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Abstract. *Escherichia coli* K-12 lysogens of three different Shiga toxin 2 (Stx2)-encoding bacteriophages were examined for variability in their pulsed-field gel electrophoresis (PFGE) fragment patterns. The PFGE fragment patterns could be classified into 3 types (i.e. PFGE types B, C, and D).   For the PFGE type D, a 255 kbp fragment present in the original K-12 strain was apparently shifted by the size of Stx 2-encoding phage genomic DNA   (ca. 65 kbp) to the position at 320 kbp. In contrast, the types B and C showed the above fragment shift plus a further 6 and 10 fragment differences, respectively, from the original K-12 strain. The evidence suggests that even a single genetic event like lysogeny can cause marked genotypic modification of the host strain.

In order to determine relatedness among bacterial isolates involved in an outbreak of infectious disease, the isolates should be typed strain level after identification at species level. With recent progress in molecular biology, several newer molecular methods have been developed for the strain typing. Among them, pulsed-field gel electrophoresis (PFGE) [1] is considered to be an excellent approach, and is employed in many epidemiological investigations of food or water-borne outbreaks and nosocomial infections caused by bacterial strains such as methicillin-resistant *Staphylococcus aureus* [9, 19] and enterohemorrhagic *Escherichia coli* (EHEC) [2, 6]. A minor variation in PFGE fragment patterns in clonally related bacterial strains has been reported elsewhere. For example, On [12] demonstrated that multiple subculturing in vitro resulted in appreciable changes in PFGE profiles of *Camplylobacter coli* strains due to spontaneous genomic rearrangements. Lysogeny of bacteriophages also caused minor variations in PFGE fragment patterns in staphylococci [8, 15].

EHEC O157:H7 is an emerging pathogen that causes hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura [5, 7]. Shiga toxin (Stx) produced by EHEC O157:H7 is considered to be a major virulence factor causing these clinical symptoms [3]. Stx is presently classified into two broad types, Stx1 and Stx2 [3], and the structural genes encoding these toxins are carried on bacteriophages, and are apparently

transferred to the chromosomal DNA of host *E. coli* by lysogeny [11, 16]. Recently, Murase *et al.* [10] reported that pulsed-field gel electrophoresis (PFGE) fragment patterns of EHEC O157:H7 isolates were changed by loss of single fragments during maintenance or subculturing, which was possibly due to curing of Stx-encoding bacteriophages. However, it remains unclear to what extent lysogeny of the phages affects genomic arrangements of the host strain, and thus potentially affects its PFGE fragment pattern. We here describe variation in PFGE profiles of experimentally created *E. coli* K-12 lysogens of Stx2-encoding bacteriophages, which had been derived from EHEC O157:H7 isolates.

Materials and Methods

An *E. coli* K-12 derivative, LE392 [14], was used as a host strain for Stx 2-encoding bacteriophages. Three strains of EHEC O157:H7, from which the bacteriophages had been derived, and a strain of EHEC O157:H7, EDL933, were used as reference strains in the present study.. The strains were maintained on heart infusion agar (HIA; Difico) until use. Three Stx 2-encoding bacteriophages, ϕCDC-8, ϕ19V2, and ϕYO-12, which had been isolated previously and described elsewhere [13], were used for the production of lysogens. The bacteriophages were propagated on another *E. coli* K-12 derivative, MC1061, and the resulting phage lysates were maintained in T-broth which consisted of 10 g tryptone (Oxoid), 8 g NaCl, 10 mM $MgCl_2$ and 10 mM

CaCl$_2$ per litter until use.

Twenty-microliters of appropriately diluted lysate of each phage was spotted on T-agar plate (T-broth with 1.2% agar) covered with 100 μl of an overnight culture of LE392. The plates were incubated for 18 h at 37 $^{\circ}$C, and bacteria that had grown within the lysis zones were further subcultured to single colonies on a T-agar plate. Subsequently, 14 single colonies on the plate were randomly selected, and were examined for Stx 2-specific DNA sequences by the polymerase chain reaction (PCR) method with a set of primers, 5'-ATCCTATTCCCGGGAGTTTACG-3' and 5'-GCGTCATCGTATACACAGGAGC-3', as described elsewhere [4] in order to obtain the lysogens.   Meanwhile, we spotted 20 μl of T-broth were spotted onto another T-agar plate covered with an overnight culture of LE392, and incubated for 18 h at 37 $^{\circ}$C. After incubation, bacteria that had grown within the spotted zone were further subcultured to single colonies. Among them, 14 well-isolated colonies were randomly selected, and these isolates used as non-lysogenized LE392 strains for subsequent genotypic comparisons with the lysogens.

PFGE was performed as described elsewhere [6], with minor modifications. Briefly, bacterial cells on HIA were directly embedded in low-melting-temperature agarose (FMC BioProducts). After appropriate preparations for restriction endonuclease digestion were made, the DNAs in each plug were digested with 30 U of *Xba* I (Takara) at 37°C for 4 h. PFGE was performed with a 1% agarose gel by using a CHEF DRII apparatus (Bio-Rad) in

0.5 x TBE (Tris-borate-EDTA) buffer at 12°C at 200V. For separation of a whole genome, a linearly ramped switching time from 4 to 8 s was applied for 12 h and then a linearly ramped switching time from 8 to 50 s was applied for 10 h. For separation of fragments of 200 to 400 kbp, 8- to 50-s switching time was applied for 30 h. After PFGE, the gels were stained with ethidium bromide (0.2 μg/ml) and were photographed under UV transillumination. Southern blots of *Xba* I-digested total bacterial DNA were hybridized with an alkaline phosphatase labeled Stx 2-specific gene probe.   The probe was prepared from the amplicon (584 bp) of Stx 2-targeted PCR [16] performed on EDL933, and labeled with AlkPhos Direct Labelling Module (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Reactive DNA fragments were detected using a commercial color development kit (NBT/BCIP) (Kirkegaard and Perry). Furthermore, genomic DNAs of the bacteriophages were extracted and purified following the method described elsewhere [16] and the DNA preparations treated with *Xba* I or untreated were subjected to PFGE analysis in order to determine the size of phage DNAs and to confirm absence of any *Xba* I restriction sites in the DNAs.

Results and Discussion

A total of 19 lysogens were obtained in the present study.   These included 7 strains lysogenized with φCDC-8, 7 strains with φ19V2, and 5 strains with φ YO-12. PFGE analysis on *Xba*I digested DNAs of 14 randomly selected

original LE392 colonies indicated that the fragment patterns (designated as type A) were identical in all subjects except one, LE-392-4, which apparently lost a 55 kbp-fragment but acquired a new 43 kbp-fragment (designated as type A') compared with the type A (Fig. 1a). PFGE fragment patterns under 194 kbp of the lysogens with the type A could be classified into 3 types; one without a 55 kbp-fragment (designated as type B), one without 55 kbp- and 138kbp-fragments but with a new 150 kbp-fragment (designated as type C), and one identical to type A (designated as type D)(Fig. 1a).   A comparison of the fragment patterns above 194kbp showed: type A' was identical to the type A; the type B lost 288 kbp-, 255 kbp-, and 210 kbp fragments but gained 320 kbp-, 307kbp-, and 195 kbp-fragments; type C lost 35 0kbp-, 288 kbp-, and 255 kbp-, and 210 kbp-fragments but gained 330 kbp-, 320 kbp-, 307 kbp-, and 195 kbp-fragments; type D lost a 255kbp-fragment but gained a 320 kbp-fragment (Fig. 1c).

Occurrence of the above PFGE types in the lysogens and the original LE392 strains are summarized in Table 1. It has been generally accepted that single genetic events like lysogenization by a bacteriophage can cause a minor variation in DNA restriction patterns of a host strain. This has been well documented in coagulase-negative styphylococci [8], in which one particular fragment found in the host strain was shifted upwardly by the size of total genomic DNA of a bacteriophage after lysogeny. Recently, a similar fragment shift in PFGE has been reported for *Shigella sonnei* and *E. coli* K12 lysogenized

with an Stx-encoding bacteriophage [17]. In the present study, such a readily accountable fragment shift was observed only in the PFGE type D, in which a 255 kbp-fragment was shifted by the size of Stx 2-encoding bacterophages' DNA (ca. 65 kbp) to the position at 320 kbp.

By contrast, the lysogens of the PFGE types B and C presented not only the above fragment shift but also other marked changes affected upon lysogeny. As for type B, 4 fragments that had been seen in the original host strain disappeared, and 3 fragments that had not been seen previously in the host strain were observed, which amounted to a total of 7 fragment changes from the PFGE pattern of the original host strain. As for type C, 6 fragments that had been seen in the original host strain disappeared, and 5 fragments that had not been seen in the host strain were observed, which amounted to a total of 11 fragment changes from the original PFGE pattern. The subsequent Southern blot analysis revealed that the Stx 2-targeted probe reacted with the 320 kbp-fragment of *Xba* I digest, which were present in all lysogens but absent in the original LE392 strains including the one showing the type A' (Fig. 1b). PFGE analysis of whole genomic phage DNAs indicated that all DNAs were sized approximately 65 kbp, and another analysis on the *Xba* I-treated preparations showed that the DNAs did not have any restriction site for the endonclease (data not shown).   The evidence suggests that the observed changes in PFGE patterns, except for the fragment shift from 255 kbp to 320 kbp, was not caused by the mechanical insertion of phage DNA. Furthermore, these changes could

not be explained by spontaneous rearrangements of the host genomic DNA since the occurrence of rearrangement (as seen in the PFGE type A') was found to be low.

According to the criteria for interpreting PFGE patterns as proposed by Tenover *et al.* [18], two strains in question are genetically unrelated if the number of fragment differences between them exceeds 7, corresponding to three or more implied independent genetic events. However, the criteria were not applicable to the present study since those of the PFGE type C showed more than 7 fragment differences from the genetically related host strain. The evidence thus points to a rather labile nature of the host genome, in which lysogeny might trigger multiple genetic alterations, leading to creation or loss of *Xba* I restriction sites in the host's genome.

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LEGENDS FOR FIGURE

Fig. 1. Representative PFGE patterns in *E. coli* LE392 and its lysogens of Stx 2-encoding bacteriophages and Southern blot analysis results. (a) PFGE showing variations in *Xba* I-digests under 200 kbp. (b) Southern blot analysis with the Stx 2-targeted probe on the corresponding gel. (c) PFGE showing variations in *Xba* I-digests above 200 kbp. (c) Southern blot analysis with the Stx 2-targeted probe on the corresponding gel. M, molecular size markers (Lambda Ladder PFG Marker) (New England BioLabs, Beverly, Ma.)

Table 1. Frequencies of occurrence of different PFGE types in *E. coli* LE392 and its lysogens of Stx 2-encoding bacteriophages

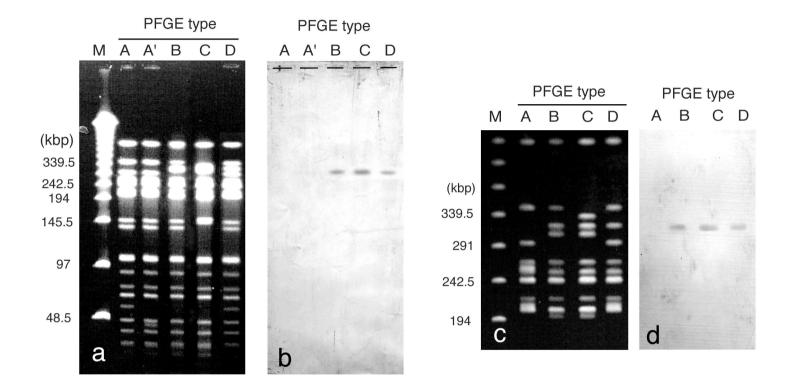| PFGE type	No. of PFGE type			
	origninal LE392 strain	Lysogens of:		
		φCDC-8	φ19V2	φYO-12
A	13/14	0/7	0/7	0/5
A'	1/14	0/7	0/7	0/5
B	0/14	4/7	3/7	0/5
C	0/14	2/7	1/7	0/5
D	0/14	1/7	3/7	5/5

PFGE type

M   A   A'   B   C   D

PFGE type

A   A'   B   C   D

(kbp)

339.5
242.5
194

145.5

97

48.5

a

b

PFGE type

M   A   B   C   D

PFGE type

A   B   C   D

(kbp)

339.5

291

242.5

194

c

d

FIG.1