Detection of an R Factor Showing Temperature-Sensitive Transfer in *Salmonella typhimurium* Isolated from Calves

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An R factor carrying tetracycline resistance, the transfer of which was observed in mating cultures at 25 but not at 37 C, was demonstrated in strains of *Salmonella typhimurium* isolated from calves.

In detecting R factor transfer in bacteria, mixed cultivation of donor and recipient strains is made, usually at 37 C (2). However, Terawaki et al. (5) detected a thermosensitive R factor (Rts1) which conferred resistance to kanamycin in *Proteus vulgaris*. The transfer of the R factor was strongly affected by the temperature at which the mating culture was kept; the transfer frequency of the R factor at 25 C was about 10^6 times higher than at 37 C. We report here the isolation of strains of *Salmonella typhimurium* which harbor an R factor which is detectable only after mixed cultivation at lower temperatures.

A group of 36 calves was introduced into a feedlot. Among them, four excreted *S. typhimurium* once or repeatedly during a period of 8 to 20 days after arrival. Of 11 strains isolated from the four calves described, 10 were resistant to tetracycline (TC; 400 µg/ml), streptomycin (SM; 800 µg/ml or more than 1,600 µg/ml), and sulfadimethoxine (SA; more than 1,600 µg/ml). The resistance pattern of each strain is shown in Table 1. The ten strains were examined for the presence of an R factor by mixed cultivation at 37 C (4). Four of the TC-resistant strains (No. 35, 36, 41, and 42) could transfer the triple (TC-SM-SA) resistance to *Escherichia coli* K-12 (ML1410) (4), which was used as recipient. However, the remaining six strains from two calves housed separately did not transfer the drug resistance to the recipient when mixed culture was made at 37 C for 6 h or overnight. To confirm the result, the next day the mixed broth cultures which had been left at room temperature (about 25 C) were reinoculated onto selective media containing nalidixic acid (50 µg/ml) and the drug under test (TC or SM, 25 µg/ml; SA, 200 µg/ml). DHL agar (Eiken) was used as basal medium for the

test of TC or SM resistance, and Mueller Hinton medium (Eiken), to which 1.5 g of lactose and 4 ml of 0.2% bromothymol blue per 100 ml had been added, was used for test of SA-resistance. In repeated experiments, transfer of TC-resistance to the recipient occurred.

The following experiment was then conducted to confirm the result obtained above. Equal volumes (0.2 ml) of overnight broth cultures of donor and recipient strains were added to 10 ml of brain heart infusion broth (Difco). Thereafter, the broth cultures were dispensed into small test tubes and incubated at 20, 25, 30 and 37 C for 18 h. The incubated broth cultures were enumerated by plating on the selective media described above. Table 1 shows that transfer of TC resistance of the six strains (No. 38, 37, 43, 45, 46, and 47) was suppressed by the incubation temperature of more than 30 C and especially at 37 C. From these six strains, the *E. coli* ML1410 exconjugants transferred their TC resistance more readily to a secondary recipient *E. coli* strain (W3630) (4) at the incubation temperature of 25 rather than 37 C. In view of the sequential transfer of TC resistance, it is inferred that the strains harbor an R factor for TC resistance.

Next, the frequency of transfer of the R factor carrying TC resistance, at incubation temperatures of 25 and 37 C, was determined (4, 5). Of the six *S. typhimurium* strains, No. 47 was used as a donor. *E. coli* ML1410 and *S. typhimurium* LT-2 NA^r (nalidixic acid-resistant), which acquired the R factor carrying TC resistance from strain 47 at 25-C incubation, were also used as donors. These two latter strains were designated ML1410(R:TC) and LT-2 NA^r (R:TC), respectively. For these donors, ML1410 RFP^r (rifampin-resistant) and LT-2 RFP^r were employed as recipients. Equal volumes (5 ml) of Penassay

TABLE 1. *Transfer of TC resistance from Salmonella typhimurium at various incubation temperatures*

No. of isolate	Name of calf	Resistance pattern	Resistance pattern of R factor	Transfer of TC resistance after incubation (18 h) at			
				37 C	30 C	25 C	20 C
35	A (8) ^a	TC-SM-SA	TC-SM-SA	++ ^b	++	++	++
36	B (8)	TC-SM-SA	TC-SM-SA	++	++	++	++
41	B (14)	TC-SM-SA	TC-SM-SA	++	++	++	++
42	B (16)	TC-SM-SA	TC-SM-SA	++	++	++	++
38	D (8)	TC-SM-SA	TC	- ^b	+ ^b	++	++
37	C (8)	TC-SM-SA	TC	-	+	++	++
43	C (12)	TC-SM-SA	TC	-	+	++	++
45	C (14)	TC-SM-SA	TC	-	+	++	++
46	C (18)	TC-SM-SA	TC	-	+	++	++
47	C (20)	TC-SM-SA	TC	-	+	++	++

^a Day (after introduction to the feedlot) on which isolation was made.

^b A dose of 0.02 ml of mixed broth culture was dropped onto selective media. Symbols: ++, growth with complete or partial confluence; +, 2 to 40 colonies; -, no growth.

broth (Difco) cultures of donor and recipient strains (precultured at 37 C for 3 h with shaking) were mixed and then divided equally into two tubes. The mixed cultures were incubated at 25 and 37 C without shaking. An appropriate dilution of the mixtures was plated on selective media periodically after 1 to 6 h of incubation. The frequency of transfer was calculated relative to the number of donors (as transfer per donor cell). When the mixed cultivation was made at 37 C, no transfer to the recipient was observed (transfer frequency of less than 10^{-9} /donor cell) (Table 2). On the other hand, the transfer in the mixed cultures at 25 C occurred at a frequency of 10^{-8} to 10^{-9} /donor cell. Thus, temperature-sensitive transfer of the R factor carrying TC resistance was

demonstrated in all combinations of donor and recipient strains used.

Elimination frequency of the R factor carrying TC resistance from host cells grown at 25, 37, and 42 C was examined (5). Preculture of host cells was performed at 25 C for 18 h with gentle shaking. A volume of 10^{-8} dilution of each culture was subcultured into drug-free broth and incubated at 25, 37, or 42 C with gentle shaking. After 20 h of incubation, elimination frequency was determined by checking the TC resistance marker of more than 100 colonies by the replica-plating method (3). When *S. typhimurium* 47 was cultured at 42 C, 81.5% of the total cells tested became sensitive to TC (Table 3). It was noted that the spontaneous loss of the R factor was also observed in 7%

TABLE 2. *Transfer frequency of the R factor carrying TC resistance at 25 and 37 C*

Donor	Recipient	Incubation temp	Time in mixed cultivation (h)			
			1	2	4	6
<i>S. typhimurium</i> 47 (R:TC)	<i>E. coli</i> ML1410	25 C	10^{-7a}	10^{-4}	10^{-3}	10^{-3}
		37 C	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$
	<i>S. typhimurium</i> LT-2 NA ^r	25 C	NT ^b	NT	NT	10^{-4}
		37 C	NT	NT	NT	$<10^{-9}$
ML1410 (R:TC)	ML1410 RFP ^r	25 C	10^{-8}	10^{-8}	10^{-7}	10^{-5}
		37 C	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$
	LT-2 RFP ^r	25 C	10^{-8}	10^{-7}	10^{-6}	10^{-6}
		37 C	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$
LT-2 NA ^r (R:TC)	ML1410 RFP ^r	25 C	NT	NT	NT	10^{-5}
		37 C	NT	NT	NT	$<10^{-9}$
	LT-2 RFP ^r	25 C	NT	NT	NT	10^{-5}
		37 C	NT	NT	NT	$<10^{-9}$

^a Transfer frequency was calculated as the number of exconjugant recipients, which acquired drug resistance, divided by that of donor cells.

^b NT, not tested.

TABLE 3. Elimination of the R factor carrying TC resistance from cells grown at various cultivation temperatures

Cultiva- tion temp (C)	Donor strain tested	No. of total cells tested	Rate of TC-sensi- tive cells (%)
25	<i>S. typhimurium</i> 47 (R:TC)	122	7.4
	<i>E. coli</i> ML1410 (R:TC)	228	0
	<i>S. typhimurium</i> LT-2 NA ^r (R:TC)	162	0
37	47 (R:TC)	308	18.2
	ML1410 (R:TC)	110	0
	LT-2 NA ^r (R:TC)	365	0
42	47 (R:TC)	248	81.5
	ML1410 (R:TC)	90	8.9
	LT-2 NA ^r (R:TC)	166	2.4

of *S. typhimurium* 47 strain even at 25 C, indicating that the R factor is relatively labile in the original host cell. When the ML1410 or LT-2 NA^r strain was used as host, spontaneous loss of the R factor was observed only at 42 C, but the

frequency was very low compared with that of *S. typhimurium* 47 strain as host.

The data described above indicate that the thermosensitive transfer and the accumulated elimination frequency of the R factor carrying TC resistance at elevated temperature might depend upon thermosensitive replication of this R factor itself. However, it is not clear whether this R factor belongs to the same compatibility group as Rts1 (5) and other thermosensitive R factors reported by Coetzee et al. (1).

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