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Note

Association of Cell-adhesion Activities with Virulence in Shiga toxin-producing *Escherichia coli* O103：H2

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The characteristics of 11 strains of Stx1-producing and Stx2-non-producing STEC O103：H2 were analyzed to investigate the differences in virulence in a single serotype of Shiga toxin（Stx）-producing *Escherichia coli*（STEC）. Differences in the cell-adhesion activity to Caco-2 cells were observed among the strains. The activity of the one strain, isolated from a patient with hemolytic uremic syndrome was 4-20-fold higher than those of the other strains. Although the strains with high cell-adhesion activity showed high expressions of *eae*, *espB*, *espD*, and *tir* in the locus of enterocyte effacement related with cell-adhesion, those were not specific for this strain. In addition, the Stx1 production level of the strain was not particularly high. It was indicated that the high adhesion activity might be a potential factor to associate serious symptom.

Key words ： Shiga toxin-producing *Escherichia coli* / Serotype O103：H2 / Virulence / Adhesion activity / Caco-2 cells.

Shiga toxin（Stx）-producing *Escherichia coli*（STEC） is one of the most important foodborne pathogens, and is known to cause gastroenteritis and serious symptoms such as bloody diarrhea and hemolytic uremic syndrome（HUS）（Mainil and Daube, 2005）. It is easy to assume that the human immune strength strongly affects the risk and severity of STEC infection；however, the virulence of the strain is also regarded as an important factor for the severity of the symptoms. The most important virulence factors of STEC are Stx1 and Stx2；in particular, epidemiological and experimental evidence suggests that the ability to produce Stx2 is associated with the severity of disease（Boerlin et al., 1999；Gyles, 2007）. In addition to Stx, the factors needed for initial attachment to the intestinal epithelium are also thought to be important for STEC virulence, since adherence is crucial for the STEC pathogenic process. It is known that major high-virulent STECs harbor the locus of enterocyte effacement（LEE）, which is associated with intimate adherence to the intestinal epithelium. The LEE encodes numerous adhesion factors, including intimin（Eae）, translocated intimin receptor（Tir）, and the type III secretion apparatus（EspB and EspD）（Dean and Kenny, 2009；Steyert et al., 2012）. Non-LEE STECs also possess some kinds of adhesion factors such as iron-regulated gene A（IrgA）homolog adhesion（Iha）（Tarr et al., 2000）and STEC autoagglutinating adhesion（Saa）（Paton et al., 2001）. Furthermore, certain enzymes assisting the colonization of STEC, such as catalase/peroxidase（KatP）（Brunder et al., 1996）, metalloprotease（StcE）（Grys et al., 2005）, and serine protease（EspP）（Brunder et al., 1997）are also considered to be associated with STEC virulence.

Although STEC has various O serogroups, a part of O serogroups are frequently isolated from patients with serious symptoms, and obvious associations between the serogroups and virulence are indicated（Karmali et

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al., 2003). Particularly, STEC O157 has been demonstrated to be strongly associated with outbreaks and patients with serious symptoms outcome in many countries (Meng et al., 2013). Furthermore, a subset of non-O157 STECs, including O26, O111, and O103, has also been recognized as a main public health concern worldwide (Brooks et al., 2005; Eklund et al., 2001; National Institute of Infectious Diseases, 2014). On the other hand, many strains of the serogroup associated with serious diseases, such as O157, are also isolated from asymptomatic carriers, suggesting variations of virulence within a single serogroup. However, there are a few data regarding the virulence variations within a single serotype (Lee et al., 2011).

In this study, we investigated the potential factors of the virulence variations of STEC, such as the cell-adhesion activity, and Stx-productivity among Stx1-producing and Stx2-non-producing STEC O103:H2 strains. It has been reported that a large subset of serogroup O103 produce Stx1 but not Stx2 (Beutin et al., 2004; Eklund et al., 2001). Although Stx1 is thought to have a lesser impact than Stx2, cases of HUS have been shown to result from Stx1-producing and Stx2-non-producing STEC.

In this study, 11 strains of STEC O103:H2 isolated from five patients with serious or mild, and six asymptomatic carriers, who were examined based on guideline for cook, were used (Table 1). First, we examined the variations in adhesion activity to Caco-2 cells among the strains. Abu-Ali et al. (Abu-Ali et al., 2010) showed differences in the level of adhesion to epithelial cells between two strains of STEC O157 and concluded that the difference implied variation of their ability to colonize or to initiate the disease process. The adherence of strains to Caco-2 cells (ATCC HTB37, American Type Culture Collection, Manassas, VA, USA), a human epithelial colorectal adenocarcinoma cell line, was evaluated as previously described with minor modifications (Vikram et al., 2012). Briefly, Caco-2 cells cultured in 24-well plates coated with Cellmatrix (Nitta Gelatin Inc., Osaka, Japan) were washed with phosphate buffered saline (PBS) twice and reconstituted with 1 ml Dulbecco's Minimal Essential Medium (DMEM) without antibiotics. Subsequently, the cells were infected with 50 μl bacterial culture with tryptic soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at a concentration of 1×10^{8} cfu/ml. Following incubation for 2 h at 37℃, the cells were washed with PBS five times to remove unbound bacteria. For microscopic observation of adherence, the cells were fixed with 10% formalin in PBS for 20 min and stained with Giemsa for 40 min. In the next, the cells were lysed with 0.1% Triton-X in PBS and serial dilutions were plated on tryptic soy agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The experiments were repeated with three independent cultures of each strain. Student's *t*-test was used to determine the difference in cell-adherence activity between ESC531 vs. other strains.

Among the strains used in this study, there was wide variation in the cell-adhesion activity, with up to 20-fold differences in recovery from Caco-2 cells observed

TABLE 1. Characteristics of STEC O103:H2 strains isolated from human.

Strain no.	Host		Prevalence of adhesion genes[a]									Stx1 production (mean±SD)[c]
			LEE				Adhesin		Enzyme			
	Clinical symptoms	Age	*eae*[b]	*espB*	*espD*	*tir*	*iha*	*saa*	*espP*	*katP*	*stcE*	
ESC531	HUS	<5	+	+	+	+	−	−	−	+	−	112±32
ESC570	Bloody stool	22	+	+	+	+	−	−	−	−	+	NT
ESC572	Bloody stool	2	+	+	+	+	−	+	−	−	−	NT
ESC554	Loose stool	35	+	+	+	+	−	−	−	+	−	256
ESC569	Loose stool	56	+	+	+	+	−	+	−	−	−	448±128
ESC548	Asymptom	20	+	+	+	+	+	−	−	−	−	64
ESC549	Asymptom	62	+	+	+	+	+	−	−	−	−	80±32
ESC552	Asymptom	37	+	+	+	+	+	−	−	−	−	80±32
ESC553	Asymptom	61	+	+	+	+	+	−	−	−	−	256
ESC574	Asymptom	13	+	+	+	+	+	−	−	−	−	512
ESC575	Asymptom	24	+	+	+	+	+	−	+	+	−	NT

[a] Prevalence of each gene is shown by + (present) or - (absent).
[b] All strains were positive for eae (ε).
[c] Maximum titers of positive reaction in reversed-passive agglutination assay.

between the strains (Fig.1A). ESC531, which was isolated from a less than 5 year-old child with HUS, showed significantly higher adhesion activity than the other strains (ESC531 vs. ESC574: $p<0.05$, ESC531 vs. all other strains: $p<0.01$). There, however, were no significant differences among adhesion activities of the other strains isolated from patients with bloody stool and loose stool, and six asymptomatic carriers. Observations of a part of strains under a confocal microscope were shown in Fig.1B.

To reveal the genetic factor likely responsible for the varying cell-adhesion activity among the strains, we investigated the presence of several genes associated with cell-adhesion by the method described by Kobayashi et al. (2013). Because all strains of STEC O103:H2 used in this study possessed the genes located on LEE, namely *eae*, *espB*, *espD*, and *tir*, the prevalence of these genes was considered not to be associated with the variation in cell-adhesion activity (Table 1). Adhesion genes (*iha* and *saa*) and the genes coding enzymes that assist in the colonization of STEC (*espP*, *katP*, and *stcE*) were sporadically possessed. However, the prevalence of these genes was not correlated with the level of symptom and the cell-adhesion activity (Table 1).

On the other hand, it has been reported that there is variation in the expression of the genes on LEE among STEC O157 strains with differing cell-adhesion activity (Abu-Ali et al., 2010; Tobe et al., 2005). Accordingly, we next analyzed the expression of these genes (*eae*, *espB*, *espD*, and *tir*) among six strains (ESC531, ESC574, ESC552, ESC553, ESC554, ESC569). Monolayers of Caco-2 cells cultured in 6-cm dishes with 5 ml DMEM were inoculated with 250 μl bacterial strains at a concentration of 1×10^{8} cfu/ml. After incubation for 30 min at 37℃ in a 5% CO_2 atmosphere, the culture were collected into tubes and bacteria were harvested by centrifugation. Total RNA was isolated using illustra RNAspin Mini RNA Isolation Kit (GC Healthcare, Buckinghamshire, UK) and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). First-strand cDNA was synthesized from 1 μg of RNA using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan). One microliter of 500-fold diluted cDNA solution was subjected to polymerase chain reaction (PCR) amplification (Gene Amp PCR System 9700, Applied Biosystems, CA, USA) in a 20-μl final volume containing 10 μl of 2×SYBR *Premix Ex Taq* (TaKaRa) and 800 nM of each primer. The amplification condition was one cycle at 95℃ for 30 s, followed by 40 cycles at 95℃ for 5 s and 60℃ for 30 s. Melting curve analysis was performed to ensure the specificity of the PCR products. The PCR for each target gene were performed in duplicate for every sample. The amplifications of *eae* and 16S rRNA gene were performed with the primers previously described (Bergholz et al., 2007; Nielsen and Andersen, 2003). The primers for other genes used in this study were as follows: espB-qF1 (5'-ATGATATCGCTAGCCGTCTG) and espB-qR1 (5'-AGCTAAGCGAACCGATTGAC) for *espB*, espD-qF1 (5'-AACGAGATCTACACGGATGG) and espD-qR1 (5'-CAACCAACCAAAGACCTGAC) for *espD*, and tir-qF1 (5'-CAGCCTACGAATAACGCATC) and tir-qR1 (5'-AGCATACGGATTCTCTACCG) for *tir*. Data were analyzed by use of the comparative critical threshold (ΔΔCT) method, as previously described (Pfaffl, 2001). The experiments were repeated with three independent cultures of each strain. Student's *t*-test was used to determine the differences in the expression levels of each strain against that of ESC569. The two

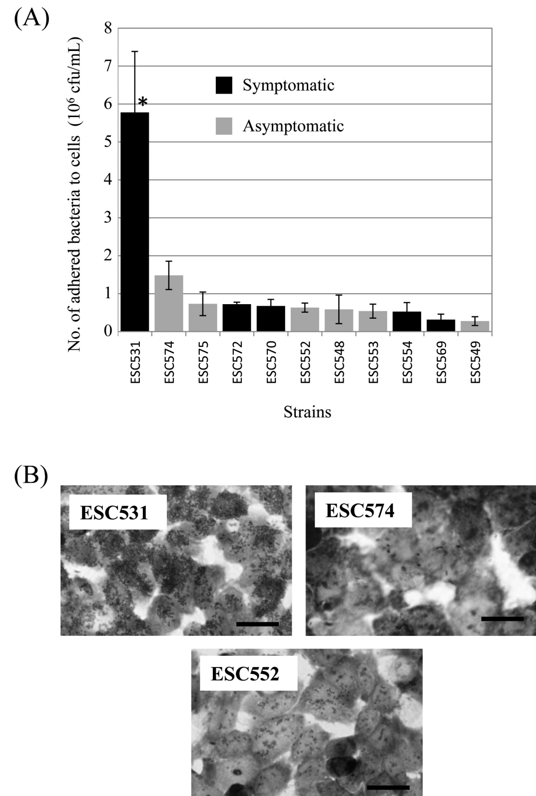

FIG. 1. Adherence activity to Caco-2 cells of Shiga toxin-producing *Escherichia coli* (STEC) O103:H2.
(A) Adhered bacterial cell numbers of STEC O103:H2 were calculated by recovery from Caco-2 cells. Error bars indicate the standard deviations. *ESC531 vs. ESC574: $p<0.05$, ESC531 vs. other strains: $p<0.01$.
(B) Caco-2 cells cultured with three strains of STEC O103:H2 were stained by Giemsa. ESC531: an isolate from a hemolytic uremic syndrome patient, ESC552 and ESC574: isolates from an asymptomatic carriers. Scale bars represent 50 μm.

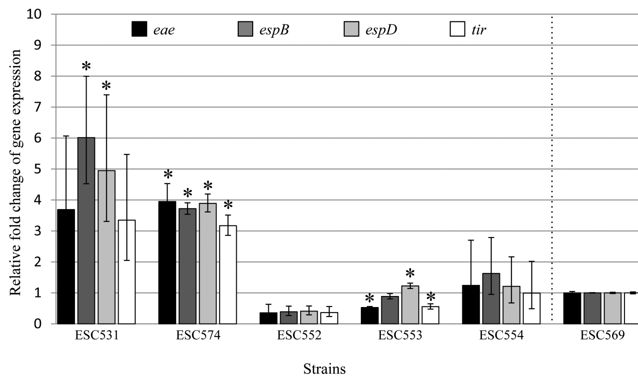

FIG. 2. Expressions of locus of enterocyte effacement (LEE)-genes of Shiga toxin-producing *Escherichia coli* (STEC) O103:H2.
The *x*-axis denotes the strains sorted in ascending order of the cell-adherence activity and the *y*-axis indicates the relative expression level of each gene. Expression levels appear as fold-changes relative to the expression level of ESC569. ESC531: an isolate from a hemolytic uremic syndrome patient, ESC574, ESC552 and ESC553: isolates from an asymptomatic carriers, ESC554 and ESC569: isolates from loose stool patients. Error bars indicate the standard deviations. $^{*}p<0.01$.

strains (ESC531 and ESC574) with the first and second of high cell-adhesion activity were found to express these genes more than three times higher than the strain (ESC569) with the second lowest cell-adhesion activity (Fig.2). Because expression level of ESC574, a strain isolated from no symptom patient, was larger than those of ESC554 and 569, strains from patients with loose stool, expression level was not adequate indicator of cell-adhesion.

The strains analyzed for gene expression were further studied by quantifying the amount of Stx1 production using the VTEC-RPLA kit (Denka Seiken Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. The experiments were repeated with four independent cultures of each strain. ESC574, which was isolated from an asymptomatic 13 years old patient, produced the highest levels of Stx1 (Table 1). Further, ESC554 and ESC569, isolated from patients with loose stool as mild symptoms, produced Stx1 at relatively high levels. ESC531, isolated from a patient with HUS as serious symptoms, produced less than half the level of ESC574, ESC554, and ESC569. These suggest that the Stx1 production ability is not important to the virulence of STEC O103:H2.

Since disease severity is likely to be a function of a complex interplay between the pathogen and host factor(s), it can likely not be explained on the basis of the pathogen attributes alone. It is known that STEC disproportionately affects children under 5-yearold and senior adults (National Institute of Infectious Diseases, 2014). In this study, ESC531, isolated from a less than 5 year-old patient with HUS, showed significantly higher cell-adhesion activity than those of the other strains (Fig.1), and the serious symptoms in this case might have been caused by the high cell-adhesion activity of this strain. Adhesion activities of strains from patients with symptoms other than ESC531, however, were similar to those of strains from an asymptomatic carrier. Thus it is not suggested that low level of adhesion activity indicates low virulence. Other factors might affect virulence of strains. Whereas ESC531 and ESC554 show the same prevalence pattern of adhesion genes (Table 1), ESC531 and ESC554 were isolated from patients with serious and mild symptoms, respectively. Moreover, while the Stx1 production of ESC554 was twice as high as that of ESC531 (Table 1). It suggests that Stx1 production is not related to the patient symptoms.

The results of this study indicate that marked high adhesion activity might be associated with serious symptoms such as HUS in patients with STEC O103:H2 infection, whereas the Stx1 production level and prevalence pattern of adhesion genes might be minor factors. However, this study investigated only serotype O103:H2, so that further studies are needed to reveal the virulence of other STEC serotypes and strains.

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