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Sequencing of the Gene Encoding the Major Pilin of Pilus Colonization Factor Antigen III (CFA/III) of Human Enterotoxigenic *Escherichia coli* and Evidence that CFA/III Is Related to Type IV Pili

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The plasmid-encoded structural gene *cofA* necessary for the production of the major pilin subunit of pilus colonization factor antigen III (CFA/III) of human enterotoxigenic *Escherichia coli* was identified, and the nucleotide sequence of the gene was determined. *cofA* consists of 714 nucleotides encoding a 238-amino-acid protein (molecular weight of 25,309). CofA seems to be a precursor of CFA/III pilin, because the first 23 residues of the N-terminal amino acid sequence of the purified CFA/III pili coincided with the deduced amino acid sequence for residues 32 to 54 of CofA. Western blot (immunoblot) analysis of CofA also indicated its processing to form mature pilin in the presence of the downstream region of *cofA*. These results suggest that the major pilin of CFA/III pili is produced as a precursor form which is posttranslationally modified to the mature pilin and forms morphological pili after cleavage of the Gly-30–Met-31 junction, probably by a protease encoded by an as-yet-unknown gene located downstream of *cofA*. Interestingly, the N-terminal 30-amino-acid sequence of mature CFA/III shows the highest identity (76.7%) to TcpA pilin of *Vibrio cholerae*, which is a type IV class B pilin.

The ability of enterotoxigenic *Escherichia coli* (ETEC), an important cause of diarrhea with a worldwide distribution, to adhere to and colonize intestinal epithelium is an essential step for pathogenicity in addition to the ability to produce heat-labile and/or heat-stable enterotoxins. The colonizing ability of human ETEC depends on the presence of colonization factor antigens (CFAs) on the surface of the cells, which form pili (or fimbriae). Distinct types of CFAs, such as CFA/I, CFA/II, CFA/III, and CFA/IV, have been described for human ETEC strains. CFA/I consists of a single antigen (10), whereas some types such as CFA/II (5, 23, 27, 37) and CFA/IV (25, 40) consist of a complex of different antigens named *E. coli* surface (CS) antigens. Cloning and sequencing of genes encoding CFA/I (15, 21), CS1 and CS3 of CFA/II (2, 31), and CS5 of CFA/IV (4, 20) have been reported previously. Although CFA/I- and CFA/II-carrying ETEC seem to be the most prevalent, a wide variation in the prevalence of ETEC strains harboring CFAs in different parts of the world has been reported (5, 9, 10, 14, 19). According to our survey (19), 8% of ETEC strains isolated from patients with traveler's diarrhea in Japan were found to carry CFA/III pili. However, no information on CFA/III genetic determinants has yet been reported. We and colleagues have previously cloned the gene necessary for expressing the CFA/III pili (35). In the present study, we report the sequencing of the gene encoding CFA/III pilin and evidence that the CFA/III pilus is related to type IV pili, especially the class B pili as defined in reference 13.

A 55-kb plasmid controlling the expression of CFA/III was isolated from *E. coli* 260-1 (18, 19) after it was marked with ampicillin-resistant transposon Tn*3* (35). The 17.4-kb region of the Tn*3*-marked plasmid pSH1001 responsible for CFA/III formation was determined as described previously (35). The partially overlapping 11.5- and 12.4-kb fragments of the region have been separately cloned to compatible plasmid vectors, resulting in pTT202 and pTT206, respectively (35). The simultaneous presence of pTT202 and pTT206 in *E. coli* was necessary for cell agglutination with anti-CFA/III antiserum (19) and pilus formation. Cells were grown on CFA agar plates at 37°C for CFA/III production (18). Long, straight pili were observed only on *E. coli* harboring both pTT202 and pTT206 but not on *E. coli* harboring two cloning vectors (pACYC 184 and pMW119) by electron microscopic examination (19). To determine which region is responsible for production of the major pilin, Western blot (immunoblot) analysis of the cell extracts was carried out (Fig. 1). *E. coli* HB101 harboring both pTT202 and pTT206 produced CFA/III with a molecular mass of 20.5 kDa which was identical to the purified CFA/III. No CFA/III was observed in *E. coli* harboring only pTT206. *E. coli* carrying only pTT202, however, produced a cross-reacting material with an apparent molecular mass of 26.5 kDa although no pilus formation was observed on the cells. These results suggest that the structural gene for CFA/III pilin is located on pTT202 and that CFA/III pilin may be produced as a 26.5-kDa precursor and then proteolytically processed to form the 20.5-kDa mature pilin.

Thus, we further subcloned the fragments of pTT202 into vector plasmid pMW119 (42) and the products of *E. coli* harboring a series of clones were analyzed by Western blotting (41) with CFA/III antiserum (19). As shown in Fig. 2, the cell extracts of pTT201, pTT217, and pTT213, all of which shared the 1.1-kb *Eco*RI-*Sal*I fragment, produced 26.5-kDa antigens but the clones (pTT210 and pTT220) which did not have the fragment produced no antigen. This suggests that the struc-

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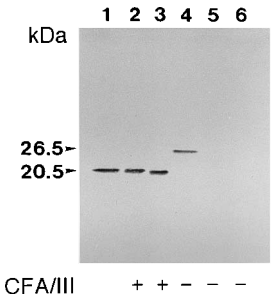

FIG. 1. Western blotting analysis of *E. coli* extracts using anti-CFA/III rabbit antiserum. Lane 1, purified CFA/III; lane 2, wild-type strain (260-1); lane 3, HB101 harboring pTT206 and pTT202; lane 4, HB101 harboring pTT202; lane 5, HB101 harboring pTT206; lane 6, HB101. The symbols below the gel (CFA/III) represent the results of bacterial slide agglutination tests with CFA/III antiserum. The 20.5- and 26.5-kDa bands are indicated (arrowheads).

tural gene for the pilin subunit is located in the 1.1-kb *Eco*RI-*Sal*I region.

From these findings, we determined the DNA sequence of the 1.4-kb *Ava*I-*Sal*I fragment of pTT213 (Fig. 2). The DNA fragment obtained after subcloning was finally cloned into the M13mp18 vector (43) and then digested by exonuclease III to generate DNA fragments of various lengths (16). The nucleotide sequences of cloned fragments were determined by the dideoxy chain termination method using a commercial DNA Sequencing Kit (Takara Shuzo Co., Kyoto, Japan) (33). Sequence analysis revealed a 714-bp open reading frame (ORF), ORF1 (Fig. 2). In ORF1, a Shine-Dalgarno sequence (36) in the 10-bp region upstream of the start codon and a transcriptional terminator-like inverted repeat sequence (32) in the 10-bp region downstream of the stop codon were observed (Fig. 3). ORF1 encodes 238 amino acids with a calculated molecular weight of 25,309, which agrees well with the size of

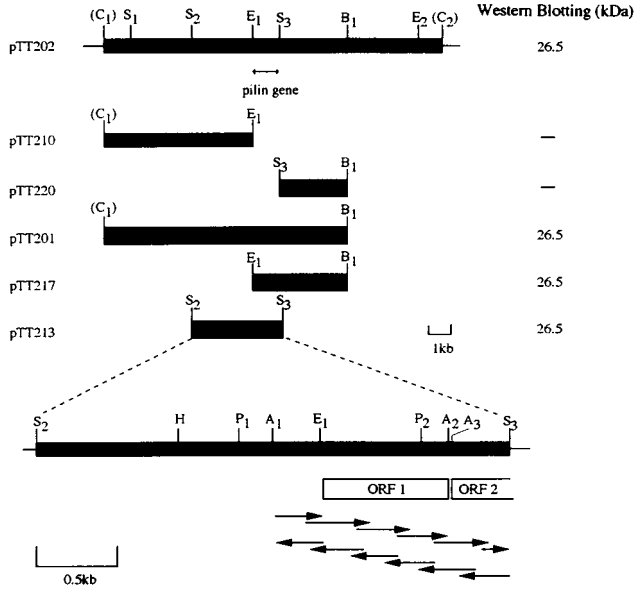

FIG. 2. Subcloning of the gene encoding CFA/III pilin from pTT202 and strategy for DNA sequencing of pTT213. General cloning techniques, digestion of DNA with restriction enzyme, gel electrophoresis, ligation, and transformation were performed as previously described (3, 35). Results of Western blotting analysis are shown on the right. The arrows represent sequenced fragments and their direction of reading. S, *Sal*I; E, *Eco*RI, B; *Bam*HI; H, *Hin*dIII; P, *Pst*I; A, *Ava*I; C, *Cla*I.

the precursor-like protein (26.5 kDa) identified by Western blot analysis (Fig. 1). Furthermore, the sequence of the 23 amino acids beginning at Ser-32 was completely identical to that obtained sequencing data (the N-terminal 23 amino acids) of the purified 20.5-kDa mature pilin protein (39), indicating that ORF1 is a structural gene (designated *cofA*) of the major pilin of CFA/III. We found that the sequence of the 30 amino acids from the N terminus of the mature pilin had the highest identity (76.7%) to the sequence of TcpA pili of *Vibrio cholerae* (11, 34) by a sequence homology search and the pili were highly homologous to other type IV pili expressed in several other organisms (Fig. 4) (8, 11, 12, 17, 24, 26, 28). No significant homology of the amino acid sequence in the residual region of CFA/III to those of type IV pili was observed. Similar observations of various type IV pili have been reported previously (8, 11, 12, 17, 24, 26, 28).

Several distinct types of pilus colonization factors for human and animal ETEC have been described. Among these, K88 and K99 found in animal ETEC have been intensively characterized at the molecular level (1, 6, 29, 30). Genes for K88 and K99 have been demonstrated to consist of operons, and each gene product has also been characterized in detail (1, 6, 29, 30). On the other hand, genetic information regarding CFAs of human ETEC is still limited. Only the structural genes for the major pilin subunit of CFA/I and for CS1, CS3, and CS5 have been reported (2, 4, 15, 20, 21, 31).

In the present paper, we describe the complete nucleotide sequence of the structural gene for CFA/III pilin. In our experience with subcloning, construction of the mature pili (morphological pilus formation on *E. coli*) required a large (21-kb) DNA fragment ($ClaI_1$-$BamHI_2$) (35), suggesting the requirement of a complex of genes, probably operons. Judging from our present results, there may be a gene for a protease which cleaves (or processes) a precursor of CFA/III pilin (238 amino acids, 26.5 kDa) to the mature pilin (20.5 kDa). Comparison of the amino acid sequences of the precursor CFA/III pilin and mature pilin (39) suggests a cleavage of the precursor between Gly-30 and Met-31.

Interestingly, the N-terminal 30-amino-acid sequence of the mature CFA/III pilus is highly hydrophobic and has homology to those of type IV pili such as TcpA of *V. cholerae* O395 (11, 34) and the pili of *Neisseria gonorrhoeae* (MS11) (26), *Pseudomonas aeruginosa* PAK (28), *Moraxella bovis* EPP63 (24) and *Bacteroides nodosus* 265 (8). Overall identity between amino acid sequences of CFA/III and sequences of these type 4 pilins was about 30%. Among these, the highest identity (76.7%) was found between CFA/III pilin and TcpA pilin, which is believed to be an important colonization factor of *V. cholerae* (22). Not only amino acid sequence homology but also morphological similarities (long and straight pili) and functional similarities (attachment to intestinal cells [18]) between CFA/III and type IV pili (TcpA) were observed. In addition to these similarities CofA was processed to form the mature pilin (CFA/III) only in the presence of the downstream region of *cofA*. Processing of the TcpA precursor to the mature pilin is believed to be carried out by a protease encoded in the downstream region of *tcpA* (22), suggesting that the genes for CFA/III and TcpA pili may be evolutionarily closely related.

Along with ORF1 (*cofA*), another ORF (ORF2) was observed in the 61-bp region downstream of ORF1, although the entire sequence of ORF2 has not yet been cloned (Fig. 2). A Shine-Dalgarno sequence (GGAG) was observed 7 bp upstream of ORF2, and some similarity (about 20 to 30%) of the deduced amino acid sequence of ORF2 to the accessory fimbrial proteins of *V. cholerae* (11, 34), *B. nodosus* (17), and *M. bovis* (12) was observed at least in the N-terminal 113-amino-

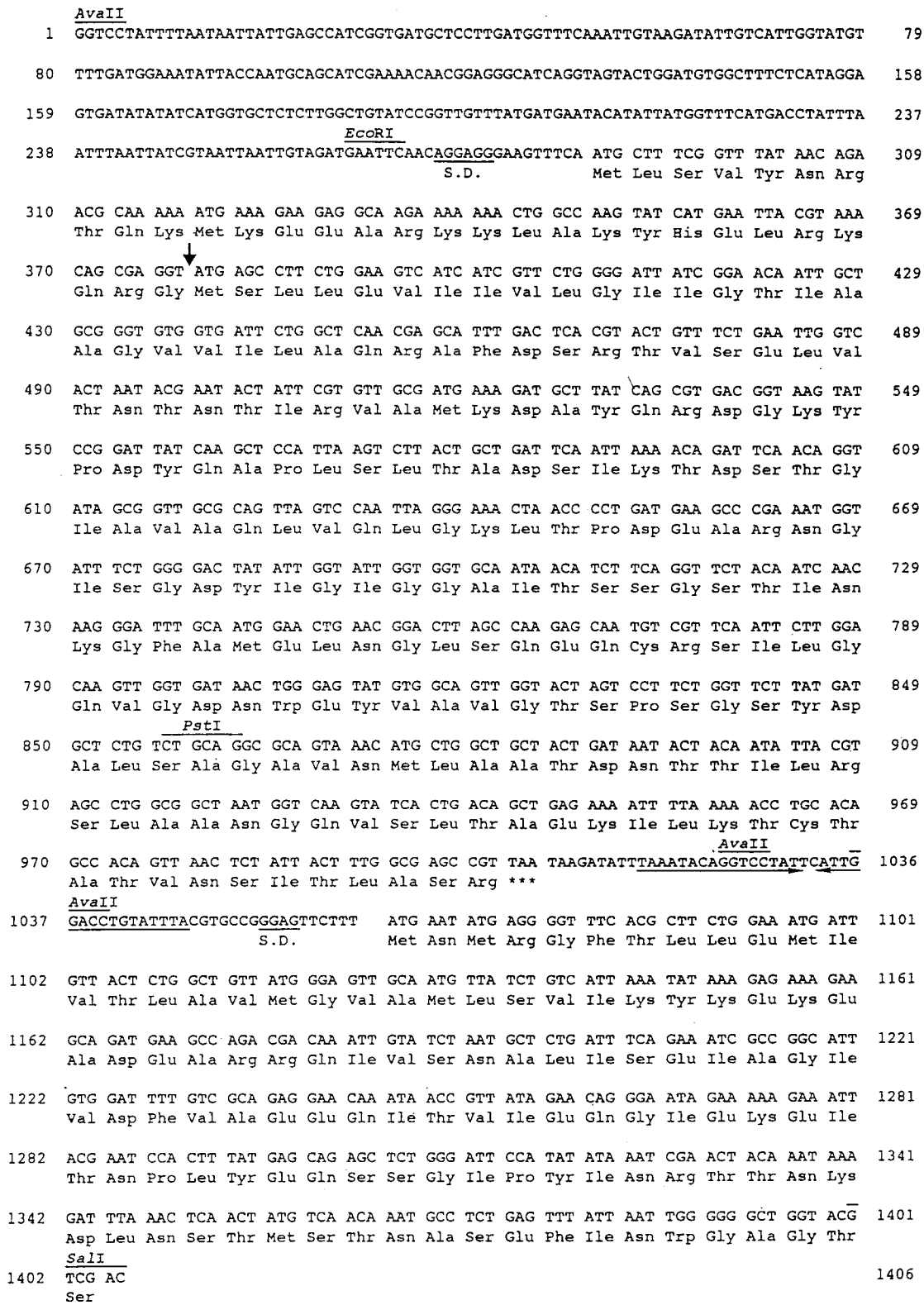

FIG. 3. Nucleotide and amino acid sequencing of the gene encoding CFA/III pilin. The underlined Shine-Dalgarno sequences (S.D.) and the proteolytic site (vertical arrow) are indicated. Inverted repeat sequences and restriction enzyme-recognizing sequences are represented by horizontal arrows under and lines above the nucleotides, respectively. The nucleotide sequence numbers are shown in the margins.

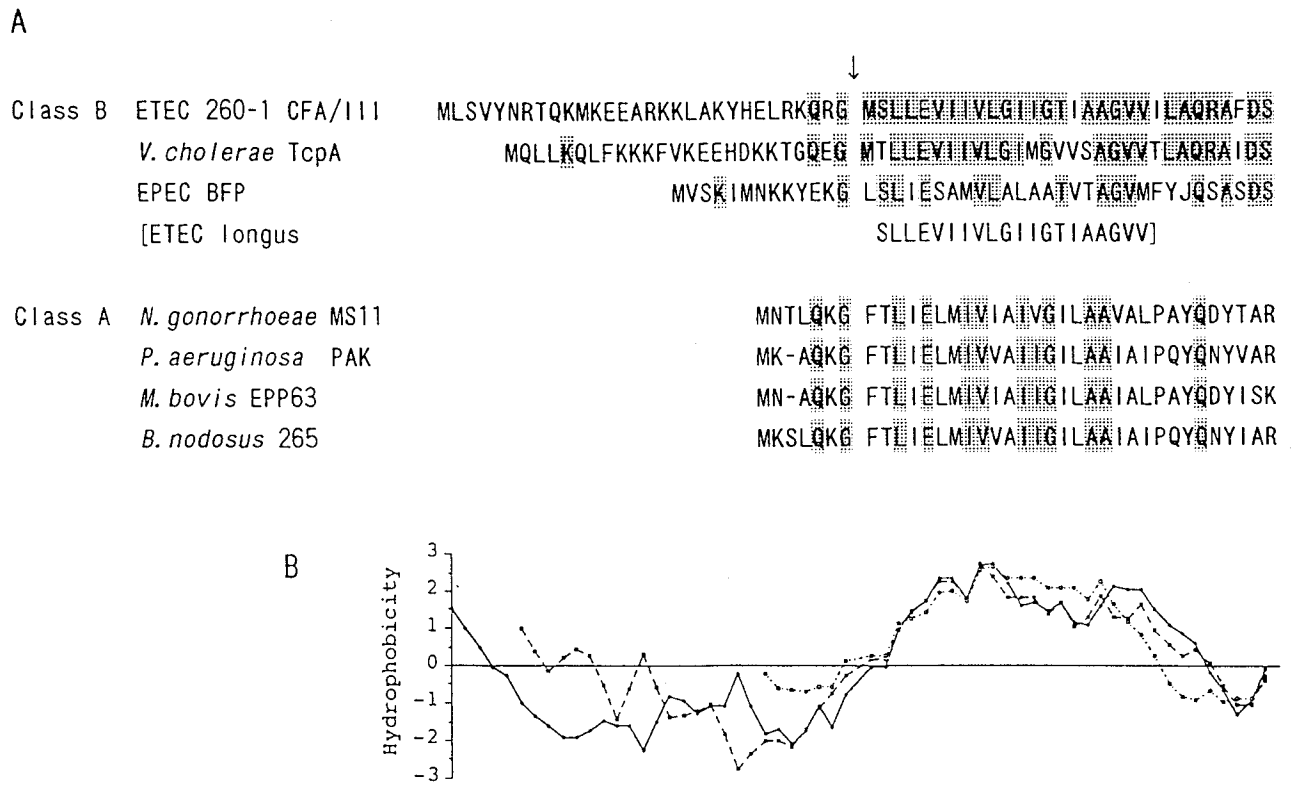

FIG. 4. Comparison of N-terminal amino acid sequence of CFA/III, which has a high homology with those of other type IV pili, including TcpA of *V. cholerae*. (A) N-terminal amino acid sequences of type IV pili of various organisms. The sequences were aligned through the use of the multialignment FASTA program from the Genetics Computer Group (University of Wisconsin, Madison, Wis.) sequence analysis software package. Class A and B pilins are classified according to the definition proposed in reference 13. Identical residues are shaded. The arrow denotes the signal peptidase cleavage site. The N-terminal amino acid sequence of ETEC longus is shown in brackets as a reference, since it was obtained only from peptide analysis (B). (B) Hydrophobicity profiles of CFA/III (●——●), TcpA (■----■), and other type IV pili (□ · — · □).

acid sequence which we deduced in this study. Although we need the complete sequence of the ORF2 protein to have conclusive evidence, we suppose from the sequence similarity that the ORF2 protein is an accessory (minor) component of CFA/III.

Recently, a new pilus, termed longus, produced by ETEC was reported (13) and it was demonstrated that longus is a new member of the type IV pilus family on the basis of its high degree of N-terminal amino acid sequence homology with other type IV pili, including the bundle-forming pili of enteropathogenic *E. coli* (7, 38). The N-terminal amino acid sequence of CFA/III was identical to that reported for longus (13) in the first 20 amino acids. On the basis of the N-terminal similarities of CofA to Tcp and longus found in the enteric pathogens, CofA (CFA/III pilin) can be classified as a class B type IV pilin as proposed in reference 13. Although it is possible that CFA/III and longus are identical, it is also possible that they are different molecules, because CFA/III production is usually limited to heat-labile enterotoxin-producing ETEC which has O25, whereas longus is produced by a rather heterologous ETEC including O25 producing heat-labile enterotoxin (13). The complete amino acid sequence of longus has not yet been reported, and so further discussion of the degree of identity between CFA/III and longus is at present premature.

However, we believe that this is the first report of a complete sequence of type IV pili found in human ETEC and this information will be an important consideration for development of a vaccine against ETEC diarrhea.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number D37957.

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