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Detection and characterization of Shiga toxin-producing *Escherichia coli* from seagulls

S. MAKINO[1]*, H. KOBORI[1], H. ASAKURA[1], M. WATARAI[1], T. SHIRAHATA[1], T. IKEDA[2], K. TAKESHI[2] AND T. TSUKAMOTO[3]

[1] *Department of Veterinary Microbiology, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*
[2] *Hokkaido Institute of Public Health, Sapporo, Hokkaido 060-0819, Japan*
[3] *Osaka Prefectural Institute of Public Health, Osaka, Osaka 537-0025, Japan*

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SUMMARY

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains isolated from a seagull in Japan were examined. A total of 50 faecal samples was collected on a harbour bank in Hokkaido, Japan, in July 1998. Two different STEC strains, whose serotypes were O136:H16 and O153:H$^-$, were isolated from the same individual by PCR screening; both of them were confirmed by ELISA and Vero cell cytotoxicity assay to be producing active Stx2 and Stx1, respectively. They harboured large plasmids, but did not carry the haemolysin or *eaeA* genes of STEC O157:H7. Based on their plasmid profiles, antibiotic resistance patterns, pulsed-field gel electrophoresis analysis (PFGE), and the *stx* genes sequences, the isolates were different. Phylogenic analysis of the deduced Stx amino acid sequences demonstrated that the Stx toxins of seagull-origin STEC were closely associated with those of the human-origin, but not those of other animal-origin STEC. In addition, Stx2Φ-K7 phage purified from O136 STEC resembled Stx2Φ-II from human-origin O157:H7, and was able to convert non-toxigenic *E. coli* to STEC. These results suggest that birds may be one of the important carriers in terms of the distribution of STEC.

INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are frequently isolated from humans and animals in a variety of serotypes. Although the predominant STEC serotype associated with human infection is O157:H7 [1], non-O157 STEC also cause human disease [2, 3]. However, while several STEC serotypes can cause disease in pigs and calves [4, 5], most are non-pathogenic and are harboured by asymptomatic, healthy animals including a variety of ruminants and non-ruminants [6–14]. Thus, STEC exist as normal resident flora in environments closely associated with humans, and may thereby constitute a risk of human STEC infection.

We have reported the isolation of STEC from sheep and houseflies close to human habitation and also from wild deer [15–17]. In those reports, we reported that the *stx* genes of those STEC isolates were closely related to those of human-origin STEC through the examination of their toxin activities and genetic analyses. These findings indicate that STEC, which produce active toxin for humans, are widely distributed in the environment. However, their precise distribution is unclear. Although we have discussed the possibility that houseflies physically transmit STEC to humans [17], we also regarded birds as

* Author for correspondence.

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potential carriers of STEC because birds have a wider ranging living area than houseflies and have been implicated in the transmission of various pathogenic bacteria, such as *Campylobacter* spp., *Salmonella* spp., etc. [14, 18–22]. Therefore, we tried to isolate STEC from seagulls living in a harbour in Hokkaido.

It is known that lysogenic bacteriophage in STEC encodes the *stx* genes. This toxin gene may transfer to toxin-negative enteric bacteria in the environment and *in vivo* by the phage conversion [23–25]. In order to examine the possibility that this genetic transmission was actually occurring, the Stx-phage particles from seagull-origin STEC were purified and compared with human-origin phage. In this paper, we discuss the distribution of STEC in the environment through the above genetical and biological properties. In addition, we propose the possibility of physical spread of STEC among animals and horizontal transmission of the toxin gene.

MATERIALS AND METHODS

Faecal samples

A total of 50 fresh faecal samples from seagulls was collected on the bank of a harbour in Hokkaido, Japan, in July 1998.

Strains and media

Strains used in this study are listed in Table 1. STEC O157:H7 Obi-1, which was a representative from the mass outbreak that occurred in Obihiro, Japan, 1996 [26], and *E. coli* C600 are used as positive and negative control strains for all experiments, respectively. Stx-converting phage-lysogenized C600 strains used in this study [27] are listed in Table 2. Brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) and Trypticase soy broth (TSB; BBL/Becton Dickinson, Detroit, MI, USA) were used as rich media. DHL agar (Eiken Co. Ltd, Japan) was used as selective medium for STEC.

PCR

PCR was performed essentially as described previously [28]. Oligonucleotide primers for PCR were as follows: *stx* common primers [28], 5′-GAGCGAAATAATTTATATGTG-3′ and 5′-TGATGATGGCAATTCAGTAT-3′; *stx1* specific primers [28], 5′-GCAGTTCGTGGCAAGAGCG-3′ and 5′-GCGTCGCCAGCGCACTTG-3′; *stx2* specific primers [28]; 5′-AATTTATATGTGGCAGGGTTC-3′ and 5′-CTTCACTGTAAATGTGTCATC-3′, *eaeA* specific primers [29], 5′-GTGGCGAATACTGGCGAGACT-3′ and 5′-CCCCATTCTTTTTCACCGTCG-3′; *hlyA* specific primers [30], 5′-GGTGCAGCAGAAAAAGTTGTAG-3′ and 5′-TCTCGCCTGATAGTGTTTGGTA-3′. The sizes of DNA fragments amplified with each primer set were 518, 522, 435, 890 and 1551 base-pair (bp), respectively. To isolate the entire both toxin genes by PCR (5′-CGTATGGTGCTCAAGGAG-3′ and CGATAAGACTCAGTTGC-3′) for *stx1* and (5′-GGAACACCTGTATATGAAGTG-3′ and CTCAGTCATTATTAAACTGCAC-3′) for *stx2* were used. To determine the DNA sequences of the *stx1* gene, common primers, M13 primer, RV primer, *stx1* specific primers, and other primers (5′-GCAATTCTGGGAAGCGTG-3′, 5′-GTCCTGTTAACAAATCC-3′, 5′-GTCACAGTAACAAACCG-3′ and 5′-CAAGTGCGCTGGCGACGC-3′) were used. To determine the DNA sequences of the *stx2* genes, common primers, M13 primer, RV primer, and other primers (5′-GTTATACTGAATTGCCATCATC-3′, 5′-TAACTGCTGTCCGTTGTCATG-3′ and 5′-CAGTTATCTGACATTCTGGTTG-3′) were used.

Isolation of Stx-producing organisms

STEC from the faecal samples were isolated essentially as described previously [15, 16]. Briefly, 1·0 g of fresh faeces was incubated in 10 ml of BHI broth supplemented with vancomycin at a final concentration of 6 μg/ml at 37 °C for 6 h with shaking, then an aliquot of the bacterial culture was inoculated into BHI broth with vancomycin, followed by incubating at 37 °C for 20 h with shaking. Then, 10 μl of the enrichment culture were diluted with 100 μl of sterile distilled water, followed by heating at 95 °C for 15 min and centrifugation at 15000 rpm for 5 min. One μl of the supernatant was used for PCR with *stx* common primers. PCR-positive cultures were plated on BHI agar to produce discrete colonies, 500 of which were examined by colony hybridization using a 518 bp DNA fragment amplified with *stx* common primers labelled with DIG (Boehringer–Mannheim, Germany).

Other techniques

Biochemical characteristics and serotyping of STEC, Vero cell cytotoxicity assays, detection of *E. coli*

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Table 1. *Characteristics of STEC isolates*

Strain	Serotype	PCR				RPLA		Vero cytotoxicity
		Stx1	*Stx2*	*hlyA*	*eaeA*	Stx1	Stx2	
K-7	O136:H16	−	+	−	−	−	+	+
K-10	O153:H⁻	+	−	−	−	+	−	+
C600	nd	−	−	−	−	−	−	−
Obi-1	O157:H7	−	+	+	+	−	+	+

Table 2. *Capacity of phage Stx2Φ-K7 to form plaques on various* E. coli *hosts*

Strains	Phage titre by Stx2Φ-K7
C600	$2{\cdot}4 \times 10^7$
C600 (Stx2Φ-I)	$2{\cdot}2 \times 10^7$
C600 (Stx2Φ-II)	nd
C600 (933W)	$2{\cdot}5 \times 10^7$
C600 (Stx2Φ-K7)	nd*

* nd, Not detectable.

verotoxin by reverse passive latex agglutination (VTEC-RPLA 'Seiken'®; Denka Seiken Co. Ltd, Japan), disk diffusion susceptibility tests using Sensi-Disc® (Becton Dickinson, USA), pulsed-field gel electrophoresis (PFGE), isolation of total DNA and DNA cycle sequencing were performed according to the methods described previously [15, 16]. Nucleotide sequences determined have been submitted to the DDBJ data bank as accession numbers AB030484 and AB030485. Isolation of phage and its genetic study were performed by the methods described by Watarai and colleagues [27].

RESULTS

Isolation of STEC and their toxin types

From a total of 50 fresh faecal samples of seagulls, two kinds of PCR-positive colonies were obtained from a single sample by screening with the *stx* common primers. Both were identified as *E. coli* serotypes O136:H16 and O153:H⁻ and were designated K-7 and K-10, respectively (Table 1). The toxin types and the ability to produce active toxin of those both STEC isolates were examined by PCR, Vero cell assay and RPLA and showed that K-7 produced Stx2 while K-10 produced Stx1 (Table 1). Although the detection of other virulence genes reported in O157:H7 was examined by PCR, neither STEC isolate possessed *hlyA* or *eaeA* genes of STEC O157:H7 (Table 1).

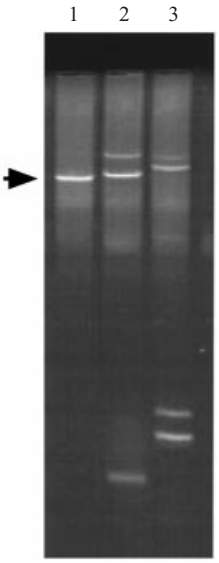

Fig. 1. Plasmid profiles of STEC isolates from seagulls. Lanes: 1, Obi-1; 2, K-7; 3, K-10. The 90 kb large plasmid of STEC O157:H7 [29] is arrowed.

Genetic relatedness among STEC isolates

In order to compare the phenotypes of both STEC isolates, antibiotic resistance patterns were examined by disk diffusion susceptibility tests with 14 antibiotics: fosfomycin, tetracycline, minocycline, streptomycin, chloramphenicol, kanamycin, nalidixic acid, norfloxacin, cefdinir, sulphonamide, spectinomycin, streptomycin, rifampicin and trimethoprim. K-7 was resistant to spectinomycin, sulphonamide, ampicillin and penicillin G, while K-10 was resistant to spectinomycin, sulphonamide, ampicillin, penicillin G and tetracycline. Next, an examination of their plasmid profiles revealed that both isolates carried large plasmids but that those large plasmids were not identical to the 90 kb virulence plasmid [31] associated with pathogenicity in O157:H7 (Fig. 1) because PCR using *hlyA* specific primers was negative (Table 1).

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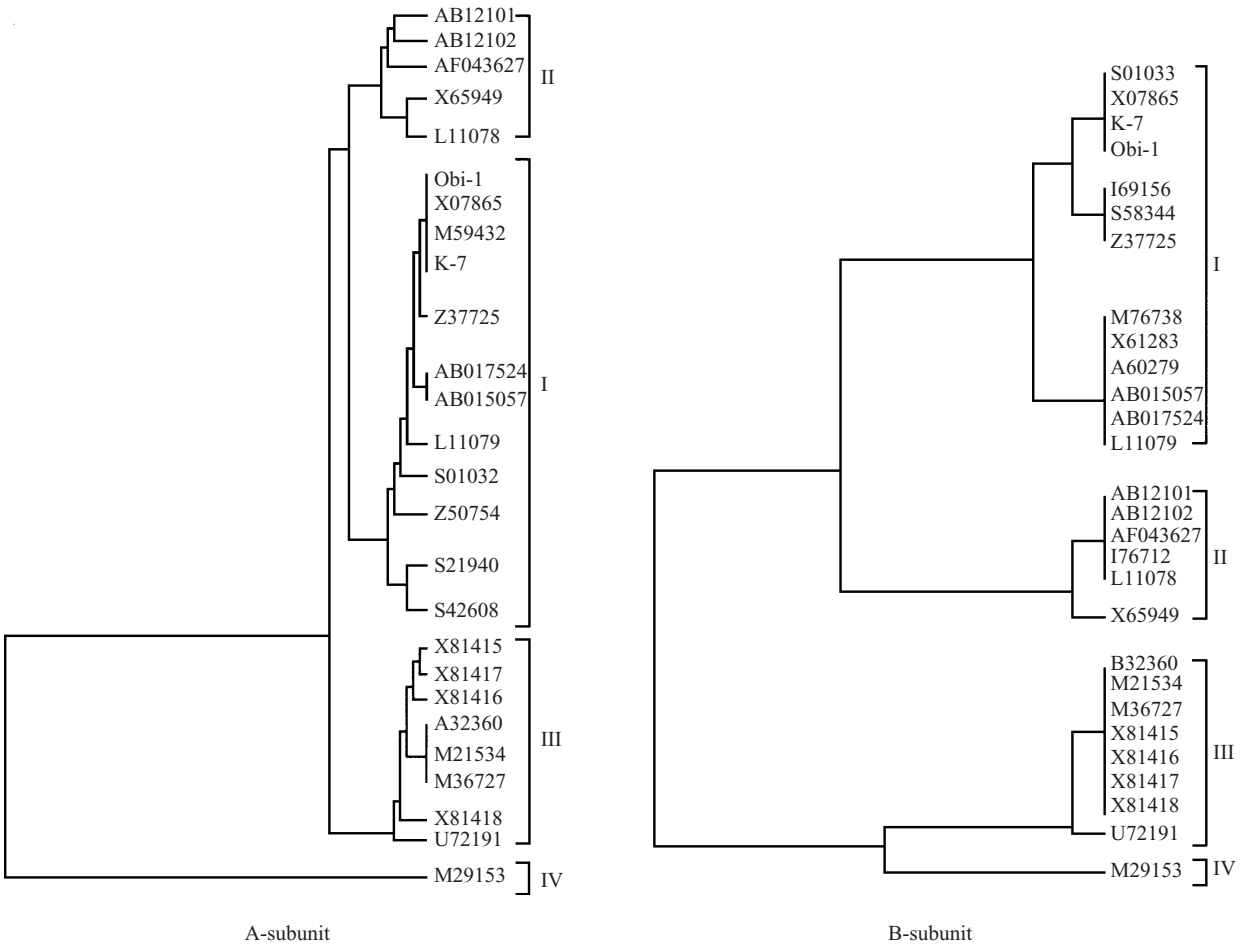

Fig. 2. Phylogenic trees of amino acid sequence of Stx2. Accession numbers in SWISS-PROT and PIR databases are indicated.

Although both STEC were derived from the same individual, they were different from each other by the above criteria and also by PFGE (data not shown), showing that a single seagull was carrying the different types of STEC.

The DNA sequences of the toxin genes in K-7 and K-10 were determined and both amino acid sequences were compared with other toxin genes by using evolutional tree analysis with the UPGMA method by Genetyx Ver. 10.1 (Software Development Co. Ltd, Japan). Analysis of phylogenic trees of each of the A- and B-subunits in the *stx2* genes revealed four clusters (Fig. 2), while that of the *stx1* genes revealed two clusters (Fig. 3).

Genetic properties of Stx2-encoding phage

Since the bacteriophage-borne *stx* genes in STEC might transmit horizontally to toxin-non-producing *E. coli* by phage-conversion to yield STEC, Stx-phage particles from seagull-origin STEC were purified and compared with human-origin phage to examine the possibility that this genetic transmission was actually occurring. First an *E. coli* lysogenized strain C600 (Stx2Φ-K7) was isolated from K-7. To assess the properties of Stx2Φ-K7, its lysogenic immunity against human-origin Stx2-phages [27] was examined. Stx2Φ-K7 showed no immunity to 933W and Stx2Φ-I, which were shown to resemble each other [27], but showed immunity to Stx2Φ-II (Table 2), which had properties distinct from 933W and Stx2Φ-I [27], suggesting that Stx2Φ-K7 resembled Stx2Φ-II. Next, phage DNAs purified from those four phage particles was digested with *Bam*HI and *Xho*I to compare their restriction patterns and also hybridized with the 518 bp DIG-labelled *stx*-gene probe. As shown in Figure 4A, digestion patterns of Stx2Φ-I, Stx2Φ-II and 933W DNAs were similar, but slightly different from Stx2Φ-K7. The full length of Stx2Φ-K7 was about 7 kilobase-pairs (kb) shorter than Stx2Φ-I [27].

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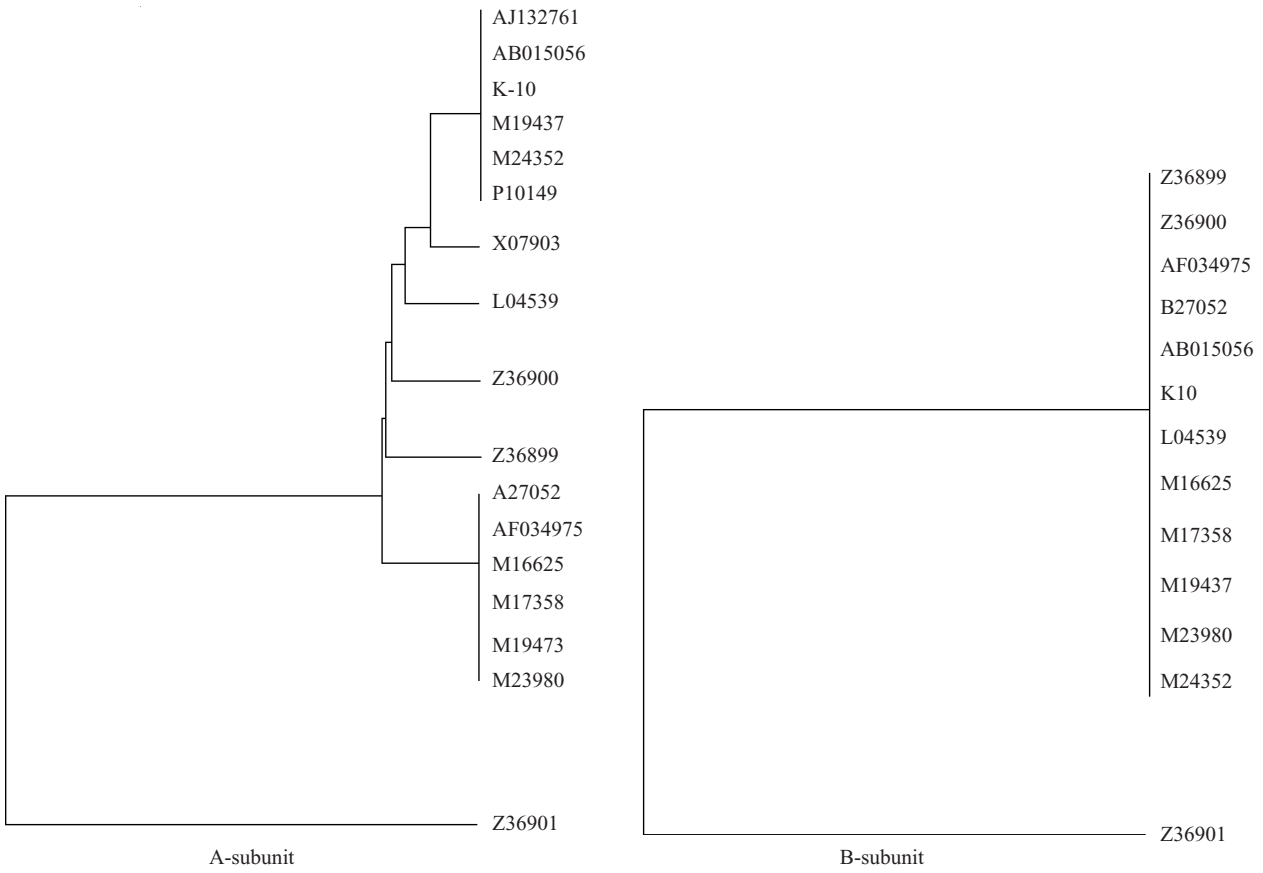

Fig. 3. Phylogenic trees of amino acid sequence of Stx1. Accession numbers in SWISS-PROT and PIR databases are indicated.

Moreover, the sizes of *Bam*HI-*Xho*I fragments containing the *stx* genes in Stx2Φ-K7 and Stx2Φ-II appeared to be identical (Fig. 4B, lanes 1 and 2).

DISCUSSION

STEC has been widely isolated from domestic and wild animals, pets and houseflies [6–17, 29], and also from gulls in England [14]. We showed here that STEC was also isolated from seagulls in Japan. Although the frequencies appear to be low, the potential for birds such as seagulls to transmit STEC to cattle and other animals may be considerable. We collected faecal samples for this study in a harbour in which there were tens of thousand of seagulls. Many of the seagulls appeared to roost in nearby farmland, thereby creating a potential to contaminate water through their faeces. Although it is not clear that seagull-origin STEC have pathogenicity for humans, the Stx1 and Stx2 toxins produced by them would probably be toxic to humans because they had the Vero cell cytotoxicity similar to human-origin STEC (Table 1). These results suggested that both seagull-origin STEC isolates might potentially be pathogenic for humans. However, both were belonging to serotypes O136 and O153, which had been isolated from sheep and cattle [6, 7, 9, 10, 15], suggesting that such serotypes might be spread among animals. Simultaneously, even if seagull-origin STEC could produce the active toxin and be transmitted to humans, their pathogenicity for humans may well be reduced because they did not carry virulence genes such as *hlyA* and *eaeA*. Infact, O136 and O153 STEC from animals were *eae*- and *hly*-negative, respectively [6, 9, 10].

Analysis of phylogenic trees of the A- and B-subunits of the *stx2* genes revealed four clusters (Fig. 2). In the A-subunit essential for the toxin activity, groups 1 and 2 were derived from one branch, suggesting that group 1 is more closely related to group 2 than groups 3 and 4. In the B-subunit, four groups, whose members were the same as those in the A-subunit, were also observed (Fig. 2). STEC isolates in groups 1 and 2 contained deer-origin STEC (accession nos. AB12101 and AB12102) and fly-origin STEC (accession no. AB015057) [16, 17], but other

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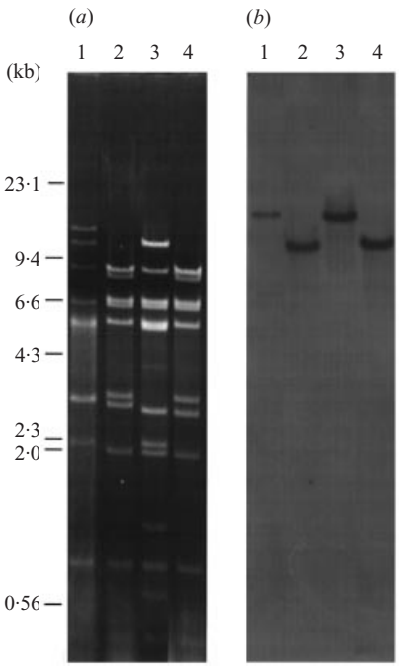

Fig. 4. *Bam*HI and *Xho*I restriction patterns of Stx2-phage DNA. Lanes: 1, Stx2Φ-K7 DNA; 2, Stx2Φ-I DNA; 3, Stx2Φ-II DNA, 4, 933W DNA. The positions of *Hin*dIII-digested λDNA size markers are shown at the left. (*a*) Digestion patterns; (*b*) Southern hybridization patterns using a 518 bp *stx* gene probe.

STEC in both groups consisted mainly of human related STEC. Group 3 consisted of pig- and rabbit-origin STEC and formed a different branch from groups 1 and 2. An STEC isolate in group IV originated from a human infant with diarrhoea [33] but was most closely related to pig-origin STEC based on its B-subunit (Fig. 2). The *stx2* gene in K-7 was a member of group 1, suggesting that it was closely related to human-origin toxin genes rather than pig- and rabbit-origin toxin genes. In addition, amino acid sequence of the *stx2* gene of K-7 was 100% identical to the *stx2* gene of Obi-1, which was O157:H7 from the outbreak in Obihiro, Hokkaido, Japan in 1996 [26]. The amino acid sequence of the *stx1* gene of K-10 was more than 99·7% identical to other *stx1* genes but 97·5% identical to an STEC strain of accession number Z36901 regarding the A-subunit (Fig. 3). Regarding the B-subunit, it was 100% identical to other *stx1* genes but 96·6% identical to Z36901 (Fig. 3). Our results are consisted with reports that the amino acid sequences of *stx1* genes of STEC are well conserved [32, 34]. The *stx1* gene of K-10 was almost identical to human-origin toxin genes.

Although STEC strains are widely distributed in the environment, the precise distribution is unclear. It is possibly that the *stx* genes of STEC have spread by phage conversion among many kinds of bacterial species *in vitro* or in sewage [23, 24]. We demonstrated in this study that the *stx2* gene of a seagull-origin STEC, K-7, transferred to Stx-non-producing *E. coli* C600 by phage-conversion, and that Stx2Φ-K7 resembled Stx2Φ-II encoding human-origin *stx* gene [27] and also produced active Stx toxin. In this sense, Stx2Φ-K7 seemed to have the capacity to change non-toxigenic enteric *E. coli* to STEC. Such phage conversion may be a major mechanism for the widespread environmental dissemination of STEC. Simultaneously, it may be useful to know if the seagull have non-toxigenic *E. coli* serotype O136 and O153 because it is interesting to know whether the Stx2Φ-K7 phage is able to convert them to STEC in their body. But we could not isolate such non-toxic *E. coli*. Also, it may be interesting to examine whether the phage is able to convert non-toxigenic *E. coli* O157 to STEC pathogenic to humans. However, since the *Bam*HI–*Xho*I digestion pattern of Stx2Φ-K7 DNA and its full length were slightly different from human-origin Stx-phages (Fig. 4), the structure of Stx2Φ-K7 might be different from human-origin phages. Further studies are necessary to analyse the genetic structure of Stx2Φ-K7. In this context, we must wait the DNA sequences of the whole genomes of Stx2Φ-I and Stx2Φ-II, which are currently being undertaken (Yamazaki, personal communications). These data may well reveal phage-specific sequences and allow comparison with phage genomes from various origins.

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