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Identification of a Shiga-toxin type I variant containing an IS*1203*-like element, from Shiga-toxin producing *Escherichia coli* O157:H7

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Abstract

We found two Shiga toxin producing *Escherichia coli* O157:H7 strains isolated from humans carrying the stx_1 gene with an IS*1203*-like element (designated as IS*1203*v_1). The IS*1203*v_1 was inserted into the coding region of the A subunit 7 bp upstream from the TGA termination codon, resulting in a loss of two amino acid residues (Ser–Ser) from its C terminus. Toxicity of the Stx1 was confirmed by Vero cell assay. IS*1203*v_1 hardly affected the stx_1 gene in either its expression or the toxicity of its product.

Keywords: Shiga toxin 1 variant; IS*1203*; Shiga toxin-producing *Eschericia coli* O157

1. Introduction

Shiga toxins 1 and 2 (Stx1 and 2) are cytotoxins produced by the *Escherichia coli* strains known as Shiga toxin-producing *E. coli* (STEC). These organisms cause diarrhea, often bloody and sometimes complicated by hemorrhagic colitis and/or a hemolytic uremic syndrome. Shiga toxins are classified into two major classes, Stx1 and Stx2, based on a toxin-neutralization test and DNA sequences.

Although a number of stx_2 variants have been reported, their stx_1 variants are few compared with the stx_2 gene. There are at least two types of prototype Stx1, namely one is identical to the Shiga toxin produced by *Shigella dysenteriae* type 1 (referred to as $Stx_{\text{-shigella}}$ in this study) [1,2] and the other differs one amino acid residue at 67 from $Stx_{\text{-shigella}}$ (referred to as $Stx_{1\text{-O157}}$ in this study) [3]. The amino acid residue at 67 is threonine in $Stx_{\text{-shigella}}$, and serine in $Stx_{1\text{-O157}}$. At least five stx_1 variants have been identified so far, one (*stx1v51*) revealed 26, one (slt_{1OX3}) revealed nine, and three (slt_{1CB} slt_{1PH} and slt_{1O48}) revealed two amino acid changes from $stx_{1\text{-O157}}$ [4–7]. The nucleotide sequences of these variants were closer to $stx_{\text{-shigella}}$ than to $stx_{1\text{-O157}}$.

The genomes of most bacteria contain many distinct classes of insertion sequences (IS), one of which (IS*1203*) was first reported by Paton and Paton [8] from *E. coli* O111:H-strain PH. IS*1203* is a 1312-nucleotide (nt) long, with imperfect 26-bp terminal inverted repeats and is closely related to IS*629* of *Shigella sonnei* [9] and IS*3411* [10] of the *E. coli*. At least 12 copies of IS*1203* were found in the genome of the *E. coli* O111:H-strain PH [8]. IS*1203* was also detected in the lambdoid phage of *E. coli*, which encodes the stx_2 gene [5]. Kusumoto et al. [11] reported an insertion sequence with an extremely high homology to IS*1203* in the stx_2 genes of some isolates of STEC O157:H7, they named this insertion sequence IS*1203* variant (IS*1203*v), which was inserted in the region encoding the amino-terminus of the B subunit with a duplication of 3 bp at the target site,

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resulting in the inactivation of the *stx*$_2$ gene. In this case, *stx*$_2$ genes were found in *E. coli* strains by polymerase chain reaction (PCR), but no mature protein were detected.

Recently, we found two STEC O157:H7 strains producing both Stx1 and 2. However, we could not detect the *stx*$_1$ gene using commercial primers, and extremely larger amplicons were obtained using the other primer pair for *stx*$_1$. In the present study, we reported an *stx*$_1$ gene containing a 1.3-kb insertion sequence which showed an extremely high homology with *IS1203*v. This is the first report of the insertion into the *stx*$_1$ gene by an IS element.

2. Materials and methods

2.1. Bacterial strains

Two STEC O157:H7 (2000-182, 2000-188) strains were isolated from independent sporadic cases in Aichi Prefecture, Japan, in November 2000. Both strains were obtained from healthy children whose family members were infected with STEC.

2.2. Detection of Shiga toxins 1 and 2

Expressions of Stx1 and Stx2 of these strains were confirmed by a reverse passive latex-agglutination test (RPLA). To obtain culture filtrates, the strains were cultured at 37 °C overnight with shaking (120 rpm) in casamino acid yeast extract (CAYE) broth [12]. The culture supernatant was examined by an RPLA test performed using the *E. coli* Verotoxin Detection Kit (Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions.

Detection of *stx*$_1$ and *stx*$_2$ genes was performed by PCR using commercial primers EVT1/2 for *stx*$_1$ and EVS1/2 for *stx*$_2$ (Takara Bio, Otsu, Japan). The positions and sequences of these primers are not open to the public. PCR was performed in a total volume of a 50 μl mixture containing heat-extracted template DNA, EX-Taq buffer (2 mM Mg^{2+}), dNTPs, 0.2 μM of each primer, and 1 U of EX-Taq (Takara Bio, Otsu, Japan). The PCR cycles were 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min, with PCR performed over a total of 30 cycles using the GeneAmp PCR System 9600 (Applied Biosystems Japan, Tokyo, Japan).

2.3. Sequence analysis of stx$_1$ gene containing IS1203

To obtain whole length *stx*$_1$ gene, we performed PCR on upstream (nt 58–926) and downstream region (nt 404–2735). The primer pair for upstream region was VT111 (5′-GGC GTG GAG GAT GTC AAG-3′) and VT122 (5′-ATC AGA ATT GCC CCC AGA G-3′), and that for downstream region was VT121 (5′-GCT ATA CCA CGT TAC AGC G-3′) and VT142 (5′-CCA CCA AAC CCG CGT ATT C-3′). PCR was performed under the same conditions as described above. The nucleotide sequences of amplified *stx*$_1$ genes were determined directly using CEQ2000 (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions.

2.4. Molecular weight determination of the Stx1 variant

The molecular weight of this Stx1 variant was determined by high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) according to our previous study [13]. Strain 2000-188 was cultured in 1000 ml of CAYE broth at 37 °C overnight with shaking (100 rpm). Supernatant was collected by centrifugation of 6000 rpm at 4 °C for 30 min and filtrated with 0.22 μm PES membrane filter system (Asahi Techno Glass, Tokyo, Japan). A total of 561 g of ammonium sulfate was added to the supernatant and stirred at 4 °C overnight. After centrifugation the precipitate was dissolved in 10 ml of phosphate-buffered saline (PBS), pH 7.0 and dialyzed against PBS three times. The dialyzed solution (about 20 ml) was centrifuged at 12,000 rpm for 15 min. The supernatant was concentrated to about 5 ml by amicon ultra 15 (MWCO 30K). The maximum dilutions of Stx1 and Stx2 detected by RPLA of this concentrated sample were 1:4000 and 1:64,000, respectively. Then, 0.6 ml of Anti-Stx1 polyclonal antibody prepared in our laboratory [13] was added to this concentrated sample and incubated at 4 °C overnight with mild stirring. The sample was centrifuged at 12,000 rpm for 15 min. The precipitate was washed with PBS and distilled water and then dissolved in 0.05 ml of 0.2% aqueous formic acid containing 10% acetonitrile. The molecular weight of Stx1 was measured by a Quattro II triple quadrupole mass spectrometer (Micromass UK, Altrincham, UK) with a PLRP-S HPLC column (150 mm × 2 mm ID, 8 μm, 1000 Å, Michrom BioResources, Inc).

2.5. Vero cell assay of the Stx1 variant

The toxicity of the Stx1 variant found in this study was tested by Vero cell assay, using an supernatant of strain 2000-188 produced through ammonium sulfate precipitation. Stx2 in 5 ml of the sample was absorbed twice, with 0.5 ml of anti-Stx2 polyclonal antibody prepared in our laboratory (details of which are being prepared for submission). The Stx2 absorbed sample was diluted twofold serially to a final dilution range of 1:500 to 1:128,000. A neutralization test was performed using a sample absorbed with both anti-Stx1 and Stx2 antibody at a dilution of 1:10. We also used culture filtrate of Stx1 producing strain of STEC O26:HNM (2000-38) for positive control. The amino acid sequence

of this Stx_1 was homologous to $Stx_{1\text{-O157}}$ (unpublished data). To obtain culture filtrates, the strain 2000-038 was cultured at 37 °C overnight with shaking (120 rpm) in tripticase soy broth. The amounts of Stx expression in these samples were estimated by RPLA. Vero cell assay was performed according to the methods reported by Konowalchuk et al. [14] with a slight modification as follows. Vero cells were grown in MEM medium with 5% fetal calf serum (FCS). Toxin activity was assayed in plastic 24-well dishes (Falcon 3047, Becton Dickinson, Franklin Lakes, USA) with 0.5 ml of cell culture in each well. The monolayers were obtained by seeding 10^5 cells in MEM medium with 2% FCS per well 1 day before use. On the day of the assay, 0.05 ml of each sample was added to 0.5 ml of cell culture.

3. Results

3.1. PCR products and RPLA titer of Stx1 and Stx2 in strain 2000-182 and -188

Although no amplicon of the *stx*$_1$ gene was detected by PCR from either strain using EVT1/2 primers, *stx*$_2$ amplicons were detected. The maximum dilution of Stx1 and Stx2 detected by RPLA were 1:256 and 1:16,000, respectively, in strain 2000-182, and 1:1000 and 1:8000, respectively, in strain 2000-188.

3.2. Sequencing analysis of the stx_1 variant

The same sequences were obtained from both STEC O157:H7 strains, 2000-182 and 2000-188. The sizes of PCR products of these *stx*$_1$ genes were about 1 kbp for VT111 and VT122 primer pair and about 2.3 kbp for VT121 and VT142 primer pair. Sequence data are shown in Fig. 1. Sequencing analysis showed that an insertion sequence (referred to as IS*1203*v$_1$) was inserted at 7 bp upstream of the TGA termination codon of the *stx*$_1$ gene (*stx*$_1$::IS*1203*v$_1$). The IS*1203*v$_1$ was 1313 bp in length and had a 25 bp long imperfect terminal inverted repeats with the three bases mismatched and was flanked by direct repeats "ATT". The IS*1203*v$_1$ created a new termination codon at the insertion site. Compared with *stx*$_{1\text{-O157}}$ the substitution of adenine for thymine at nt 199 in the A subunit altered the deduced amino acid codon serine to threonine. As a result of the IS*1203*v$_1$ insertion, two amino acids (Ser–Ser) were lost from the C terminal of the A subunit. There were three and one same sense mutations in the A subunit and B subunit coding regions, respectively.

3.3. Molecular weight determination

In order to determine the molecular weight of the mature protein of *stx*$_1$::IS*1203*v$_1$ a culture supernatant of strain 2000-188 produced through ammonium sulfate precipitation was mixed with anti-Stx1 polyclonal antibody, and the resulting precipitate was analyzed by HPLC-ESI/MS. As shown in the reconstructed ion chromatogram (RIC) at m/z 500–1950, five peaks were observed (Fig. 2). Peaks 2 and 4 have the same retention times as those of Stx1 B and A subunits, respectively. The ESI mass spectra of peaks 2 and 4 shown in Figs. 3(a) and (b) are the sums of the scans in the range marked by an X-axis. Figs. 3(c) and (d) show the molecular weight spectra of peaks 2 and 4 calculated from the ESI mass spectra of the respective peaks. The observed average molecular weight of peak 2 is 7686.8 ± 1.7 (Fig. 3(c)), a value in close agreement with the theoretical value of Stx1 B subunit (7690.3) calculated from the deduced amino acid sequence of Stx1. The molecular weight spectrum of peak 4 shows two ion signals, 32049.3 ± 5.3 and 31680.0 ± 2.8 (Fig. 3(d)). The former is almost the same as that of the theoretical molecular weight of mature protein calculated from the deduced amino acid sequence of *stx*$_1$::IS*1203*v$_1$ (32051.6). These findings verified the sequencing result, which indicated that A subunit of Stx1::IS*1203*v$_1$ lost two amino acid residues (Ser–Ser) from the C terminal. Another ion signal shows the molecular mass 31680.0 ± 2.8, with the difference in the molecular mass of two peaks being 369. The molecular structure of the latter peak remains to be elucidated. Peaks 1 and 5

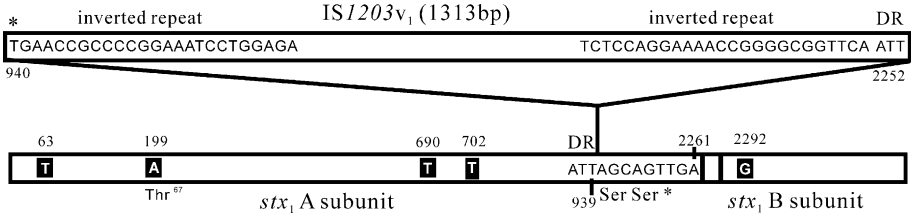

Fig. 1. Schematic illustration of *stx*$_1$::IS*1203*v$_1$. The upper bar represents IS*1203*v$_1$ and the lower bar represents *stx*$_1$ gene. The nucleotides that changed from the prototype *stx*$_1$ gene of STEC O157 are indicated in white letters. The amino acid residues that differed from Stx1 of STEC O157 is Thr^{67} of the Stx1 A subunit. The inverted repeats and the direct repeats (DR) are shown in the IS*1203*v$_1$. The new termination codon created by the insertion of IS*1203*v$_1$ is indicated by asterisk at nt 940. The *stx*$_1$ A subunit has been truncated by IS*1203*v$_1$ (nt 2253–2261), resulting in the loss of two amino acid residues (Ser–Ser) from its C terminal. The nucleotide sequence of the *stx*$_1$::IS*1203*v$_1$ gene has been registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. AB083043.

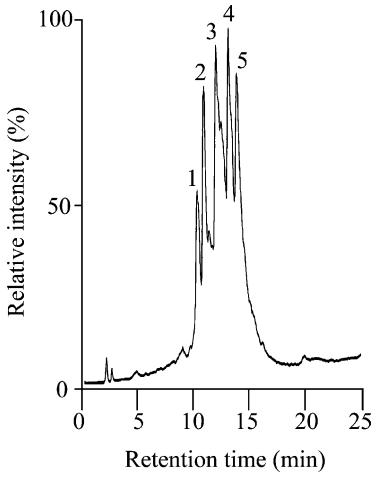

Fig. 2. HPLC/ESI-MS analysis of Shiga toxins prepared from STEC O157 strain 2000-188 by immunoprecipitation. Peaks 2 and 4 have the same retention times as those of Stx1 B and A subunits, respectively. Peaks 1 and 5 correspond to Stx2 B and A subunits, respectively.

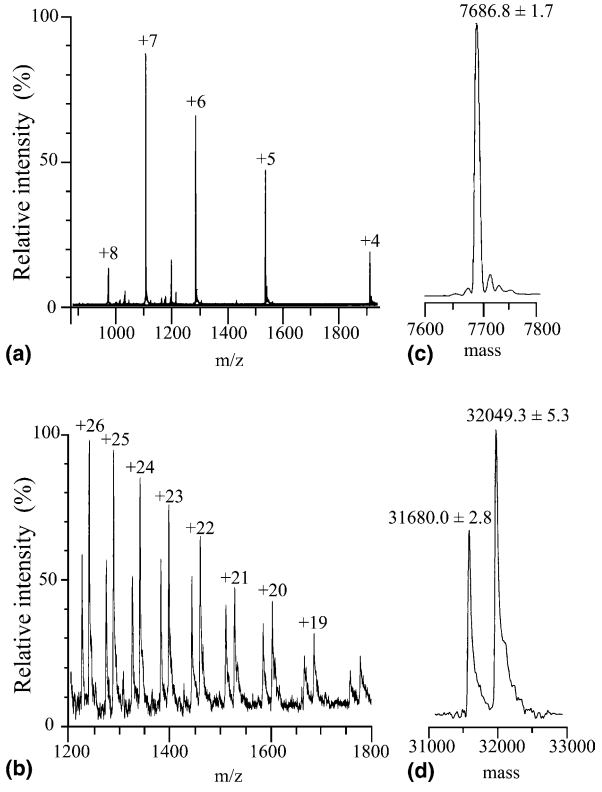

Fig. 3. HPLC-ESI mass spectra of peaks 1 (a) and 4 (b), and transformed mass spectra of peaks 2 (c) and 4 (d), respectively. The observed average molecular weight of peak 2 (c) (7686.8 ± 1.7) is in close agreement with the theoretical value of the Stx1 B subunit (7690.3). The molecular weight spectrum of peak 4 (d) (32049.3 ± 5.3) is almost the same as that of Stx1-IS*1203*v$_1$ A subunit (32051.6).

(Fig. 2) correspond to the Stx2 B and Stx2 A subunits, respectively. The explanation why the peaks corresponding to Stx2 subunits are detected in the sample is that anti-Stx1 antibody might react slightly with Stx2. The observed average molecular weight of peak 3 (Fig. 2) was 59809.0 ± 29.5. The molecular structure of peak 3 remains to be elucidated.

Table 1
Maximum dilution and relative activity of Stx1 and its variant where positive reaction was detected in Vero cell assay and RPLA

Toxin preparation	Vero cell assay	RPLA	Relative specific activity
Stx1::IS*1203*v$_1$	1:1000	1:4000	0.25
Prototype Stx1	1:320	1:320	1

3.4. Vero cell assay (Table 1)

The toxicity of the Stx1 variant was examined by a Vero cell assay using the sample whose Stx2 component was removed by anti-Stx2 antibody (Table 1). Vero cells were not killed in the neutralization test. The maximum dilutions of Stx1 and Stx2 detected by RPLA were both <1:1 in the neutralization test. Relative specific activity of Stx1::IS*1203*v$_1$ estimated from Vero cell assay and RPLA was one-fourth that of positive control sample.

4. Discussion

The IS*1203*v$_1$ of the *stx*$_1$::IS*1203*v$_1$ was highly homologous to the IS*1203*v reported by Kusumoto et al. [11]. In the majority of cases, insertion of IS elements causes inactivation of genes [5,11]. In this study, in spite of IS*1203* insertion, *stx*$_1$ gene was not inactivated but two amino acids (Ser–Ser) were lost from the C terminus of the A subunit. The HPLC-ESI/MS analysis supported this result. Though the distance between the *stx*$_1$-A and *stx*$_1$-B genes became long as the result of IS*1203*v$_1$ insertion, the toxin activity is retained. As shown in Figs. 2 and 3, an intact B subunit was purified from the mutant strain. Since IS*1203*v$_1$ insertion occurred at the terminal region of the A subunit, it did not affect the SD sequence nor the coding sequence of the B subunit. In the inserted IS*1203*v$_1$ sequence, there was no candidate for a possible terminator [15]. It is conceivable that a long polycistronic mRNA was synthesized without being interrupted by such sequence, and translated into the functional proteins.

Although, there is possibility of residual Stx2 activity in the Vero cell assay, we considered that most Stx2 was removed because Vero cells were not killed in the neutralization test. Therefore, Vero cell assay and RPLA test demonstrated that the toxicity of Stx1::IS*1203*v$_1$ was slightly lower than that of prototype Stx1. However, Stx1::IS*1203*v$_1$ was probably lost to some extent and possibly decomposed during purification and absorption steps, and the relative specific activity of Stx1::IS*1203*v$_1$ estimated from the maximum dilution of CD50 and RPLA is practically the same or slightly lower than that of positive control (Stx1 produced from strain 2000-38).

As far as we know, this is the first report of insertion into the stx_1 gene by IS*1203*. Previous studies revealed that IS*1203* was inserted into the stx_2 gene. Kusumoto et al. [11] reported that the stx_{2vhd} gene [16], one of the stx_2 variants, was interrupted by IS*1203*v, resulting in the inactivation of the stx_2 gene. Moreover, Okitsu et al. [17] indicated that IS*1203*v was inserted into the stx_2 gene at the same site as that of Kusumoto et al. [11]. IS*1203* was detected in the region near the stx_2 gene in the chromosome [18]. In addition, many IS*1203*s were found in the O157 genome, whose sequence has recently been published by Perna et al. [19] and Hayashi et al. [20]. The sequences of the reported insertion sites varied, and no insertion hot spot appears to exist. Kusumoto et al. [21] reported that stx_2::IS*1203* was reverted to the wild type stx_2 gene by transposase activity of IS*1203*. Consequently, stx_1::IS*1203* in this study can be reverted to the wild type. Kusumoto et al. [21] also reported that variant stx_2 genes were made by excision of IS*1203* from stx_2::IS*1203* and these variant genes contained a part of IS*1203* [21]. However, IS*1203* might not participate in creating stx_1 variant genes such as *stx1v51*, slt_{1OX3} and others [4–7], because IS*1203* fragment is not found in these variant genes. To elucidate why there is no report about the stx_1 gene inserted by IS*1203* except for our study, further studies are required.

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