Distribution and Diversity of Shiga Toxin 2 Gene in Urban Rivers

Katsuji Tani,*[,a,b] Misuzu Kaneshige,[b] and Masao Nasu[b]

[a]*Environmental Science and Microbiology Faculty of Pharmacy, Osaka Ohtani University, 3–11–1 Nishikiori-kita, Tondabayashi, Osaka 584–8540, Japan and* [b]*Environmental Science and Microbiology Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan*

(Received January 17, 2007; Accepted May 10, 2007; Published online May 23, 2007)

In urban rivers in Osaka, Japan, DNA containing the *stx*$_2$ gene, which encodes the Shiga toxin 2 (Stx$_2$), was found to be present in sediment, even when it was not detected in the surface water. A DNA sequence similar to that of bacteriophage 933W and the Sakai strain was detected at every sampling location. Two strains of *Escherichia coli* O157 carrying the *stx*$_2$ gene were independently isolated from sediment. These results show that river sediment is a potential reservoir of the *stx*$_2$ gene and Shiga toxin-producing bacteria in the natural environment.

Key words —— Shiga toxin 2 gene, river, distribution, diversity, most probable number-nested polymerase chain reaction

INTRODUCTION

Shiga toxin is one of the most important pathogenicity factors known to affect humans,[1] and Shiga toxin-producing bacteria such as *Escherichia coli* O157 are a significant cause of hemorrhagic diarrhea and hemolytic uremic syndrome. Shiga toxins fall into two groups: Stx$_1$ and Stx$_2$. The gene encoding Stx$_1$, *stx*$_1$, is highly conserved, whereas that encoding Stx$_2$, *stx*$_2$, has 11 distinct variants.[2] These genes are encoded by a bacteriophage genome.[3]

Farm ruminants such as cattle and sheep are the major reservoirs of these bacteria[4] and can excrete them in their feces, resulting in contamination of soil and water. Most outbreaks are linked to the consumption of undercooked bovine food products, fecal-contaminated vegetables, and other contaminated foodstuffs.[5–8] It has recently been found that *E. coli* O157:H7 may possibly be transmitted via contaminated slugs.[9] Transmission through contaminated drinking or swimming water is also an important pathway of *E. coli* O157 infection.[10–12] *E. coli* can survive in aquatic environments,[13–15] and both Shiga toxin-producing bacteria and bacteriophages carrying the Shiga toxin gene have been isolated from aquatic environments.[4, 16–20] It is possible that aquatic environments are a reservoir for Shiga toxin-producing bacteria.

In the present study, we measured the concentration of *stx*$_2$ DNA in river surface water and sediment, and compared the partial sequences of the genes found. *E. coli* O157 carrying the gene was also isolated.

MATERIALS AND METHODS

Sampling —— Sampling stations were shown in Fig. 1. Surface water and sediment samples were taken from Kurumatsukuri, Minami-takahama, Juhachijo (in the Aigawa-Kazakigawa River system), Shiromi (Neyagawa River) and Higashikuwazu (Inagawa River) in the northern part of Osaka, Japan, from June to December, 2004 (Table 1). Surface water samples were collected in a sterilized polycarbonate bottle. Sediment samples were collected using the Eggman.

Quantification of *stx*$_2$ DNA —— Volumes of 10, 1 or 0.1 ml of surface water, or 10, 1 or 0.1 g (wet-weight) of sediment were placed into tubes with 10 ml of R2A liquid medium[21] in triplicate. After overnight incubation at 30°C with shaking, bacterial cells were collected from 1 ml of culture and washed. Then bacterial DNA was extracted by three repeats of freezing in liquid nitrogen and thawing at room temperature. After purification using phenol-chloroform and concentration by ethanol precipitation, DNA was resolved in 50 µl

*To whom correspondence should be addressed: Environmental Science and Microbiology School of Pharmacy, Osaka Ohtani University, 3–11–1 Nishikiori-kita, Tondabayashi, Osaka 584–8540, Japan. Tel. & Fax: +81-721-24-9742; E-mail: tanika@osaka-ohtani.ac.jp

of Tris- EDTA (TE) buffer, and 1 µl aliquots were used for polymerase chain reaction (PCR) amplification. The stx_2 DNA was detected by nested PCR. Primers were designed as follows: first round primer set, 5′-CCATGACAACGGACAGCAGTT-3′ and 5′-CCTGTCAACTGAGCACTTTG-3′; second round PCR primer set, 5′-ATCAGTCGTCACTCACTGGT-3′ and 5′-CCAGTTATCTGACATTCTG-3′. The PCR reaction mixtures contained 5 U of Amplitaq Gold, 2 mM $MgCl_2$ and 0.2 mM deoxy ribonucleotide triphosphate mixture (dNTP). The volume of the PCR mixture was 20 µl. PCR conditions were as follows: one cycle of 95°C for 9 min (hot start); 30 cycles of 94°C for 30 sec (denaturation), 65–55°C (touchdown, −0.5°C per cycle) for 30 sec (annealing), 72°C for 1 min (extension); then one cycle of 72°C for 7 min. One microliter of reaction mixture of the first PCR was used for the second PCR. The reaction mixture and the conditions for the second PCR were the same as used for the first, except that the concentration of $MgCl_2$ was 2.5 mM. The number of copies of stx_2 DNA present was estimated by using the most probable number (MPN) method.[22)]

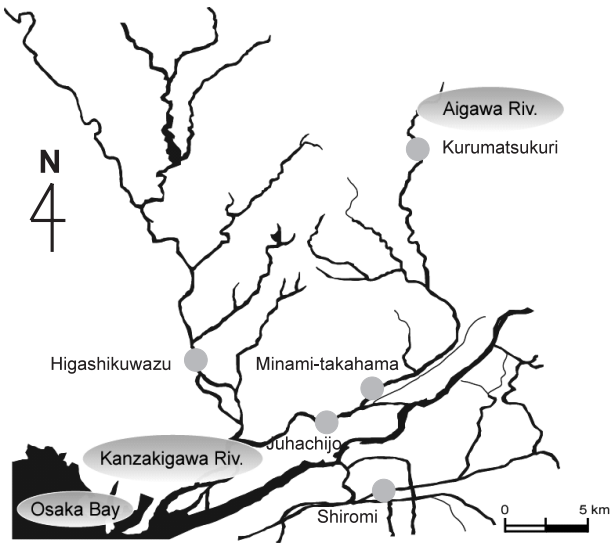

Fig. 1. Sampling Station
Each station is located in Osaka prefecture, Japan.

Isolation of *E. coli* O157 —— *E. coli* O157 was isolated using Ogden's method[20)] with slight modifications. Ten grams of river sediment was inoculated into 250 ml of R2A liquid medium[21)] and incubated at 30°C with shaking overnight. One milliliter of the culture was transferred into 50 ml of buffered-peptone water containing 8 mg/ml vancomycin, then incubated at 42°C for 6 hr with shaking. One milliliter of the culture was used for immunomagnetic isolation.

Sequencing —— The PCR products obtained from the isolates and environmental samples were cloned into plasmid pGEM-T Easy (Madison, WI, U.S.A.). Sequencing was performed using CEQ Terminator Cycle Sequencing with the Quick Start Kit (Beckman Coulter, Fullerton, CA, U.S.A.) in a CEQ800 DNA Analyzer (Beckman Coulter, Fullerton, CA, U.S.A.) in accordance with the manufacturer's instructions. Nucleotide sequence analysis of homologous DNA sequences from the DDBJ and GenBank databases was performed with ClustalX.

Table 1. Abundance of stx_2 Gene in River Surface Water and Sediment in Osaka

Sampling date	Kurumatsukuri		Minami-takahama		Juhachijo	
	Water (MPN/100 ml)	Sediment (MPN/100 g)	Water (MPN/100 ml)	Sediment (MPN/100 g)	Water (MPN/100 ml)	Sediment (MPN/100 g)
Jun 9	3	—	—	—	43	—
23	—	—	—	—	9.1	—
30	—	—	—	—	< 3.0	—
Jul 6	3.6	—	—	—	< 3.0	—
13	—	—	—	—	< 3.0	43
Aug 24	—	—	—	460	—	44
Sep 15	—	3	—	—	14	43
Oct 19	3.6	3	—	—	3	—
28	< 3.0	< 3.0	< 3.0	3.6	< 3.0	43
Nov 9	3	< 3.0	3.6	3.6	< 3.0	7.2
30	< 3.0	< 3.0	< 3.0	3.6	3.6	3.6
Dec 14	< 3.0	< 3.0	< 3.0	9.1	3	3.6

Sampling was carrying out from June to December in 2004. —: not done

RESULTS AND DISCUSSION

In the present study, we obtained samples from five sampling locations (Fig. 1). Kurumatsukuri is located in an agricultural area, and at that point the river is narrow, shallow and fast-flowing. Minami-takahama and Juhachijo are located in an industrial area, and the river at these points is considered to be highly polluted. Shiromi is in a commercial area in Osaka City, and is considered to be the most polluted of the four sampling stations. Higashikuwazu is located in an industrial area. The abundance of DNA containing the stx_2 gene at three sampling stations is shown in Table 1. At Juhachijo, the concentration of stx_2 DNA in surface water ranged from less than 3 to 43 MPN/100 ml. At this sampling location, stx_2 DNA was detected in the sediment at every sampling time, even when it was not detectable in the surface water. At Minami-takahama, the concentration of stx_2 DNA in surface water was less than 3.6 MPN/100 ml, whereas in the sediment the concentration ranged from 3.6 to 460 MPN/100 g.

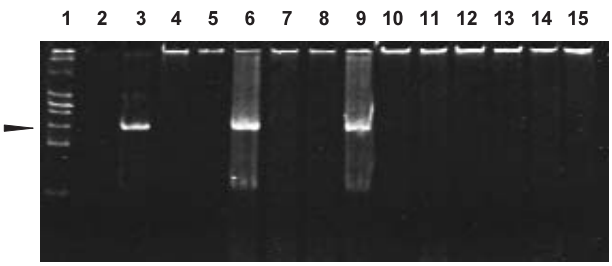

Fig. 2. Isolation of Bacteria Carrying stx_2 Gene
Lane 1: molecular size marker, 2: negative control (no DNA), 3: positive control (EHEC Sakai strain), 4: isolate (Kurumatsukuri), 5–15: isolates (Juhachijo). Arrow shows the position of the nested-PCR products originated from the stx_2 gene.

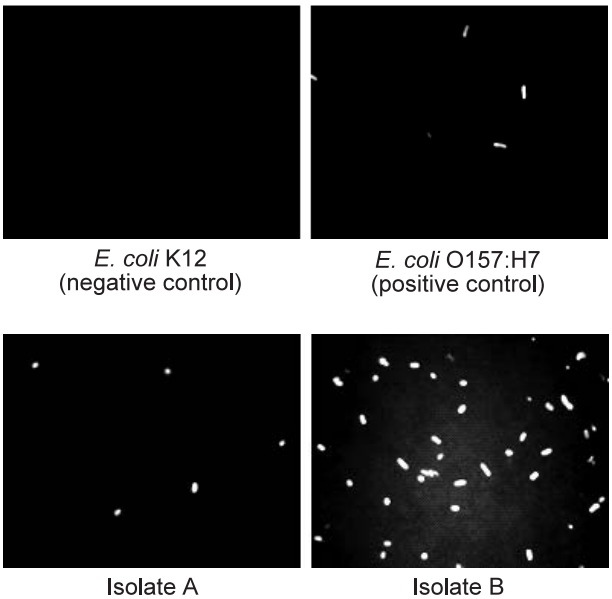

Fig. 3. Identification of *E. coli* O157 by FITC-labeled Anti *E. coli* O157 Monoclonal Antibody Staining
Isolate A and B were isolated from the sediment of Juhachijo.

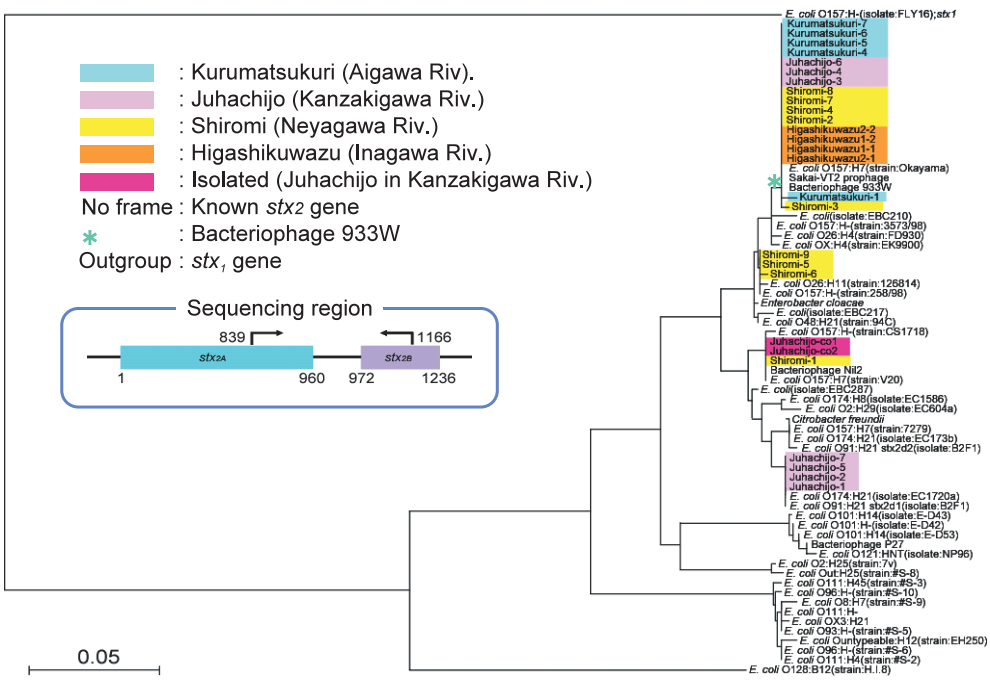

Fig. 4. Phylogenetic Relationship of Partial stx_2 Gene Sequence

At Kurumatsukuri, an agricultural area, the concentration of stx_2 DNA was less than 4 MPN/100 ml or g in both the surface water and sediment during the study period. These results suggest that stx_2 DNA may be ubiquitous in the sediment of an urban river, but was transient in a suburban river in this study.

Two strains of *E. coli* O157 carrying stx_2 DNA were independently isolated from sediment at Juhachijo. Before use of selective media, samples were incubated in liquid R2A medium.[21] R2A medium is suitable for the cultivation of bacteria that occur in the natural environment, and we were not able to isolate bacteria carrying the stx_2 gene without using this medium. PCR amplification and fluorescent antibody staining showed that these strains were carrying the stx_2 gene and the O157 cell surface antigen (Figs. 2, 3). Viable *E. coli* O157 was present in urban river sediment.

Nucleotide sequences at position 839–1166, a variable region in the stx_{2A}–stx_{2B} gene, were compared for 5, 7, 10 and 11 sequenced clones obtained from Kurumatsukuri, Juhachijo, Shiromi and Higashikuwazu. The entire stx_2 gene for two isolates from Juhachijo were also sequenced. Figure 4 shows the phylogenetic relationships of the partial stx_2 gene sequences of our samples and representatives. There was a low level of diversity in the stx_2 gene among the four sampling locations. Eleven distinct variants of the stx_2 gene are known to exist;[2] in the present study four variants were present in Shiromi, three in Juhachijo, and all clones obtained from Kurumatsukuri and Higashikuwazu (Inagawa River) belonged to the same group. Sequences that were similar to those of bacteriophage 933W and the Sakai strain were also detected.

To prevent infection, it is important to understand the ecology of pathogenic bacteria. Farm ruminants are known to be major reservoirs of Shiga toxin-producing bacteria.[4] In the present study, we found that *E. coli* O157 carrying the stx_2 gene lived in river sediment and that stx_2 DNA was present in sediment even when it was not detectable in surface water. Though we did not determine whether the stx_2 DNA was present in bacterial cells or bacteriophages alone, it is clear that river sediment is a potential reservoir for the stx_2 gene in the natural environment. If sediment is stirred, the river water may come to contain a high level of bacteria carrying the stx_2 gene, which may spread downstream. Therefore, river water should be considered a potential source of Shiga toxin-producing Phylogenetic Relationship of Partial stx_2 Gene Sequence. bacteria.

REFERENCES

1) Nakano, H. and Takeda, T. (2000) *Escherichia coli* Shiga toxin. *J. Nat. Toxin.*, **9**, 299–313.
2) Brett, K. N., Hornitzky, M. A., Bettelheim, K. A., Walker, M. J. and Djordjevic, S. P. (2003) Bovine non-O157 Shiga toxin 2-containing *Escherichia coli* isolates commonly possess stx_2-EDL933 and/or stx_2vhb subtypes. *J. Clin. Microbiol.*, **41**, 2716–2722.
3) Jackson, M. P., Neill, R. J., O'Brein, A. D., Holmes, R. K. and Newland, J. W. (1987) Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophage from *Escherichia coli* 933. *FEMS Microbiol. lett.*, **44**, 109–114.
4) Heuvelink, A. E., van den Biggelaar, F. L., de Boer, E., Herbes, R. G., Melchers, W. J., Huis in't Veld, J. H. and Monnens, L. A. (1998) Isolation and characterization of verocytotoxin-producing *Escherichia coli* O157 strains from Dutch cattle and sheep. *J. Clin. Microbiol.*, **36**, 878–882.
5) Center for Disease Control and Prevention (1993) Multistate outbreak of *Escherichia coli* O157:H7 infections from hamburger-western United State, 1992–1993, Morb. Mortal Wkly. Rep. **42**, 258.
6) Cieslak, P. R., Barrett, T. J., Griffin, P. M., Gensheimer, K. F., Beckett, G., Buffington, J. and Smith, M. G. (1993) *Escherichia coli* infection from a manured garden. *Lancet*, **342**, 367.
7) Hilborn, E. D., Mermin, J. H., Mshar, P. A., Hadler, J. L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.-A., Farrar, J. A., Glynn, K. and Slutsker, L. (1999) A multistate outbreak of *Escherichia coli* O157:H7 infection associated with consumption of mesclun lettuce. *Arch. Intern. Med.*, **159**, 1758–1764.
8) Michino, H., Akira, K., Minami, S., Takaya, S., Miyazaki, N., Ono, A. and Yanagawa, H. (1999) Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. *Am. J. Epidemiol.*, **150**, 787–796.
9) Sproston, E. L., Macrae, M., Ogden, I. D., Wilson, M. J. and Strachan, J. C. (2006) Slugs: Potential novel vectors of *Escherichia coli* O157. *Appl. Environ. Microbiol.*, **72**, 144–149.
10) Ackman, D., Marks, S., Mack, P., Caldwell, M., Root, T. and Brikhead, D. (1997) Swimming associated haemorrhagic colitis due to in *Escherichia coli* O157 infection: evidence of prolonged contamination of a fresh water lake. *Epidemiol. Infect.*, **119**, 1–8.

11) Jackson, S., Goodbrand, R. B., Johnson, R. P., Odorico, V. G., Alves, D., K. Rahn, K., Wilson, J. B., Welch, M. K. and Khakhria, R. (1998) *Escherichia coli* O157:H7 diarrhoea associated with well water and infected cattle on an Ontario farm. *Epidemiol. Infect.*, **120**, 17–20.
12) Swerdlow, D. I., Woodruff, B. A., Brady, R. C., Griffin, P. M., Tippen, S., Donnell, H. D., Geldreich, E., Payne, B. J., Meyer, A., Wells, J. G., Greene, K. D., Bright, M., Bean, N. H. and Blake, P. A. (1992) A waterborne outbreak in Missouri of *Escherichia coli* O157:H7 associated with bloody diarrhea and death. *Ann. Intern. Med.*, **117**, 812–819.
13) Byappanahalli, M., Fowler, M., Shively, D. and Whitman, R. (2003) Ubiquity and persistence of *Escherichia coli* in a Midwestern coastal stream. *Appl. Environ. Microbiol.*, **69**, 4549–4555.
14) Wang, G. and Doyle, M. P. (1998) Survival of enterohemorrhagic *Escherichia coli* O157:H7 in water. *J. Food. Prot.*, **61**, 662–667.
15) Whitman, R. L., Shively, D. A., Pawlik, H., Nevers, M. B. and Byappanahalli, M. N. (2003) Occurrence of *Escherichia coli* and Enterococci *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Appl. Environ. Microbiol.*, **69**, 4714–4719.
16) Dumke, R., Schröter-Bobsin, U., Jacobs, E. and Roske, I. (2006) Detection of phages carrying Shiga toxin 1 and Shiga toxin 2 genes in waste water and river samples. *Lett. Appl. Microbiol.*, **42**, 48–53.
17) Garcia-Aljaro, C., Muniesa, M., Jofre, J. and Blanch, A. R. (2004) Prevalence of the stx_2 gene in coliform populations from aquatic environments. *Appl. Environ. Microbiol.*, **70**, 3535–3540.
18) Higgins, J. A., Belt, K. T., Karns, J. S., Russell-Anelli, J. and Shelton, D. R. (2005) *tir*- and *stx*-positive *Escherichia coli* in stream waters in a metropolitan area. *Appl. Environ. Microbiol.*, **71**, 2511–2519.
19) Muniesa, M. and Jofre, J. (1998) Abundance in sewage of bacteriophages that infect *Escherichia coli* O157:H7 and that carry the Shiga toxin 2 gene. *Appl. Environ. Microbiol.*, **64**, 2443–2448.
20) Odgen, I. D., Hepburn, N. F. and MacRae, M. (2001) The optimization of isolation media used in immunomagnetic separation methods for the detection of *Escherichia coli* O157 in food. *J. Appl. Microbiol.*, **91**, 373–379.
21) Reasoner, D. J. and Geldreich, E. F. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.*, **49**, 1–7.
22) De Man, J. C. (1975) The probability of the most probable number. *Eur. J. Appl. Microbiol.*, **1**, 72–77.