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 reacting 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 referred to as enterohemorrhagic E. coli (EHEC), and 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). Hattori and colleagues (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 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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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 serogroup O26, one O118, and one O123 strain among the EHEC 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.

**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 were designed based on the sequence of toxB gene of EHEC O157 strain EDL933 (GenBank accession no. AB024946) (33).

**TABLE 1. PCR primers used in this study**

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Sequence</th>
<th>Primer location</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>toxB.911F</td>
<td>5’-ATA CCT ACC TGC TCT GGA TTG A-3’</td>
<td>79431–79452</td>
<td>30</td>
</tr>
<tr>
<td>toxB.1468R</td>
<td>5’-TTT TCG CGT AAT GAT GC-3’</td>
<td>80030–80009</td>
<td>30</td>
</tr>
<tr>
<td>toxB 5’-int-up</td>
<td>5’-TTT TCG CGT AAT GAT GC-3’</td>
<td>81278–81294</td>
<td>This study</td>
</tr>
<tr>
<td>toxB 5’-int-lo</td>
<td>5’-ACG CCG TGA GAA TAA TGT C-3’</td>
<td>81954–81936</td>
<td>This study</td>
</tr>
<tr>
<td>toxB 3’-int-up</td>
<td>5’-CAA CAG CCC CTT CAT TCC ATT C-3’</td>
<td>83708–83729</td>
<td>This study</td>
</tr>
<tr>
<td>toxB 3’-int-lo</td>
<td>5’-TTT TGC CAC ATT GCT AAG ATA ACG-3’</td>
<td>84277–84254</td>
<td>This study</td>
</tr>
<tr>
<td>toxB 5’-upper</td>
<td>5’-AAA ATA ATT CAT CCC CCA GTT CT-3’</td>
<td>78906–78928</td>
<td>27</td>
</tr>
<tr>
<td>toxB 5’-lower</td>
<td>5’-CCG CAC CAA AGG CAT TAG-3’</td>
<td>79472–79455</td>
<td>27</td>
</tr>
<tr>
<td>toxB 3’-upper</td>
<td>5’-TAG CGG AAA GAA TAT TGG TAG TCA-3’</td>
<td>84703–84726</td>
<td>27</td>
</tr>
<tr>
<td>toxB 3’-lower</td>
<td>5’-CTG TAG TGT GGC GGG AAC G-3’</td>
<td>85555–85537</td>
<td>27</td>
</tr>
</tbody>
</table>

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

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.

**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.

**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.

**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 were 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.
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.

![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.](http://jcm.asm.org/)
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 O125) 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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REFERENCES


