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Molecular epidemiological study on tetracycline resistance R plasmids in enterohaemorrhagic *Escherichia coli* O157:H7

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SUMMARY

Restriction patterns obtained with *Eco*RI and Southern hybridization were used for the differentiation of tetracycline-resistant (Tet^r) R plasmids in enterobaemorrhagic *Escherichia coli* (EHEC) O157:H7 isolates from a mass outbreak at a kindergarten in Obihiro-City, Hokkaido, Japan, 1996. Two kinds of Tet^r R plasmids of 50 and 95 kb were detected. The 50-kb plasmids were identical to each other, while the 93-kb plasmids were of three types that were very similar to each other. The *tet* genes of both 50- and 95-kb R plasmids were 100% identical to the *tet* gene of pSC101 and all plasmids hybridized to a probe for *tet*. Because food-origin O157 strains were sensitive to tetracycline, we concluded that such Tet^r R-plasmids might transfer to drug-sensitive O157 strains in the infected individuals.

INTRODUCTION

A mass outbreak of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 infection occurred at a kindergarten in Obihiro, Hokkaido, Japan, in 1996. In previous reports [1, 2], we concluded that the causative food was potato salad contaminated with EHEC O157:H7, because O157 isolates from foods and patients were genetically and biochemically identical to each other. In that outbreak, we also observed patients who continued to excrete O157 for several months after the occurrence (so-called continuous-infection), and person-to-person infection in a family (so-called family-infection). We also reported the isolation on Sorbitol MacConkey agar plates without any antibiotics 142 O157 strains originating from patients (patient-origin O157). Examination of drug resistance patterns using 12 antibiotics (fosfomycin, tetracycline, minocycline, streptomycin, ampicillin, penicillin G, chloramphenicol, kanamycin, nalidixic acid, norfloxacin, cefdinir, and trimethoprim) showed that 21 isolates (14·8%) were resistant only to tetracycline (Tet^r) and 15 isolates (10·6%) were resistant to both tetracycline (Tet^r) and streptomycin (Str^r). The plasmid profiles detected in that study were divided into four types, A to D, which carried 93 and 38-kb plasmids, and B, C and D types which had additional 82-, 50- and 45-kb plasmids, respectively (Fig. 1) [2]. The Tet^r isolates were of type A or C. Moreover, since all O157 isolates originating from potato salad as the causative food (food-origin O157) were sensitive to the above mentioned 12 antibiotics and their plasmid profiles were type A, we also speculated that the acquisition of drug-resistance and plasmids by those patient-origin O157 isolates might occur *in vivo* during infection.

In this study, we sought to clarify the relationship between the variations of the plasmid profiles and the emergence of Tet^r phenotypes in that mass infection, we studied the genetical similarities of the *tet* gene and plasmid.

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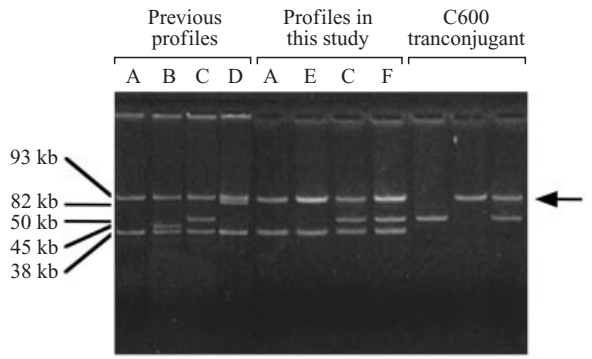

Fig. 1. Representative plasmid profiles in *E. coli* O157 isolates and Tet^r *E. coli* C600 transconjugants. The arrow shows a plasmid at the same position as pO157 [6].

MATERIALS AND METHODS

Strains and media

Table 1 lists 36 Tet^r *E. coli* O157:H7 isolates used in this study, which were isolated from the mass outbreak in Obihiro City, Hokkaido, Japan, In 1996 [1]. *E. coli* C600 rifampicin resistant (Rif^r)-derivative and MC1061 were used for the host strains to detect R plasmids and to isolate the *tet* gene, respectively. An L-broth was used to cultivate the *E. coli*, and agar plates contained 1·5% (w/v) agar. Tetracycline, streptomycin and rifampicin were used at final concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml, respectively.

Other techniques

Total *E. coli* DNA was purified as previously described [3]. Large plasmid DNA was isolated and agarose gel electrophoresis was performed as previously described [4]. The R plasmids were detected by filter mating at 37 °C using *E. coli* C600 Rif^r as the recipient strain and Tet^r O157 isolates as the donor strains as previously described [5]. DNA sequencing was performed using an ABI PRISM 377 (Applied Biosystems). The nucleotide sequence determined was assigned the accession number AB023657 by the DNA Data Bank of Japan (DDBJ). Southern hybridization was performed using the DIG system (Boehringer–Mannheim, Japan).

RESULTS

Plasmid profiles and drug-resistance patterns in O157:H7

As described in the Introduction, plasmid profiles in a total of 142 isolates from a mass outbreak in Obihiro were divided into four types (types A to D) (Fig. 1) [2]. However, when the densities of the DNA bands detected in all isolates were carefully compared with each other, the DNA fragment present only in 16 Tet^r patient-origin O157 (Fig. 1, arrow) showed a stronger density than in other isolates, indicating that those 16 isolates constitute two additional groups, types E and F (Fig. 1, Table 1). In both types, the DNA band (arrowed) appeared to contain at least two co-migrating plasmids, one of which is the 93-kb virulence plasmid pO157 detected commonly in EHEC O157 [6, 7]. Moreover, 15 of the 16 isolates were both Tet^r and Str^r (Table 1). The other 20 Tet^r isolates were type C plasmid profile (Table 1, Fig. 1).

Isolation of R plasmids

To examine if Tet resistance was associated with R plasmids, conjugation tests were performed at 37 °C by filter mating using Tet^r O157 isolates as the donor strains and *E. coli* C600 Rif^r as the recipient strain. Tet^r transconjugants were isolated from all 36 Tet^r O157 strains (Table 1) at frequencies of 10^{-1}/recipient. When the plasmid profiles of all 36 *E. coli* transconjugants were examined, three patterns were detected, corresponding to strains which had a 93 kb-plasmid, a 50-kb plasmid or both plasmids (Fig. 1). The 50-kb plasmid was found in plasmid profile types C and F, and the 93-kb plasmid was at the identical position to the plasmid indicated by the arrow (Fig. 1). When a pO157-specific polymerase chain reaction [7] was performed using all C600 Tet^r transconjugants, DNA amplification occurred only in EHEC O157, but not in these transconjugants (data not shown). Thus two different plasmids with the same size, pO157 and the 93-kb plasmid, coexist in O157 isolates of types E and F. In addition, all Tet^r transconjugants originating from Str^r O157 isolates were also Str^r and had a single plasmid except strain number 1141, indicating that both resistance markers were encoded on the 93-kb plasmid.

Isolation of the *tet* gene and similarities of R plasmids

Total DNA from the O157:H7 strain number 871 was digested with *Eco*RI, followed by cloning into pBluescript, transformation of *E. coli* MC1061 and selection of Tet^r transformants. One Tet^r recombinant plasmid was selected, and various deletion derivatives were constructed, resulting in the identification of a

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Table 1. *Tet^r* E. coli *O157:H7 strains and their* E. coli *C600 transconjugants*

	O157:H7				C600 transconjugants	
Strain number	Plasmid profile	Drug resistance	Continuous infection†	Family infection†	Plasmid	Drug resistance
866	C	Tet	942		pTR50	Tet
884	C	Tet	–		pTR50	Tet
1011	C	Tet	–		pTR50	Tet
1026	C	Tet	–		pTR50	Tet
1068	C	Tet	–		pTR50	Tet
1128	C	Tet	–		pTR50	Tet
1181	C	Tet	–	939*	pTR50	Tet
1218	C	Tet	–		pTR50	Tet
1318	C	Tet	–		pTR50	Tet
1347*	C	Tet	3382	2732	pTR50	Tet
1456	C	Tet	–		pTR50	Tet
1477	C	Tet	1814		pTR50	Tet
1517	C	Tet	–		pTR50	Tet
1605	C	Tet	–		pTR50	Tet
1635	C	Tet	–		pTR50	Tet
1715	C	Tet	–		pTR50	Tet
1735	C	Tet	–	1247*	pTR50	Tet
1813*	C	Tet	871	2439	pTR50	Tet
1814	C	Tet	1477		pTR50	Tet
2631	C	Tet	1607, 2531		pTR50	Tet
871*	E	TetStr	1813	2439	pTR93-1	TetStr
872	E	TetStr	–		pTR93-1	TetStr
874	F	Tet	1268		pTR93-3, pTR50	Tet
934	F	TetStr	–		pTR93-2	TetStr
935	F	TetStr	1108		pTR93-2	TetStr
1050*	E	TetStr	–	1030	pTR93-1	TetStr
1055	E	TetStr	–		pTR93-1	TetStr
1141	F	TetStr	2593		pTR93-2, pTR50	TetStr
1188	F	TetStr	–		pTR93-2	TetStr
1247*	F	TetStr	–	1735	pTR93-1	TetStr
1302	F	TetStr	–		pTR93-1	TetStr
1693*	E	TetStr	–	1864	pTR93-1	TetStr
1703	F	TetStr	–		pTR93-1	TetStr
1706	E	TetStr	–		pTR93-1	TetStr
1707	E	TetStr	–		pTR93-1	TetStr
1710	E	TetStr	–		pTR93-1	TetStr

* Showing O157 isolates from the patients of the primary infection.
† Underlined strains are Tet^r strains.

minimum region for Tet^r on a 1·7 kb *Eco*RI–*Pst*I DNA fragment, whose nucleotide sequence was determined. One open reading frame (ORF) consisting of 1191 bp, which was 100% identical to the *tet* structural gene of pSC101 [7] (data not shown), was detected. DNA sequences of the *tet* genes isolated from four other O157 isolates, numbers 866, 2631, 874, and 1141 were also determined to be identical to number 871 (data not shown).

To examine the similarities of R plasmids in C600 transconjugants, plasmid DNAs were purified and their *Eco*RI restriction patterns were compared. Southern hybridization was also performed using a 277 bp *Bam*HI–*Sal*I internal fragment within the cloned Tet^r structural gene as a probe. Five restriction patterns were detected (Fig. 2). The 50-kb plasmids were identical to each other by both restriction and hybridization analysis (Fig. 3, Table 1) and this plasmid was designated pTR50. The 93-kb plasmids all hybridized with the *tet* probe but comprised 3 kinds of plasmids based on digestion patterns and resistance profiles (Fig. 2, Fig. 3*c*, Table 1), in which there was

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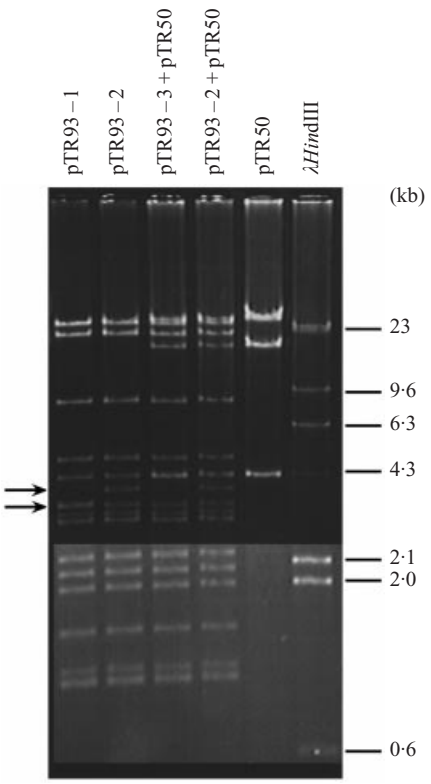

Fig. 2. Representative *Eco*RI restriction patterns in *E. coli* C600 transconjugants. The arrows show different DNA fragments in the 93-kb plasmids. λ *Hin*dIII, λ DNA digested with *Hin*dIII as molecular size markers. To show the restriction patterns clearly, the bottom and top sections of the gel were subjected to different exposures.

some difference indicated by arrows in Fig. 2, especially indicated by a lower arrow in lanes 1 and 3 (Fig. 2), and resistance profiles (Table 1). Thus we designated the three kinds of 93-kb plasmids as pTR93-1, pTR93-2 and pTR93-3, respectively (Table 1). The transconjugants of O157 numbers 874 and 1141 contained pTR50, and additionally pTR93-3 and pTR93-2, respectively (Fig. 2, Table 1).

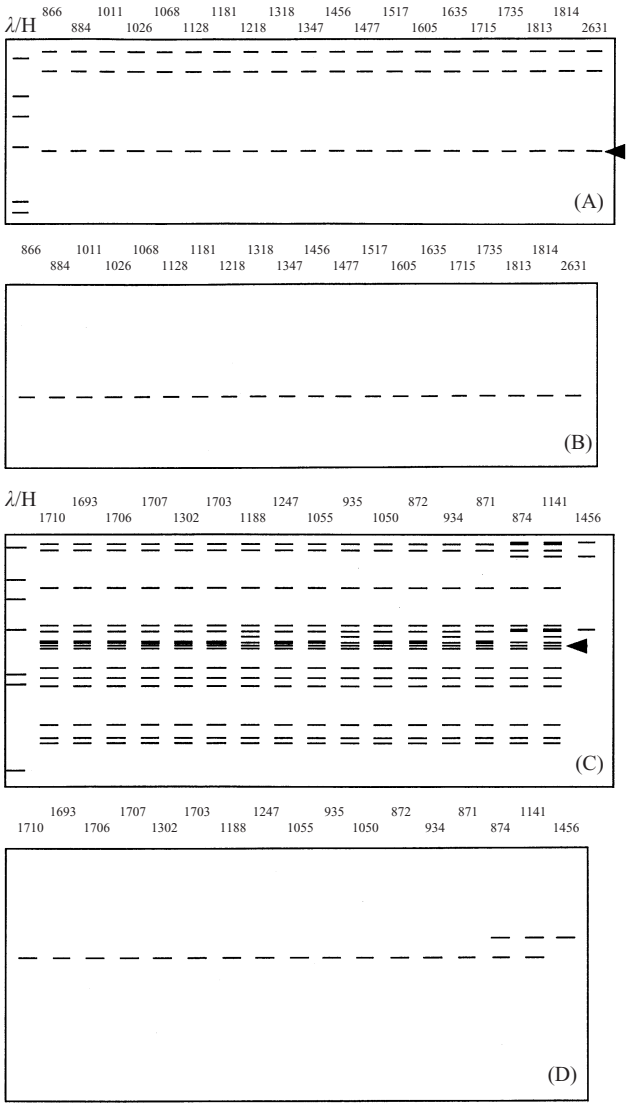

Fig. 3. *Eco*RI restriction profiles in all R plasmids and their Southern hybridization using a *tet* gene probe. Numbers shown at the top of the figures are listed in Table 1. Arrowheads in panels A and C show hybridizing DNA fragments (B and D). λ/H, λ DNA digested with *Hin*dIII as molecular size marker.

DISCUSSION

In this study, all 36 Tet^r O157:H7 isolates from a mass outbreak in Obihiro were found to have Tet^r R plasmids: 20 Tet^r isolates having pTR50, 11 Tet^r Str^r isolates having pTR93-1, 3 Tet^r Str^r isolates having pTR93-2, 1 Tet^r Str^r isolate having pTR50 and pTR93-2, and 1 Tet^r isolate having pTR50 and pTR93-3. In addition, Str^r in Str^r O157 isolates was also encoded on one of R plasmids. However, drug-resistant O157 isolates were not detected from the causative food in this mass outbreak, and the plasmid profiles detected in all food-origin O157 isolates were only type A but not types B, C and D [2], that is, the 93- and 38-kb plasmids in plasmid profile type A were detected in all patient-origin O157 (Fig. 1). Therefore, we speculate that the R plasmid might have been acquired *in vivo* by drug-sensitive food-origin O157 strains, which resulted in the appearance of patients with Tet^r O157. If so, the R plasmid appeared to have transferred to O157 at a very high frequency, because Tet^r O157 was detected from 4 (26·6%) of 128 O157 positive patients in the mass outbreak [2]. Since the transfer frequency of all R plasmids from O157 isolates to *E. coli* C600 was about

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10^{-1}/recipient, the transfer of R plasmid to O157 *in vivo* might have occurred at a similar high frequency. In this outbreak, fosfomycin (Fom) was used for treatment, but neither tetracycline nor streptomycin were used [1]. Therefore, we do not believe that Tet^r and Tet^r Str^r O157 appeared as a result of selective pressure from using Fom because all O157 isolates were sensitive to Fom. We also speculate that 45- and 82-kb cryptic plasmids that were detected by types B (9·2% of total patient-origin O157) and D (7·7% of total patient-origin O157) transferred *in vivo* to O157. Transfer of plasmids among *Enterobacteriaceae* has been reported to occur normally within animal intestinal flora [9, 10], with the result that plasmids carrying various phenotypes are spreading even in environments where there is no selective pressure, e.g. from use of antibiotics.

Moreover, we detected four kinds of R plasmids, pTR50, pTR93-1, pTR93-2 and pTR93-3, from 36 patient-origin O157 isolates by the analysis of *Eco*RI digestion patterns, Southern hybridization and antibiotic resistance (Fig. 3, Table 1). If these R plasmids transferred *in vivo* to O157 in the intestine, it is likely that patients originally possessed Tet^r bacterial strains carrying such R plasmics in their intestine before the outbreak. Although we were unable to clarify the origin of the R plasmid, it may have originated from salmonella because pSC101 was a cryptic plasmic in salmonella [8] and its *tet* gene was 100% identical to the *tet* genes shown here.

In this study, we indicated that the 93-kb plasmids were subdivided into PTR93-1, pTR93-2 and pTR93-3, which were very similar to each other by the restriction and resistance patterns, suggesting that those basic replicon might be same. Since all 16 Tet^r transconjugants with the 93 kb-plasmids were resistant to sulphonamide, they might have the *sul1* genes on the plasmids. Moreover, pTR93-1 and pTR93-2 might have the *aadA* genes because all 15 Str^r Tet^r transconjugants were resistant to spectinomycin. The results indicate that the variability in the 93 kg-plasmid may indicate the presence of a class 1 integron (11) and that the integrons in pTR93-1 and pTR93-2 may contain the *aadA* gene cassettes.

Plasmid profiles in O157 isolates derived from the same individual in the continuous-infections were different from each other except number 1477 and 1814 (Table 1), and, in six cases, R plasmids were isolated only once. In the family-infections, O157 isolates derived from the same family had the different plasmid profiles from each other (Table 1), and, in six cases, the drug-resistance patterns of O157 isolates from patients of the primary and secondary infections were different from each other (Table 1). Interestingly, in one case of family infection, an O157 isolate (number 939) from a kindergarten child of the primary infection had no R plasmid, but a secondary O157 isolate (number 1181) from its mother had a R plasmid pTR50 (Table 1). These data suggest that the plasmid profiles of O157 in humans are variable, and also that several kinds of conjugative plasmids transfer very readily between bacterial cells in the enterobacterial flora. It is possible therefore that the transmission of R plasmids might occur not only *in vivo*, but also in the field, resulting in the spread of drug-resistant bacterial cells.

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