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Detection of *toxB*, a Plasmid Virulence Gene of *Escherichia coli* O157, in Enterohemorrhagic and Enteropathogenic *E. coli*

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The virulence plasmid of *Escherichia coli* O157 strain EDL933 carries a 10-kb putative virulence gene designated *toxB*. Little is known about the distribution of this gene among *E. coli* O157 strains or its presence in other enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) strains. We developed PCR and hybridization tools for the detection of the entire *toxB* sequence and investigated its presence in a collection of EHEC O157 strains and other EHEC and EPEC strains belonging to different serogroups and isolated from different sources. The EHEC O157 strains reacted with all of the PCR primers and probes used, thus indicating the presence of a complete *toxB* gene regardless of the human or bovine origin of the isolates. Similar positive reactions were observed for about 50% of the EHEC O26 strains tested and a few other EHEC and EPEC strains. However, the size of the DNA fragments hybridizing with the *toxB* probes differed from that of the positive fragments from EHEC O157, suggesting a polymorphism in the *toxB* genes present in the different *E. coli* serogroups. Moreover, several EHEC and EPEC strains belonging to different serogroups reacted with only some of the genetic tools used, suggesting either the existence of major variants of *toxB* or the presence of fragments of the gene. Southern blotting analysis showed that *toxB* sequences were located on large plasmids in EHEC and EPEC O26 as well.

Escherichia coli O157 is a zoonotic pathogen which represents worldwide an important cause of diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) (10). The pathogenicity of *E. coli* O157 mainly relies upon the production of Shiga toxins (Stx) and the capability of colonizing the intestinal mucosa of the host with a characteristic attaching and effacing (A/E) mechanism of adhesion (18). The production of Stx is due to the presence of lysogenic bacteriophages carrying *stx* genes (20), while the formation of A/E lesions is genetically governed by a pathogenicity island (PAI) termed the locus of enterocyte effacement (LEE) (14). This locus comprises genes coding for several effectors involved in the pathogenesis, such as the adhesin intimin, its translocated receptor, and a type III secretion system (9). The capability to induce A/E lesions is shared by other Stx-producing *E. coli* strains, which belong to a restricted number of serogroups and are usually referred to as enterohemorrhagic *E. coli* (EHEC), and by enteropathogenic *E. coli* (EPEC) strains, which do not produce Stx and represent a common cause of infantile diarrhea (18). These two groups of pathogens have also been termed attaching and effacing *E. coli* (AEEC).

In addition to the LEE and Stx-converting phages, the pathogenesis of EHEC O157 infections likely involves virulence factors encoded by other PAIs and other phages and by a large plasmid referred to as pO157. This plasmid is consistently present in EHEC O157 strains (25) and carries the genes governing the production of enterohemolysin (*ehxA*) (24, 26) and other putative virulence factors, such as a catalase-peroxidase (*katP*) (5) and a serine protease (*espP*) (6). Sequencing of the whole pO157 plasmid showed the presence of another large putative virulence gene, named L7095 in the EHEC O157 EDL933 strain (7) and *toxB* in the RIMD 0509952 strain (13). This gene is 9.5 kb in size, and the deduced amino acid sequence of its product shows 20% similarity with toxin B of *Clostridium difficile* (2, 13), which belongs to the large-clostridial-toxin family (34). Tatsuno and collegues (32) showed that the product of the *toxB* gene contributes to the adherence of EHEC O157 to Caco-2 cells through the promotion of the production and/or the secretion of type III secreted proteins. Moreover, the ToxB protein shares a considerable homology (28% of identical amino acids and 47% of similar amino acids) with the product of *efa-1*/*lifA*, another virulence gene frequently found in AEEC isolates (17). This gene is located in a PAI designated O#122 in the sequence of the EHEC O157 EDL933 strain (22) and has a role in enhancing the adhesion of EHEC O111 and EPEC O127 to cultured cells (1, 19). Moreover, its presence inhibits the activation of human and murine gastrointestinal lymphocytes (12) and influences the intestinal colonization and enteropathogenicity in calves experimentally infected with an EHEC O111 strain (28). *efa-1*/*lifA* has been detected in EHEC strains belonging to serogroups other than O157 (non-O157) and in EPEC strains (17), while EHEC O157 strains possess only a fragment of this gene, corresponding to the first 1,300 bp of the 5′ region (11, 17, 22).

As far as the distribution of *toxB* is concerned, its presence has been demonstrated only in the two pO157 plasmids that have been sequenced (7, 13), and little is known about its presence in EHEC non-O157 and EPEC strains. Recently, the presence of a 600-bp region of the 5′ terminus of *toxB* in EHEC isolates belonging to serogroups O121, O26, O103, and O145 was detected by PCR (30). In addition, a DNA sequence (open reading frames 35 and 36) showing 97% identity with a 1,900-bp fragment of the 3′ region of *toxB* is present on the

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TABLE 1. PCR primers used in this study

Primer designation	Sequence	Primer location[a]	Reference or source
toxB.911F	5′-ATA CCT ACC TGC TCT GGA TTG A-3′	79431–79452	30
toxB.1468R	5′-TTC TTA CCT GAT CTG ATG CAG C-3′	80030–80009	30
toxB 5′int-up	5′-TTT TCG CGT AAT GAT GC-3′	81278–81294	This study
toxB 5′int-lo	5′-ACG CCG TGA GAA TAA TGT C-3′	81954–81936	This study
toxB 3′int-up	5′-CAA CAG CCC CTT CAT TCC ATT C-3′	83708–83729	This study
toxB 3′int-lo	5′-TTT TGC CAC ATT GCT AAG ATA ACG-3′	84277–84254	This study
toxB 5′ upper	5′-AAA ATA ATT CAT CCC CCA GTT CT-3′	78906–78298	27
toxB 5′ lower	5′-CCG CAC CAA AGG CAT TAG-3′	79472–79455	27
toxB 3′ upper	5′-TAG CGG AAA GAA TAT TGG TAG TCA-3′	84703–84726	27
toxB 3′ lower	5′-CTG TAG TGT GGC GGG AAC G-3′	85555–85537	27

[a] Primer locations refer to nucleotides in the pO157 *toxB* sequence (GenBank accession no. AF074613).

virulence plasmid of an EPEC O111 strain (GenBank accession no. AB024946) (33).

toxB is a large gene, and the data mentioned above refer to the presence of only portions of its sequence. Therefore, we decided to develop PCR and hybridization tools for the detection of most of the *toxB* sequence. The investigation on the presence of *toxB* was conducted with a collection of EHEC O157 strains belonging to different phage types and other EHEC and EPEC strains belonging to different serogroups and isolated from different sources.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* isolates examined included 23 EHEC O157, 37 EHEC non-O157, and 36 EPEC strains. Many of them have been described previously (16, 17, 21). The EHEC O157 strains included 17 strains isolated from cattle and 6 from human infections. Eight strains belonged to phage type 8 (PT8), three to PT14, four to PT21, and two to PT49, while the phage types of the remaining strains were not determined. Twenty-one EHEC non-O157 and 30 EPEC strains were isolated from humans, while the others were from different animal species. All of the isolates possessed the intimin-encoding *eae* gene (21). The EHEC strains produced Stx, as assessed by Vero cell cytotoxicity assay and PCR amplification of *stx* genes (15). Twenty-nine out of the 37 EHEC non-O157 strains and 24 out of the 39 EPEC strains were also positive for *efa-1/lifA*, while all of the EHEC O157 strains had only the 5′ region of this gene (17).

PCR analyses. The primer pair toxB.911F/toxB.1468R described by Tarr and colleagues (30) was used to amplify a fragment corresponding to the 5′ region of *toxB*, under the conditions described by the authors. Primer pairs toxB 5′int-up/toxB 5′int-lo and toxB 3′int-up/toxB 3′int-lo were designed based on the sequence of the *toxB* gene of EHEC O157 strain EDL933 (GenBank accession no. AF074613) and used to amplify two fragments corresponding to the internal region of the gene. The sequences of the primers and their locations on the pO157 sequence are listed in Table 1. All PCRs were performed using 50 ng of total DNA as the template, 200 μM deoxynucleoside triphosphate, 1 μM of each primer, and 5 U of *Taq* polymerase. The amplifications with the primer pairs designed in this study were conducted with the following thermal cycle, which was preceded by a 5-min denaturation at 94°C and run for 30 cycles: 94°C for 30 s, 49°C (toxB 5′int-up/toxB 5′int-lo) or 53°C (toxB 3′int-up/toxB 3′int-lo) for 30 s, and 72°C for 45 s. The cycles were followed by a final extension step of 72°C for 7 min.

Hybridization experiments with *toxB* probes. Two DNA probes corresponding to fragments located in the 5′ and 3′ regions of *toxB* were prepared by PCR with the primer pairs toxB 5′ upper/toxB 5′lower and toxB 3′ upper/toxB 3′ lower (27) (Table 1), respectively, and total DNA from the EDL933 strain as the template. For hybridization experiments, total DNA was extracted from the bacterial strains by using a NucleoSpin tissue kit (Macherey-Nagel GmbH, Duren, Germany) under the conditions indicated by the manufacturer, and 1 μg of DNA was then treated overnight with 10 U of HindIII restriction endonuclease. DNA fragments were separated by electrophoresis in 1% agarose gel and transferred to nylon membranes by Southern blotting (23). Probe labeling, hybridization, stringency washes, and detection were performed by using an enhanced chemiluminescence direct labeling and detection system under the conditions indicated in the kit manual (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

Plasmid analysis. High-molecular-weight plasmids were extracted by using a Marligen Bioscience high-purity plasmid purification kit (Marligen Bioscience, Inc., Maryland). One microgram of plasmid DNA was digested overnight with 10 U of EcoRI and run onto a 1% agarose gel. DNA fragments were transferred to nylon membranes and hybridized with the 5′ and 3′ *toxB* probes as described above.

RESULTS

The study was conducted with a collection of *E. coli* isolates selected to comprise a large number of EHEC and EPEC serogroups, including those involved mainly in human and animal infections.

PCR amplification of *toxB* sequences in AEEC. The PCR strategy to detect the presence of *toxB* included the use of three distinct reactions. The targeted regions of *toxB* are shown in Fig. 1, and the results obtained with EHEC and EPEC strains of different serogroups are shown in Tables 2 and 3.

All of the EHEC O157 isolates were positive by the three reactions and gave amplification products of the expected sizes. Positive reactions with the three primer pairs were also observed with eight EHEC O26 strains and two EPEC O26 strains. Six other *E. coli* O26 strains and a few strains belonging to serogroups O111, O86, O118, O127, O121, O123, and O145 were positive with at least one of the primer pairs used.

Hybridization analysis with *toxB* probes. A hybridization strategy involving the use of two probes corresponding to the 5′ and 3′ regions of *toxB* (*D* and *E*, respectively, in Fig. 1) was also used, and the results obtained are reported in Tables 2 and 3.

All of the EHEC O157 strains hybridized with both the 5′ and 3′ probes, with hybridization signals corresponding to HindIII fragments of 6.1 kb and 3.2 kb, respectively, as expected from the sequence of pO157 *toxB*.

Hybridization with both probes was also obtained with seven O26, one O118, and one O123 strain among the EHEC strains and with one O26 and one O86 strain among the EPEC strains. One EHEC O26 strain hybridized with the 5′ probe only, and four EHEC strains and three EPEC strains of different serogroups reacted with the 3′ probe only. The size of the fragments hybridizing with the 5′ probe was 5.8 kb for all of the positive non-O157 EHEC and EPEC strains. The 3′ probe recognized a 2.5-kb fragment with all of the positive strains

TABLE 2. Presence of *toxB* sequences in EHEC strains, listed by serogroup

Serogroup	No. of strains	Results of[a]: PCR with *toxB* primer pair			Hybridization with *toxB* probe[b]	
		A	*B*	*C*	*D*	*E*
O18	2	−	−	−	−	−
O26	6	+	+	+	5.8	2.5
O26	2	+	+	+	−	2.5
O26	1	+	+	−	5.8	2.5
O26	1	+	+	−	5.8	−
O26	1	+	−	+	−	−
O26	1	−	−	−	−	−
O45	2	−	−	−	−	−
O75	1	−	−	−	−	−
O86	1	−	−	−	−	−
O103	3	−	−	−	−	−
O111	2	+	−	−	−	−
O111	6	−	−	−	−	−
O118	1	+	+	+	5.8	2.5
O118	2	−	−	−	−	−
O121	1	+	+	+	−	2.5
O123	1	+	+	+	5.8	2.5
O128	1	−	−	−	−	−
O145	1	+	+	+	−	2.5
O152	1	−	−	−	−	−
O157	23	+	+	+	6.1	3.2

[a] Primer pairs used for PCR amplifications (*A*, *B*, and *C*) and probes used for Southern hybridizations (*D* and *E*) are defined in the legend for Fig. 1.
[b] The numbers are the sizes (in kilobases) of the DNA fragments hybridizing with the probe.

TABLE 3. Presence of *toxB* sequences in EPEC strains, listed by serogroups

Serogroup	No. of strains	Results of[a]: PCR with *toxB* primer pair			Hybridization with *toxB* probe[b]	
		A	*B*	*C*	*D*	*E*
O26	1	+	+	+	5.8	2.5
O26	1	+	+	+	−	2.5
O26	2	+	−	−	−	−
O26	1	−	−	+	−	−
O26	4	−	−	−	−	−
O45	1	−	−	−	−	−
O55	4	−	−	−	−	−
O86	1	+	+	+	5.8	5
O86	1	+	+	−	−	5
O103	2	−	−	−	−	−
O111	1	+	+	+	−	−
O111	2	−	−	−	−	−
O114	1	+	−	+	−	−
O125	3	−	−	−	−	−
O127	1	−	+	+	−	2.5
O127	4	−	−	−	−	−
O128	6	−	−	−	−	−

[a] Primer pairs used for PCR amplifications (*A*, *B*, and *C*) and probes used for Southern hybridizations (*D* and *E*) are defined in the legend for Fig. 1.
[b] The numbers are the sizes (in kilobases) of the DNA fragments hybridizing with the probe.

except for the two O86 EPEC strains, which showed a 5.0-kb band.

Positive reactions with all of the PCR primer pairs and probes were observed with six O26, one O118, and one O123 EHEC strain and with one O26 and one O86 EPEC strain.

Localization of *toxB* gene in *E. coli* O26 strains. High-molecular-weight plasmid DNA was purified from an EHEC O26 strain and an EPEC O26 strain that reacted positively with all of the PCR primers and probes. Both strains harbored plasmids that hybridized with both the 3′ and 5′ *toxB* probes.

DISCUSSION

toxB has recently been described as a new virulence gene located on the large virulence plasmid of EHEC O157. Its presence has been associated with an enhancement of bacterial adhesion to cultured cells (31) and with the inhibition of the host lymphocyte activation (12). Moreover, it has been shown to influence the expression and secretion of the LEE-encoded proteins (29, 31). *toxB* is 9.5 kb in size (7, 13), and the presence of its complete coding sequence has been demonstrated only in the two pO157 plasmids which have been fully sequenced so far (7, 13). Little is known about the frequency of this gene among EHEC O157 strains or its presence in other EHEC and EPEC strains. The available information refers to the presence of DNA fragments corresponding to limited regions of *toxB* in some EHEC serogroups (30) and in the plasmid of an EPEC O111 strain (33). In this study, we investigated the presence of the *toxB* gene in EHEC and EPEC strains belonging to different serogroups by using a combination of three PCRs and two DNA probes, altogether spanning most of the full length of the gene.

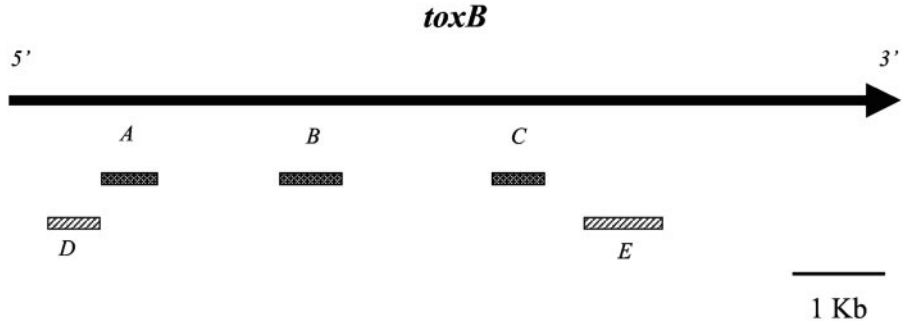

FIG. 1. Locations of the PCR products obtained with the primer pairs used in this work and listed in Table 1 (based on the sequence of the pO157 plasmid of strain EDL933 [GenBank accession no. AF074613]). *A*, toxB.911F/toxB.1468R (30); *B*, toxB 5′int; *C*, toxB 3′int; *D*, toxB 5′ probe; *E*, toxB 3′ probe.

All of the EHEC O157 strains tested reacted with the three PCR primer pairs and the two probes, thus indicating the presence of a complete *toxB* gene regardless of whether the isolates were from human disease or from animal sources. Moreover, the strains belonged to different phage types and had been isolated in different years and different Italian regions, thus indicating that they were not clonally related. This suggests that *toxB* is a stable component of the pO157 plasmid.

The presence of a presumably entire *toxB* gene was not restricted to EHEC O157, as positive reactions with all of the genetic tools employed in the study were also observed in a considerable proportion (50%) of EHEC O26 strains and in a few other EHEC (O118 and O123) and EPEC (O26 and O86) strains. *E. coli* O26 probably represents the most important non-O157 EHEC serogroup in human infections (3, 4). Furthermore, EHEC O118 (35) and O123 (8) have been associated with severe infections in calves. So, the presence of *toxB* in EHEC serogroups causing severe infections in both humans and calves may support the hypothesis that this gene has an important role in the pathogenesis of EHEC infections. Conversely, *toxB* sequences were not found in other EHEC serogroups, like O111 and O103, that are often associated with severe human infections.

Interestingly, we observed a difference between EHEC O157 and the other *toxB*-positive EHEC and EPEC strains in the sizes of the DNA fragments hybridizing with both of our probes. This indicates the existence of at least a polymorphism in the *toxB* genes present in different *E. coli* serogroups. Sequencing of the 5′ region of the *toxB* gene of an EPEC O26 strain indicated an 86% homology (data not shown) with the corresponding region of the EHEC O157 *toxB* gene (7). This preliminary observation suggests that the polymorphism observed in the hybridization experiments is the result of an extensive sequence variation and is not limited to the restriction sites.

Several EHEC and EPEC isolates belonging to different serogroups showed positive reactions with at least one of the PCRs and/or probes. This finding could be explained by the presence of either *toxB*-like genes with a higher degree of polymorphism or truncated forms of *toxB*, like that present in the plasmid of the EPEC O111 strain B171 (GenBank accession no. AB024946) (33).

In conclusion, this study indicates that the pO157-harbored gene *toxB* is consistently present in EHEC O157 strains, regardless of their human or bovine source. DNA sequences closely related to *toxB* are frequent in EHEC O26 and have also been detected in a few other EHEC and EPEC serogroups. The presence of strains reacting with only some of the genetic tools used suggests either the existence of major variants of *toxB* or the presence of portions of the gene only. Cloning and sequencing of *toxB* from EHEC O26 and other EHEC and EPEC serogroups will help in elucidating these matters.

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