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Identification and Characterization of Transferable Integron-Mediated Antibiotic Resistance among *Salmonella* serovar Typhimurium and *Salmonella* serovar Infantis Isolates from 1991 to 2002

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Salmonella serovar Typhimurium and *Salmonella* serovar Infantis have been major causes of *Salmonella* infections in Japan during the past decades, though in 1989 *Salmonella* serovar Enteritidis suddenly emerged and its epidemic still prevails (1). However, the drug-resistant *S*. Enteritidis has been rare (2). We report here transferable class 1 integrons which conferred drug resistance on non-DT104 *S*. Typhimurium and *S*. Infantis. Class 1 integrons are predominant among *Enterobacteriaceae* (3), *Vibrionaceae* (4,5), and some non-fermenting gram-negative bacteria (3). Integrons contain gene cassettes that can be mobilized via their association with transposons or conjugative plasmids (3). They are important sources for the spread of antibiotic resistance among bacteria (3).

We analyzed a total of 30 *S*. Typhimurium isolates obtained in 1991-2002 in Hyogo Prefecture, 29 from humans and one from chicken, and 11 *S*. Infantis isolates, which were all multidrug resistant (MDR) (submitted for publication). The 29 isolates of *S*. Typhimurium from humans were obtained from six outbreaks and 23 sporadic cases. One *S*. Typhimurium isolate was judged as DT104 based on the drug resistance pattern, but others were not.

Antibiotic sensitivity was assayed by means of the disk diffusion method by using commercial antibiotic disks (Becton Dickinson Microbiology Systems, Cocksville, Md., USA) or by the agar dilution method on Mueller-Hinton agar plates following the recommendation by the National Committee for Clinical Laboratory Standards (6). Antibiotic disks were those of ampicillin (Am), cefotaxime, kanamycin (Km), gentamicin, streptomycin (Sm), tetracycline (Tc), trimethoprim (Tm), ciplofloxacin, fosfomycin, chloramphenicol (Cm), Sulfamethoxazole-trimethoprim (Su-Tm), and nalidixic acid (Na). The plates used for the agar dillution method contained Tc (25 μg/ml), Am (30 μg/ml), Km (25 μg/ml), Sm (25 or 50 μg/ml), Cm (25 μg/ml), Tm (25 μg/ml), or Su (100 μg/ml). MaConkey agar plates containing 0.5% lactose (Lac-MAC) were also used for the agar dilution method.

Class 1 integrons were detected by a consensus sequence for PCR primer pair, int I-F (5′-GGCATCCAAGCAGCAAGC-3′) and int I-R (5′-AAGCAGACTTGACCTGAT-3′), and PCR amplification was carried out as reported previously

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(6). The pulsed-field gel electrophoresis (PFGE) patterns of DNA from *S.* Typhimurium isolates and trasconjugants were analyzed by using a Gene Path Typing System (Program No. 5/No. 10; Bio-Rad Laboratories, Hercules, Calif., USA). Bacterial chromosomal DNAs were digested with *Xba*I or B*ln*I (Takara Shuzo Co., Ltd., Kyoto) and the method for isolation of plasmid DNA and its detection on PFGE were the same as reported (submitted for publication). The molecular size markers were 100 bp DNA ladder and lambda (λ) -*Hin*dIII digest (Takara Shuzo), pGEM® marker (Promega Corporation, Madison, Wis., USA), 48.5 kb (λ) DNA 1adder (Roche Diagnostics GbmH, Mannheim, Germany).

PFGE patterns of *Xba*I-digested chromosomal DNA of 30 *S.* Typhimurium strains are shown in Fig. 1 (Figs. 1A and B and lanes 1-6 in Fig. 1C). The overall patterns were similar among the isolates. There were four mutually identical pairs, Tyml3 and Tym15 (lanes 1, 3 in Fig. 1B), Tym21 and Tym22 (lanes 9, 10 in Fig. 1B), Tym23 and Tym24 (lanes 11, 12 in Fig. 1B), and Tym26 and Tym30 (lanes 2, 6 in Fig. 1C). However, others were dissimilar, i.e., the *S.* Typhimuriun isolates used had different genetic constitutions. The PFGE patterns of *Bln*I-digests appeared less variable among the isolates (lanes 7-12 in Fig. 1C).

Among the 30 *S.* Typhimurium isolates, 22 strains were drug resistant (Table 1) and the remaining eight were sensitive to all the drugs tested (see the legend to Fig. 1). Out of the 22 drug-resistant *S.* Typhimurium strains, 19 strains exhibited MDR patterns while three strains (Tym02, Tym09, and Tym11) were resistant to Tc only (Table 1). The MDR patterns exhibited by the 19 strains varied widely while those in *S.* Infantis were relatively homogeneous (submitted for publication). Class I integrons were present in the MDR strains of *S.* Typhimurium and *S.* Infantis (Table 1 and Fig. 2). Twelve strains among 21 drug-resistant *S.* Typhimurium and all of the 11 MDR strains of *S.* Infantis had class 1 integrons. All the 11 *S.* Infantis had the same-sized integrons (1.0 kb long, C type) (Fig. 2). On the other hand, *S.* Typhimurium had two kinds of integrons different in size; seven strains had 1.0- and 1.2-kb integrons (A type), while five strains had a 2.0-kb integron only (B type) (Fig. 2). The strains that harbored integrons were Tc-, Am-, and Cm-resistant (Tc^r, Am^r, Cm^r). All the strains with type A integron were Sm-resistant (Sm^r), while all the strains that harbored type B integron were Sm-sensitive (Sm^s). All the isolates with type A integron were Km-sensitive (Km^s), while all the strains with B-type integrons except Tym13 were Km-resistant (Km^r) (Table 1).

The 11 MDR *S.* Infantis strains were known to have antibiotic resistance genes, along with a 1.0-kb integron, on a 300-kb R plasmid (submitted for publication). In the present study we examined 21 strains of *S.* Typhymurium for their capacity to transfer drug resistance by conjugation to *S.* Litchfield strain AO Lac^+Nal^r-01 (Lac^+Nal^r) (7). When the donor strain was Na-resistant, the rifampicin-resistant (Rif^r) derivative recipient was used in place of the parental strain. The mating time was 4 h. The transconjugants were selected on Lac-MAC plates containing either Tc, Am, Km, Sm, or Cm (concentrations were the same as those described above). The donor strains were eliminated by Na (25 μg/ml) or by Rif (25 μg/ml) depending upon the combination of donors and recipients.

We found that, among 21 strains, Tym04, Tym13, Tym15, and Tym20 had an ability of conjugal transfer (Tables 1 and 2). Among them, Tym04 strain had no integrons, while Tym13, Tym15, and Tym20 had B type integrons. Transfer of anti-

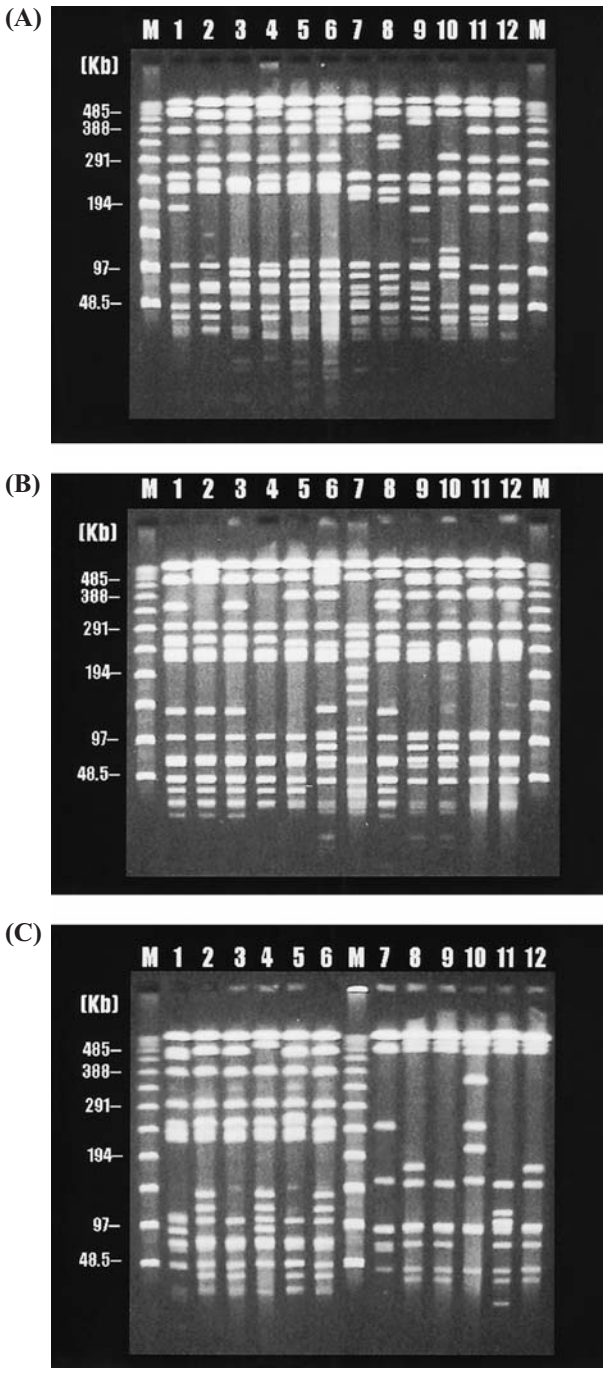

Fig. 1. PFGE patterns (using Gene Path Typing System, Program No. 5) of *Xba*I- or *Bln*I-digests of chromosomal DNA of *Salmonella* Typhimurium isolates in 1991-2002. For the eight strains that showed resistant to none of the drugs tested, their origin and year of isolation are described in parentheses. *Bln*I-digests are lanes 7-12 in C, and other lane wells all contain *Xba*I-digests. M in each figure indicates the λDNA ladder.

(A) Lane 1: Tym01 (from sporadic infection in 1991). Lane 2: Tym02. Lane 3: Tym03. Lane 4: Tym04. Lane 5: Tym05. Lane 6: Tym06. Lane 7: Tym07. Lane 8: Tym 08. Lane 9: Tym09. Lane 10: Tym10. Lane 11: Tym11. Lane 12: Tym12.

(B) Lane 1: Tym13. Lane 2: Tym14. Lane 3: Tym15. Lane 4: Tym16. Lane 5: Tym17 (from sporadic infection in 1994). Lane 6: Tym18. Lane 7: Tym19. Lane 8: Tym20. Lane 9: Tym21. Lane 10: Tym22. Lane 11: Tym23 (from sporadic infection in 1997). Lane 12: Tym24 (same as Tym23).

(C) Lanes 1 and 7: Tym25. Lanes 2 and 8: Tym26 (from a food poisoning outbreak in 2000). Lanes 3 and 9: Tym27 (same as Tym26, but a different outbreak). Lanes 4 and 10: Tym28 (from chicken in 2001). Lanes 5 and 11: Tym29. Lanes 6 and 12: Tym30 (from sporadic infection in 2002).

Table 1. List of drug-resistant *S.* Typhimurium isolates showing their antibiograms, integrons, and conjugal transferability

Strain	Year of isolation	Source[1]	Antibiogram[2]	Class I-integron type[3]	Ability of conjugal transfer
Tym 02	1991	S	Tc	–	–
Tym 03	1992	F	Tc Am Sm Cm Na	A	–
Tym 04	1992	F	Tc Am Km Sm	–	+
Tym 05	1992	S	Tc Am Sm Cm	A	–
Tym 06	1992	S	Tc Am Sm Cm	A	–
Tym 07	1993	S	Tc Cm	–	–
Tym 08	1993	S	Tc Cm	–	–
Tym 09	1993	S	Tc	–	–
Tym 10	1993	S	Tc Cm	–	–
Tym 11	1993	S	Tc	–	–
Tym 12	1993	S	Tc Am Sm	–	–
Tym 13	1993	S	Tc Am Cm	B	+
Tym 14	1993	S	Tc Am Km Cm	B	–
Tym 15	1993	S	Tc Am Km Cm	B	+
Tym 16	1993	S	Tc Am Km Cm	B	–
Tym 18	1994	S	Tc Am Sm Cm	A	–
Tym 19	1995	F	Tc Cm	–	–
Tym 20	1996	S	Tc Am Km Cm	B	+
Tym 21	1996	S	Tc Am Sm Cm	A	–
Tym 22[4]	1997	S	Tc Am Sm Cm Tm Su-Tm	A	–
Tym 25	1997	F	Tc Am Sm Cm	A	–
Tym 29	2001	S	Tc Km	NT[5]	NT

[1] All the strains were from humans. S: sporadic infection, F: food poisoning outbreak.
[2] Assay was performed by means of the disk diffusion method. Tc: tetracycline, Am: ampicillin, Km: kanamycin, Sm: streptomycin, Cm: chloramphenicol, Tm: trimethoprim, Su-Tm: sulfamethoxazole-trimethoprim, Na: nalidixic acid. But Sm-resistance was finally judged on plates containing 50 μg/ml of the drug.
[3] Type A: two kinds of class 1 integrons of 1.0 and 1.2 kb in size and B: one integron of 2.0 kb in size, and -: negative.
[4] Showed a phage type of DT104. Others listed above were not tested.
[5] Not tested.

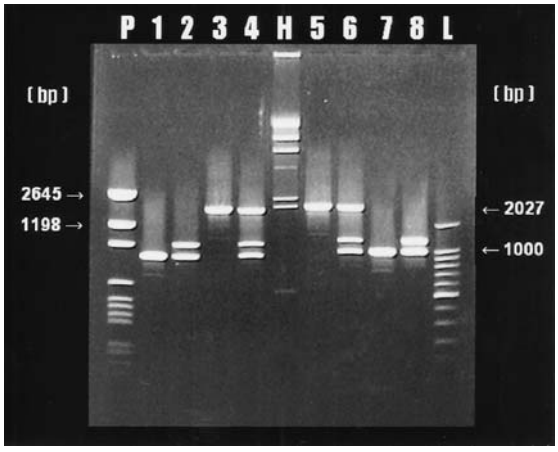

Fig. 2. Different sizes of PCR products obtained with a primer pair targeting the conserved segments of class 1 integrons in *Salmonella* Infantis and *S.* Typhimurium isolates in 1991-2002. The PCR products were separated using conventional agarose gel electrophoresis. P, H, and L in the figure indicate pGEM® marker (Promega Corp.), λ-*Hin*d III digest (Takara Shuzo), and 100 bp DNA ladder (Takara Shuzo), respectively.
Lanes 1 and 7: 1-kb amplicons (type C) obtained from 11 MDR strains of *S.* Infantis.
Lanes 2 and 8: 1- and 1.2-kb amplicons (type A) from *S.* Typhimurium.
Lanes 3 and 5: 2.0-kb amplicons (type B) from *S.* Typhimurium.
Lanes 4 and 6: a mixture of PCR products of types A and B.

biotic resistance markers and integrons by means of conjugation are shown in Table 2.

Mating experiments using Tym04 as a donor gave a result which suggested that Tc^r, Km^r and Sm^r were linked but Am^r and $Tc^rKm^rSm^r$ were not (Table 2), i.e., it was possible that Am^r and $Tc^rKm^rSm^r$ were on separate plasmids or a plasmid and chromosome. To test this possibility, competent *Escherichia coli* K-12 DH5 α cells (Takara Shuzo) were transfected with plasmid DNA fraction prepared from Tym04. The transfectants were selected on Lac-MAC plates containing Tc, Am, Km, or Sm. Seventy-four transfectants were obtained. Eight colonies selected on Tc plates, 25 colonies selected on Km plates and 18 colonies selected on Sm plates were all Tc^r Km^r Sm^r Am^s, while 23 colonies selected on Am plates were all Tc^s Km^s Sm^s Am^r. No Tc^r Km^r Sm^r Am^r transfectants were obtained. The data supported the hypothesis that Tym04 starin had two plasmids, one with Tc^r Km^r Sm^r and the other with Am^r.

When Tym13, Tym15, and Tym20 as donors were used as donors, the order of transfer of drug resistance genes was suggested to be Cm^r, Tm^r, and Km^r/Am^r. This result was best explained by postulating that these drug resistance genes were present on the bacterial chromosomes in that order, because, if the drug resistance genes were on a plasmid, all the genes should have been transferred together simultaneously.

In this study we demonstrated the transferability of integron-mediated drug resistance in non-DT104 *S.* Typhimurium and *S.* Infantis. The gene cassette structures of the integrons of

Table 2. Contrasferred integrons along with the antibiotic resistance determinants in three matings

Cross of conjugal transfer[1]			Number of transconjugants obtained	Antibiogram[3]	Class I-integron[4] positive/tested
donor	selection[2]	frequency/h			
Tym 04				Tc Am Km Sm	–
	Tc	1.6×10^{-5}	15	Tc Km Sm Na	NT[5]
			2	Tc Am Km Sm Na	NT
	Am	4.3×10^{-6}	9	Tc Am Km Sm Na	NT
			8	Am Na	NT
	Km	1.4×10^{-5}	16	Tc Km Sm Na	NT
			1	Tc Am Km Sm Na	NT
	Sm	NT	1	Tc Km Sm Na	NT
Tym 13				Tc Am Cm	+ (B type)
	Tc	2.1×10^{-9}	2	Tc Am Cm Na	1/2
			2	Tc Cm Na	0/2
	Am	1.0×10^{-9}	1	Tc Am Cm Na	1/1
	Cm	NT	10	Tc Cm Na	0/3
			1	Am Cm Na	0/1
Tym 15				Tc Am Km Cm	+ (B type)
	Tc	1.5×10^{-8}	3	Tc Am Km Cm Na	3/3
			3	Tc Km Cm Na	0/3
			1	Tc Cm Na	0/1
	Am	1.5×10^{-8}	6	Tc Am Km Cm Na	6/6
	Km	1.2×10^{-8}	5	Tc Am Km Cm Na	5/5
	Cm	NT	2	Tc Am Km Cm Na	2/2
			2	Tc Km Cm Na	0/2
			3	Tc Cm Na	0/3
Tym 20				Tc Am Km Cm	+ (B type)
	Tc	NT	2	Tc Am Km Cm Na	0/2
	Am	NT	4	Tc Am Km Cm Na	2/4
	Km	NT	5	Tc Am Km Cm Na	5/5
	Cm	NT	8	Tc Am Km Cm Na	3/3
			5	Tc Cm Na	0/3

[1] Transconjugants were obtained from the crosses, *S*. Typhimurium isolates x AO Lac^+Nal^r-01 which possessed no class 1-integrons. The recipient showed spontaneous frequencies of $<7.1 \times 10^{-11}$ to each of Tc^r, Am^r, Km^r, and Cm^r.
[2] Coexsistence of Na in common with the selective agents indicataed.
[3] Assay was performed by means of the agar dilution method. Tc: tetracycline, Am: ampicillin, Km: kanamycin, Sm: streptomycin, Cm: chloramphenicol, Na: nalidixic acid.
[4] Positive strains harbored the same type of integrons as the donors.
[5] Not tested.

these bacteria remain to be clarified in comparison with those of the well-characterized DT104 (8).

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