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Molecular Cloning and Expression of Shiga-Like Toxin II Gene (*slt*-II) from an Isolate of Healthy Korean Native Bovine Feces, *Escherichia coli* KSC109

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By PCR amplification using the sequence of the previously cloned shiga-like toxin II DNA, a gene encoding it has been cloned from an isolate of healthy Korean native bovine feces, *Escherichia coli* KSC109. The nucleotide sequences included two open reading frames coding for 319 and 89 amino acids corresponding to A and B subunits, respectively. Comparsion of the nucleotide and predicted amino acid sequences of newly cloned gene (*slt*-II) with those of others in the SLT-II family revealed completely identical homology with SLT-II cloned previously from bacteriophage DNA of *E. coli* 933 derived from a patient with hemorrhagic colitis. In addition, the sequence homology of SLT-II with SLT-II variant from bovine was more than 95% at both the nucleotide and protein levels. Overexpression of SLT-II recombinant gene by induction with IPTG using an *E. coli* host-vector system was conducted and the correctly processed products with active mature form exhibited 1000-fold higher cytotoxicity for Vero cells than that from original strain.

Key words: Shiga like toxin II gene, bovine feces, *E. coli*

Some *Escherichia coli* strains, associated with diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS), produce protein cytotoxins with similar biological activities that are related to the Shiga toxin (STX) produced by *Shigella dysenteriae* type I (12). These toxins are called Shiga-like toxins (SLTs) or verotoxins (VTs) due to their cytotoxic activity on Vero cells and two major types (SLT-I and-II, or VT I and II) have been distinguished by serological methods as well as nucleotide sequence analysis (4); *i.e.*, SLT-I and VT I can be neutralized by antiserum prepared against purified Shiga toxin, whereas SLT-II and VT II are not neutralized by antiserum to Shiga toxin (22). The SLT-I and-II structural genes shared 58% overall nucleotide and 56 % amino acid sequence homologies (4). As a variant of SLT-II, SLT-IIv (or VTe) is produced by *E. coli* strains associated with edema disease and is neutralized by antiserum to SLT-II (10). SLT-II and SLT-IIv cross-react immunologically with each other and share 91% homology in nucleotide sequence (3, 4, 15~18, 20, 21, 23). While Shiga toxin, SLT-I and SLT-II are predominantly cell associated in *E. coli* and are equally cytotoxic for Vero and HeLa cells, SLT-IIv is predominantly extracellular and is cytotoxic for Vero cells but not for HeLa cells (10, 23). Moreover, SLT-I and-II genes are generally encoded on bacteriophages of *E. coli* producing their toxins (13), but SLT-IIv genes are encoded on the chromosomal DNA like STX (3, 15~18, 20, 21, 23).

SLTs and STX are subunit toxins, consisting of one noncovalently active A subunit, which inhibits eukaryotic protein synthesis, and five B subunits responsible for binding to glycolipid receptors in target cell membranes (5). The oligomeric B subunits of STX, SLT-I and-II bind to globotriaosylceramide (Gb_3) glycolipid receptor, while the B subunits of SLT-II varients predominantly bind to the larger globotetraosylceramide (Gb_4) receptor (7, 8). To date, a variety of SLT-II variant genes have been cloned from the chromosomal DNA and

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show a very high homology (more than 80~90%) in both nucleotide and amino acid sequences among them except for SLT-IIva (1, 3, 15~18, 20, 21, 23).

In this study, we report the existance of SLT-II gene in a Korean native bovine isolate, *E. coli* KSC109 (O157 : H7) strain which considered as an important member of VTEC (Verotoxin-producing *Escherichia coli*) and expression of its structural gene using *E. coli* host-vector system.

Materials and Methods

Bacterial strains and plasmids

E. coli KSC109 isolated from feces of healthy Korean native bovine as described by Cha *et al.* (in press, 1996. *Kor. J. Vet. Res.*), which is an O157 : H7 serogroup, was used for the isolation of SLT-II-converting bacteriophage. *E. coli* JM109 was used as a host strain for the cloning, subcloning and expression of recombinant DNA. Plasmids pUC118 and 119, and pUC18 were used as the vector plasmids for cloning, subcloning and expression, respectively. M13KO7 helper phage was used in single-stranded DNA preparation for DNA sequencing. *E. coli* C600 was used as the indicator strain for plaque assays and as the host strain in phage conversion experiments.

Isolation of SLT-II-converting bacteriophage from *E. coli* KSC109 strain and preparation of phage DNA

Isolation of the bacteriophage from *E. coli* KSC109 was originally carried out by the procedure (13) as described previously. *E. coli* KSC109 cells were grown in 5 ml of the modified LB broth (1% Bacto-tryptone, 0.5% yeast extract, 0.25% NaCl, 10 mM $CaCl_2$ and 0.001% thiamine) to an optical density of 0.5 at wavelength 600 nm. Cells were then harvested by centrifugation, resuspended in 5 ml of 10 mM $CaCl_2$, and irradiated with UV light for 1 min to induce phage from *E. coli* KSC109 cells. The irradiated cells were incubated for 5 h at 37°C in the modified LB broth. After centrifugation, the lysate was sterilized by membrane filtration (0.45 μm) and stored at 4°C. The sterilized solution containing phage was adsorbed onto *E. coli* C600 cells, which were prepared by the same procedure as *E. coli* KSC109 described above and resuspended with 10 mM $CaCl_2$, for 20 min at 37°C in the modified LB broth. LB soft agar containing the adsorption mixture (100 μl) was overlayed onto LB agar plate, and incubated for 18 h at 37°C. Plaques were obtained as the phage solution for phage DNA isolation by suspension with SM buffer (NaCl 5.8 g, $MgSO_4$ 2 g, 1 M Tris (pH 7. 5) 50 ml and 2% gelatin per liter). Isolation and purification of phage DNA were carried out by the same procedure using cesium chloride density gradients as previously described (9).

Cloning of the *slt*-II by polymerase chain reaction (PCR) and DNA sequence analysis

Cloning of the *slt*-II was carried out by PCR using the purified phage DNA as template and oligonucleotide primers as to the sequence of the SLT-II gene (*slt*-II) cloned previously from phage DNA derived from *E. coli* 933 (4). The sense primer; 5'-CGGTCGAATTCTCATGCGTCCATTATC-3' (*Eco*RI site is underlined) and the antisense primer; 5'-AGATTAAGCTTGTTACCCACATACCAC-3' (*Hind*III site is underlined) were used for cloning. PCR amplification was carried out using a DNA thermal cycler (Perkin-Elmer Cetus), with 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR product was purified from 1.5% agarose gel, and then digested with *Eco*RI and *Hind*III. The resulting product (1.5 kb) was subcloned into the corresponding sites on pUC118 and 119 vector plasmids, and single-stranded DNAs produced on infection with a helper phage (M13KO7) were used as templates for DNA sequencing. DNA sequences were determined by the dideoxynucleotide chain-termination method (19) using an Autocycle DNA sequencing kit and A. L. F. DNA sequencer (Pharmacia). Sequences were analyzed using PC/Gene (Teijin System Technology, Japan).

Construction of an expression plasmid pKSC101

For overexpression of the newly cloned SLT-II gene in *E. coli* host strain, an expression plasmid, pKSC101, was constructed according to the following procedure. The structural gene coding for A and B subunits of SLT-II was amplified by PCR with the cloned 1.5 kb fragment as template using two oligonucleotide primers with *Eco*RI and *Hind*III sites on the sense and antisense primers, respectively: E101, 5'-TGCTGAATTCTTCAGCCAAAAGGAACA-3' (*Eco*RI site is underlined) and the antisense primer, 5'-AGATTAAGCTTGTTACCCACATACCAC-3' (*Hind*III site is underlined). PCR amplification was performed by the same method as described above. The PCR product was purified from 1.5% agarose gel, and then digested with *Eco*RI and *Hind*III. The resulting product (1.3 kb) was subcloned into the corresponding sites on pUC18 to generate plasmid pKSC101. Finally, the structural gene (1.3 kb) of SLT-II introduced into pUC18 was confirmed by DNA sequencing.

Expression of the newly cloned SLT-II gene in *E. coli* cells

E. coli JM109 cells harboring pKSC101, and pUC18 as control were cultured at 37°C overnight in LB medium

(1% Bacto-tryptone, 0.3% yeast extract, 0.5% NaCl) containing 0.5% glucose and ampicillin (100 μg/ml of medium). Precultured cells were inoculated (1%) into the same medium and cultured at 37°C. At $A_{600\,nm}$=0.7, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the culture to a final concentration of 0.5 mM, and then cultured for 3 h in order to induce expression of SLT-II gene. After harvesting by centrifugation, the supernatant was filtered with membrane (0.45 μm), concentrated 10-fold on Centricon 30 filter (Amicon) and used for assaying of cytotoxicity.

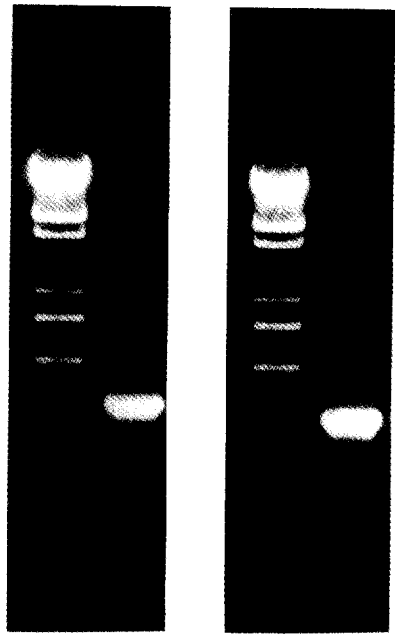
Cytotoxicity assays on Vero and HeLa cells

Microcytotoxicity assays were performed as described previously (2). In brief, Vero and HeLa cells were cultured in the minimal essential medium (MEM, Sigma) containing 5% calf bovine serum, 100 μg/ml of gentamycin and 100 U/ml of penicillin. The concentrated supernatants were added to freshly seeded Vero or HeLa cells in 96-well microtiter plates (1 μl of the supernatant per well in duplicate). The last dilution of the samples in which greater than or equal to 50% of the Vero or HeLa cells detached from the plastic as assessed by A_{620} measurements was considered the 50% cytotoxic dose (CD_{50}) per ml of culture. For comparsion of the cytotoxicity for Vero cells, the original strain, *E. coli* KSC109 cells were cultured in the same condition as in the case of *E. coli* JM109 cells carrying pKSC101 until optical density at wavelength of 600 nm was identical for both strains. The same volumes (10 ml) of cell cultures were harvested, and the supernatants were filtered, concentrated 10-fold and used for assaying of cytotoxicity.

Results and Discussion

Cloning of the SLT-II gene

Two oligonucleotides were designed and used as PCR primers for amplification of *slt*-II from SLT-II-converting phage DNA. The sense primer is homologous to a region 190~215 nucleotides upstream from the ATG start codon of the gene encoding the A subunit of SLT-II, while the antisense primer is complementary to a region 16~31 nucleotides downstream from the stop codon of the SLT-II B subunit (4). Thus these primers direct the amplification of a 1,460 bp fragment which contains the entire *slt*-II operon, including the putative -10 and -35 promoter region. When PCR was carried out using these primers and the purified phage DNA as template, a single band corresponding to about 1.5 kb was amplified(Fig. 1A). Restriction enzyme analysis for 1.5 kb showed the same pattern as that of the SLT-II gene (slt-II) cloned previously by Jackson *et al.* (data not shown).



(A) (B)

Fig. 1. Comparison of cytotoxicity of the SLT-II produced in *E. coli* cells carrying pKSC101 (lane 1) and in the original strain, *E. coli* KSC109 (lane 2).

Nucleotide and amino acid sequence analyses

The complete nucleotide and deduced amino acid sequences of the 1.5 kb was determined (Fig. 2). Two open reading frames (ORFs) corresponding to the genes (*slt*-IIA and *slt*-IIB) encoding the A and B subunits were located from nucleotides 198-1,157 and 1,170-1,438, respectively, within this fragment. Putative Shine-Dalgarno (SD) sequences were located immediately upstream from each ORF. Putative -10 and -35 promoter regions with homology to the consensus hexamers as reported previously (10) were also located approximately 85~117 bp upstream from the A subunit initiation codon. Therefore, it is likely that the A and B subunit genes of the SLT-II are independently translated from a polycistronic messenger RNA synthesized under the control of a promoter located 5' to the sequence encoding the A subunit as in the case of SLT-II family.

Comparsion of the nucleotide and predicted amino acid sequences of *slt*-IIA and *slt*-IIB with those of others in the SLT-II family revealed completely identical homology with SLT-II cloned from bacteriophage DNA of *E. coli* 933 derived from a patient with hemorrhagic colitis (Table 1). However, compared with other SLT-II genes cloned from the chromosomal DNAs of VETC, SLT-II gene shared very high homologies except for SLT-IIva, with more than 94% and 83% identities for A and B subunits, respectively, at the nucleotide and protein levels. Of them, the sequence

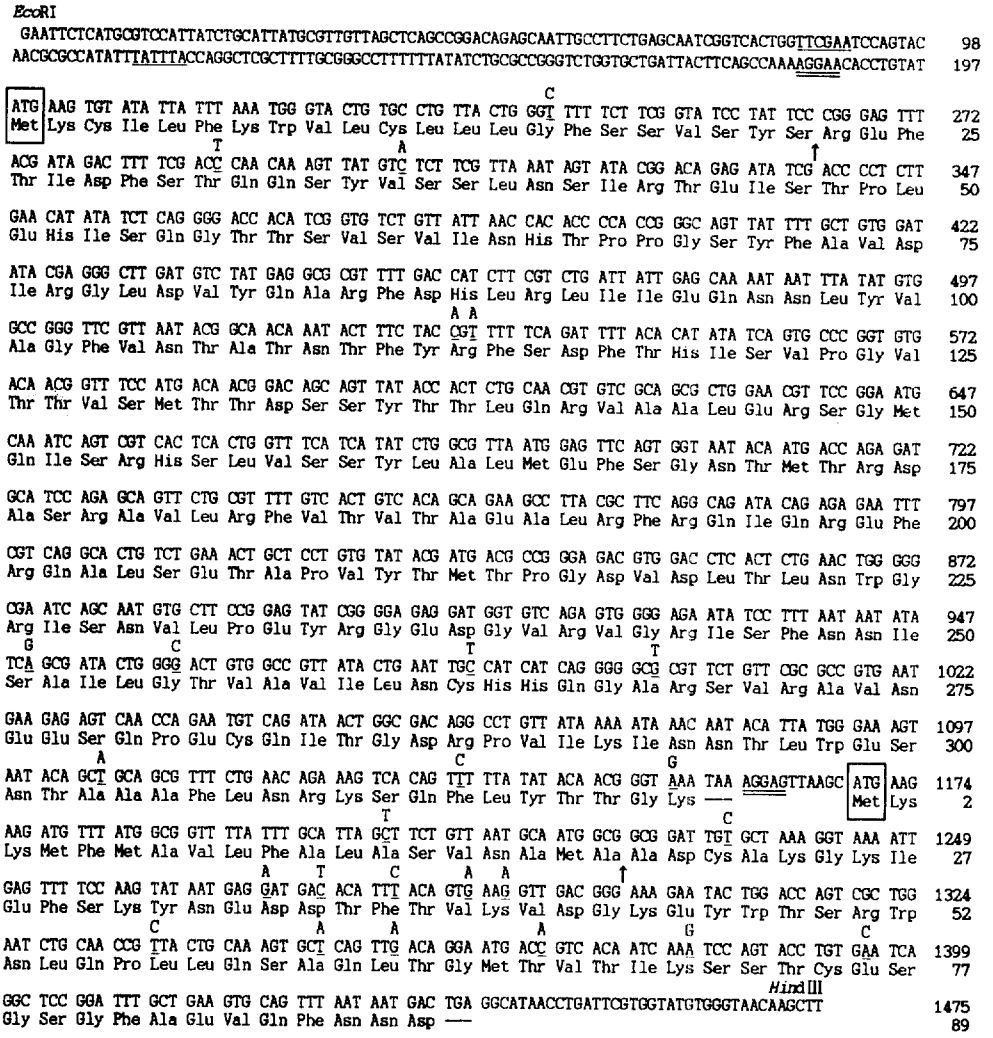


Fig. 2. Agarose gel (1.5%) electrophoresis and ethidium bromide staining of PCR products amplified for cloning (A) and expression (B) of SLT-II gene. The size of the PCR products was determined by their relative electrophoretic mobilities compared to λDNA fragments digested with *Sty*I.

homology of SLT-II with SLT-II variant from bovine was more than 95% at both the nucleotide and protein levels. This result indicates a difference in the sequences of SLT-II genes between bacteriophage and chromosomal DNAs. The A and B subunits of SLT-II possessed signal peptides of 22 and 19 amino acids, respectively (shown as vertical arrows in Fig. 2), which were identical to the signal peptides of the SLT-II family. Separation of the signal peptides from the mature polypeptides at putative *E. coli* signal peptidase cleavage sites (13) resulted in the processed SLT-IIA and B subunits of 297 and 70 amino acids with a calculated molecular weight of 33,135 and 7,817, respectively.

Expression of the SLT-II gene in *E. coli*

In order to induce overexpression of the SLT-II gene using *E. coli* host-vector system, the structural gene of SLT-II was amplified as a single band with size of 1.3 kb by PCR (Fig. 1B). This product includes the structural gene of SLT-II except for promoter region. This fragment was introduced into pET-3d vector plasmid which includes T7 promoter with the strongest transcription activity, and transformed into *E. coli* BAL21(Lys) which is a host strain for pET-3d vector. However, it was impossible to obtain transformant containing the plasmid inserted with SLT-II gene. Although there is no direct evidence to explain this problem, this may be due to an expression of SLT-II gene by the leakage of T7 promoter activity before host cells harbouring the plasmid inserted with SLT-II gene proliferate completely. Therefore, The 1.3 kb fragment was introduced into pUC18 vector plasmid with the *lac* promoter, and an expression vector for the SLT-II gene, plasmid pKSC101, was constructed. Consequently, the SLT-II gene was expressed not as a

Table 1. Comparison of the nucleotide and predicted amino acid sequences of the A and B subunits of the SLT-II gene from *E. coli* KSC109 with those of the SLT-II family

SLT-II family[a]	% Homology[b]			
	Nucleotide sequence		predicted amino acid	
	A subunit	B subunit	A subunit	B subunit
SLT-II (*E. colit* KSC109)	100	100	100	100
SLT-II (*E. coli* 933)[c]	100	100	100	100
SLT-IIv	94.4	83.5	94.1	86.4
SLT-IIva	68.1	79.3	68.7	82.9
SLT-IIc	99.5	95.5	100	96.7
SLT-OX3	96.0	86.6	95.6	88.6
SLT-OX3/2	99.3	95.2	99.4	96.6
SLT-II/0111	95.9	86.6	95.6	88.6
SLT-IIc (*c. freundii*)	98.8	05.9	98.4	96.7
SLT-II/048	99.6	98.9	100	98.9
SLT-II/Ent	99.2	99.3	99.1	98.9
VT2vha	99.2	95.9	99.1	96.7
VT2 variant (pKTN1054)	98.7	95.0	99.3	96.6

[a] References indicated SLT-II family are follows : SLT-II (4), SLT-IIv (22), SLT-IIva (1), SLT-IIc (19), SLT-OX3 (16), SLT-OX3/2 and SLT-II/0111 (17), SLT-IIc from *C. freundii* (20), SLT-II/048 (14), SLT-Ii/Ent (15), VT2vha (3), VT2 variant from pKTN1054 (6).

[b] Values represent percent homology with the indicated sequences from the A and B subunits genes of SLT-II from *E. coli* KSC109.

[c] The revised nucleotide sequence is deposited with the EMBL/GenBank/DDBJ databases under accession number X07865.

protein fused with β-galactosidase but as a homogeneous protein in *E. coli* cells harboring pKSC101, and expression of the gene in pKSC101 is able to be induced by the addition of an inducer, IPTG. *E. coli* cells carrying pKSC101 grew well in medium containing 0.5% glucose

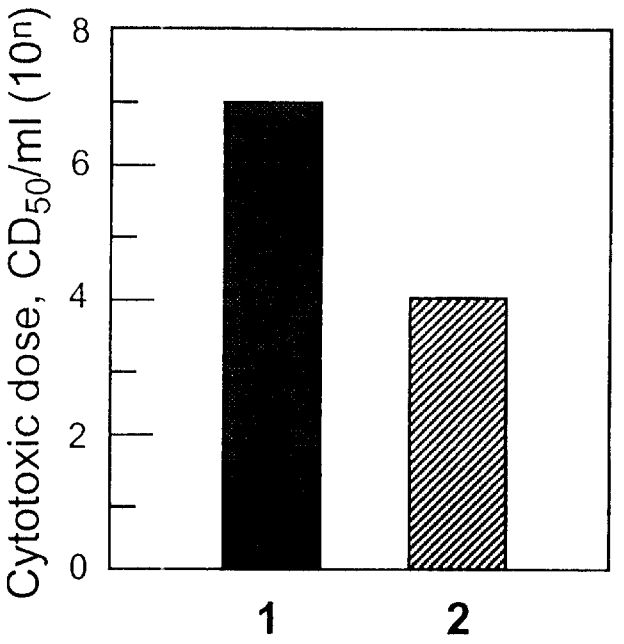


Fig. 4. Cytotoxicity of the SLT-II for Vero (A) and HeLa cells (B). Cytotoxicity assays were carried out using SLT-II produced in *E. coli* cell carrying pKSC101 or pUC18 as control.

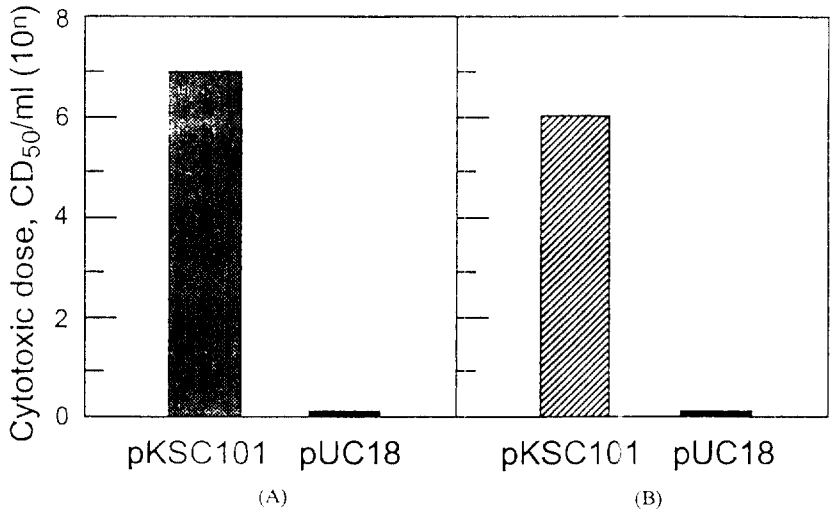


Fig. 3. Nucleotide and the deduced amino acid sequences of SLT-II. Structural gene encoding SLT-II is composed of A subunit (nucleotide sequence from 198 to 1,157) and B subunit (nucleotide sequence from 1,170 to 1,438). The numbering of nucleotides starts at the 5' terminus of the gene encoding SLT-II, and that of amino acids at the N-terminus of SLT-II. The deduced amino acid sequence is shown beneath the nucleotide sequence. The column of numbers at the right indicates the position in the nucleotide and amino acid sequence at the end of each line. Single underlines indicate a promoter sequences (-35 and -10 regions) and double underlines represent a Shine-Dalgarno sequence (SD). Rectangles indicate an initiation codon (ATG) of A and B subunits. Nucleotides shown above the underlined one indicate a position different from the sequence of SLT-II variant (*E. coli* KY-019) from bovine (6). Vertical arrows indicate cleavage sites of A and B subunits by signal peptidase.

in the absense of IPTG, and the production of SLT-II was induced with IPTG for 3 h. The SLT-II expressed by the induction of IPTG was secreted into the culture medium and the supernatant showed a strong cytotoxicity for Vero and HeLa cells, while no cytotoxicity was detected in *E. coli* cells harboring only the vector plasmid (Fig. 3). In addition, as shown in Fig. 4, SLT-II overproduced in *E. coli* cells carrying pKSC101 exhibited 1000-fold higher cytotoxicity for Vero cells than that from original strain, *E. coli* KSC109. This result indicates that SLT-II produced in *E. coli* cells carrying pKSC101 was correctly processed to an active mature form by cleavage of SLT-II signal peptide and secreted extracellularly.

In conclusion, this study indicates that *E. coli* KSC109 isolated from Korean native bovine feces is carrying the SLT-II gene responsible for HUS and infant diarrhea, and healthy domestic animals may serve as an important reservior of human pathogens.

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