EPISOME-MEDIATED TRANSFER OF DRUG RESISTANCE IN *ENTEROBACTERIACEAE*

V. Spontaneous Segregation and Recombination of Resistance Factors in *Salmonella Typhimurium*

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ABSTRACT

WATANABE, TSUTOMU (Keio University, Tokyo, Japan) AND KEONG W. LYANG. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. V. Spontaneous segregation and recombination of resistance factors in *Salmonella typhimurium*. J. Bacteriol. **84**:422–430. 1962.—It was found that spontaneous segregation of the transmissible multiple drug resistance [resistance to streptomycin (Sm), chloramphenicol (Cm), tetracycline (TC), and sulfonamide (Su)] takes place so frequently in *Salmonella typhimurium* strain LT-2 (Su, Sm, Cm, Tc) as to allow a clone analysis of the segregation of resistance factors with replica plating. Su-, Sm-, and Cm- resistance factors together were spontaneously lost most frequently, whereas the spontaneous loss of Tc-resistance factor alone was rather infrequent. The complete loss of the resistance factors was also noted with low frequencies. The mechanism of the spontaneous segregation of the resistance factors was assumed to be due to either genetic exchange between the resistance factors and host genome or incomplete replication of the transmissible resistance factors (composed of resistance factors and resistance-transfer factor). In relation to the mechanism of spontaneous segregation of the transmissible resistance factors, circular models of the transmissible resistance factors are presented. Four-drug-resistant strains were produced by transferring segregant types of transmissible resistance factors (Su, Sm, Cm) and (Tc) to the strains with (Tc) and (Su, Sm, Cm), respectively. In these four-drug-resistant strains, the two types of transmissible resistance factors were found to be in two different states; in one state the two types of transmissible resistance factors exist in the same cells independently of each other, and in the other state a transmissible resistance factor, which was produced as a result of recombination of the two types of transmissible resistance factors, is present. Two unusual strains of LT-2 with Su-, Sm-, and Cm-resistance factors and with four resistance factors were found. These strains could not transfer their resistance factors to *Escherichia coli* K-12 by conjugation. Their resistance factors were spontaneously segregated as frequently as those of LT-2 (Su, Sm, Cm) and LT-2 (Su, Sm, Cm, Tc), and the transfer of (Tc) to strain LT-2 with nontransmissible Su-, Sm-, and Cm-resistance factors gave rise to two types of clones; one could transfer the four resistance factors as a unit to K-12 by conjugation, and another could transfer only (Tc) and frequently segregated two types of clones; one had (Tc) and another had nontransmissible Su, Sm-, and Cm-resistance factors. From these findings, the unusual strains with nontransmissible resistance factors are assumed to have a "defective" resistance-transfer factor.

It has been reported by Watanabe and Fukasawa (1960, 1961*a*, *b*, *c*, 1962) that the resistance factors of the multiply drug-resistant *Enterobacteriaceae* [resistant to streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), and sulfonamide (Su)] are carried by an episome, resistance-transfer factor (RTF), and transferred from cell to cell by conjugation. S. Iseki later proposed the term "R-factors" for the transmissible resistance factors. On the basis of the results of Watanabe and Fukasawa, R-factors can be understood to imply the units composed of resistance factors and RTF. R-factors behave as episomes because of the episomality of RTF. Watanabe and Fukasawa (1960, 1961*a*, *b*, *c*) further reported that the resistance factors can

be segregated both spontaneously and by transduction. In the spontaneous segregation of the resistance factors, Tc-resistance factor alone, Su-, Sm-, and Cm-resistance factors together, or all the resistance factors together are lost. No other possible combinations of resistance factors are segregated spontaneously as far as studied. In those workers' studies, spontaneous segregation of the resistance factors took place most frequently in *Salmonella typhimurium* and least frequently in *Escherichia coli*, the frequency of segregation in *Shigella flexneri* being between those of the other two species. The frequency of spontaneous segregation of resistance factors in *S. typhimurium* is so high that we have found it possible to study the segregation of resistance factors in this bacterium by a clone analysis, the results of which are reported here.

Watanabe and Fukasawa (1960) also reported that four-drug-resistant strains can be obtained by transferring an R-factor (Tc) to the strain with an R-factor (Su, Sm, Cm) and by transferring an R-factor (Su, Sm, Cm) to the strain with an R-factor (Tc) by conjugation. They did not study how the two types of R-factors are present in the same cells. We have since found that there are two different states of the two types of R-factors in these cells; one is the state where the two types of R-factors (Tc) and (Su, Sm, Cm) exist independently of each other without close association, and another state is that in which the two types of R-factors are closely associated with each other. Analysis of the artificially combined resistance factors is also reported here.

In the course of the present study, two clones which have resistance factors but which cannot transfer them by conjugation were isolated. Studies of these clones suggested that they have a sort of defective mutant R-factors which are able to replicate autonomously but cannot be transferred by conjugation.

After our preliminary reports (Watanabe and Lyang, 1961*a*, *b*) were published, Mitsuhashi, Harada, and Hashimoto (1961) and Hashimoto, Harada, and Mitsuhashi (1962) also reported the interactions of two different types of R-factors in the same cells of *E. coli* K-12.

MATERIALS AND METHODS

The media employed were the same as those used by Watanabe and Fukasawa (1961*a*, *b*, *c*). The drugs used were described by Watanabe and Fukasawa (1961*a*).

S. flexneri 2b strain 222 (Su, Sm, Cm, Tc), *Salmonella typhimurium* LT-2, and substrains of *E. coli* K-12 were used. The substrains of K-12 used are CSH-2 ($Meth^- F^-$), W-677/$Pro^-T6^rSm^r$ ($Thr^-Leu^-B_1^-Man^-Xyl^-Mal^-Gal^-Lac^-Pro^-$-$T6^rSm^rF^-$), and 58-161 ($Meth^-Bi^-F^+$).

The cultivation method, conjugation conditions, and method of selection for clones that received resistance factors were the same as those described by Watanabe and Fukasawa (1961*a*).

Ultraviolet (UV) irradiation was the same as that described by Watanabe and Fukasawa (1961*b*).

The replica-plating technique of Lederberg and Lederberg (1952) was employed, using sterile velvet.

The procedure for transduction with phage Plkc was the same as that described by Watanabe and Fukasawa (1961*c*).

RESULTS

Detection of spontaneous segregants of S. typhimurium LT-2 with multiple drug resistance. Multiple drug resistance was transferred from *Shigella flexneri* 2b strain 222 (Su, Sm, Cm, Tc) by conjugation to *Salmonella typhimurium* LT-2. The LT-2 cells that received multiple drug resistance were selected on minimal agar (Davis and Mingioli, 1950) containing 5 μg of Cm per ml by utilizing the requirement of strain 222 for nicotinic acid, tryptophan, and methionine, and the prototrophy of strain LT-2. A Penassay Broth (Difco) culture of LT-2 (Su, Sm, Cm, Tc) was diluted and plated on drug-free Nutrient Agar (Difco) for colony isolation, and the inoculated plates were incubated at 37 C overnight. The resulting well-isolated colonies were replica-plated onto Nutrient Agar containing 25 μg per ml of Sm, Cm, and Tc and 500 μg per ml of Su. Four of the colonies which were thus confirmed to be resistant to the four drugs were used as starting materials. Penassay Broth (5 ml) in a test tube was inoculated with each colony and incubated stationary at 37 C for 24 hr. Each culture was then diluted with Penassay Broth to 10^{-5}, and 0.1 ml of this dilution was transferred to 5 ml of fresh Penassay Broth and subcultures were prepared thereafter in a similar fashion. At each transfer, the foregoing culture

TABLE 1. *Spontaneous segregation of resistance factors of Salmonella typhimurium strain LT-2 (Su, Sm, Cm, Tc) in its successive transfer in Penassay Broth**

Colony number	Drug in replica plates	Percentage of drug-resistant colonies among those developed on drug-free Nutrient Agar inoculated with each subculture									
		1	2	3	4	5	6	7	8	9	10
I	Tc	100	100	—	100	100	99	—	99	100	100
	Cm	100	75	—	65	26	7	—	2	3	1
	Sm	100	75	—	65	26	7	—	2	3	1
	Su	100	75	—	65	26	7	—	2	3	1
II	Tc	—	100	100	100	100	100	100	100	100	100
	Cm	—	91	85	70	28	20	12	9	4	3
	Sm	—	91	85	70	28	20	12	9	4	3
	Su	—	91	85	70	28	20	12	9	4	3
III	Tc	100	100	100	100	100	100	100	98	99	98
	Cm	84	93	87	41	25	15	2	4	1	1
	Sm	84	93	87	41	25	15	2	4	1	1
	Su	84	93	87	41	25	15	2	4	1	1
IV	Tc	100	100	100	100	100	100	100	97	100	100
	Cm	95	92	80	55	33	23	6	3	1	0
	Sm	95	92	80	55	33	23	6	3	1	0
	Su	95	92	80	55	33	23	6	3	1	0

* Among the ten Tc-sensitive segregants in this table, two clones were resistant to Su, Sm, and Cm, and eight were sensitive to all the drugs. Su-, Sm-, and Cm-resistance factors were segregated together in all instances.

was diluted to 10^{-5} and 10^{-6}, and 0.1 ml of these dilutions was plated on drug-free Nutrient Agar. The patterns of drug resistance of the developed colonies were studied with a replica-plating technique. Su-, Sm-, and Cm-resistance factors together were spontaneously lost with very high frequencies, whereas the segregation of Tc-resistance factor was rather low (Table 1). In addition to these two types of segregation, the complete loss of the resistance factors was also found, although with low frequencies. The ratios of segregant types increased in parallel with the number of subcultures.

Ability of the spontaneous segregants to transfer their resistance factors by conjugation. Segregant colonies (150) with Tc-resistance factor alone were taken at random from those obtained in the above experiment and were studied for their ability to transfer their Tc-resistance factor to strain CSH-2 through conjugation by utilizing nonfermentation of lactose by LT-2 and fermentation of lactose by CSH-2. By plating the mixed cultures of donor and recipient on bromothymol blue-lactose-Nutrient Agar containing 25 μg of Tc per ml, the CSH-2 cells which received Tc-resistance factor were detected as yellow colonies. All of the donor clones tested were found to transfer their Tc-resistance factor but no other resistance factors. This finding indicates that the phenotypically Tc-resistant segregants have only Tc-resistance factor. This result is in accordance with those obtained by Watanabe and Fukasawa (1961*b*). The sensitive segregants with no drug resistance all failed to convert sensitive recipient CSH-2 to resistance to any drugs by mixed cultivation.

One of the two segregants with Su-, Sm-, and Cm-resistance factors and without Tc-resistance factor was found to be able to transfer these resistance factors by conjugation but another could not transfer them at all. We will denote the latter strain as LT-2 ≪Su, Sm, Cm≫. UV irradiation of this strain could not induce the transfer of resistance factors by conjugation.

Ability of the four-drug-resistant clones to transfer their resistance factors by conjugation. Colonies (100) with four-drug-resistance were taken at random from those obtained in the above experi-

ment. Their ability to transfer the resistance factors by conjugation was studied; 99 colonies were found to transfer the four resistance factors to CSH-2 (the frequencies of transfer were 10^{-2} to 10^{-3} per donor cell in the experiment with several donor clones) but 1 colony was found to be unable to transfer any of the resistance factors to CSH-2 even in low frequencies (less than 10^{-8} per donor cell). This clone was named LT-2 ≪Su, Sm, Cm, Tc≫. UV irradiation of this strain could not induce the transfer of resistance factors by conjugation.

Genetic stability of LT-2 (Su, Sm, Cm), LT-2 ≪Su, Sm, Cm≫, and LT-2 ≪Su, Sm, Cm, Tc≫. One colony isolated from each of these strains was checked for its resistance to these drugs by replica plating; they were then inoculated into 5 ml of Penassay Broth and subcultured each day for 12 days. The 1st, 5th, and 12th subcultures were diluted and plated on Nutrient Agar without any drug. The resultant colonies were studied for their drug resistance by a replica-plating technique. Strains LT-2 ≪Su, Sm, Cm≫ and LT-2 ≪Su, Sm, Cm, Tc≫, which cannot transfer their resistance factors by conjugation, also segregated their resistance factors as frequently (Table 2) as strains LT-2 (Su, Sm, Cm) and LT-2 (Su, Sm, Cm, Tc).

TABLE 2. *Spontaneous segregation of resistance factors of Salmonella typhimurium strains LT-2 (Su, Sm, Cm, Tc), LT-2 (Su, Sm, Cm), LT-2 ≪Su, Sm, Cm, Tc≫, and LT-2 ≪Su, Sm, Cm≫ in their successive transfer in Penassay Broth*

Strain	Drug in replica plates	Percentage of drug-resistant colonies among those developed on drug-free Nutrient Agar inoculated with the following subculture		
		1st	5th	12th
LT-2 (Su, Sm, Cm, Tc)	Tc	100	99	97
	Cm	85	18	0.7
	Sm	85	18	0.7
	Su	85	18	0.7
LT-2 (Su, Sm, Cm)	Tc	0	0	0
	Cm	92	30	15
	Sm	92	30	15
	Su	92	30	15
LT-2 ≪Su, Sm, Cm, Tc≫	Tc	99	100	100
	Cm	94	53	5
	Sm	94	53	5
	Su	94	53	5
LT-2 ≪Su, Sm, Cm≫	Tc	0	0	0
	Cm	98	64	8
	Sm	98	64	8
	Su	98	64	8

TABLE 3. *Additional transfer of (Tc) to Salmonella typhimurium strains LT-2 (Su, Sm, Cm) and LT-2 ≪Su, Sm, Cm≫ from Escherichia coli strain CSH-2 (Tc) by conjugation*

Donor	Recipient	Recipient clones which received (Tc)	
		No. of clones resistant to Su, Sm, and Cm/no. studied	No. of clones able to transfer four resistance factors by conjugation/no. studied
CSH-2 (Tc)	LT-2 (Su, Sm, Cm)	3/10	3/10
CSH-2 (Tc)	LT-2 ≪Su, Sm, Cm≫	6/10	4/10

Transfer of Tc-resistance factor from CSH-2 (Tc) to LT-2 (Su, Sm, Cm) and LT-2 ≪Su, Sm, Cm≫ by conjugation. A spontaneous segregant strain LT-2 (Tc) was grown together with CSH-2 in Penassay Broth, and CSH-2 (Tc) was obtained. CSH-2 (Tc) and LT-2 (Su, Sm, Cm) or LT-2 ≪Su, Sm, Cm≫ were grown together in Penassay Broth, and the LT-2 cells which received Tc-resistance factor were selected on minimal agar containing 1 μg of Tc per ml, by utilizing the methionine-requirement of CSH-2. This low concentration of Tc was chosen because Tc was found to be more effective in minimal agar than in Nutrient Agar. Ten colonies were picked at random out of those developed, in each combination of donor and recipient and after being reisolated on minimal agar containing 1 μg of Tc per ml. Each of them was grown together with CSH-2 in Penassay Broth overnight and plated on bromothymol blue-lactose-Nutrient Agar containing each of the four drugs. Thus, the resistance factors and their transmissibility by conjugation could be studied. Four of ten clones of LT-2 ≪Su, Sm, Cm≫ which received Tc-resistance factor were able to transfer not only Tc-resistance factor but also the other three resistance factors by conjugation (Table 3). Two other clones had four-drug-resistance but could transfer only Tc-resistance factor. Seven of ten clones of LT-2 (Su, Sm, Cm) and four of ten clones of LT-2 ≪Su, Sm, Cm≫ which received

Tc-resistance factor were found to have lost the other three resistance factors.

Transfer of resistance factors through conjugation by the four-drug-resistant strains artificially made by combining (Tc) and (Su, Sm, Cm) or ≪Su, Sm, Cm≫. As mentioned above, some of the four-drug-resistant LT-2 strains artificially made by combining resistance factors were found to be able to transfer the four resistance factors to the recipient population, but it is not known whether the four resistance factors are transferred together as a unit. Donor cells (artificially made four-drug-resistant LT-2 cells by combining (Tc) and (Su, Sm, Cm) or ≪Su, Sm, Cm≫) and recipient cells (CSH-2) were mixed in a ratio of about 1:10 and incubated at 37 C for 30 min. The mixed cultures were plated on bromothymol blue-lactose-Nutrient Agar containing Cm or Tc. The resultant colonies were tested for their resistance to the other drugs. Many of the colonies selected on Tc were found sensitive to the other drugs, whereas many of those selected on Cm and Su were resistant to Tc as well (Table 4). As described above, Su-, Sm-, and Cm-resistance factors are very frequently segregated as a unit by LT-2 (Su, Sm, Cm, Tc), and Tc-resistance factor is less frequently segregated. Accordingly, the finding that segregated transfer of resistance factors occurred in the artificially combined four-drug-resistant strains might have resulted from the heterogeneity of the donor cultures used.

Transfer of resistance factors through conjugation by CSH-2 cells which received four resistance factors from artificially combined four-drug-resistant LT-2. Two colonies each of CSH-2 which received four resistance factors from the four-drug-resistant LT-2 strains, which had been artificially formed by transferring (Tc) to LT-2 (Su, Sm, Cm) and LT-2 ≪Su, Sm, Cm≫, were used as donors, and their resistance factors were further transferred to W-677/Pro⁻ T6ʳSmʳ by conjugation. The mixed cultures were plated on Nutrient Agar containing 1,000 μg of Sm and 25 μg of Cm or Tc per ml. The resultant colonies were replica-plated on Nutrient Agar containing another drug for testing their resistance factors. Plates containing Su and Sm were omitted because spontaneous segregation between Su-, Sm-, and Cm-resistance factors usually does not take place. One of the two colonies of CSH-2 which received four resistanee factors from the artificially combined four-drug-resistant LT-2 derived from LT-2 (Su, Sm, Cm) was found not to transfer the four resistance factors together (Table 5).

TABLE 4. *Ability of Salmonella typhimurium strains LT-2 (Su, Sm, Cm) and LT-2 ≪Su, Sm, Cm≫, which received (Tc), to transfer four resistance factors to Escherichia coli strain CSH-2 by conjugation*

Donor	Recipient	No. of clones able to transfer four resistance factors/no. of clones with four resistance factors		
		Clones selected on Su	Clones selected on Cm	Clones selected on Tc
LT-2 (Su, Sm, Cm) + (Tc)	CSH-2	6/9	5/9	5/9
LT-2 ≪Su, Sm, Cm≫ + (Tc)	CSH-2	12/12	12/12	1/12

TABLE 5. *Ability of Escherichia coli strain CSH-2 with four transmissible resistance factors, which were received from Salmonella typhimurium strains LT-2 (Su, Sm, Cm) + (Tc), LT-2 ≪Su, Sm, Cm≫ + (Tc), and LT-2 (Su, Sm, Cm, Tc), to transfer the four resistance factors to E. coli W-677/Pro⁻T6ʳSmʳ as a unit by conjugation*

Initial donor of four resistance factors	Clone of CSH-2 with four resistance factors	No. of clones of W-677/Pro⁻ T6ʳSmʳ which received resistance factors			
		Colony count on master plate containing		Colony count on replica plate containing	
		Tc	Cm	Tc	Cm
LT-2 (Su, Sm, Cm) + (Tc)	A	18			18
			36	18	
	B	83			83
			90	90	
LT-2 ≪Su, Sm, Cm≫ + (Tc)	A′	277			277
			255	255	
	B′	257			257
			265	265	
LT-2 (Su, Sm, Cm, Tc)		384			384
			295	295	

Transduction of resistance factors by phage Plkc using artificially combined four-drug-resistant strain. Resistance factors of LT-2 strains with four resistance factors, which had been artificially formed by transferring (Tc) to LT-2 (Su, Sm, Cm) and LT-2 ≪Su, Sm, Cm≫, were transferred

to 58-161 by way of CSH-2 and W-677/Pro⁻T6^{r}Sm^r through conjugation. Phage Plkc was propagated on 58-161, which thus received the four resistance factors and was used as donor for transduction of resistance factors employing CSH-2 as a recipient. For the control, the strain 58-161 which received (Su, Sm, Cm, Tc) through conjugation from strain 222 (Su, Sm, Cm, Tc) by way of LT-2, CSH-2, and then W-677/Pro⁻T6^{r}Sm^r was used. Only 20 transductants were studied for each donor in this experiment. All of the transductants studied were resistant to all of the four drugs and were also able to transfer their resistance factors further by conjugation. Segregated transfer of resistance factors, as reported by Watanabe and Fukasawa (1961*c*), could not be found, but this failure is quite understandable because the frequency of segregated transduction of resistance factors with phage Plkc is rather low (Watanabe and Fukasawa, 1961*c*).

DISCUSSION

In the present study, we have found that the spontaneous segregation of resistance factors in *S. typhimurium* LT-2 (Su, Sm, Cm, Tc) takes place with unusually high frequencies, enabling us to conduct a clone analysis of the spontaneous segregation of the resistance factors with replica plating. The types of spontaneous segregants were those with Tc-resistance factor alone, with Su-, Sm-, and Cm-resistance factors, and with no resistance factor. No other possible combinations of resistance factors could be found among the spontaneous segregants. Su-, Sm-, and Cm-resistance factors were spontaneously lost most frequently. Spontaneous segregants with no resistance factor were found with much lower frequencies, and those with Su-, Sm-, and Cm-resistance factors and no Tc-resistance factor were found with the lowest frequency. As Watanabe and Fukasawa (1961*a*) reported, *Salmonella* strains are not good recipients of R-factors. It is quite conceivable that this fact plays an important role in causing high frequencies of segregation, because the infection of the sensitive segregants with R-factors by the surrounding cells presumably does not occur easily.

Watanabe and Fukasawa (1961*c*) presented two linear models for the structure of an R-factor (Su, Sm, Cm, Tc) on the basis of their results of transduction in *E. coli* K-12 with phage Plkc and in *S. typhimurium* LT-2 with phage P-22. Those models are both compatible with the results of spontaneous segregation of resistance factors of the R-factor, as these authors pointed out. They have also presented a hypothesis that the mechanism of spontaneous segregation of resistance factors is due to the genetic exchange between the resistance factors carried by RTF and host genome; they suspected that the genetic homology between the resistance factors and the genome of *S. typhimurium* is very high. Since Watanabe and Fukasawa (1961*d*), in the kinetic studies of transfer of host chromosomal markers and R-factors by Hfr strains of K-12 carrying R-factors, have shown that R-factors can be attached to host chromosome, it can be safely assumed that the genetic exchange between R-factors and host genome takes place after R-factors are integrated. The integration of R-factors is assumed to suppress the autonomous replication of the R-factors, because they are episomes (Jacob, Schaeffer, and Wollman, 1960). Since the cells of enteric bacteria are usually multinucleate, and the number of particles of R-factors in each cell is assumed to be rather small, as discussed below, there may be a stage such that the cells with integrated R-factor possibly still have nuclei with no integrated R-factor. Thus, cells with no R-factor can be segregated by subsequent cell divisions. If genetic exchange occurs between R-factors and host chromosome at the integrated state of R-factors, cells with a part of the resistance factors can be produced. So far we have been unable to isolate cells with chromosome-incorporated resistance factors which cannot be transferred by conjugation because of the absence of RTF. This failure might be due to the fact that these cells are masked by the R-factors, with complementary resistance factors replicating autonomously in these cells. Strains LT-2 ≪Su, Sm, Cm≫ and LT-2 ≪Su, Sm, Cm, Tc≫ which cannot transfer their resistance factors by conjugation are interpreted to have a "defective" RTF, as discussed below.

Ochiai et al. (1959), Akiba et al. (1960), Nakaya, Nakamura, and Murata (1960), and Mitsuhashi et al. (1961) isolated *Shigella* and *E. coli* strains with various types of R-factors from natural sources. They are (Su, Cm, Tc), (Cm, Tc), (Su, Sm), and (Sm) in addition to (Su, Sm, Cm, Tc), (Su, Sm, Cm), and (Tc). (Cm) was isolated as a segregant of (Cm, Tc) by Mitsuhashi et al. (1961). Watanabe and Fuka-

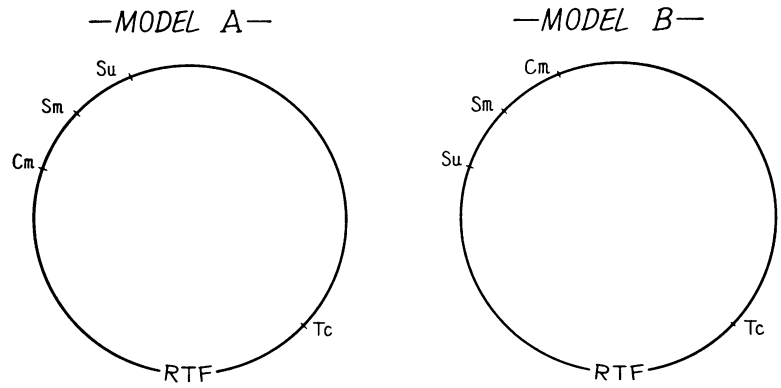

FIG. 1. *Circular models of four-drug-resistant R-factor. R-factor is a unit composed of RTF (resistance transfer factor) and resistance factors: sulfonamide-resistance factor (Su), streptomycin-resistance factor (Sm), chloramphenicol-resistance factor (Cm), and tetracycline-resistance factor (Tc). The relative distances between the markers do not represent the degree of linkage between them.*

sawa (1961*c*) assumed that the four-drug-resistant R-factor had possibly developed in a single step and not by multiple steps through the addition of resistance factors, in view of the epidemiological findings. The above-mentioned various R-factors are assumed to have arisen through segregation of resistance factors of the four-drug-resistant R-factor. The development of these R-factors can be accounted for on the hypothesis of genetic exchange between the four-drug-resistant R-factor and host genome, if we assume multiple crossovers between this R-factor and host genome. It may be possible that genetic exchange takes place between the segregant type of R-factor (Su, Sm, Cm) and host genome. It is also conceivable that RTF picks up only a part of the resistance factors from the original bacterial chromosome (Watanabe and Fukasawa, 1961*c*).

Campbell (*in press*) presented a hypothesis that the episomes in general have circular structures when they are in an autonomous state. This hypothesis was especially successful in explaining the anomalous linkage data of markers of vegetative phage and prophage of λ, which were reported by Calef and Licciardello (1960). Campbell succeeded in accounting for the mechanism of formation of λdg without assuming the presence of genetic homology between the galactose region of the host chromosome and phage λ. If we follow this hypothesis in R-factors, the structure of the R-factor (Su, Sm, Cm, Tc) may be presented as in Fig. 1. The circular structures of R-factors may be stretched to linear forms, when they are integrated, as was assumed by Campbell for phage λ. When they revert to the autonomous state, they will form circles again. When they reform circles, they may usually form the original structures but may rarely enclose a part of the host genome, leaving a part of the structures of the R-factors on the host chromosome. They might form smaller circles than the original ones without incorporating a part of the host genome. Both of the linear and circular models of R-factors seem to be compatible with our data of transduction and spontaneous segregation of resistance factors. We are currently studying the linkage relationship of the resistance factors and RTF of the integrated R-factors by transduction. By comparing the results of this study with those of the autonomous R-factors, we might be able to reach some significant conclusion about the feasibility of the circular models presented here.

We have also shown in the present study that the infection of the cells carrying an R-factor with another type of R-factor gives rise to cells with two types of R-factors. The two different types of R-factors can exist in the same cells independently of each other. These cells with two types of R-factors usually transfer one of the R-factors to the recipient cells at once but rarely transfer two different types of R-factors to the recipient cells at once. The cells with two different types of R-factors frequently segregate the cells with either type of the R-factors. Our finding that many of the clones of strains LT-2 (Su, Sm, Cm) and LT-2 ≪Su, Sm, Cm≫ that received

(Tc) did not have their original Su-, Sm-, and Cm-resistance factors is assumed to have been brought about by this mechanism. It is also possible that the spontaneous sensitive segregants contained in the recipient populations of LT-2 (Su, Sm, Cm) and LT-2 ≪Su, Sm, Cm≫ received (Tc) more easily than the recipient cells with the resistance factors (Watanabe and Fukasawa, 1962). At any rate, the frequent segregation of either one of the two types of R-factors may indicate that the number of R-factor particles that can be present in a cell is rather limited. The same situation may hold true for the cells with a single type of R-factor.

The cells with two different types of R-factors are occasionally converted to cells that can stably inherit the combinations of the resistance factors of the two R-factors. They transfer these resistance factors as a unit by conjugation and also by transduction with phage Plkc. The stable combination of the two types of R-factors is assumed to result from their recombination due to crossover.

In the course of the present study, two unusual strains of LT-2 with nontransmissible resistance factors were isolated; one of them was resistant to the four drugs and another was resistant to Su, Sm, and Cm. It was found that these strains segregate their resistance factors as frequently as LT-2 (Su, Sm, Cm, Tc) and LT-2 (Su, Sm, Cm). UV irradiation could not induce the transfer of the resistance factors by conjugation in these unusual strains but the transfer of a segregant type R-factor (Tc) to strain LT-2 ≪Su, Sm, Cm≫ converted some of the recipient cells to forms able to transfer all of the four resistance factors as a unit by conjugation. Some other recipient clones were able to transfer only (Tc). These clones with the four resistance factors were found to segregate the clones with either (Tc) or ≪Su, Sm, Cm≫ with high frequencies. These results indicate that LT-2 ≪Su, Sm, Cm≫ has autonomously replicating R-factors but cannot transfer them by conjugation on account of some defect of the R-factors. This defect apparently can be complemented by close association of the defective R-factor with a segregant R-factor (Tc), suggesting that the defect is possibly in RTF. We can consider that this defective RTF is comparable to the defective mutants of phages (Jacob, 1960). We cannot test this hypothesis with strain LT-2 ≪Su, Sm, Cm, Tc≫, but because of its similarity to LT-2 ≪Su, Sm, Cm≫ in its spontaneous segregation of resistance factors, it is also assumed to have a defective mutant of RTF.

According to H. Ozeki (*personal communication*), we might not have to assume the genetic exchange between R-factors and host genome to account for the mechanism of the spontaneous segregation of resistance factors. If the replication of the R-factor starts at one end of it and stops on its way for some unknown reason, segregant types of R-factors could be produced. A difficulty of this hypothesis is the lack of explanation for the surprisingly high frequencies of spontaneous segregation of the cells with segregant types of R-factors in *S. typhimurium* without postulating the suppression of the original type of R-factor. However, this difficulty can be overcome if we assume that the number of particles of R-factors that can be present in a cell of *S. typhimurium* is small.

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