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Prevalence and Association of the Longus Pilus Structural Gene (*lngA*) with Colonization Factor Antigens, Enterotoxin Types, and Serotypes of Enterotoxigenic *Escherichia coli*

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Human enterotoxigenic *Escherichia coli* (ETEC) produces a plasmid-encoded type IV pilus termed longus (for long pilus). Regardless of the geographic origins of ETEC strains, the longus structural gene *lngA* was found to have the highest level of association with ETEC producing colonization factor antigen (CFA) CFA/II, followed by ETEC producing CFA/I and CFA/IV. ETEC bearing the less prevalent CFA/III and putative colonization factors and ETEC negative for CFA and putative colonization factor also contained *lngA*-related sequences. *lngA* was found in a considerable number of ETEC serotypes and was more often associated with ETEC producing heat-stable enterotoxins than with ETEC producing both heat-labile and heat-stable enterotoxins or heat-labile enterotoxin alone. *lngA* was found more often in strains isolated from children with diarrhea than in strains from healthy children, suggesting an association with intestinal disease. We conclude that longus is a widely distributed antigenic determinant in ETEC that is highly associated with known plasmid-encoded virulence factors, namely, CFAs and enterotoxins. A longus-specific probe may be a helpful epidemiological tool to assist in the identification of ETEC.

Diarrheal disease due to enterotoxigenic *Escherichia coli* (ETEC) continues to be an important health problem in developing countries, especially among pediatric populations and travellers to these countries (2, 4, 14, 20, 23, 30, 38). ETEC adheres to and colonizes the gut mucosa by means of a repertoire of fimbrial antigens called colonization factor antigens (CFAs) (7, 8, 21). ETEC infections are manifested by watery diarrhea due to the activity of heat-labile and/or heat-stable enterotoxin (LT and ST, respectively). Among the CFAs described, CFA/I, CFA/II, and CFA/IV are the best characterized and the most prevalent (1, 6, 14, 25, 26). CFA/II comprises a family of coli surface antigens, CS1, CS2, and CS3; CS3 may be found alone or associated with CS1 or CS2 (5, 22, 35). Similarly, the CFA/IV family of coli surface antigens consists of CS4, CS5, and CS6 fimbrial determinants; CS6 may be found alone or associated with CS4 or CS5 (21, 35). Less important epidemiologically are CFA/III, which has been found only in LT-producing strains of the O25:H16 and O25H$^-$ serotypes and in some instances has been associated with CS6 (26), and a variety of putative colonization factors (PCFO9, PCFO20, PCFO148, PCFO159, PCFO166, CS7, and CS17) which are restricted to fewer serotypes and are generally found in LT-producing strains (1, 14, 16, 21, 25, 26, 36, 37). Since natural and experimental infections of ETEC elicit immunity through antibodies directed to CFAs and LT, a pilus-based ETEC vaccine should contain those CFAs which are most prevalent (3, 21, 32).

Recently, a new plasmid-encoded pilus antigen termed longus (for long pilus) in human ETEC strains was identified and characterized after growth on CDC anaerobe blood agar (12). Longus is characterized by its length (>20 μm long), its polar distribution, and its tendency to form large bundles of pilus aggregates (12). In contrast, CFAs are conventionally sought after growth on CFA agar and are pili 1 to 2 μm long, distributed peritrichously on the bacterial cell surface (7). Production of both CFAs and longus is temperature dependent and is mediated or controlled by large plasmids generally also encoding ST and/or LT (8, 12, 28). Longus is composed of a repeating structural subunit of 22 kDa, and its N-terminal amino acid sequence shows similarity with the toxin-coregulated pilus (TCP) of *Vibrio cholerae*, the bundle-forming pilus of enteropathogenic *E. coli*, and to a lesser extent with the type IV, or methylphenylalanine, pilins of some gram-negative pathogens (10, 12, 31, 34). A subclassification of the growing family of type IV pili was proposed; in this classification the methylphenylalanine pili of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella bovis*, *Moraxella lacunata*, *Moraxella nonliquefaciens*, *Dichelobacter nodosus*, *Pseudomonas aeruginosa*, *Branhamella catarrhalis*, *Kingella denitrificans*, *Kingella kingae*, and *Eikenella corrodens* are grouped into class A, and the nonmethylphenylalanine pili such as TCP, the bundle-forming pilus, and longus found in intestinal pathogens are grouped into class B (12, 31). Recently, elucidation of the DNA sequence of the gene encoding the major pilin subunit of CFA/III (*cofA*) of ETEC revealed that CFA/III has protein and DNA similarities to type IV pili, especially with TCP and longus, which places CFA/III in type IV pilus class B (33). Thus, CFA/III is the only member in type IV pilus class B that is produced peritrichously on the cell surface. The overall resemblance of longus to the latter intestinal colonization determinants suggests an analogous role of longus in the pathogenesis of ETEC.

To learn about the epidemiology of this putative colonization factor (PCF), we studied the prevalence of the gene encoding the major repeating subunit of longus, *lngA*, among ETEC of different geographic origins, as well as its association

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TABLE 1. Prevalence of longus among ETEC isolates from different geographic locations

Origin of collection	Total no. of strains studied	Total no. (%) longus positive[a]	Total no. (%) longus negative
CVD[b]	102	39 (38)	63 (62)
Egypt	215	67 (31)	148 (69)
Chile	340	95 (28)	245 (72)
Bangladesh	74	14 (19)	60 (81)
Total	731	215 (29)	516 (70)

[a] Strains that hybridized with longus oligonucleotide probe K100 were considered positive.
[b] The CVD collection contains ETEC strains from all around the world.

with the most prevalent CFAs, PCFs, ST and LT, and O:H serotypes of ETEC by using DNA hybridization. The ETEC strains analyzed for the presence of longus-related sequences were obtained from different sources (Table 1). The collection of ETEC from the Center for Vaccine Development (CVD) includes strains from a variety of countries and that produce various CFAs and enterotoxins. The ETEC strains from Egypt were isolated from diarrheal stool samples from U.S. troops serving in training exercises in the vicinity of Cairo and were previously described (38). The ETEC strains from Chile were isolated in the course of an epidemiological study of *E. coli* diarrheal infections in children (0 to 47 months of age) from a low-socioeconomical-level periurban community in Santiago (20). The ETEC strains from Bangladesh were isolated from 36 and 38 children with and without diarrhea, respectively. To determine the association of *lngA* and less prevalent CFAs and PCFs, a collection of 59 strains comprising 5 CFA/III-, 5 CS7-, 5 CS17-, 1 PCFO20-, 4 PCFO159-, and 9 PCFO166-producing ETEC strains and 30 CFA- and PCF-negative ETEC strains reported previously (1, 18, 36, 37) was studied. The ETEC strains, which had been maintained at −70°C in Luria broth and 15% glycerol, were cultured on Luria agar for colony DNA hybridization.

Previously, we showed that an oligonucleotide probe [K100; 5′-CTGTGGAA(G)GTA(T)ATCATCGTA(T)CTG-3′] derived from the N-terminal amino acid sequence of the structural subunit of longus specifically hybridized with human ETEC strains that express longus (12). Animal ETEC and nonenterotoxigenic *E. coli*, as well as other type IV pilus-producing bacteria such as *V. cholerae* and enteropathogenic *E. coli*, did not hybridize with the longus probe (9, 11, 12, 34). The K100 probe was end labeled with [γ-^{32}P]dATP by using polynucleotide kinase. To confirm the presence of ST, LT, and CS3 genes in prototype strains, specific DNA probes were used and labeled by nick translation. The ST (0.216-kbp) and LT (1-kbp) gene probes were obtained from pCVD427 and pCVD403, respectively, as previously described (20). The CS3 probe consisted of a 4.5-kb *Hin*dIII fragment containing the operon of CS3, which was obtained from pCS100 (13). All DNA probes were purified by using a G-25 quick-spin column (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (24). DNA extraction and hybridization conditions were as previously described (9, 12, 24). The filters incubated with K100 were washed at 50°C for 10 min with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% sodium dodecyl sulfate (SDS), for 10 min with 1× SSC containing 0.1% SDS, and for 10 min with 1× SSC containing 0.5% Triton X-100 and were given a final rinse with 1× SSC at room temperature. Filters incubated with double-stranded DNA probes were hybridized at 37°C and washed at 65°C in 5× SSC containing 0.5% SDS (12). All filters were then exposed to X-ray film at −70°C.

Among a total of 731 ETEC strains studied, longus-related sequences were found in 29% (Table 1). This frequency is not negligible considering the reported frequency of CFA/I (11 to 56%) and CFA/II (13 to 33%) in ETEC strains from previous epidemiological studies (14, 21, 25, 38). ETEC were analyzed on the basis of geographic origin; *lngA* sequences were found in 31, 28, and 19% of ETEC strains from Egypt, Chile, and Bangladesh, respectively. In the collection of ETEC strains from the CVD, which contains strains from throughout the world, *lngA* was found in 38% of the strains. In light of the high level of prevalence of longus among the ETEC strains studied, we determined the association of the presence of longus-related sequences with CFAs and PCFs among ETEC strains. In all the groups of ETEC strains studied, *lngA* was more frequently found among strains that produced CFA/II, followed by CFA/I and CFA/IV (Table 2), suggesting that in contrast to CFAs which are not found to be associated with other CFAs, *lngA* may be a very common feature of ETEC. In addition, a considerable percentage of ETEC isolates with no known CFA hybridized with the *lngA* probe (Table 2). All six CFA/III- and one PCFO159-producing ETEC isolate hybridized with the longus probe (data not shown). The genetic relationship between CFA/III and longus was reported by Taniguchi et al. (33). One of the six CFA/III-producing, longus probe-positive ETEC isolates is isolate E34420A, also reported as 31-10 (16, 17) and previously noted by McConnell and Rowe to also produce CS6 (26). These data suggest that ETEC strains bearing some of the less prevalent PCFs may also be able to produce longus.

A wealth of evidence supports the association of CFAs with enterotoxins (1, 6, 21, 27, 28, 38). In this study, we found *lngA* to have the highest prevalence among ETEC strains producing ST only, followed by ETEC strains producing both LT and ST (Table 2). In the collection of ETEC isolates from Bangladesh, 14 of 14 (100%) LT-only-producing ETEC isolates hybridized with longus probe. This collection contained 47 of 74 (63.5%) ETEC isolates producing LT only and 27 of 74 (36.5%) ETEC isolates producing ST only, differences which may account for the high frequency of *lngA* among the Bangladeshi LT-producing strains. Among ST-only-producing ETEC strains isolated from children with diarrhea in Chile, circa 70% of ST-producing ETEC isolates hybridized with the longus probe (data not shown). The high level of association of ST and longus found in this study suggests that longus and ST are borne on the same

TABLE 2. Association of longus with other fimbrial antigens and enterotoxins of ETEC

CFA or enterotoxin	No. (%) of longus-positive ETEC strains from each location associated with virulence factor			
	CVD (n = 39)	Egypt (n = 67)	Chile (n = 95)	Bangladesh (n = 14)
CFA/I	8 (21)	8 (12)	32 (34)	ND[a]
CFA/II[b]	24 (62)	28 (42)	39 (41)	ND
CFA/IV[c]	4 (10)	9 (13)	1 (1)	ND
None	3 (7)	22 (33)	23 (24)	ND
LT	ND	6 (9)	7 (7)	14 (100)
ST	ND	40 (60)	48 (50)	0 (0)
LT and ST	ND	21 (31)	45 (43)	0 (0)

[a] ND, not determined.
[b] Includes CS1, CS2, and CS3.
[c] Includes CS4, CS5, and CS6.

plasmid or that two separate plasmids, one containing the ST gene and the other containing the longus gene, are compatible and frequently coexist among these strains. However, in the course of this study, we noted that some strains lost the ST and/or LT gene while remaining *lngA*$^+$, suggesting that ST and *lngA* may not be necessarily encoded on the same plasmids (data not shown). The loss of ST genes may be explained by deletions within or loss of the large virulence plasmid (26–28). To learn about the association of longus with disease, we analyzed a subset of strains from Bangladesh that were isolated from symptomatic and asymptomatic children. Longus-related sequences were found in 33.3% of strains isolated from children with diarrhea, whereas only 5.2% of ETEC strains from healthy children contained *lngA* sequences. Since all the strains from Egypt, Chile, and the CVD were isolated from children or adults with ETEC infections, it appears that longus is highly associated with diarrheal disease. Serotyping data were available for all the ETEC strains studied except for the strains from Bangladesh. Similar to CFA/I and CFA/II antigens, which are widely distributed among ETEC serotypes, *lngA* was also found in a considerable number of serotypes (O2:NT, O6:NM, O6:H$^-$, O6:H16, O6:H48, O8:H$^-$, O8:H6, O8:H21, O8:H29, O20:H$^-$, O25:NM, O25:H42, O49:NM, O128:NM, O139:H28, O148:H28, O153:H$^-$, O153:H45, O158:H10, and O159:H4). These data provide further evidence of the wide distribution of longus among ETEC strains.

A general criterion for the identification of CFAs is the positivity of CFA agar-grown ETEC on colony dot blots, slide agglutinations, enzyme-linked immunosorbent assays, or Western blots (immunoblots) utilizing specific pilus antisera (1, 6, 7, 14, 20, 23, 26). However, these assays rely on phenotypic expression, which in turn depends on the compositions of media and growth conditions; therefore, morphological differences among the different pili, like CFA/III and longus, may be overlooked. Thus, it is possible that some of CFA/III$^+$ ETEC strains isolated in epidemiological studies may have been inadvertently misclassified. There exists the possibility that some of the strains examined in this study that hybridized with the *lngA* probe are indeed CFA/III$^+$ ETEC strains. Several findings argue against this possibility. For example, CFA/III was originally reported to occur in strains 31-10, 260-1, and TH31 as long (6- to 10-μm), semiflexible, peritrichous pili that exhibit some flexures and which are produced on CFA agar (16, 17, 19, 37). Shinagawa et al. (29) noted that when the genes that control the formation of CFA/III were mobilized into *E. coli* K-12, the transconjugant strain produced long, peritrichous pili similar to those produced by the parent strain. The morphology of CFA/III in other ETEC strains has not been reported. On the other hand, longus-positive ETEC strains produce long (>20-μm), polar pili which are not produced on CFA agar (12). It is obvious that genetic factors that control the length and the peritrichous and polar distribution of these pili on the bacterial cell surface exist. Moreover, CFA/III production is mostly found in the O25:H$^-$ and O25:H16 ETEC serotypes, which are associated with LT production, while longus *lngA* appears to be widely distributed among heterologous ETEC serotypes, mainly those producing ST (12).

In view of the DNA and protein similarities observed between longus, CFA/III, and TCP (33), monoclonal antibodies 121-42B (17) and CFA/III 3:3 (37) against CFA/III of ETEC TH31 and 31-10, respectively, and polyclonal antilongus (12) and anti-TCP antibodies were used to determine antigenic cross-reactivities between these two pili by slide agglutination (12, 37). CFA/III-producing ETEC strains E3442OA (O25:H$^-$), Z14-2 (rough:H33), Z26-5 (O25:H$^-$), Z84-1 (O25:H$^-$), Z128-6 (O?:H$^-$), and D117-5 (rough:H33) (16–19) were grown on CFA agar, and longus-positive ETEC strains E9034A, B2C, B7A, 633C1, M4447, and 9964 (12) were grown on CDC anaerobe blood agar and Trypticase soy agar with 5% defibrinated sheep blood at 37°C. CFA/III-producing ETEC did not agglutinate with anti-TCP or antilongus antibodies, and longus-producing ETEC did not agglutinate with anti-CFA/III or anti-TCP antibodies. These data suggest that there is no cross-reactivity between natives epitopes of these pili. The genetic relatedness between longus and CFA/III will be addressed elsewhere (10).

The study of the prevalence of CFAs in ETEC is a prerequisite for the design of a pilus-based vaccine for use in humans (1, 3, 5, 21, 32). Among the known CFAs of ETEC, CFA/I and CS3 (the common antigen in the CFA/II family of pilus antigens) are the most prevalent pilus antigens found in clinical isolates but are never expressed together by the same wild-type strain (21). In nature, ETEC strains expressing any known CFA do not express a second CFA. However, the less prevalent CFA/III may be found to be associated with CS6-producing ETEC, but the determinants for these antigens are encoded by different plasmids (26). Recently, Viboud et al. found that a number of ETEC isolates which lacked CFA/I, CFA/II, and CFA/IV possessed other less common PCFs, namely, CFA/III, CS7, CS17, PCFO20, and PCFO166 (36, 37).

In this study, the gene encoding the repeating subunit of longus (*lngA*) was found to be highly associated with ETEC isolates that bear the ST gene alone or both the ST and LT genes and with ETEC isolates producing CFA/II and CFA/I (the most prevalent PCFs) and was more frequently found in ETEC isolated from children with diarrhea than in ETEC from healthy children, suggesting that longus may be associated with intestinal colonization and diarrhea. While the production of longus pilus was not sought in all strains analyzed here, we believe that the presence of the *lngA* is an indicator of the presence of the potential genetic machinery necessary to produce longus. The association of production of longus pilus and hybridization with the longus probe has been shown earlier (12). The wide distribution of *lngA* among ETEC serotypes demonstrated in this study suggests that at some time in evolution, this factor may have been acquired by ETEC strains from a compatible genetic element containing the longus gene or a longus-related gene(s) and that longus-negative strains may have lost this element or never had it. Whether ETEC longus sequences were acquired from *V. cholerae*, enteropathogenic *E. coli*, or other type IV piliated organisms or vice versa is an interesting evolutionary question that remains to be explored. It appears that within ETEC type IV pili, genetic divergences have occurred and that these divergences have yielded pilus proteins with similar biochemical, genetic, and functional properties that nevertheless show distinct morphological properties and are under the control of different regulatory elements. The N-terminal sequence similarities between longus and type IV pilins of some gram-negative pathogens suggest an association with colonization and virulence (11, 15, 31).

The high frequency of *lngA* among ETEC strains indicates that the plasmid encoding *lngA* is quite stable among ETEC strains, and considering that bacterial passages promote the loss of virulence markers (8, 27, 28), we predict that the frequency of *lngA* in fresh epidemic isolates would be higher. Longus may constitute an additional or accessory colonization factor of ETEC or may be important for the survival of ETEC in certain biological niches. Elucidation of the role of longus as a colonization factor and as an immunogenic antigen is essential before the true significance of longus can be determined. The wide distribution of *lngA* among ETEC isolates is an

important consideration for the development of a pilus-based vaccine against ETEC.

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