletions from each clone. The single-stranded DNA templates were prepared according to the standard procedure (31), and the nucleotide sequences were determined by the dideoxy-chain termination method (31) with a 7-DEAZA sequencing kit (Takara Shuzo Co., Ltd., Kyoto, Japan).

Preparation of the periplasmic extract. *E. coli* strains on CFA agar plates were harvested in phosphate-buffered saline (PBS), and then the cells were collected by centrifugation at 12,000 × *g* for 5 min. To prepare the periplasmic extract, the cells were treated with polymyxin B (5,000 U/ml in PBS) at 37°C for 10 min and centrifuged at 12,000 × *g* for 5 min. The supernatant obtained was used as the periplasmic fraction.

SDS-PAGE and Western blot analysis. Whole-cell lysates and periplasmic extracts were denatured by boiling for 5 min in a running buffer containing 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 50 mM Tris-HCl (pH 7.5). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12.5% acrylamide (31). The proteins in the gels were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) using a semidry blotting apparatus and analyzed by Western blotting (31). Membranes were blocked for 1 h in Tris-buffered saline with 0.05% Tween-20 (TBS-T) containing 5% skim milk. The blocked membranes were incubated for 1 h with a 1:1,000 dilution of rabbit antiserum against purified CFA/III (13) in TBS-T, washed with TBS-T, and incubated for 1 h with a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories, West Chester, Pa.) in TBS-T containing 5% skim milk. Following another wash with TBS-T, the enzyme activity was detected with the substrate of 4-chloro-1-naphthol.

CFA/III detection. *E. coli* strains on CFA agar plates were harvested in PBS. A 10-μl sample of the bacterial suspension (ca. 10^8 bacteria/ml) was mixed with 10 μl of anti-CFA/III antiserum on a glass slide. The mixture was gently rotated for 2 min, and then bacterial agglutination was observed by the naked eye (13). Pilus formation on the cells was also observed with a transmission electron microscope after staining with 1% (wt/vol) ammonium phosphotungstate (pH 7.0) as described previously (12).

Bacterial adhesion assay. Caco-2, a human colonic carcinoma cell line, was used. Caco-2 cells were maintained in Dulbecco modified Eagle medium (Life Technologies, Inc., Rockville, Md.) supplemented with 10% fetal calf serum (Life Technologies, Inc., Rockville, Md.) at 37°C in 5% CO_2. Caco-2 cells were seeded onto the glass coverslips in six-well tissue culture plates at a concentration of about 10^5 cells/cm^2. The cultures were used at postconfluence after 15 days of incubation, which is the condition for well-mature Caco-2 cells, as previously described (3, 45). Prior to the adhesion assay, Caco-2 cells were washed in PBS (pH 7.0). A suspension of about 10^6 bacteria/ml (grown on CFA agar) in the culture medium containing 1% D-mannose was prepared, 2 ml of the suspension was added to the washed Caco-2 cells, and the mixture was incubated for 3 h at 37°C in 5% CO_2. The samples were washed three times with PBS (pH 7.0), fixed in methanol, stained with 10% Giemsa solution, and examined by oil immersion light microscopy to assess bacterial adherence. The adhesion indices were presented as the percentage of Caco-2 cells with at least one adhering bacterium (index 1) and the average number of bacteria/cell (index 2) by counting 10 randomly chosen fields in three separate experiments.

DNA and protein data analyses. The analyses of nucleotide and deduced amino acid sequences were performed with GENETYX-MAC version 8.0 (Software Development Co., Ltd., Tokyo, Japan) and the multialignment FASTA program from the Genetics Computer Group (University of Wisconsin, Madison, Wis.) sequence analysis software package. Computer-assisted open reading frame (ORF) search was performed by the following criteria: an ORF would encode a polypeptide of 100 or more translated amino acids; ATG as the

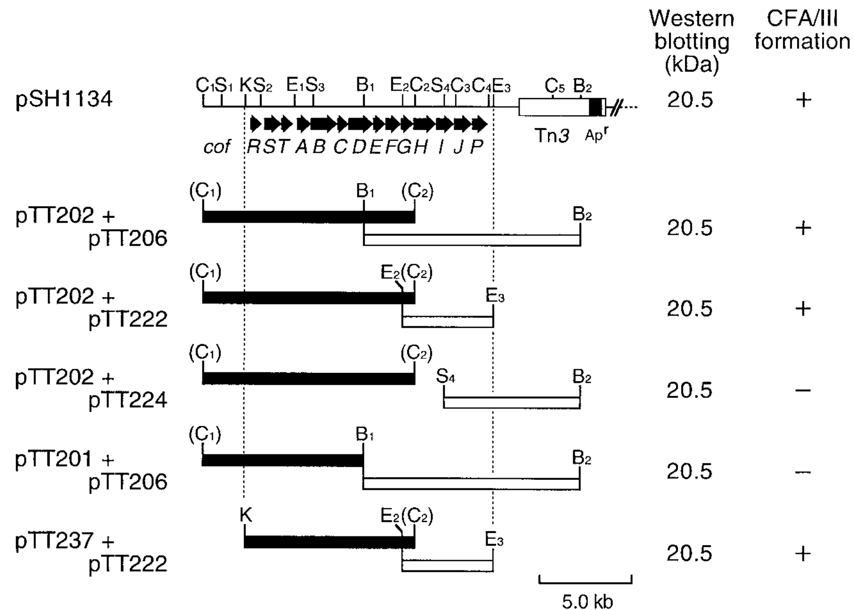

FIG. 1. Restriction maps of pSH1134 and CFA/III clones and their expression. pSH1134, pTT202, and pTT206 were digested with the appropriate restriction endonucleases and the fragments (solid and open boxes) were cloned into pMW119 and pACYC184, respectively. The cloned genes were expressed under the control of their own promoter or promoter on the cloning vector. The proposed organization of the *cof* gene cluster is illustrated in the upper part of the figure. The values represent the molecular mass (in kilodaltons) on Western blot analysis with anti-CFA/III antiserum. The symbols (+ and −) on the right side show the results of CFA/III formation. + and − represent the formation and the nonformation of CFA/III, respectively. Restriction endonuclease sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; and S, *Sal*I.

translational initiation codon; and an *E. coli* consensus ribosome-binding site (RBS), which was located at an optimal distance upstream of the ATG (29).

Nucleotide sequence accession number. The nucleotide sequence reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number AB049751.

RESULTS

Region of *cof* genes for CFA/III formation. *E. coli* HB101 harboring both pTT202 (carrying *cofA*) and pTT206 (carrying *cofP*) was agglutinated with anti-CFA/III antiserum, and pilus formation on the cells was also observed (32). Moreover, the whole-cell extract was revealed to produce a 20.5-kDa protein (pilin) which was identical to the purified CFA/III on Western blot analysis (40). To define the minimum region responsible for CFA/III formation, we subcloned various restriction fragments of pTT202 and pTT206 into vector plasmid pMW119 and pACYC184, and a series of plasmids were introduced into *E. coli* HB101. As shown in Fig. 1, *E. coli* HB101 harboring pTT202 and pTT222 or harboring pTT237 and pTT222 produced the 20.5-kDa processed pilin, and the CFA/III formation on the cells was observed. These results suggested that the region needed for CFA/III formation was restricted to the 14-kb region between the *Kpn*I site and the *Eco*RI_3 site in pSH1134.

Western blot analysis of CofA. *E. coli* HB101 harboring pTT202 and pTT224 or harboring pTT201 and pTT206 produced the 20.5-kDa processed pilin, but no pilus formation was observed on the cells (Fig. 1). To determine the location of the expressed antigen (pilin) in *E. coli* HB101, we attempted Western blot analysis of CofA. As shown in Fig. 2, a 20.5-kDa protein (pilin) was detected in the periplasm. On the other hand, whole-cell lysates of *E. coli* HB101 harboring only pTT202 contained the 26.5-kDa prepilin, but no cross-reacting materials (26.5- or 20.5-kDa protein) were detected in the periplasm.

Nucleotide sequence of *cof* gene cluster. We determined the nucleotide sequence of the 14-kb *Kpn*I-*Eco*RI_3 region in pSH1134. The sequencing analysis revealed the presence of 14 tandemly arranged potential ORFs with the same transcriptional orientation, which may constitute an operon (Fig. 1). This gene cluster contained the previously reported *cofA* and *cofP* encoding major pilin and prepilin peptidase, respectively. The G+C content of the *cof* gene cluster was 37%, which was significantly lower than normally found in *E. coli* (50%). This

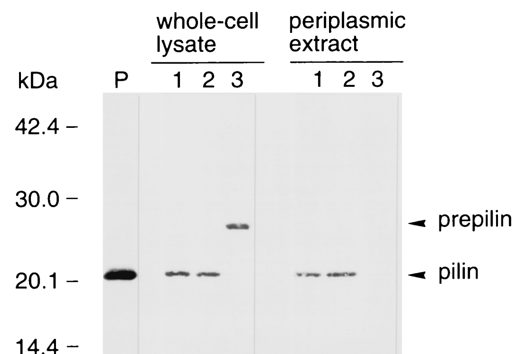

FIG. 2. Western blot analysis of *E. coli* HB101 whole-cell lysates (left) and periplasmic extracts (right) using anti-CFA/III antiserum. Lane P, purified CFA/III; lane 1, *E. coli* HB101 harboring pTT202 and pTT224; lane 2, *E. coli* HB101 harboring pTT201 and pTT206; lane 3, *E. coli* HB101 harboring pTT202. The prepilin and processed pilin bands are indicated by arrowheads. Molecular mass markers are noted on the left side.

TABLE 2. Features of the *cof* genes and deduced proteins

Gene				Deduced protein			
Name	Position (start→stop)	Length (bp)	G+C content (%)	Length (amino acids)	Size (kDa)	Predicted function or location	Closest homology by FASTA (% identity, overlap [no. of amino acids])
cofR	635→935	300	33.2	100	11.7	Regulation	Serovar Typhimurium PefB (53.8, 80); ETEC FaeB (48.1, 79)
cofS	1228→2077	849	39.2	283	32.0	Regulation	ETEC FapR (25.0, 260); ETEC CfaD (24.6, 264)
cofT	2092→2533	441	43.8	147	16.7	Unknown	Serovar Typhi IagB (41.2, 136); EPEC BfpH (31.2, 138)
cofA	2836→3550	714	42.3	238	25.3	Type IV pilin	ETEC LngA (78.2, 238); *V. cholerae* TcpA (34.7, 225)
cofB	3613→5182	1,569	36.7	523	57.1	Type IV pilin-like protein	*V. cholerae* TcpB (22.1, 77)
cofC	5201→5612	411	34.2	137	15.7	Unknown	EPEC BfpG (26.9, 134); *V. cholerae* TcpQ (24.3, 70)
cofD	5632→7087	1,455	35.4	485	54.2	Outer membrane lipoprotein	EPEC BfpB (24.3, 437); *V. cholerae* TcpC (22.5, 485)
cofE	7091→7649	558	36.0	186	21.8	Inner membrane	None
cofF	7653→8478	825	32.8	275	31.2	Inner membrane	*V. cholerae* TcpD (24.4, 270)
cofG	8468→8951	483	39.2	161	17.7	Unknown	None
cofH	9151→10483	1,332	39.0	444	50.0	Inner membrane nucleotide-binding protein	*V. cholerae* TcpT (44.2, 441); EPEC BfpD (26.9, 431)
cofI	10469→11492	1,023	36.6	341	37.8	Inner membrane	*V. cholerae* TcpE (38.0, 337); EPEC BfpE (23.6, 343)
cofJ	11508→12552	1,044	35.4	348	39.6	Unknown	None
cofP	12557→13376	819	35.5	273	30.5	Prepilin peptidase	*V. cholerae* TcpJ (35.7, 266); EPEC BfpP (31.7, 262)

low G+C content is common for virulence-associated genes of *E. coli*. The potential promoter sequences corresponding to the −35 (TTTACA, nucleotide positions 535 to 540) and −10 (TA CTAT, nucleotide positions 558 to 563) regions were found upstream of *cofR*, the first gene in the *cof* gene cluster. These sequences have a high degree of identity to the σ^{70} promoter −35 (TTGACA) and −10 (TATAAT) regions for *E. coli* RNA polymerase (11). The spacing of 17 nucleotides between the two regions is optimal. There is no potential promoter sequence downstream of *cofR*. A potential stem-loop structure which acts as a transcriptional terminator was observed between *cofA* and *cofB* (nucleotide positions 3562 to 3595) with the structural free energy ΔG (25°C) of −23.3 kcal/mol (40, 43). Other CF operons also have stem-loop structures downstream of the gene encoding the major pilin (2, 15). This is considered a regulatory mechanism for overexpression of the major pilin gene relative to other genes in the operons. With the exception of *cofP*, all the genes were preceded by the consensus RBS (29). Although *cofP* lacks a consensus RBS, *cofP* is preceded by a nucleotide sequence (GATTA) similar to the proposed RBS of the *E. coli sdaA* gene (38, 41).

Properties of *cof* genes and deduced proteins. The major features of the *cof* genes and deduced proteins are summarized in Table 2.

cofR encodes a 100-amino-acid protein (11,739 Da) lacking a signal peptide. The deduced amino acid sequence of CofR is homologous with several bacterial proteins such as PefB (53.8% identity) of *Salmonella enterica* serovar Typhimurium, FaeB (48.1% identity) for K88 of animal ETEC, AfaA (46.8% identity) for afimbrial adhesin (AFA-III) of uropathogenic and diarrhea-associated *E. coli*, and PapB (46.3% identity) for P pili of uropathogenic *E. coli* which have been reported as positive regulators concerning the biogenesis of the pili (Fig. 3A).

cofS encodes a 283-amino-acid protein (32,049 Da) lacking a signal peptide. The deduced amino acid sequence of CofS has homology with a number of bacterial transcriptional regulators belonging to the family of proteins represented by AraC, the regulator of the *E. coli* and serovar Typhimurium arabinose operons (8), e.g., FapR (25.0% identity) for 987P of animal ETEC, CfaD (24.6% identity) for CFA/I, Rns (23.7% identity) for CS1 and CS2 of CFA/II, and ToxT (20.1% identity) for TCP of *V. cholerae*. All members of the AraC family are most homologous in the C terminal region of the sequence. This region contains a helix-turn-helix motif associated with the DNA-binding activity (Fig. 3B).

cofT encodes a 147-amino-acid protein (16,717 Da). CofT has a potential signal peptide of 20 amino acids which is the most hydrophobic region of the protein. Consequently, because the mature CofT is markedly hydrophilic, it may be in the periplasm or exported out of the cell. The deduced amino acid sequence of CofT has homology with the product of gene X (51.4% identity) for the conjugative transfer of the IncF plasmids (F, R1, and R100) of *E. coli*, IagB (41.2% identity) of *Salmonella enterica* serovar Typhi, IpgF (35.2% identity) of *Shigella flexneri*, BfpH (31.2% identity) for BFP of EPEC, and Slt (31.5% identity) of *E. coli* (Fig. 3C). In serovar Typhi and *S. flexneri*, IagB and IpgF are involved in the invasion of the eukaryotic host cells by the bacterial cells (1, 21). The *E. coli slt* gene encodes a 70-kDa soluble lytic transglycosylase (4). X-ray crystallography revealed that the C-terminal region of Slt has a three-dimensional structure similar to those of hen egg-white lysozyme and bacteriophage T4 lysozyme, associated with a peptidoglycan-lytic activity (42).

cofA encodes a 238-amino-acid protein (25,309 Da) which is the major pilin belonging to the type IV class B pilin family as reported previously (39, 40). The signal peptide of CofA is 30 amino acids long. The N-terminal 30-amino-acid sequence of the mature CofA is the most conserved and hydrophobic region of the protein (Fig. 3D).

cofB encodes a 523-amino-acid protein (57,089 Da). The N-terminal amino acid sequence of CofB is similar to that of type IV pilin. The completely conserved glycine, leucine, and glutamic acid residues in the N terminus of type IV prepilins appear at the 5th, 8th, and 10th amino acids of CofB, respectively. It is likely that the Gly-5–Phe-6 junction of CofB is cleaved by the type IV prepilin peptidase (CofP). The deduced amino acid sequence of CofB has homology with LngB for Longus of ETEC (9) and TcpB (22.1% identity) for TCP of *V. cholerae* (Fig. 3E).

cofC encodes a 137-amino-acid protein (15,658 Da) contain-

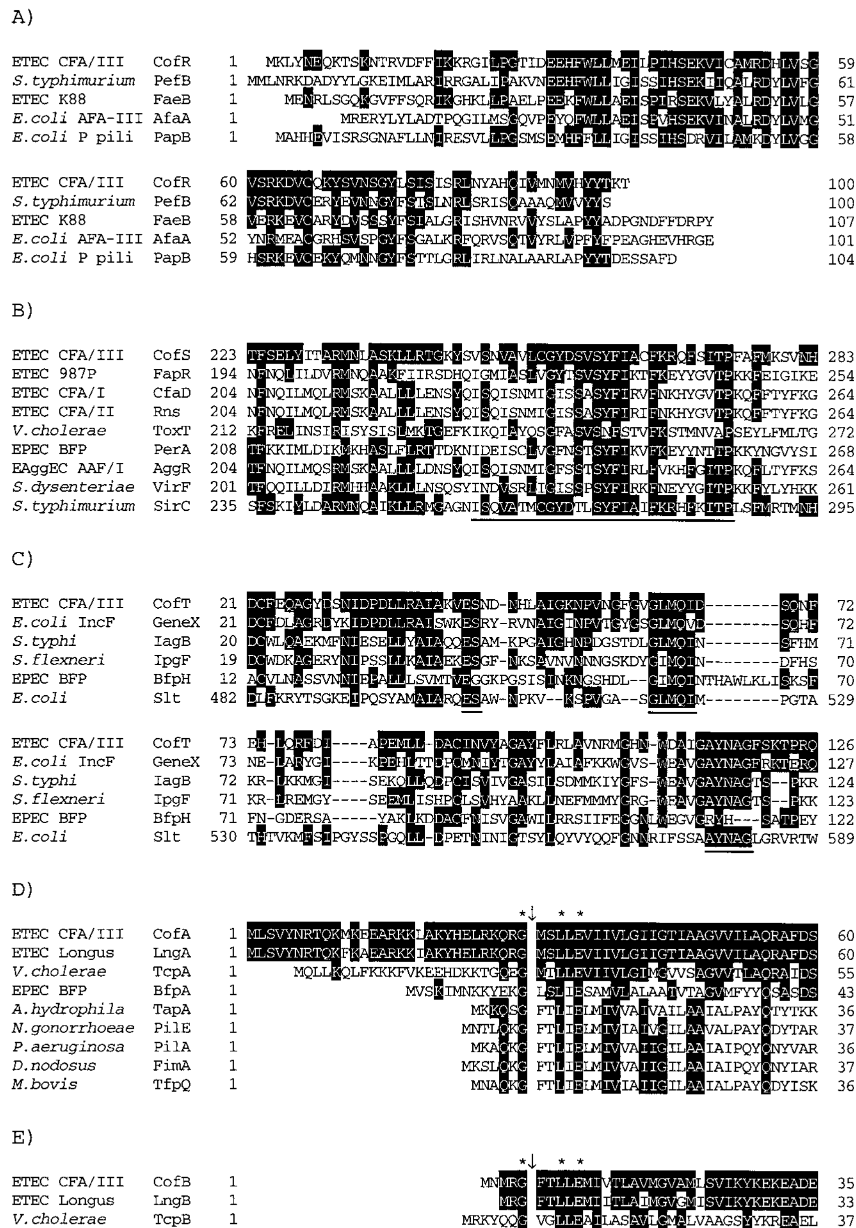

FIG. 3. Partial alignment of the deduced amino acid sequences of the Cof proteins with those of known proteins. Identical amino acids are indicated by a black background. Gaps introduced for alignment are represented by dashes. (A) Alignment of the deduced amino acid sequences of CofR, *Salmonella* serovar Typhimurium PefB (GenBank accession no. L08613), ETEC FaeB (Z11709), *E. coli* AfaA (X76688), and *E. coli* PapB (X03391). (B) Alignment of the deduced amino acid sequences of CofS, ETEC FapR (X53494), ETEC CfaD (M55609), ETEC Rns (J04166), *V. cholerae* ToxT (X64098), EPEC PerA (Z48561), enteroaggregative *E. coli* (EAggEC) AggR (Z32523), *S. dysenteriae* VirF (X58464), and serovar Typhimurium SirC (AF134856) in the region containing the DNA-binding domain (underline). (C) Alignment of the deduced amino acid sequences of CofT, *E. coli* Gene X (X07264), *Salmonella* serovar Typhi IagB (X80892), *S. flexneri* IpgF (L04309), EPEC BfpH (Z68186), and *E. coli* Slt (M69185). Three motifs conserved in putative lytic transglycosylases are underlined. (D) Alignment of the deduced amino acid sequences of CofA, ETEC LngA (AF004308), *V. cholerae* TcpA (X64098), EPEC BfpA (Z68186), *A. hydrophila* TapA (U20255), *N. gonorrhoeae* PilE (X66144), *P. aeruginosa* PilA (M14849), *Dichelobacter nodosus* FimA (X52405), and *Moraxella bovis* TfpQ (M59712) in the N-terminal region. The cleavage site of type IV pilins is shown by a downward arrow. The conserved glycine, leucine, and glutamic acid residues are marked by asterisks. (E) Alignment of the deduced amino acid sequences of CofB, ETEC LngB, and *V. cholerae* TcpB (X64098) in the N-terminal region. The type IV pilin-like cleavage site is shown by a downward arrow. The conserved glycine, leucine, and glutamic acid residues are marked by asterisks. (F) Alignment of the deduced amino acid sequences of CofD, *V. cholerae* TcpC (X64098), and EPEC BfpB (Z68186) in the N-terminal region. The lipoprotein-cleavage site is shown by a downward arrow. (G) Alignment of the deduced amino acid sequences of CofH, *V. cholerae* TcpT (X64098), EPEC BfpD (Z68186), *A. hydrophila* TapB (U20255), *N. gonorrhoeae* PilF (U32588), *N. gonorrhoeae* PilT (S72391), *P. aeruginosa* PilB (M32066), *P. aeruginosa* PilT (M55524), and *K. pneumoniae* PulE (M32613) in the region containing the nucleotide-binding domain. The Walker box A, Asp boxes, and Walker box B are underlined. The conserved CXXC motifs are marked by asterisks. (H) Alignment of the deduced amino acid sequences of CofP, *V. cholerae* TcpJ (M74708), EPEC BfpP (Z68186), *A. hydrophila* TapD (U20255), *N. gonorrhoeae* PilD (U32588), *P. aeruginosa* PilD (M32066), *D. nodosus* FimP (U17138), *K. pneumoniae* PulO (M32613), and *Erwinia chrysanthemi* OutO (L02214) in the region containing the conserved CXXC motifs (asterisks).

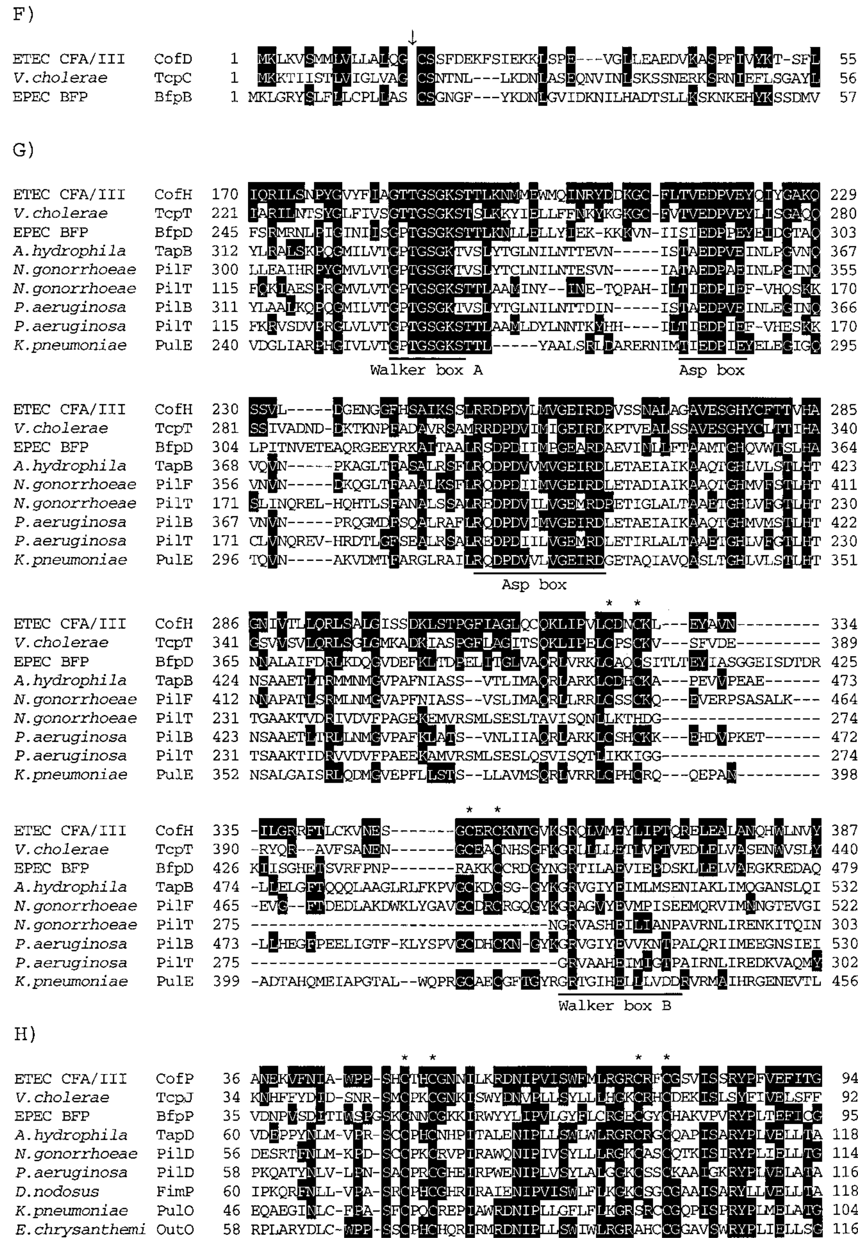

FIG. 3—*Continued.*

ing a typical signal peptide of 25 amino acids. The mature CofC is markedly hydrophilic, suggesting that it may be localized in the periplasm or exported out of the cell. The deduced amino acid sequence of CofC is homologous with BfpG (26.9% identity) for BFP and TcpQ (24.3% identity) for TCP.

cofD encodes 485-amino-acid protein (54,236 Da). Analysis of the deduced amino acid sequence of CofD revealed that the N-terminal sequence conforms to the lipoprotein signal peptidase recognition site, including the presence of the essential cysteine residue to which a glyceride fatty acid lipid would be attached (Fig. 3F). CofD is homologous with BfpB (24.3% identity) for BFP and TcpC (22.5% identity) for TCP. The BfpB and TcpC have been reported as outer membrane lipoproteins related to the biogenesis of BFP and TCP, respectively (25, 28).

cofE encodes a 186-amino-acid protein (21,773 Da). CofE has a markedly hydrophobic C terminus (amino acid positions 159 to 186) which may function as a potential membrane-anchoring domain. No known protein containing an amino acid sequence with significant similarity to that of CofE was found in the GenBank database.

cofF encodes a 175-amino-acid protein (31,188 Da). CofF has a markedly hydrophobic region (amino acid positions 21 to 42) which may be a membrane-spanning domain. The deduced amino acid sequence of CofF is homologous with TcpD (24.4% identity) for TCP of *V. cholerae*.

cofG encodes a 161-amino-acid protein (17,681 Da). CofG has a typical signal peptide of 25 amino acids. Consequently, because the mature CofG is markedly hydrophilic, it may be in

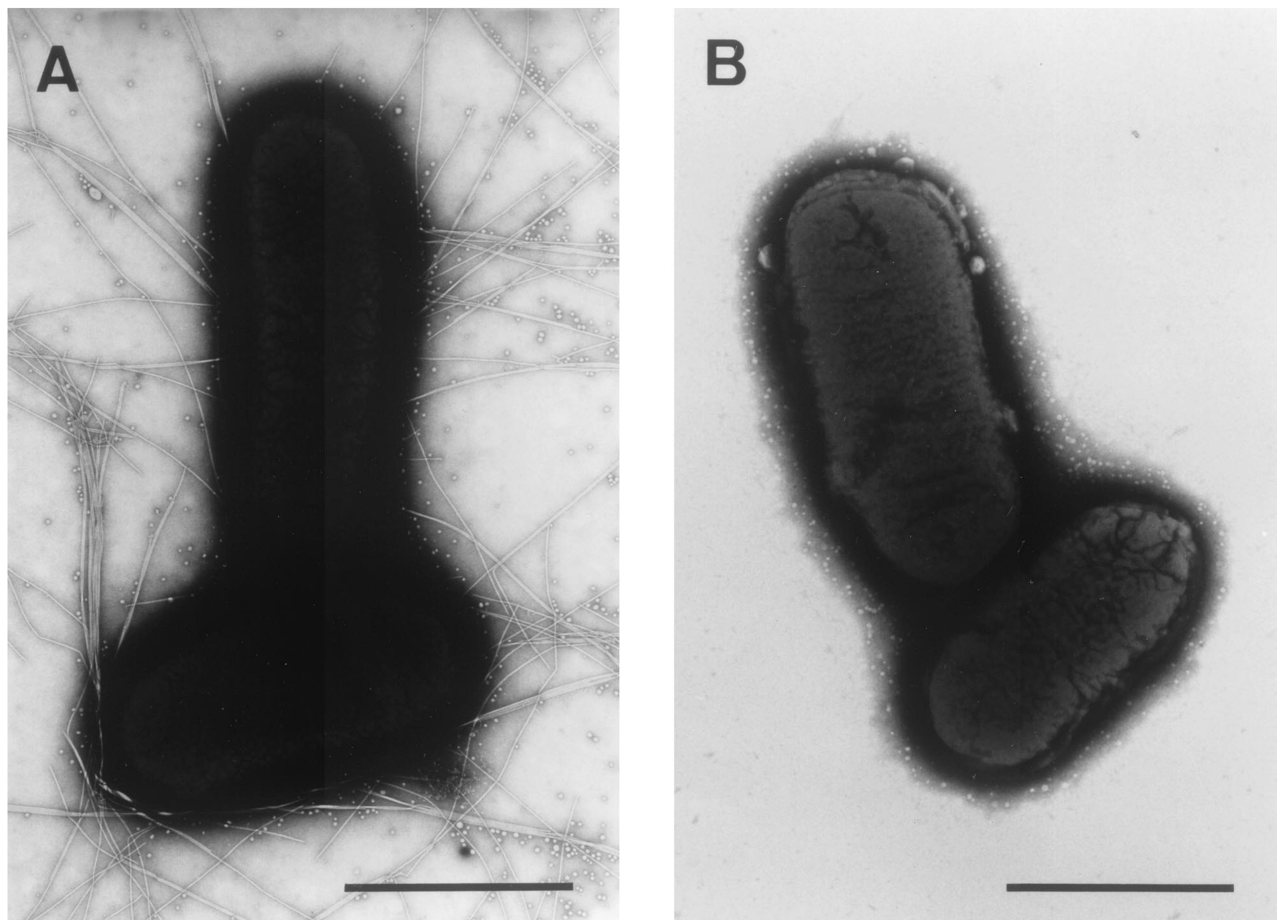

FIG. 4. Electron micrographs of *E. coli* HB101 after growth on CFA agar plates at 37°C for 20 h. (A) *E. coli* HB101 harboring pTT237 and pTT222. (B) *E. coli* HB101 harboring pMW119 and pACYC184. Bar, 1 μm.

the periplasm or exported out of the cell. No known protein similar to the CofG could be found in the GenBank database.

cofH encodes a 444-amino-acid protein (50,009 Da) lacking a signal peptide. The deduced amino acid sequence of CofH is homologous with several bacterial proteins such as TcpT (44.2% identity) for TCP, BfpD (26.9% identity) for BFP, TapB (28.3% identity) of *Aeromonas hydrophila*, PilF (27.6% identity) of *Neisseria gonorrhoeae*, PilB (28.0% identity) of *Pseudomonas aeruginosa*, and PulE (27.6% identity) of *Klebsiella pneumoniae*, which are related to the biogenesis of the type IV pili and extracellular protein secretion (26). These proteins carry conserved Walker boxes A and B, Asp boxes, and two pairs of cysteine residues (CXXC motifs) associated with a nucleotide-binding activity (47). They may act in the energy fueling steps of the biogenesis of the type VI pili and the protein secretion system (Fig. 3G).

cofI encodes a 341-amino-acid protein (37,847 Da) lacking a signal peptide. CofI may be an integral cytoplasmic membrane protein since it has three putative transmembrane domains (amino acid positions 108 to 131, 164 to 184, and 316 to 332). The deduced amino acid sequence of CofI is homologous with several bacterial proteins related to the biogenesis of the type IV pili including TcpE (38.0% identity) for TCP, BfpE (23.6% identity) for BFP, and PilC (22.3% identity) of *P. aeruginosa*.

cofJ encodes a 348-amino-acid protein (39,579 Da). The N terminus of CofJ conforms to a typical signal peptide of 22 amino acids. No known protein similar to the CofJ could be found in the GenBank database.

cofP encodes a 273-amino-acid protein (30,533 Da) which is the type IV prepilin peptidase of the CFA/III as reported previously (41). The type IV prepilin peptidases, including CofP, are homologous over the entire amino acid sequence. Notably, two pairs of cysteine residues (CXXC motifs) that have been shown to be required for the enzymatic activity of *P. aeruginosa* PilD (36, 37) are present in all type IV prepilin peptidases (Fig. 3H).

Expression of *cof* gene cluster in *E. coli* HB101 and adhesive function of CFA/III. To examine whether the 14-kb *Kpn*I-*Eco*RI$_3$ region contains all of the information needed for the biogenesis of the functional CFA/III, *E. coli* HB101 harboring pTT237 and pTT222 was observed by electron microscopy and tested for the ability to adhere to the Caco-2 cells, an established cell culture model for ETEC colonization. As shown in Fig. 4, *E. coli* HB101 harboring pTT237 and pTT222 produced long rod-like pili with a diameter of 7 nm, but *E. coli* HB101 harboring pMW119 and pACYC184 did not produce pili as expected. The ability of *E. coli* strains to adhere to the Caco-2 cells is shown in Fig. 5. The wild-type strain (*E. coli* 31-10) and *E. coli* HB101 harboring pTT237 and pTT222 adhered to the Caco-2 cells with indices (index 1) of 84.8 and 89.4% and with

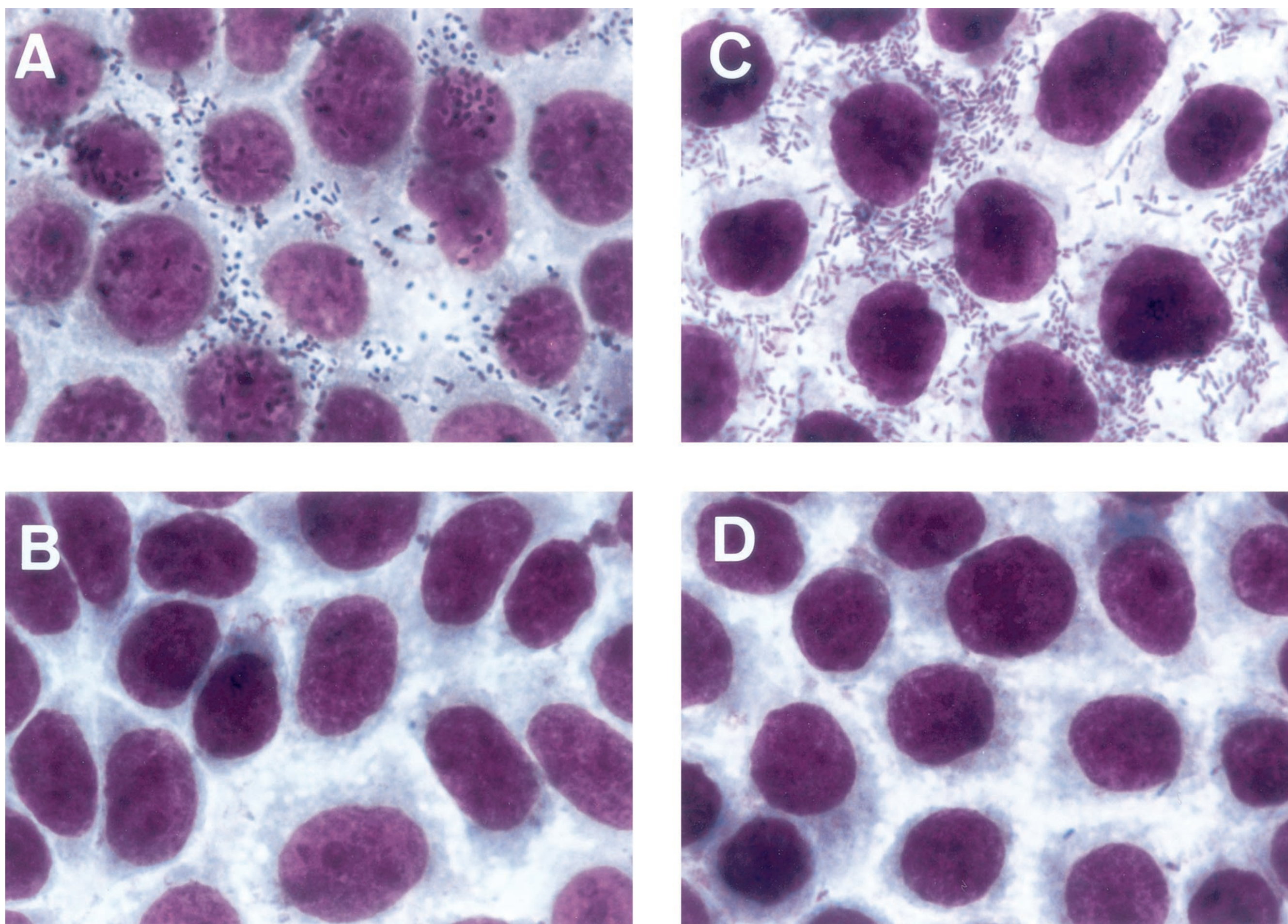

FIG. 5. Micrographs showing adhesion of *E. coli* strains to Caco-2 cells. (A) ETEC 31-10 (CFA/III-positive strain). (B) ETEC 31-10P (CFA/III-negative strain). (C) *E. coli* HB101 harboring pTT237 and pTT222. (D) *E. coli* HB101 harboring pMW119 and pACYC184.

the average numbers of bacteria/cell (index 2) of 22.8 and 54.6, respectively. On the other hand, *E. coli* 31-10P and *E. coli* HB101 harboring pMW119 and pACYC184 showed no adherence to the Caco-2 cells with indices (index 1) of 5.6 and 4.8% and with the average numbers of bacteria/cell (index 2) of 0.07 and 0.08, respectively. These results suggest that the sequenced region contains all information required for the formation of a functional CFA/III on the surface of *E. coli* HB101.

DISCUSSION

We report here the nucleotide sequence of the minimal region (14-kb *Kpn*I-*Eco*RI$_3$ region) for CFA/III formation of ETEC. This region contains 14 *cof* genes which are thought to constitute an operon. Several proteins encoded by the *cof* genes are homologous with the proteins involved in the BFP biogenesis of EPEC and the TCP biogenesis of *V. cholerae* (19, 34, 35). The gene organization of the *cof* genes was compared to those of the *bfp* and *tcp* operons (Fig. 6). Both the *bfp* and the *tcp* operons are also comprised of 14 genes. The organizations of these gene clusters have some similarity to each other. Especially, the relative positions of the *cofA, cofB, cofC, cofD, cofF, cofH, cofI*, and *cofP* genes are conserved in both *cof* and *tcp* gene clusters. The major pilin genes (*cofA* and *tcpA*) are located in the upstream regions, and the prepilin peptidase genes (*cofP* and *tcpJ*) are located at the last positions of these gene clusters. The conservation of gene organizations and the similarity of amino acid sequences suggest that CFA/III and TCP biogenesis systems have evolved from a common ancestral gene system.

Pilus operons are generally shown to encode one or two positive regulatory proteins (local regulators) (7, 22). The *cof* gene cluster also contains two genes (*cofR* and *cofS*) encoding regulatory proteins, which are located upstream of the major pilin gene (*cofA*). These gene products probably act as transcriptional activators of the *cof* gene cluster, but their precise modes of action are not yet known.

Many virulence gene clusters appear to have been imported as a unit into bacteria that may not have previously been pathogenic (17, 23). This is deduced from their unusual G+C content and/or the presence of insertion sequence flanking them. The G+C content of the *cof* gene cluster is 37%, which is significantly lower than the average for *E. coli* (50%). A region homologous with part of the sequence of the transposable element IS*630* (20) is observed downstream of the *cof* gene cluster (nucleotide positions 13587 to 13651). Recent studies (17, 18, 46) of *V. cholerae* have shown that the *tcp* gene cluster

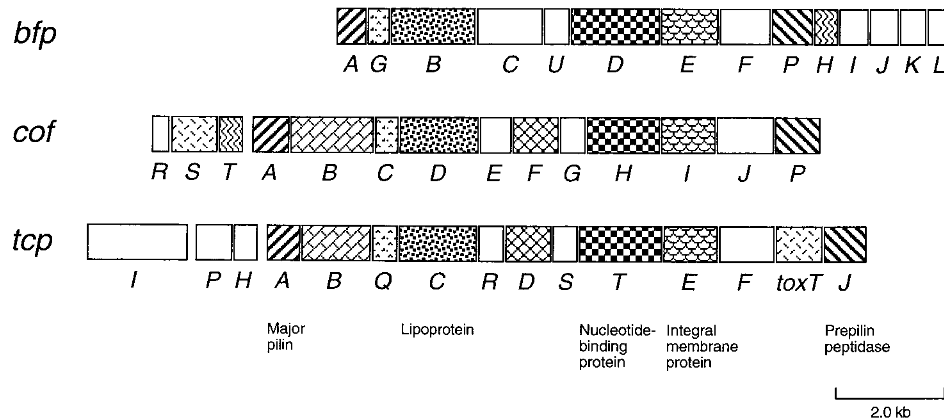

FIG. 6. Genetic organizations of *cof, bfp,* and *tcp* gene clusters. All genes except *tcpI* are transcribed rightward. The homologous genes are indicated by the same shading patterns. Predicted similar functions are indicated in the bottom of the figure.

is located on a *Vibrio* pathogenicity island which includes the genes of lysogenic filamentous phage (VPIΦ), and TCP functions as a receptor for cholera toxin phage (CTXΦ). This information suggests the interesting possibilities that the *cof* gene cluster might have been transferred into *E. coli* via phage(s) or plasmid(s) from another unknown organism and that CFA/III might function as a receptor for unknown phage(s).

In our earlier report (41), we found a close relation between the processing of prepilin and CFA/III pilus formation. However, *E. coli* HB101 harboring pTT202 and pTT224 or harboring pTT201 and pTT206 produced 20.5-kDa processed pilin in the periplasm, but no pilus formation was observed on the cells. The gene lacking in these plasmids may be required for the pilus formation on the cells. The *cofD* lacking in pTT201 and pTT206 is homologous with *tcpC* and *bfpB* encoding outer membrane lipoproteins for TCP and BFP biogenesis, respectively (25, 28). The protein products of *tcpC* and *bfpB* are required for each pilus formation. The genes lacking in pTT202 and pTT224 are *cofH* and *cofI*. The CofH and CofI are homologous with the nucleotide-binding proteins and the integral membrane proteins, respectively, related to other type IV pilus biogenesis. Although further study is needed, these *cof* gene products may have an important role for the pilus formation, probably via lack of the basal apparatus of the pili. We also found that CFA/III itself possessed adhesive function on human colonic epithelial cells. This is in agreement with the previous findings in the suckling mice experiment (12). CFA/III is a complex extracellular organelle involved with several proteins such as minor pilin (adhesin), periplasmic transporter, outer membrane channel, and regulatory protein and is characterized as gene clusters similar to other CFs and type IV pili. Therefore, further studies on the functions of *cof* gene products are in progress in our laboratory. This knowledge should help in the development of an ideal pilus vaccine against ETEC diarrhea.

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