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Type IV Longus Pilus of Enterotoxigenic *Escherichia coli*: Occurrence and Association with Toxin Types and Colonization Factors among Strains Isolated in Argentina

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The longus type IV pilus structural gene (*lngA*) was sought among 217 clinical enterotoxigenic *Escherichia coli* (ETEC) strains isolated in Argentina. *lngA* was present in 20.7% of the isolates and was highly associated with ETEC producing heat-stable toxin and the most common colonization factors. The prevalence of longus among ETEC strains in Argentina was comparable to that of colonization factor antigen I (CFA/I), CFA/II, and CFA/IV in other regions of the world.

Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of infantile diarrhea in developing countries, as well as an important etiologic agent of traveler's diarrhea (14, 16, 19, 21, 23, 26, 28, 33). Adhesion to the small intestine is essential in the pathogenesis of ETEC, allowing the bacteria to overcome the defense mechanisms of the host and colonize epithelial cells. Subsequently, the microorganisms produce the heat-labile (LT) and heat-stable (ST) enterotoxins responsible for inducing watery diarrhea (13). ETEC adheres to enterocytes by means of appendages termed colonization factors (CFs), which include the colonization factor antigens (CFAs) and putative colonization factors (PCFs). So far, 21 different CFs have been described; they can be grouped into families according to amino acid sequence similarities of their structural subunits (6, 24). In addition, a large proportion of ETEC strains produce a type IV pilus called longus (9). Unlike all other pili of ETEC, longus has a distinct morphology, forming long filaments that can extend over 20 μm from the cell surface (9). Type IV pili are considered to function as CFs or as receptors for different phages harboring enterotoxin or other virulence genes, and they are found among several gram-negative bacterial pathogens, including *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, enteropathogenic *E. coli* (8), and *Vibrio cholerae* (31).

It has been shown that ETEC CFs are good immunogens that can elicit a protective antibody response (27, 29). However, to be effective, a pilus-based vaccine should include the most prevalent CFs as well as a toxin component (15, 30). During the last decade, many epidemiological studies have been carried out to assess the prevalence and characteristics of ETEC strains in several areas of the world (2, 16, 21, 23, 28). Despite the vast antigenic variation and geographic distribution observed, particular associations of phenotypic traits (i.e., serotype, toxin, and CF profile) seem to prevail (35); these include O6:H16 CFA/II LT-ST, O153:H45 CFA/I ST, and O78:H12 CFA/I ST, among others. These studies have also shown that a large proportion of ETEC strains do not produce any of the CFs for which tests are performed. Although longus has only been sought in a limited number of studies, it is apparent that this pilus is widely distributed among ETEC isolates since it has been found in strains of various serotypes, both in isolates producing other CFs and in strains lacking these adhesins (7, 12, 25). The prevalence of longus among ETEC strains isolated from different areas suggests that longus should be considered a potentially important component of ETEC vaccines (35).

In Argentina, ETEC constitutes one of the main causes of infantile diarrhea, accounting for 10 to 15% of the cases of bacterial intestinal infections (20, 32). Several epidemiological studies have shown that a large proportion (40%) of ETEC strains isolated from patients with diarrhea do not produce any of the known CFs (2, 33, 34). In this study, we assessed the prevalence of the longus type IV pilus gene among ETEC strains isolated in Argentina and evaluated the expression of this pilus and its association with other virulence factors.

A total of 217 ETEC strains isolated from children with diarrhea in nine different areas of Argentina were included in the study. The group comprised isolates recovered in epidemiological studies conducted from 1988 to 1993 (2, 33, 34). All of the strains were kept at −70°C in the culture collection of the Bacteriology Department of the National Institute of Infectious Diseases—Administración Nacional de Laboratories e Institutos de Salud "Carlos G. Malbrán." The CF profiles of these strains had been determined previously by dot blot enzyme-linked immunosorbent assay with specific monoclonal antibodies (MAbs) against CFA/I, CS1 to CS8, CS12, CS14, and CS17 (provided by Ann-Mari Svennerholm [18, 34]) or with polyclonal antisera against CS15 (provided by Arlette Darfeuille-Michaud [1]), CS18, and CS22. Strain E9034A was used as a positive control for the presence and expression of the longus gene (9).

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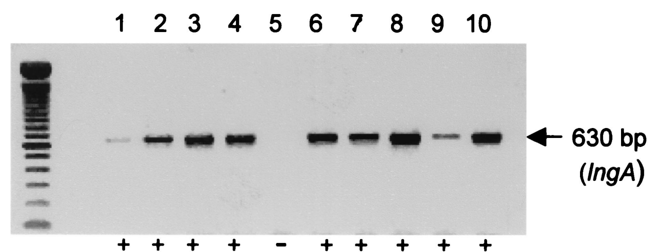

FIG. 1. Amplification of *lngA* in ETEC strains isolated in Argentina. Lanes: 1, Z14-d1 (LT-ST, CFA/II); 2, D18-2 (ST, CFA/I); 3, D25-2 (ST, CFA/I); 4, D40-6 (LT-ST, CFA/II); 5, Z45-4 (LT, CF negative); 6, C261-3 (ST, CFA/I); 7, T567-5 (LT, ST, CFA/II); 8, LP 17592-2 (LT, CF negative); 9, M1777-4 (ST, CF negative); 10, MP196-6 (ST, CFA/I).

Occurrence of the longus gene in Argentinean ETEC isolates. To assess the presence of the longus gene (*lngA*), a DNA probe consisting of the entire *lngA* gene, obtained by *Eco*RI digestion of plasmid pZG1, was employed to detect this gene by colony blot DNA hybridization as previously described (10). A total of 45 isolates (20.7%) carried the *lngA* gene, as determined by DNA hybridization. These isolates were analyzed by an *lngA*-based PCR protocol as follows. For the amplification reaction, a suitable amount of bacterial growth was removed from a colony on a Luria agar plate and suspended in 50 μl of a PCR mixture containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate, and 0.5 U of *Taq* polymerase. The oligonucleotide primers derived from the *lngA* DNA sequence were JG1 (5′-CGGAATTCATGAGCCTGCTGGAAGTTATCA-3′) and JG2 (5′-CGGAATTCCGGCTACCTAAAGTAATTGAGT-3′) (10, 12). The PCR consisted of 10 min of heating at 95°C, 35 cycles of 1 min of denaturation at 95°C, 3 min of annealing at 50°C, 3 min of primer extension at 72°C, and a final extension of 10 min at 72°C (12). The amplified 0.63-kbp DNA fragments were resolved by 1% agarose gel electrophoresis and visualized under UV transillumination after ethidium bromide staining. All of the *lngA* probe-positive strains were confirmed by PCR to contain *lngA* (Fig. 1).

The high prevalence of longus among the Argentinean ETEC isolates is in agreement with that observed in other regions, including Egypt, Chile, and Mexico (7, 12), and it favors the idea that this pilus is a widely distributed antigen among ETEC strains. A recent surveillance of diarrhea in Bangladesh showed that longus was found in only 8.5% of the ETEC strains analyzed (25). This lower frequency might be attributed to the use of MAbs for detecting longus-producing ETEC as opposed to determination of the presence of the longus structural gene, as was done in other studies. It is possible that some isolates bearing longus genes were missed in that study. The high prevalence of longus, in combination with the fact that longus has been reported to elicit immune responses in patients with diarrhea (25), supports the notion that this pilus may represent a good candidate for inclusion in a vaccine.

Association with serogroups, enterotoxins, and CFs. The presence of longus was determined to be associated with various O groups, the most common being serogroups O6, O153, and O78, among others (Table 1). When the *lngA*-positive ETEC strains were analyzed for their association with the production of enterotoxins and CFs, we found that 29 of the 45 isolates (64.4%) produced ST, whereas 12 strains (26.7%) produced both LT and ST and 4 isolates (8.9%) produced only LT (Table 2). Several studies have demonstrated a strong association between the presence of CFs and toxin type (2, 17, 22, 35). In a collection of strains from all over the world, the *lngA* gene showed the highest degree of association with the ST and LT-ST phenotypes (7). Nevertheless, *lngA* can be found, albeit at a low frequency, in ETEC producing only LT. The strong degree of association between ST and longus suggests that the two are encoded on the same plasmid or, alternatively, that the genes are present on coexisting, compatible plasmids.In our collection of 217 ETEC strains, 95 isolates (43.8%) were pos-

TABLE 1. Prevalence of longus among ETEC strains isolated in Argentina and association with serotypes and CF profiles

CF	Serotype (no. of strains)[a]	
	Longus positive	Longus negative
CFA/I	O78:H12 (n = 6) O78:H$^-$ (n = 1) O78:H? (n = 1) O153:H45 (n = 3) ND (n = 5) Other (n = 2)	ND (n = 4)
Total	18	4
CFA/II	O6:H16 (n = 4) O6:H$^-$ (n = 2) O6:H? (n = 2) O8:H9 (n = 1) ND (n = 2)	O6:H16 (n = 2) O6:H? (n = 1) ND (n = 1)
Total	11	4
CFA/IV	O128:H21 (n = 1) O148:H28 (n = 1) ND (n = 5)	O128:H21 (n = 4) O128:H? (n = 2) ND (n = 4) Other (n = 7)
Total	7	17
CS14	O78:H$^-$ (n = 1)	O78:H? (n = 5) O78:H$^-$ (n = 2) O78:H18 (n = 1)
Total	1	8
CF negative	O27:H7 (n = 2) ND (n = 1) Other (n = 5)	O9:H$^-$ (n = 4) O8:H10 (n = 3) O15:H40 (n = 3) ND (n = 67) Other (n = 37)
Total	8	114

[a] ND, not determined; H$^-$, nonmotile; H?, flagellar antigen unknown.

TABLE 2. Association of longus with toxin profile among ETEC strains from Argentina

Toxin(s)	No. of strains (%)	
	Longus positive (n = 45)	Longus negative (n = 172)
ST	29 (64.4)	49 (28.5)
LT-ST	12 (26.7)	16 (9.3)
LT	4 (8.9)	107 (62.2)

itive for one or more CF antigens, among which 37 (38.9%) contained the longus gene. Thus, 18 (40%) of the 45 longus-positive ETEC strains produced CFA/I, 11 (24.4%) produced CFA/II, 7 (15.5%) produced CFA/IV, 1 (2.2%) produced PCFO166, and 8 (17.7%) CF-negative ETEC strains contained *lngA*. These results show that longus can be found in ETEC strains expressing the most common CFs or in ETEC strains that have been determined to express none of the CFs so far identified. In other regions of the world, longus was highly associated with CFA/II followed by CFA/I and CFA/IV (7). Therefore, it is apparent that the association of longus with other CFs in ETEC differs according to the geographical region studied. We observed that longus was most highly associated with bacteria expressing CFA/I and CFA/II, probably because most of the isolates possessed these determinants. A recent study has shown that CFA/I, CFA/II, and CFA/IV are the most prevalent fimbrial antigens in Dhaka (26), where longus was found to be associated with CFA/II and CFA/I (25). Here we found that 7 of the 24 CFA/IV-positive isolates tested were longus positive. It is known that there are variations in the CF pattern and toxin types in ETEC strains from various geographic areas, which could partly explain the differences in coexpression of various pili (11, 16, 19, 23). We detected longus in one isolate that expressed the less prevalent PCFO166. These data support the view that longus is generally encoded alone or together with other CF antigens, suggesting an important role as an adhesive determinant or as an accessory CF contributing to the adhesive attributes of ETEC (35).To evaluate the expression of longus, all of the PCR *lngA*-positive strains identified, including those producing other CFs, were grown on trypticase soy agar with 5% sheep blood at 37°C in air, as previously described (25). The pilus was subsequently detected with specific MAb ICA39 by dot blot enzyme-linked immunosorbent assay using the appropriate secondary antibodies (25). The blots were developed with a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma). Only 12 of 45 (27%) strains that carried *lngA* expressed longus. It has been shown that phenotypic expression of ETEC CFs is affected by growth conditions and in general requires the presence of transcriptional activators (4, 5). Thus, the conditions we used for the expression of longus in vitro may not have been optimal for the production of the pili. Nevertheless, the presence of the gene suggests that it could be produced in vivo under adequate conditions.Since longus is coexpressed with other CF antigens, further studies need to be carried out to gain an understanding of how longus contributes to the adherence and colonization capacities of the bacteria in vivo. Because ETEC is a major cause of disease burden in children, leading to considerable growth retardation, malnutrition, and mortality, serious efforts should be made to develop strategies for prevention of this health problem, especially in countries of the world where ETEC infections are endemic (3). We believe the information obtained from the present study may be useful for this purpose.

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