Identification of *IngA*, the structural gene of longus type IV pilus of enterotoxigenic *Escherichia coli*

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Human enterotoxigenic *Escherichia coli* (ETEC) produces a type IV pilus termed longus which is encoded on large plasmids in association with colonization factor antigens (CFAs) and enterotoxins. A plasmid-derived 7 kbp *Bam*HI DNA fragment hybridizing with an oligonucleotide probe designed from the amino-terminal amino acid sequence of the denatured 22 kDa structural longus pilin subunit was subcloned and sequenced. DNA sequencing analysis revealed an open reading frame, designated *IngA*, whose predicted amino acid sequence matched perfectly the N-terminal sequence of LngA obtained by Edman degradation. *IngA* is the first gene described of the longus gene cluster. Cloned *IngA* encoded and expressed a prepilin protein of 236 residues with a calculated mass of 25.17 kDa. The prepilin is apparently processed into a mature pilin of 206 residues with a calculated mass of 21.5 kDa. The predicted peptide sequence of *IngA* showed 78.8 and 37% identity to CFA/III pilin (CofA) of ETEC and the toxin-coregulated pilus (TcpA) of *Vibrio cholerae*. Peptide sequence homology between *IngA* and *cofA* was more prominent towards the amino terminus than within the carboxy region. Like other type IV pilins, LngA contains two cysteine residues towards the carboxy-terminal region. Transmission electron microscopy and immunoblot analysis of ETEC strains expressing either longus or CFA/III detected antigenic differences between native and denatured epitopes of these pili. In addition, differential regulation of pilus expression was identified when ETEC strains were grown in different media. Our data indicate that longus and CFA/III are two distinct but yet highly related type IV pili of ETEC.

Keywords: longus, type IV pili, colonization factor, enterotoxigenic, ETEC

INTRODUCTION

The ability of bacterial pathogens to colonize and cause disease is attributed in part to the expression of filamentous surface factors termed pili or fimbriae, that mediate adherence directly to the host cell receptor or indirectly as bridging ultrastructures (Brinton, 1965;

Beachey, 1981). Bacterial adhesion is then the first step in the establishment of infections by pathogens that remain confined to the extracellular spaces or by invasive bacteria that mobilize into the cellular cytosol.

Pili have been classified on the basis of serology, morphology, biochemical features and receptor specificities (Klemm, 1985; Strom & Lory, 1993). The type IV pilus family comprises pilins, which share structural and functional properties such as similarities at the N-termini of their pilin structural subunits and a highly conserved N-terminal domain of 25–30 amino acid residues. They possess a leader peptide sequence of variable length, which is important for processing of the precursor prepilin into mature pilin by a prepilin

Abbreviations: Bfp, bundle-forming pilus; CAB, CDC anaerobe blood; ETEC, enterotoxigenic *Escherichia coli*; CFA, colonization factor antigen; CS, coli surface antigen; LT, labile enterotoxin of ETEC; ST, stable enterotoxin of ETEC; Tcp, toxin-coregulated pilus; TSAB, trypticase soy agar + sheep blood.

The GenBank accession number for the sequence reported in this paper is AD004308.

peptidase. The synthesis of functional pili requires the presence of several accessory genes which are involved in cleavage of the leader peptide, methylation of the newly formed N-terminal methylphenylalanine, export across the cell envelope, and anchorage and assembly of subunits into the pilus organelle.

ETEC produces a watery diarrhoea which accounts for a high rate of infantile morbidity and mortality in developing countries (Levine, 1987; Levine *et al.* 1993). Colonization of intestinal mucosa by ETEC strains is mediated by a variety of surface fimbrial appendages called colonization factor antigens (CFAs), coli surface antigens (CSs) and putative colonization factors (Evans *et al.*, 1978; McConnell, 1991; Levine *et al.*, 1995). These surface structures have been identified after growth at 37 °C on CFA agar as peritrichous pili (1–5 µm long) generally encoded on large plasmids in association with heat-stable (ST) and/or heat-labile (LT) enterotoxins which are responsible for the net secretion of electrolytes and water into the intestinal lumen (Evans *et al.*, 1978; Klemm, 1985; Levine, 1987).

A considerable number of human ETEC serotypes produce, in addition to the well-known CFAs, a plasmid-encoded type IV pilus termed longus (Girón *et al.*, 1994). The N terminus of the major repeating subunit of longus (LngA) has an apparent molecular mass of 22 kDa which shows significant N-terminal amino acid sequence similarities to TcpA of *Vibrio cholerae* and BfpA of EPEC (Taylor *et al.*, 1987; Girón *et al.*, 1991, 1994). A survey of the prevalence of the longus pilin structural gene *lngA* among ETEC showed that *lngA* was found in ETEC producing other CFAs. CFA/II-producing ETEC had the highest level of association with longus, followed by CFA/I and CFA/IV. *lngA* was more often associated with ETEC strains that produce ST only than with strains that produce ST and LT or LT alone. Some ETEC strains lacking any known CFAs were also shown to contain *lngA* and to produce longus (Girón *et al.*, 1995a).

Recently, DNA sequencing analysis of gene encoding CFA/III (*cofA*) showed that this pilus belongs to the growing type IV pilus family (Taniguchi *et al.*, 1995; Girón *et al.*, 1997). In an effort to characterize the genetic determinants involved in longus biogenesis, we report here the cloning and DNA sequencing analysis of the structural gene of longus, *lngA*. Molecular and immunological comparisons between longus and CFA/III indicate that these pili share some structural type IV pilus features but also show differences at the DNA and protein level suggesting the close relatedness of these two determinants of ETEC.

METHODS

Bacterial strains, plasmids and growth conditions. ETEC and other strains generated in this study are listed in Table 1. ETEC E9034A (O8:H9) is a wild-type strain producing longus and CS3 (Girón *et al.*, 1994) and was used as a positive control of longus production. ETEC E9034P strain is a plasmidless

derivative of E9034A unable to produce longus and was used as a negative control. Other longus-producing ETEC have been reported earlier (Girón *et al.*, 1994). ETEC E34420A (O25:H⁻), also known as 31-10, produces CFA/III, Z84-1 (O25:H⁻) also produces CFA/III, and E34420B is a plasmidless derivative of E34420A lacking CFA/III production (Knutton *et al.*, 1989; McConnell & Rowe, 1989). These strains were used as positive and negative controls of CFA/III production, respectively. pE9034A is a 90 kbp virulence plasmid of wild-type ETEC E9034A that contains *lngA* and longus-related genes, in addition to CS3 and enterotoxin genes. pJAG1 is a pACYC177 plasmid carrying a 7 kbp *Bam*HI fragment obtained from pE9034A. pOG140 is a pBluescript SK plasmid carrying a 1 kbp *Eco*RV insert containing the entire *lngA* gene. pOG309 is a pBluescript SK plasmid carrying a 0.3 kbp *Pst*I fragment containing the 3' region of *lngA*. pJAG11 is a pACYC177 plasmid carrying a 1 kbp *Pst*I fragment containing a truncated portion of *lngA*. ETEC strains were maintained at -70 °C in Luria broth and 15% (v/v) glycerol, and were cultured on CFA agar (1% Casamino acids, 0.15% yeast extract, 0.005% MgSO₄, 0.0005% MnCl₂, 2% agar; pH 7.4) for expression of CFAs (Evans *et al.*, 1978). For expression of longus, CDC anaerobe blood (CAB) agar (BBL Microbiological Systems), and trypticase soy agar supplemented with 5% (v/v) defibrinated sheep blood (TSAB) were used. Recombinant strains were grown on Luria agar or broth media with the appropriate antibiotics.

Sequencing of *lngA* and analysis. DNA sequencing of both DNA strands of *lngA* was carried out using the Sequenase kit version 2 (US Biochemical). Plasmids pJAG1, pJAG11, pOG309 and pOG140 were used as DNA templates. Sequence analysis and homology studies were performed using the programs from the Genetics Computer Group (1991).

Molecular and genetic techniques. Restriction endonuclease digestion, ligation, transformation, plasmid DNA preparations and DNA electrophoresis were performed using standard techniques (Sambrook *et al.*, 1982). Electroporation of plasmid DNA into recipient strains in 10% (v/v) glycerol was performed in 0.2 cm cuvettes using a Gene Pulser (Bio Rad) set at 400 ohms and 2.5 kV.

SDS-PAGE and immunoblotting of whole-cell extracts. To prepare whole-cell extracts, approximately 1×10^8 bacterial c.f.u. were harvested from agar plates (TSAB, CAB, or CFA media), resuspended in 50 mM PBS pH 7.4, and boiled for 5 min in SDS-PAGE sample buffer (Laemmli, 1970). After electrophoresis through 16% polyacrylamide gels, proteins were transferred onto nitrocellulose membranes and analysed by Western blotting using a 1:2000 dilution of rabbit anti-CFA/III antiserum or rabbit anti-longus antiserum. Secondary alkaline-phosphatase-conjugated IgG antibodies were used at 1:2000 dilution. The blots were developed with a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Electron microscopy. For structural and immuno-electron microscopy studies, bacteria were negatively stained with 1% phosphotungstic acid, pH 7.4 and examined in a JEOL JEM 1200 EX II transmission electron microscope at 80 kV as described previously (Girón *et al.*, 1994, 1995b). For immunological analysis of longus- or CFA/III-expressing ETEC strains, bacteria were reacted with rabbit anti-longus antiserum and/or mouse monoclonal anti-CFA/III 3:3 antibody diluted 1:100 in PBS containing 1% BSA (Sigma) for 30 min on Formvar-coated copper grids. The grids were washed three

Table 1. List of strains used or generated in this study

Strain/plasmid	Description	Reference
ETEC E9034A	O8:H9, longus and CS3	Girón <i>et al.</i> (1994)
ETEC E9034P	Plasmidless derivative of E9034A lacking longus production	CVD* collection
ETEC B2C	O6:H16, longus, CS1 and CS3	Girón <i>et al.</i> (1994)
ETEC E34420A (31-10)	O25:H ⁻ , CFA/III and CS6	Viboud <i>et al.</i> (1993)
ETEC E34420B	Plasmidless derivative of E34420A lacking CFA/III production	Viboud <i>et al.</i> (1993)
ETEC Z84-1	O25:H ⁻ , CFA/III	Viboud <i>et al.</i> (1993)
<i>E. coli</i> DH5 α	<i>supE33</i> Δ <i>lacU169</i> (080 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	BRL
pBluescriptSKII—	Cloning vector (Ap ^r)	Stratagene
pJAG1	7.0 kbp <i>Bam</i> HI fragment containing longus genes cloned into pACYC177	Girón <i>et al.</i> (1994)
pJAG11	pACYC177 with a 1.0 kbp <i>Pst</i> I insert with a 5' region of the <i>lngA</i> gene	This study
pOG140	pBluescript SKII with a 1.0 kbp <i>Eco</i> RV insert of the entire <i>lngA</i> gene	This study
pOG309	pBluescript SKII with a 0.3 kbp <i>Pst</i> I insert with the 3' region of <i>lngA</i>	This study

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times with PBS containing 1 % BSA and reacted with a 1:10 dilution of anti-rabbit and/or anti-mouse IgG conjugated to 10 or 5 nm gold particles (Amersham), respectively. The bacteria were stained and observed by electron microscopy as before.

ELISA. Production of longus or CFA/III was evaluated by ELISA after bacterial growth in different culture media. Briefly, longus-producing bacteria were grown on TSAB and CFA/III-producing bacteria were grown on CFA agar. Both strains were incubated overnight at 37 °C. E9034P and E34420B were used as negative controls for longus and CFA/III production, respectively. Bacterial suspensions were prepared in carbonate buffer pH 9.6 and adjusted to the same concentration until reaching an OD₆₀₀ of 1.0. Microtitre plates (NUNC) were coated overnight at 4 °C in quadruplicate with 100 μ l bacterial suspension, after which unbound bacteria were removed by washing with PBS containing 0.5 % Tween 20 (PBST). The wells were blocked with 1 % dry milk in PBST for 1 h at 37 °C, washed and then incubated with primary antibodies (rabbit anti-longus or rabbit anti-CFA/III) for 1 h at room temperature. After washing, secondary antibodies conjugated to alkaline phosphatase were added for 1 h at room temperature. Reactivity was measured by adding the appropriate substrate and read in an ELISA reader at 405 nm.

RESULTS

Analysis of *lngA* nucleotide sequence

ETEC expressing longus was shown to hybridize with an oligonucleotide probe specific for *lngA* (Girón *et al.*, 1994). A 7 kbp *Bam*HI fragment that hybridized with this probe in Southern blots was cloned from the large plasmid of ETEC E9034A into pACYC177 to generate

pJAG1. This plasmid and additional subclones (pJAG11, pOG140 and pOG309) generated in the present study were used to sequence the gene encoding the pilin subunit of longus. Approximately 1 kbp of DNA was sequenced from pJAG1 and a 708 bp ORF designated *lngA* was found to encode a predicted protein of 236 residues. The predicted mature product of *lngA* contains 206 amino acids, with a calculated molecular mass of 21.51 kDa, which is in agreement with the estimated mass of 22 kDa of LngA obtained by electrophoresis and Western blot analysis. The CFA/III, TCP (Rhine & Taylor, 1994) and BFP (Donnenberg *et al.*, 1992; Stone *et al.*, 1996) mature type IV pilins contain 208, 199 and 180 amino acid residues, respectively. A search through the GenBank database confirmed the sequence similarities between LngA and type IV pili. In addition, the predicted sequence of the mature pilin was identical to the N-terminal amino acid sequence (20 residues) previously obtained from the 22 kDa principal subunit of longus (Girón *et al.*, 1994) further confirming that *lngA* is the structural subunit gene of longus.

A consensus Shine–Dalgarno sequence was found 7 bp upstream of the ATG initiation codon (data not shown). No typical promoter sequences were found upstream of the *lngA* gene, suggesting that *lngA* may be part of an operon with a promoter located further upstream. The prepilin protein may require processing by a prepilin peptidase to produce a mature pilin, as is the case with other type IV pilins. LngA contains a highly basic hydrophilic amino acid leader sequence and a highly hydrophobic N-terminal domain typical of the type IV

LngA	MLS [↓] VYNRTQKF [↓] AEARKKI [↓] AKYHEL [↓] RKQ [↓] RGMSLLEVIIVLGIIGTIAAGV	50
CofA	MLS [↓] VYNRTQ [↓] KMK [↓] EEARK [↓] KLAKYHEL [↓] RKQ [↓] RGMSLLEVIIVLGIIGTIAAGV	50
LngA	VILAQRAFD [↑] SR [↑] AVTDLVTNTNTVRVRMKDAYQRD [↑] GKYPDFVDPLSLTANT	100
CofA	VILAQRAFD [↑] SRTVSELVTNTNTIRVAMKDAYQRD [↑] GKYPDYQAPLSLTADS	100
LngA	IKTDTSGIPAAQLVQLGKITPDEV [↑] RNNISGDFIAIGGALTSNGAQVKKGF	150
CofA	IKTDSTGIAVAQLVQLGKLT [↑] PDEARNISGDYIGIGGAITSSGSTINKGF	150
LngA	AIELNGLSQEQCRSILGQVGNW [↑] EYVAIGTSASGSY.AMTATGVDMSVAA	199
CofA	AMELNGLSQEQCRSILGQVGD [↑] NWEYVAVGTSPSGSYDALSAGAVNMLAAT	200
LngA	.STTVLRSLGNNGQTTLTADKILSTCTAQVNSITLGS [↑] R	236
CofA	DNTTILRSLAANGQVSLTAEKILKTCTATVNSITLAS [↑] R	238

Fig. 1. Comparison of protein sequences of LngA and CofA pilin structural subunits. Identical residues are indicated by vertical lines and conserved residues are indicated by dots. The arrows represent the sites of precursor-protein processing leading to mature LngA or CFA/III pilin proteins.

pilins (Fig. 1). While the signal peptide of class A type IV pilins is short (8–13 residues), the leader sequences of longus and CFA/III precursor are 30 residues long. TcpA and BfpA have a leader sequence of 26 and 13 residues, respectively. The Ser-Leu-Leu-Glu-Val peptide sequence found immediately after the predicted processing site of LngA is highly homologous to the consensus sequence Thr-Leu-Ile-Glu-Leu found in proteins processed by the prepilin peptidase of *Pseudomonas aeruginosa* and other members of the type IV pilus family. Previously, the N-terminal amino acid sequencing analysis of the denatured LngA showed that its first residue is a serine, indicating that the signal peptide should cleave after Met-30 (Girón *et al.*, 1994). In contrast, Taniguchi *et al.* (1995) noted that the signal peptide in CofA is cleaved between Gly-29 and Met-30. Similar to CofA, TcpA and BfpA, LngA contains two cysteines present at the C-terminal end.

LngA shows ~79% identity to CofA and ~37% identity to TcpA (from both O1 and O139 *V. cholerae*). Lower similarity is seen between LngA and BfpA. Higher homology between LngA and CofA is seen at the N-terminus of the peptides (Fig. 1). Only three conserved changes are present at the signal peptide. At the C-terminus, instead, higher diversity is observed, particularly after residue 140. It is in this area where two residues present in CofA are absent in LngA.

At the DNA level, the *lngA* and *cofA* genes are 74.2% identical (data not shown) with higher homology towards the 5' ends of the genes. There is also significant homology between *lngA* and *Erwinia chrysanthemi* *outC* (secretory component); *Eikenella corrodens* type IV N terminal methyl phenylalanine-pilin and *Xanthomonas campestris* *pef* (data not shown). These sequence similarities are concentrated at the 5' ends of these genes and less significant amino acid sequence homologies are observed between the carboxy regions of longus and other type IV pili. This is consistent with previous observations with various type IV pili (Girón *et al.*,

Fig. 2. Analysis of recombinant LngA protein expression. Whole-cell lysates of recombinant DH5α and ETEC strains were reacted with anti-longus antiserum by immunoblotting. Wild-type ETEC strain E9034A was used as a positive control of mature LngA (22 kDa) expression. DH5α and DH5α(pSK) *E. coli* strains and the plasmidless E9034P were used as negative controls. DH5α(pOG140) harbours the entire *lngA* gene in pBluescript SK and expresses a 25 kDa prepilin peptide.

1994; Strom & Lory, 1993; Taniguchi *et al.*, 1995; Taylor *et al.*, 1987).

Expression of recombinant LngA protein

To confirm that the cloned *lngA* gene encodes the structural subunit of longus pilus, a whole cell lysate of *Escherichia coli* DH5α(pOG140) carrying the *lngA* gene was evaluated by immunoblotting using the anti-longus rabbit antisera as primary antibody. As shown in Fig. 2,

two proteins of the same intensity were observed. A 25 kDa protein was observed in the lane containing the DH5 α (pOG140) recombinant strain. The molecular mass of this protein correlates well with the predicted molecular mass of 25.1 kDa for the LngA prepilin protein. The second protein of 22 kDa was observed in the lane containing the wild-type ETEC E9034A strain that was used as a positive control for expression of the mature LngA pilin. This analysis demonstrates that the sequenced *lngA* gene cloned in DH5 α expresses the prepilin LngA protein. Absence of specific prepilin peptidase and fimbrial biogenesis genes in DH5 α renders the LngA protein unable to be modified into the mature 22 kDa pilin protein.

Antigenic relatedness between longus and CFA/III pili

In view of the DNA and protein similarities observed between CFA/III and longus, antigenic cross-reactivities were examined by immunoblotting using specific anti-CFA/III and anti-longus antibodies. Towards this aim, six CFA/III⁺ ETEC strains (Honda *et al.*, 1984; Shinagawa *et al.*, 1993; Viboud *et al.*, 1993) and six longus⁺ ETEC strains (Girón *et al.*, 1994) were grown on TSAB agar and examined by immunoblotting (Fig. 3). Rabbit polyclonal anti-longus antibodies reacted strongly with longus⁺ strains and weakly with CFA/III⁺ strains (Fig. 3a). In contrast, rabbit anti-CFA/III antiserum showed a strong reaction with CFA/III pilin and no reactivity with LngA (Fig. 3b). LngA was detected after bacterial growth on TSAB (Fig. 3) or CAB agar, but not on CFA agar. In contrast, CFA/III was readily expressed after bacteria were grown on CFA, TSAB, or CAB media. While the CFA/III pilin was quite homogeneous in size, LngA showed more size variability (22–24 kDa) (data not shown). The sequence homology and the immunological cross-reactivities support the existence of common epitopes between CFA/III and longus exposed after denaturation (Girón *et al.*, 1997).

Immunogold labelling of longus and CFA/III

To further confirm the presence of cross-reacting native epitopes between longus and CFA/III pili, immunogold electron microscopy was performed on ETEC strains. Longus filaments were recognized by anti-longus antibodies (Fig. 4a) and CFA/III pili were specifically immunogold labelled with monoclonal anti-CFA/III 3:3 antibodies (Fig. 4c). Longus, however, was not labelled with anti-CFA/III monoclonal and CFA/III was not labelled with anti-longus antibodies (data not shown). To explore the possibility that both pili could be expressed at the same time by either group of bacteria, simultaneous gold labelling with monoclonal (anti-CFA/III) and rabbit (anti-longus) antibodies was performed using species-specific IgG conjugates coupled to 5 nm (anti-mouse) or 10 nm (anti-rabbit) gold conjugates. CFA/III⁺ strains were labelled only with anti-CFA/III antibodies (Fig. 4b) and longus⁺ strains were labelled only with anti-longus antibodies (data not shown). Thus, it is apparent that CFA/III and longus are

Fig. 3. Analysis of immune cross-reaction between LngA and CofA. ETEC strains were grown on TSAB agar and analysed by Western blotting using rabbit anti-longus antiserum (a) and rabbit anti-CFA/III antiserum (b). E34420A and Z84-1 are CFA/III⁺ strains while E34420B is the CFA/III-negative control; E9034A and B2C are longus⁺ strains while E9034P is a longus-negative control. Note the lack of reactivity between longus and anti-CFA/III antibodies.

not produced simultaneously by a particular ETEC strain and that no apparent cross-reactivity occurs between longus and CFA/III native epitopes. Negative controls, E9034P and E34420B, did not show gold labelling with either antiserum (data not shown).

Differential pilus expression

To learn about the level of pilus expression on different growth media by E34420A and E9034A, an ELISA was performed using rabbit polyclonal anti-longus or anti-CFA/III sera. Since the antibodies employed are specific for each pilus type, the degree of absorbance was considered as a parameter of pilus production on each of the media tested. It appears that E34420A produces ~40% more CFA/III on CFA agar than on TSAB agar. In contrast, the level of longus expression by E9034A on

Fig. 4. Immunoelectron microscopy of ETEC strains producing longus or CFA/III pili. (a) Purified longus pili produced by E9034A on CAB agar and reacted with anti-longus antibodies. Bar, 0.3 µm. (b) Dual labelling of E34420A (CFA/III⁺) with rabbit anti-longus and mouse monoclonal anti-CFA/III 3:3, followed by anti-rabbit IgG conjugated with 10 nm gold particles and anti-mouse IgG conjugated with 5 nm gold particles. Note that only CFA/III antibodies bind to the pili (large arrows). Small arrows point at background 10 nm gold particles not binding to pili. Bar, 0.175 µm. (c) E34420A producing peritrichous CFA/III pili reacted with monoclonal anti-CFA/III 3:3 and anti-mouse IgG conjugated to 10 nm gold particles. Bar, 0.175 µm.

TSAB was eightfold higher than that observed on CFA (data not shown). Thus, it is possible that different regulation systems exist for each of these pili that are triggered by as yet unidentified nutritional or environmental factor(s).

DISCUSSION

Type IV pili are colonization factors produced by a wide variety of Gram-negative bacterial pathogens including some enterobacteria (Strom & Lory, 1993; Weir & Maars, 1992; Maars, 1994). *V. cholerae* produces a TCP (Taylor *et al.*, 1987), EPEC produces BFP (Girón *et al.*, 1991) and ETEC produces longus and CFA/III (Girón *et al.*, 1994; Taniguchi *et al.*, 1995), all of which show high N-terminal amino acid sequence similarities (Girón *et al.*, 1997). Among these, longus and CFA/III are the least well characterized in terms of the genetic organization and regulation of gene expression. Recent DNA sequencing and expression analysis of an ~10 kbp *bfp* gene cluster carried on a large plasmid of EPEC showed that 14 consecutive genes located in tandem are required for expression of native BFP (Sohel *et al.*, 1996; Stone *et al.*, 1996). Taniguchi *et al.* (1995) reported that a 17.5 kbp fragment obtained from an ETEC strain directed the synthesis of CFA/III pili in an *E. coli* laboratory strain. These workers showed that the N-terminal region of LngA is identical to that of CofA, suggesting that these pili might be one pilus type produced by ETEC.

In this study, we report the nucleotide sequence of the structural gene of longus *lngA*, and show that the recombinant *lngA* gene expresses a 25 kDa LngA prepilin protein that is specifically recognized by anti-longus antiserum. The antigenic similarity between longus and CFA/III was analysed to elucidate the relatedness of these two type IV pili of ETEC. Analysis

of the *lngA* DNA sequence reveals that the predicted peptide sequence is ~79% identical to CofA and ~37% identical to TcpA and that the sequence similarities are concentrated at the N-terminal regions of the proteins. Another common characteristic with CofA and other type IV pili is the presence of two cysteine residues at the C-terminus. It has been proposed that these amino acids contribute to disulphide bond formation, which could be important for secretion of the mature pilus protein across the outer membrane (Taniguchi *et al.*, 1995). It then appears that within ETEC, genetic divergences have occurred yielding type IV pilus proteins with similar biochemical and genetic (and probably functional) characteristics that nevertheless show distinct regulatory features.

CFA/III is a semiflexible (6–10 µm long) pilus found in ETEC O25:H16 and O25:H⁻ producing LT only, while longus (>20 µm long) appears to be widely distributed among heterologous ETEC serotypes, mainly those producing ST (Girón *et al.*, 1995a). CFA/III pili are composed of a 20.5 kDa repeating subunit (Shinagawa *et al.*, 1993) while longus is composed of a major structural subunit of 22–24 kDa (Girón *et al.*, 1994). The predicted molecular mass of LngA described here correlates well with the mass determined by SDS-PAGE. In our hands, the size of CofA and LngA was almost indistinguishable on immunoblots. We examined the effect of the growth media on expression of longus or CFA/III by ELISA. ETEC E34420A expresses favourably CFA/III on TSAB and CFA agar while longus is better expressed on TSAB agar. Interestingly, longus is not produced in modified Eagle's medium (DMEM, Difco), a condition that promotes BFP expression by EPEC. The fact that expression of longus requires more defined growth media conditions suggests that CFA/III and longus pili are under the control of different regulatory elements. Further studies on the regulation of longus

gene expression will be necessary to elucidate the nature of these regulatory elements.

We tested cross-reactivities between longus and CFA/III by immunoelectron microscopy. Anti-longus antibodies did not bind to peritrichous or cell-free CFA/III pili. Similarly, a monoclonal anti-CFA/III antibody did not label longus on prototype longus⁺ strains. The lack of recognition of CFA/III by polyclonal anti-longus antibody suggests the absence of common native epitopes on these two pili. There is, however, antigenic relatedness between these two ETEC pili when examined in immunoblots. Anti-longus antibodies recognized both longus and CFA/III pilin subunits, suggesting the presence of common epitopes exposed after denaturation. Rabbit anti-CFA/III antiserum, however, did not react with lngA. CFA/III pilins produced by CFA/III⁺ strains appeared to be rather homogeneous in size while longus pilins showed more heterogeneity as determined by immunoblotting of longus⁺ strains. Similar size and antigenicity differences were observed among BfpA produced by EPEC, employing polyclonal and monoclonal anti-BFP antibodies (Girón *et al.* 1993a, 1995c). Thus, it appears that within a bacterial species or a category of pathogens such as EPEC or ETEC, a subfamily of related pilins might be produced.

Most type IV pili contribute to virulence by promoting attachment to various types of receptors during colonization of host mucosal surfaces (Strom & Lory, 1993). For example, CFA/III pili mediate adherence to intestinal epithelial cells of infant rabbits, Caco-2 cells and human enterocytes (Knutton *et al.*, 1989). However, neither CFA/III nor longus-producing ETEC haemagglutinates human red blood cells (Girón *et al.*, 1994; Honda *et al.*, 1984). The TCP was shown to be an important colonization factor in the pathogenesis of cholera (Taylor *et al.*, 1987). The demonstration of BFP within bacterial clusters adhering to HEP-2 cells and the inability of *bfpA* mutants to form localized adherence suggests that BFP is the EPEC adherence factor (Girón *et al.*, 1993a, b). With respect to longus, more genetic and functional studies are needed to elucidate the role of longus as a colonization factor. Nevertheless, an *E. coli* K-12 strain harbouring plasmid pJAG1, which contains a 7 kbp fragment encoding *lngA*, became highly aggregative after growth in liquid broth. In summary, distinction between longus and CFA/III pili can be made on the bases of morphological features, serotype distribution and association with enterotoxin genes. Our data on non-cross-reactivity at native epitopes and on differential regulation of pili expression further confirm the differences between these pili, even though *lngA* and *cofA* genes were found to be highly similar at the DNA and protein levels. Thus, it appears that longus and CFA/III are two independent type IV pili of ETEC. Due to the significant homology between longus and CFA/III pili, new genetic tools may be necessary to re-evaluate the prevalence of these pili among ETEC strains. Development of genetic tools, such as DNA probes, will be based on the analysis of DNA sequencing of *lngA* and *cofA* from multiple ETEC strains. Fur-

thermore, genetic studies will be required to elucidate the common ancestor of these pilin genes and the stage in evolution where they began to diversify.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant No. AI21657 to J.B.K. and Conacyt Grant 3485P-M9607 to J.A.G. Special thanks to Stuart Knutton for ETEC 31-10, Karen Taylor for bacterial cultures and Timothy McDaniel for critical discussions.

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Received 28 August 1998; revised 5 January 1999; accepted 19 March 1999.