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Repression of Type-1 Fimbriae in Shiga Toxin-Producing *Escherichia coli* O91:H21 Isolated from Asymptomatic Human Carriers in Korea

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Seventy-four Shiga toxin-producing *Escherichia coli* (STEC) isolates belonging to the serotype O91:H21 were isolated from 1,643 asymptomatic human carriers in a STEC outbreak at Gwangju in Korea. Although the isolates did not cause any symptoms, all of them produced Shiga toxins 1 (Stx1) and 2 (Stx2). In order to determine why these strains cause no symptoms, we explored the differences in virulence potential between the asymptomatic STEC O91:H21 isolates and symptomatic STEC O91:H21 strains (ATCC 51435 and ATCC 51434). The asymptomatic STEC O91:H21 isolates showed strongly reduced cytopathic effects compared with the symptomatic strains when intact bacterial cells were used as an inoculant. Moreover, we found a reduced adherence phenotype when testing asymptomatic strains on HeLa cells. Real-time quantitative PCR results suggest that transcriptional repression of the genes encoding type-1 fimbriae occurs in the asymptomatic isolates but not in the symptomatic strains.

Key words: Shiga toxin-producing *Escherichia coli* (STEC), asymptomatic human carriers, type 1 fimbriae

Since two independent outbreaks associated with Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 in humans were first reported in 1983, STEC has been recognized as an important foodborne pathogen that causes severe diseases such as a life-threatening hemolytic uremic syndrome [2, 16]. Whereas STEC O157:H7 was known to be the predominant serotype associated with most of the documented STEC-related outbreaks worldwide, previous epidemiological studies implied that non-O157 STEC infection can become problematic for public health [4]. The non-O157 STEC strains associated with human disease include the serotypes O8:H-, O26:H11, O91:H21, O103:H2, O111:H-, O113:H21, and O128:H2 [16].

Shiga toxins (Stxs) are major virulence factors of enterohemorrhagic *E. coli* (EHEC) and comprise a family of structurally related cytotoxins with similar biological activity. The two main groups consist of Stx1, which is nearly identical to the toxin of *Shigella dysenteriae* type 1, and Stx2, which shares less than 60% amino acid sequence homology with Stx1 [16]. Whereas Stx1 exhibits little sequence variation, the several variants of Stx2 associated with antigenic or biological characteristics have been described. Stx2 toxins include Stx2c, Stx2d, Stx2e, and Stx2f [13]. The similarity of the nucleotide sequences of these Stx2 variants to the corresponding subunits of the Stx2-encoding gene are 99.7%, 94.9%, 94.0%, and 63.4%, respectively, for the A subunit, and 95.2%, 86.6%, 79.0%, and 75.4% respectively, for the B subunit [6, 13, 15]. Furthermore, the various virulence factors of EHEC associated with diarrheagenic infections, such as EHEC hemolysin (E-Hly), type III secretion proteins (EspADB), and translocated intimin receptor (Tir) have been well documented [12, 16]. The locus of enterocyte effacement (LEE) encodes a type III secretion system and *E. coli* secreted proteins, which deliver effector molecules to the host cell and disrupt its cytoskeleton [3, 7, 12]. LEE also carries *eae*, which encodes an outer membrane protein (intimin) required for intimate attachment to epithelial cells; *eae* has proved to be a convenient diagnostic marker for LEE-positive pathogenic *E. coli* [8].

The isolation and subsequent characterization of Stx-producing *E. coli* from asymptomatic human carriers have been reported [14, 21]. In these cases, the infected individuals possessed Stx-neutralizing antibodies. However, the molecular basis of the asymptomatic phenomenon in STEC strains remains poorly understood. Previous characterization of the asymptomatic STEC O91:H21 isolates from a STEC outbreak in Korea showed that they possess both *stx1* and

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stx2, and they are genetically identified as similar on the basis of pulsed-field gel electrophoresis pattern [17]. The symptomatic reference strains of STEC O91:H21, such as the previously defined strains ATCC 51435 and ATCC 51434, are known to be highly virulent in an experimentally infected mouse model [10]; therefore, it was hypothesized that there may be some differences in virulence properties between our asymptomatic isolates and the symptomatic STEC O91:H21 strains, such as the presence or expression of known virulence factors, Stxs, and putative colonization factors. To better understand the nature of the asymptomatic phenomenon, we conducted molecular and cellular analyses of asymptomatic STEC strains isolated from an outbreak at Gwangju City in Korea.

Materials and Methods

Case Definition of an Outbreak and Isolation of STEC

On July 2004, an outbreak of STEC infections occurred at an elementary school in Gwangju, Korea. One student diagnosed with diarrhea proved to be infected with STEC. Stools (n = 1,643) were collected from asymptomatic school-related individuals. All isolates were biochemically characterized with the API20E system (bioMerieux). Of those tested, 74 people (4.5%) were positive for STEC. The 74 STEC strains isolated from stools were presently investigated [17].

Bacterial Strains and Growth

Eight asymptomatic STEC O91:H21 (ASTEC) isolates among 74 strains were chosen to be representative according to age and sex. The two symptomatic STEC O91:H21 (SSTEC) strains ATCC 51435 and ATCC 51434 were used as reference strains. All strains were grown at 37°C with aeration at 200 ×*g* in Luria–Bertani (LB; Oxoid, Ltd., Basingstoke, UK) medium containing 0.2% (w/v) glucose.

Detection of STEC-Related Virulence Genes and Toxins

After incubation, the enriched broth culture was used to isolate genomic DNA. Chromosomal DNA was purified using the GenomicPrep Cell and Tissue DNA isolation kit (Amersham Pharmacia Biotech, Sweden). The polymerase chain reaction (PCR) was performed with the Expand High Fidelity PCR System (Roche Applied Science, Switzerland), according to the manufacturer's instructions. To detect virulence genes, PCR assays were performed using the primers shown in Table 1. The production of Stx1 and Stx2 was determined using a reversed passive latex agglutination kit (VTEC-RPLA; Denka Seiken Co. Ltd., Japan), according to the manufacturer's instructions.

Cytotoxicity Assay

The cytotoxicities of symptomatic STEC ATCC 51434 and ATCC 51435 and four asymptomatic STEC isolates were determined after the growth of cells in brain heart infusion broth (Oxoid) at 37°C for 18 h and the separation of extracellular components by centrifugation. The resulting supernatant was used to treat Vero and HeLa cells. Toxicity was determined by treating cells with 100 µl of supernatant and observing the subsequent swelling, rounding, and disintegration of cell layers. The lactate dehydrogenase (LDH) activity in the culture medium was measured using a cytotoxicity detection kit (Roche Applied Science). To measure the cytotoxicity of a bacterial mixture, HeLa cells were treated with 10^5 bacterial cells per well and incubated for 6 h at 37°C. After incubation, the LDH activity of the HeLa cells was determined.

Adherence Assay

The abilities of symptomatic STEC ATCC 51434 and asymptomatic STEC1 and STEC2 isolates to adhere to HeLa cell monolayers were assessed. The cells were grown to semiconfluence at 37°C, 5% CO_2 in 24-well plates in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100,000 IU/l), and streptomycin (100 mg/l). Before use, the cells were washed with sterile phosphate-buffered saline (PBS; pH 7.4) and replenished with DMEM containing 1%

Table 1. Primers used in this study.

Target gene	Primer sequence (5' to 3')	Size of PCR product (bp)
Shiga toxin 1 (*stx1*)	CGTACGGGGATGCAGATAAATCGC	210
	CAGTCATTACATAAGAACGCCCAC	
Shiga toxin 2 (*stx2*)	GTTCTGCGTTTTGTCACTGTCAC	326
	GTCGCCAGTTATCTGACATTCTGG	
Attaching and effacing (*eaeA*)	ATGCTGGCATTTGGTCAGGTCGG	233
	TGACTCATGCCAGCCGCTCATGCG	
Hemolysin (*hlyA*)	GCATCATCAAGCGTACGTTCC	519
	AATGAGCCAAGCTGGTTAAGCT	
Type III secretion protein (*espA*)	GTTTTTCAGGCTGCGATTCT	187
	AGTTTGGCTTTCGCATTCTT	
Type III secretion protein (*espD*)	AAAAAGCAGCTCGAAGAACA	145
	CCAATGGCAACAACAGCCCA	
Type III secretion protein (*espB)*	GCCGTTTTTGAGAGCCAGAA	106
	AAAGAACCTAAGATCCCCA	
Translocated intimin receptor (*tir*)	GCTTGCAGTCCATTGATCCT	107
	GGGCTTCCGTGATATCTGA	

Table 2. Primers used to amplify the various fimbriae target genes for PCR and RT-PCR.

Target genes	Oligonucleotide sequences (5' to 3')
fimA	CGCAGTTGATGCAGGCTCTG CATCCGCATTAGCAGCACCC
fimI	GGGGACGTCATAAAAATAACGATG GAACCAGGCCTGGGCATTGG
fimC	CCCCATATGTCTAATAAAAACGTCAA CCCGGATCCTTATTCCATTACGCCC
fimD	GCGAAACACACAATGCTTGC GGAGGAATATAACCACGCGC
fimF	GTGGCTGGCGGTGAGTCACG GAGTGAAGGTAGCCGTGGC
fimG	GGCGGCAATATTGGCGCTCG TTACTGTCAATGCTCTGACC
fimH	GGCCCGATATCTGAACCCGGAGGG GGCTCGTCGACCAGCATTAGCAAT
fimB ~ A	ATGAAGAATAAGGCTGAT CAGAGCCTGCATCAACTGCG
focH	CACCGTCCTCGAGGGCTGTG TCAGTTTCCCGAAAACGCCC
papG	CCTAAATGAATAATAACTGTAATTACGG TGGCAATATCATGAGCAGCGTTGCTG
papA	GTGAAGTTTGATGGGGCGACC CGCAACTGCTGAGAAAGCACC
16S rRNA	CGGATTGGAGTCTGCAACTCG CACAAAGTGGTAAGCGCCCTC

D-mannose. The strains were grown in LB broth overnight at 37°C, and for both qualitative and quantitative assays, tissue culture cells were incubated with 10^7 bacteria per well for 3 or 6 h at 37°C. The monolayers were washed, fixed, and stained with Giemsa solution for microscopic evaluation. To quantify *E. coli* adherence, the infected monolayers were washed twice with PBS, and the adherent bacteria were recovered with 200 µl of 0.1% Triton X-100 in PBS buffer and plated on Luria agar plates containing the appropriate antibiotic.

Detection of Fimbriae Genes and Expression

To amplify the various fimbriae target genes, PCR and RT-PCR assays were performed using the primers shown in Table 2. PCR assays were performed by the method described above. RNA extracts of the strains were prepared using the Qiagen RNeasy Mini-prep Kit and RNA BacteriaProtect (Qiagen, Valencia, CA, USA), following the manufacturer's instructions for Gram-negative bacteria. Reverse transcription was conducted using the PrimeScript RT kit (TaKaRa Bio Inc., Japan). Amplification was conducted using Engine Opticon 2 (DNA Engine DYAD; MJ Research, USA) and SYBR Premix Ex Taq (TaKaRa) using a pre-denaturation step for 5 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 30 s at 72°C. The final step was an extension for 5 min at 72°C with a melting curve from 40°C–95°C read every 0.5°C, with a holding time of 1 s.

RESULTS

Virulence Genes and Toxin Production of Asymptomatic STEC O91:H21 Isolates

To test for the presence of major STEC-related virulence genes in the asymptomatic STEC O91:H21 isolates, hemolysin (*hlyA*), a well-defined adhesin intimin (*eaeA*), translocated intimin receptor Tir (*tir*), and type III secretion proteins EspADB (*espADB*) were investigated by PCR using gene-specific primers. None of the strains tested carry any of these genes (Table 3). To determine whether Stxs is present in any of the asymptomatic STEC O91:H21 isolates, they were tested using RPLA. The results show that all of these isolates possess *stx1* and *stx2* (Table 3), whose gene products (Stx1 and Stx2, respectively) were confirmed by RPLA. It is noteworthy that the symptomatic STEC O91:H21 strains ATCC 51435 and ATCC 51434 produced Stx1, in addition to the Stx2 variants previously designated as Stx2d. DNA nucleotide sequence analysis of each *stx2* gene cloned from those isolates revealed that the asymptomatic STEC O91:H21 isolates carried the *stx2d* gene with 100% nucleotide sequence identity to the corresponding genes in the symptomatic ATCC 51435 and ATCC 51434 strains (data not shown), which is known to be activated by intestinal mucus or elastase [13]. These results suggest that our asymptomatic STEC O91:H21 isolates do not differ from the symptomatic O91:H21

Table 3. Prevalence of major STEC-related virulence genes.

Target gene	Symptomatic STEC O91:H21		Asymptomatic STEC O91:H21							
	ATCC 51434	ATCC 51435	1	2	3	4	5	6	7	8
stx1	+	+	+	+	+	+	+	+	+	+
stx2	+ (*2d*)	+ (*2d*)	+ (*2d*)	+ (*2d*)	+ (*2d*)	+ (*2d*)	+ (*2d*)	+ (*2d*)	+ (*2d*)	+ (*2d*)
eaeA	-	-	-	-	-	-	-	-	-	-
hlyA	-	-	-	-	-	-	-	-	-	-
espA	-	-	-	-	-	-	-	-	-	-
espD	-	-	-	-	-	-	-	-	-	-
espB	-	-	-	-	-	-	-	-	-	-
tir	-	-	-	-	-	-	-	-	-	-

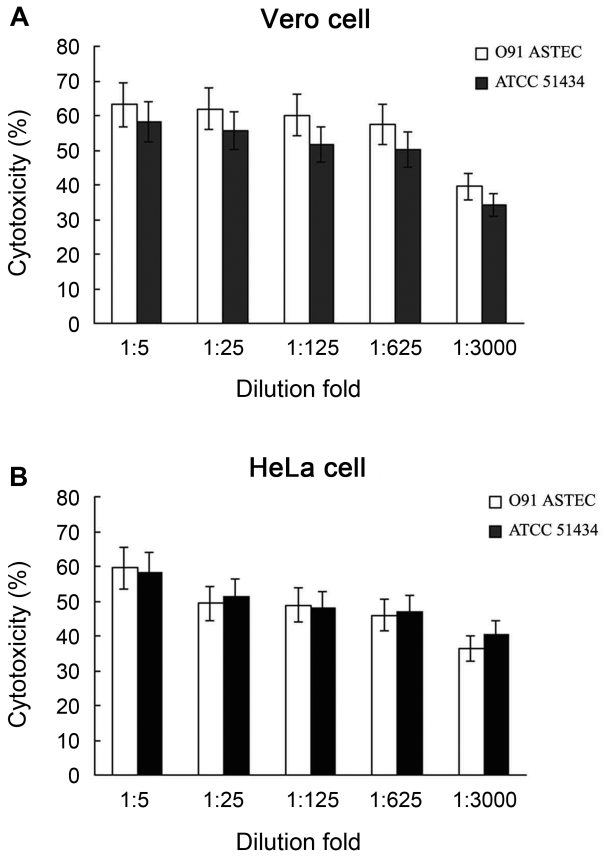

Fig. 1. Comparison of the cytotoxicities of symptomatic STEC O91:H21 ATCC51434 (SSTEC) and asymptomatic STEC O91:H21 (ASTEC) using bacterial culture supernatants.
The Y axis represents % cytotoxicity and the X axis represents the dilution of supernatant. Vero cells were used in panel **A** and HeLa cells in panel **B**.

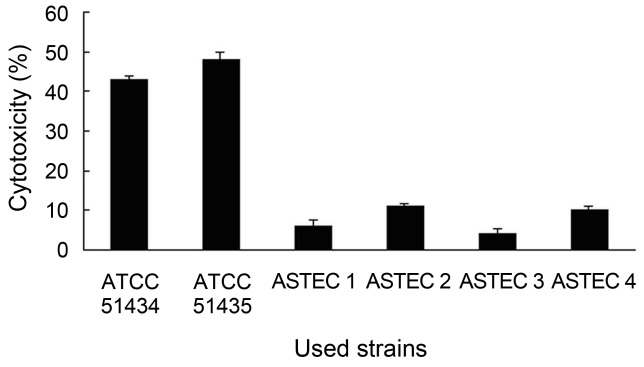

Fig. 2. Comparison of the cytotoxicities of symptomatic STEC O91:H21 strains ATCC 51434 and 51435 (SSTEC) and asymptomatic STEC O91:H21 strains (ASTEC 1 to 4) using bacterial cells.
The Y axis represents % cytotoxicity. The X axis represents the strains tested.

strains with regard to virulence gene expression or toxin production.

Cytotoxicity and Adherence of Asymptomatic STEC O91:H21 Isolates

To compare the cytotoxicities of the symptomatic and asymptomatic O91:H21 strains, we analyzed their LDH activity in human cells. Four isolated asymptomatic strains (ASTEC1 to ASTEC4) and two symptomatic strains (ATCC 51434 and 51435) were inoculated into Vero and HeLa cells and their cytotoxicity was assayed. Similar cytotoxicity was evident in both strain groups when only bacterial culture supernatants were inoculated (Fig. 1). These results indicate that Stxs production by the asymptomatic STEC O91:H21 isolates was associated with the cytotoxicity effect. However, the cytotoxicity differed when the bacterial cells of each different strain were used to inoculate cultures; decreased cytotoxicity was observed with regard to the asymptomatic strains (Fig. 2). These results suggest that the cytotoxicity effect may be influenced by other factors.

We tested the adherence of these strains using HeLa cells. The adherence assay indicated that the symptomatic strains are more adherent than the asymptomatic isolates (Fig. 3). To assess whether the reduced adherence of the asymptomatic strains is related to the surface of the strains, symptomatic and asymptomatic O91 strains recovered from agar were examined by transmission electron microscopy. The asymptomatic O91 strain possessed virtually no fimbriae on the cell surface (completely bald), whereas copious fimbriae were evident in the symptomatic strain ATCC 51434 (data not shown).

An Unusual Repression of Type 1 Fimbriae in Asymptomatic STEC O91:H21 Isolates

To confirm the presence of fimbriae genes, selected regions of chromosomally encoded type 1 fimbriae, P fimbriae, and f1C fimbriae were amplified using PCR (Table 2).

Table 4. Presence and expression of various fimbriae genes in symptomatic STEC O91:H21 strain ATCC51434 (SSTEC) and asymptomatic STEC O91:H21 strains (ASTEC 1 to 8).

Strains		*fimA/I/C/D/F/G/H*	*papB*	*papG*	*focH*
SSTEC	DNA	+	+	+	+
	RNA expression	++	-	-	-
ASTEC	DNA	+	+	+	+
	RNA expression	-	-	-	-

PCR analysis indicated that type 1 (*fimB, E, A, I, C, D, F, G*, and *H*), P (*papB* and *G*), and F1C fimbriae (*focH*) genes were present in both strains (Table 4). Sequence analysis showed that no deletions or mutations were evident in the type 1, P, or F1C fimbriae genes of asymptomatic strains compared with symptomatic STEC O91:H21 ATCC 51434 (data not shown). To determine whether these fimbriae genes were expressed, we performed quantitative real-time PCR employing the same primers used in cloning and sequencing. The mRNA expression of *fim A*, *I*, *C*, *D*, *F*, *G*, and *H* were markedly reduced in asymptomatic O91:H21 isolates compared with the symptomatic STEC O91:H21 ATCC 51434 strain. However, *papB*, *papG*, and *focH* were not expressed in either strain (Table 4).

DISCUSSION

Recent studies on virulence and pathogenicity have aimed to identify the virulence genes and mechanisms that render a STEC strain pathogenic to humans. To clarify the molecular and cellular differences between symptomatic and asymptomatic STEC strains, we analyzed asymptomatic STEC O91:H21 strains isolated during an outbreak in Korea and compared them with the symptomatic STEC O91:H21 strains ATCC 51434 and 51435. In our study, the cytotoxicity of Stxs from asymptomatic STEC O91:H21 isolates did not differ from that of Stxs from symptomatic strains. Whereas several studies have reported Stx-producing *E. coli* isolated from asymptomatic human carriers that have Stx-neutralizing antibodies (STX-Nab) [14, 21], it is difficult to reconcile the present findings with the presence of STX-Nab in every patient. Apart from the ability to produce the various forms of Stx, these kinds of pathogenic groups may possess accessory virulence factors associated with the capacity to colonize the gut, such as an LEE [5]. LEE-negative STECs are rarely isolated HUS and are not usually included among EHECs. Most STECs included in the EHEC group colonize the intestinal mucosa *via* a mechanism that subverts epithelial cell function and induces full virulence [5, 24]. The mechanism by which LEE-negative strains colonize the intestine, when the intestinal contents are flowing normally, is unclear. This prompted us to investigate the adherence factors expressed by asymptomatic and symptomatic O91:H21 strains.

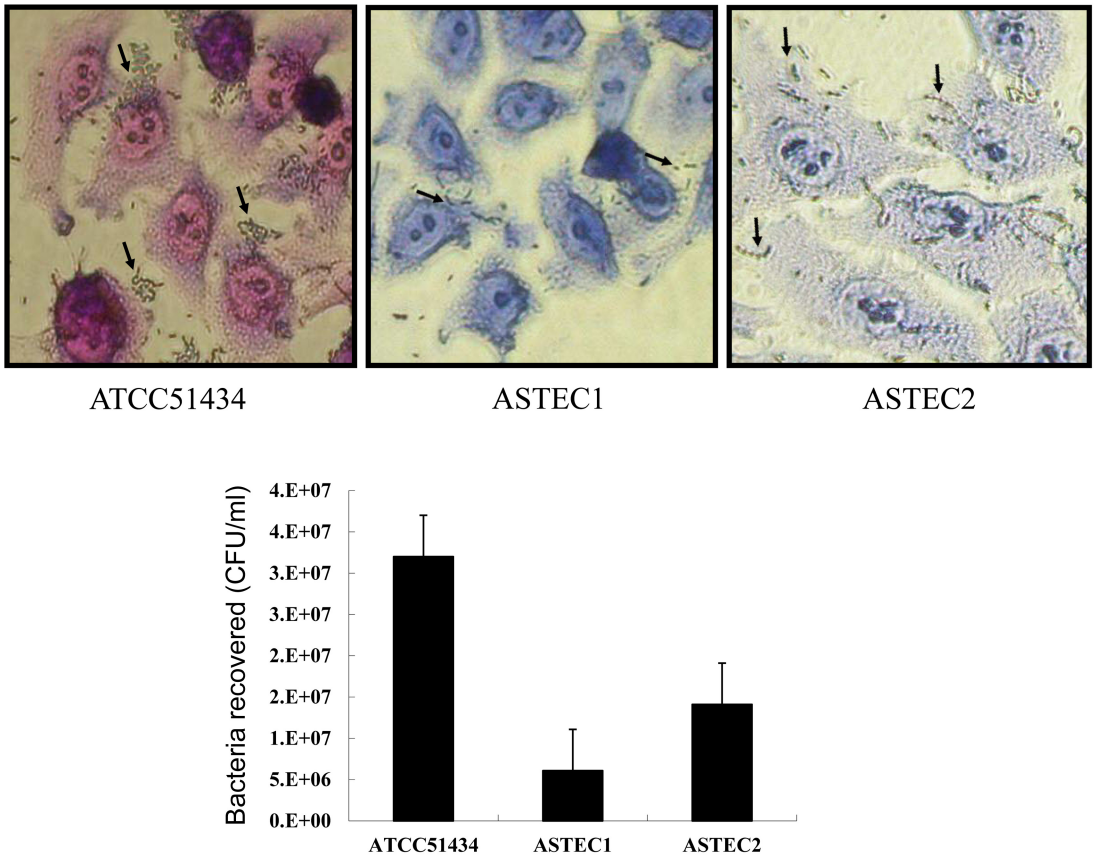

Fig. 3. Transmission electron microscopic image of adhered symptomatic STEC O91:H21 strain ATCC 51434 (SSTEC) and two asymptomatic STEC O91:H21 strains (ASTEC 1 and 2).

Molecular characterization of the asymptomatic bacterial strain ATCC 83972 in uropathogenic *E. coli* (UPEC) has been reported. It has also been reported that fimbriae are of primary influence with regard to the symptoms elicited by this strain [9, 18–20]. Type 1 fimbriae enhance colonization, induce host responses in the murine UTI model, and promote biofilm formation and invasion [9]. Receptor binding is also mediated by an adhesin located at the tips of the fimbriae (*fimH*) that binds to α-D-mannosylated proteins such as uroplakins, which are abundant in the bladder [1]. P fimbriae enhance the establishment of bacteriuria and activate the innate immune response in animal models as well as in the human urinary tract [18]. F1C fimbriae recognize receptors present in the kidney, bladder, and urethra [1]. Binding of all three types of fimbriae to epithelial cells triggers host responses that include cytokine production, inflammation, and exfoliation of infected bladder epithelial cells [1]. Most of the reported ABU have the truncated type 1 fimbriae and mutated PapG causing reduced adhesion activity on the epithelial cell surface [9]. In the asymptomatic strain ATCC 83972, type-1 fimbriae are truncated (deletion of *fim E*, *A*, *I*, and *C*), and this strain is not capable of producing these organelles. The results of our microarray and quantitative real-time PCR investigations indicated that the asymptomatic phenomenon is fundamentally associated with the possession of fimbriae by the STEC O91:H21 strains. In particular, the reduced expression of type 1 fimbriae in the STEC O91:H21 strain obviated the ability of that strain to colonize the surface of the gut. A recent report has described a similar unusual chain-like pattern of adhesion to HEp-2 cells. In this case, immunoglobulin-binding protein G (EibG) enabled the bacteria to colonize [11]. In the present study, we investigated the basis of the asymptomatic phenomenon in STEC strains. Apart from Stx, adhesin is important for establishing the initial stage of an infection. Several studies on adhesin in STEC have determined that long polar fimbriae (Lpf) are present in certain strains of various serogroups of *E. coli* [22, 23].

In conclusion, we investigated the asymptomatic phenomenon in STEC strains. Apart from Stxs, adhesin is an important factor for establishing the initial stage of infection. Our results imply that the asymptomatic phenomenon is associated with the possession of fimbriae by the STEC O91:H21 strains. To clarify the asymptomatic phenomenon, further studies on topics such as the function of *fim* genes, for example *via* restoration experiments, will be needed.

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