COMBINATION OF TWO TYPES OF TRANSMISSIBLE DRUG-RESISTANCE FACTORS IN A HOST BACTERIUM

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ABSTRACT

MITSUHASHI, SUSUMU (Gunma University, Maebashi, Japan), KENJI HARADA, HAJIME HASHIMOTO, MITSUO KAMEDA, AND MITSUE SUZUKI. Combination of two types of transmissible drug-resistance factors in a host bacterium. J. Bacteriol. **84**:9–16. 1962.—When two types of R factor, R(TC) and R (CM.SM.SA), or R(TC) and R (CM), were brought together in a host bacterium by superinfection with both factors, loss of either one or both factors was found. In the imperfectly stable existence of both factors in a host bacterium, both factors were transmitted separately by conjugation. As the result of interaction between the two types of R factor present in a host bacterium, recombinant factors were formed, R_{25} (TC.CM.SM.SA) and R_{31} (CM.TC). The recombinant factors were able to transfer their resistance by conjugation. They were also transduced as one unit into *Escherichia coli* K12 by Plkc phage in the same fashion as the original R_{11} (TC.CM.SM.SA) and R_{14} (CM.TC) factors independently isolated from dysenteric patients.

The first isolation of multiply resistant *Shigella* was reported by Kitamoto et al. (1956). This organism was resistant to four drugs: tetracycline (TC), chloramphenicol (CM), streptomycin (SM), and sulfanilamide (SA). *Escherichia coli* strains resistant to these four drugs were also isolated in an epidemic of *S. flexneri* 3a resistant to the same agents (Matsuyama et al., 1958). *S. flexneri* 2a and *E. coli*, which were resistant to CM, SM, and SA, were also isolated in another epidemic in 1958 (Mitsuhashi, Harada, and Hashimoto, 1960*a*). *E. freundii* and *E. coli*, which were resistant to TC, CM, SM, and SA, were isolated in 1959 from a dysenteric patient (Harada et al., 1959). Many shigellae isolated from human cases of dysentery in Japan have been found to be multiply resistant, and approximately 10% of the shigellae isolated in Japan in 1959 were found to be resistant to TC, CM, SM, and SA (Ochiai, 1959; Harada, Kameda, and Suzuki, 1960*a*). From the epidemiological investigation of multiply resistant strains of shigellae and *E. coli* found in the feces of human beings, we have learned that multiply drug-resistant *Shigella* strains suddenly appeared in Japan in 1956, and that shigellae, *E. coli*, and *E. freundii* were resistant to the four drugs from the very beginning (Mitsuhashi et al., 1961*a*, *b*). It was found that multiple drug resistance was transferred in vitro from resistant *E. coli* to shigellae, and also from resistant shigellae to *E. coli* (Ochiai et al., 1959; Akiba et al., 1960).

We confirmed this finding and found that transmission is not mediated by transduction, transformation, or a filtrable agent, but by cell-to-cell contact (Mitsuhashi et al., 1960*a*; Harada et al., 1961*a*). This was also confirmed by blender treatment (Watanabe and Fukasawa, 1960). We have studied the genetics of this transmissible drug resistance. Our results and the results of others may be summarized as follows. (i) F factor is not necessary for the transfer of drug resistance from resistant *Shigella* to *E. coli* K12 or the substrains of *E. coli* K12 (Mitsuhashi et al., 1960*a*). (ii) Transmissible drug resistance can be transferred to many other bacteria, including most genera of *Enterobacteriaceae* (Harada et al., 1960*c*; Nakaya and Nakamura, 1960). (iii) When TC resistance is transferred to a (CM.SM.SA) resistant recipient by conjugation, or vice versa, the recipient strain becomes resistant to (TC.CM.SM.SA) and is further able to transfer its (TC.CM.SM.SA) resistance by conjugation (Mitsuhashi et al., 1960*a*). (iv) The transmissible drug resistance can also be transferred by transduction in *E. coli* K12 with phage Plkc (Nakaya and Nakamura, 1960; Watanabe and Fukusawa, 1961*a*; Kondo, Harada, and

Mitsuhashi, 1961, 1962), in *Salmonella typhimurium* LT-2 with phage P-22 (Watanabe and Fukusawa, 1961*a*), and in *Salmonella* E group with phages ϵ_{15} and ϵ_{34} (Harada et al., 1960*b*). (v) A majority of the resistant transductants of *S. typhimurium* LT-2 produced with phage P-22 and of *Salmonella* E group produced with phage ϵ cannot transfer their resistance to sensitive recipients by conjugation, whereas the transductants of K12 with Plkc are able to transfer their resistance by conjugation. (vi) The transmissible drug resistance is eliminated spontaneously on standing, or by treatment with acridine dyes (Mitsuhashi, Harada, and Kameda, 1960*b*, 1961*c*, *d*; Watanabe and Fukusawa, 1961*b*). (vii) The term "R" (resistance) was proposed for this transmissible drug-resistance factor (Mitsuhashi, 1960). (viii) Watanabe and Fukusawa (1960) suggested the possibility that this type of drug resistance may be carried by an episome "Rtf" (resistance-transfer factor). (ix) The frequency of transfer of the intrinsic chromosomal markers of Hfr decreased markedly in the cross of Hfr $R^+ \times F^-R^-$, as if in a cross of $F^+ \times F^-$ (Nakaya, Nakamura, and Murata, 1960). (x) The resistant transductants of *Salmonella* produced with phage ϵ cannot transfer their resistance to sensitive recipients by conjugation, but again acquire the ability to transfer their resistance when infected with F′ (Hirota, 1959; Harada et al., 1961*b*). (xi) The infection by R factors of certain F^- strains results in the acquisition by the infected cells (R^+ cells) of an ability to form recombinant progeny in crosses with other F^- strains (Sugino and Hirota, *to be published*). (xii) Seven kinds of R factor are found from independently isolated bacteria: R(TC.CM.SM.SA), R(CM.SM.SA), R(TC.SM.SA), R(SM.SA), R(CM.TC), R(SM), and R(TC). The R(CM) factor is found in a segregant of a strain carrying the R(CM.TC) factor (Mitsuhashi et al., 1961*e*). (xiii) The transmission frequency of another kind of R factor to R^+ cells is relatively low, and separate loss or complete loss of both factors is found. Unstable or imperfectly stable existence of two kinds of R factor is observed in a host bacterium carrying two kinds of R factor (Harada et al., 1961*c*; Hashimoto et al., 1961*a*, *b*).

We report in this paper the combination of two kinds of R factor [R(TC) and R(CM.SM.SA); R(TC) and R(CM)] and formation of new types of R factor [R(TC.CM.SM.SA) and R(CM.TC)] in a host bacterium carrying two kinds of R factor by superinfection.

MATERIALS AND METHODS

Microorganisms. The different R factors are distinguished by a numerical suffix, and the drug-resistance pattern conferred by them is shown in parentheses. The donor of R factors was *E. coli* K12 substrain PA 200 F^- auxotroph R_{17}^+ (TC), R_{12}^+ (CM.SM.SA), R_{19}^+ (CM), and R_{20}^+ (TC). The R_{19} (CM) and R_{20} (TC) factors were obtained by segregation from *E. coli* K12 R_{14}^+ (CM.TC) strain in a transduction with phage Plkc (Kondo et al., 1962). The R_{17} (TC) and R_{12} (CM.SM.SA) factors were obtained by us from drug-resistant shigellae isolated from human beings in the field survey. The R_{14} (CM.TC) factor was obtained from *S. flexneri* 3b(N-1) resistant to (CM.TC.SA) and was supplied by K. Ochiai.

E. coli K12 F^+ prototroph R_{17}^+(TC), R_{12}^+(CM.SM.SA), R_{19}^+(CM), R_{20}^+(TC), *S. flexneri* 3a R_{17}^+(TC), R_{12}^+(CM.SM.SA), *E. coli* O-26G R_{17}^+(TC), and R_{12}^+(CM.SM.SA) strains were used as the recipients of R factors. The strains used are shown in Tables 1 and 2. Culture methods, conjugation conditions, and selection of clones that received R factors were the same as those described in the preceding paper (Harada et al., 1960*c*).

Blender treatment. A sample was withdrawn from the mixed culture of both donor and recipient strains every 5 min and diluted 1 to 100 with glycerine phosphate buffer. The diluted sample was treated in the blender at 15,000 rev/min for 15 sec in the cold and allowed to stand for 15 min at 37 C; 0.1 ml of this material was streaked on an appropriate selective medium, and the time of R factor transmission, transmission frequency, and the drug resistance of recipient strains were determined.

Transduction of R factors with Plkc phage. Transduction was conducted according to the method described by Lennox (1955). The transduction rate was defined as the number of transductants which received R factor divided by the number of adsorbed phage.

RESULTS

Transmission of R_{17} (TC) factor to R_{12}^+ (CM.SM.SA) recipient strains and vice versa. Three types of drug-resistant strains were obtained:

resistant to (TC.CM.SM.SA), (CM.SM.SA), and (TC) alone (Table 1). Either (CM.SM.SA) or (TC) resistance of the recipient strains indicates that one or both of the R_{12}(CM.SM.SA) and R_{17}(TC) factors was lost after several generations of growth of the recipient host strains, which had carried two kinds (R_{12} and R_{17}) of R factor by superinfection. Experiment no. 1–3 in Table 1 show that the loss of the R_{17}(TC) factor was more frequent than that of the R_{12}(CM.SM.SA) factor from the host strains carrying both R_{17} and R_{12} factors.

The (TC.CM.SM.SA) resistance of the host strains obtained by superinfection with both R_{17} (TC) and R_{12}(CM.SM.SA) factors. The (TC.CM.SM.SA) resistance of both *S. flexneri* 3a and *E. coli* O-26G, which occurred after superinfection with both R_{17}(TC) and R_{12}(CM.SM.SA) factors, was lost in high frequency on heart infusion (HI) agar without drugs. Complete loss, as well as separate loss, was found. In the separate loss, either (CM.SM.SA) resistance together or (TC) resistance alone was lost. In the complete loss, bacteria became sensitive to TC, CM, SM, and SA (Table 1).

The (TC.CM.SM.SA) resistance of *E. coli* K12 was more stable than that of *S. flexneri* 3a and *E. coli* O-26G, and was maintained without separate or complete loss by subculturing on HI agar without drugs. But the separate transfer of either (TC) or (CM.SM.SA) resistance of *E. coli* K12 was found in high frequency when its (TC.CM.SM.SA) resistance was transmitted to *S. flexneri* 3a R^- strain by conjugation. The unstable existence of two types of R factor in a host bacterium was reported (Hashimoto et al., 1961*b*) and will be described elsewhere (Hashimoto, Harada, and Mitsuhashi, *to be published*).

Combination of R_{17} and R_{12} factors which exist doubly in S. flexneri 3a after subculturing on HI agar containing CM and TC. *S. flexneri* 3a resistant to (TC.CM.SM.SA) was obtained by transfer of R_{17}(TC) factor from *E. coli* K12 R_{17}^+ to *S. flexneri* 3a R_{12}^+(CM.SM.SA), as shown in experiment 1 of Table 1. *S. flexneri* 3a resistant to (TC.CM.SM.SA) was subcultured three times on HI agar containing CM (25 μg/ml) and TC (25 μg/ml).

S. flexneri 3a thus obtained was resistant to (TC.CM.SM.SA), and separate loss of neither (TC) nor (CM.SM.SA) resistance was found in 875 colonies grown on HI agar without drugs.

Transmission of (TC.CM.SM.SA) resistance of S. flexneri 3a obtained by superinfection with R_{17} (TC) and R_{12}(CM.SM.SA) factors. *S. flexneri* 3a resistant to (TC.CM.SM.SA) thus obtained was subcultured three times on HI agar containing both CM (25 μg/ml) and TC (25 μg/ml). This culture was then used as the donor of drug resistance by conjugation. The transmission of the R(TC.CM.SM.SA) factor originally found in a strain of bacterium isolated from a dysenteric

TABLE 1. *Drug-resistance patterns of the recipients obtained by superinfection with R_{17} and R_{12} factors**

Expt no.	Conjugation system: Donor	Conjugation system: Recipient	Selective agent	Recipient strain	Drug-resistance patterns of recipients: (TC.CM.SM.SA)	(CM.SM.SA)	(TC)
					%	%	%
1	R_{17}(TC) × R_{12}(CM.SM.SA)		TC	*S. flexneri* 3a	95.4	1.5	3.2
				E. coli O-26G	9.5	90.5	0
				E. coli K-12	100.0	0	0
2	R_{12}(CM.SM.SA) × R_{17}(TC)		CM	*S. flexneri* 3a	16.7	83.3	0
				E. coli O-26G	60.3	39.8	0
				E. coli K-12	100.0	0	0
3	R_{12}(CM.SM.SA) × R_{17}(TC)		SM	*S. flexneri* 3a	32.2	67.7	0
			+	*E. coli* O-26G	65.5	34.3	0
			SA	*E. coli* K-12	100.0	0	0

* The donor of R factors was *E. coli* K-12 strain PA 200 R^+_{17}(TC) or R^+_{12}(CM.SM.SA), auxotrophic for threonine, thiamine, histidine, arginine, and with the markers: lac^-, gal^-, mal^-, mtl^-, xyl^-, Tr_1. The recipient strains were *S. flexneri* 3a, *E. coli* O-26G, or *E. coli* K-12 prototrophs. The cultures of both donor and recipient strains were streaked on an appropriate selective medium. The recipient colonies thus acquiring drug resistance were restreaked on HI agar for single-colony isolation, and the drug resistance of 300 colonies thus obtained was determined.

patient was compared with that of (TC.CM.SM.SA) resistance of *Shigella* made resistant by superinfection. The transmission frequency of both the R_{11}(TC.CM.SM.SA) factor and (TC.CM.SM.SA) resistance produced by superinfection with R_{17}(TC) and R_{12}(CM.SM.SA) factors was almost the same (10^{-5} to $10^{-5.2}$), and no difference in transmission frequency of (TC.CM.SM.SA) resistance thus made was found by selected markers: CM, TC, or (CM.TC). There was no difference in transmission of (TC.CM.SM.SA) resistance between the R_{11} (TC.CM.SM.SA) factor and (TC.CM.SM.SA) resistance of *S. flexneri* 3a produced by superinfection with R_{17}(TC) and R_{12}(CM.SM.SA) factors (Fig. 1 and 2).

All colonies of *E. coli* K12 which received the drug resistance of *S. flexneri* 3a produced by superinfection with R_{17}(TC) and R_{12}(CM.SM.SA) factors were resistant to TC, CM, SM, and SA, even when the mixed culture of donor and recipient strains was treated with a blender for

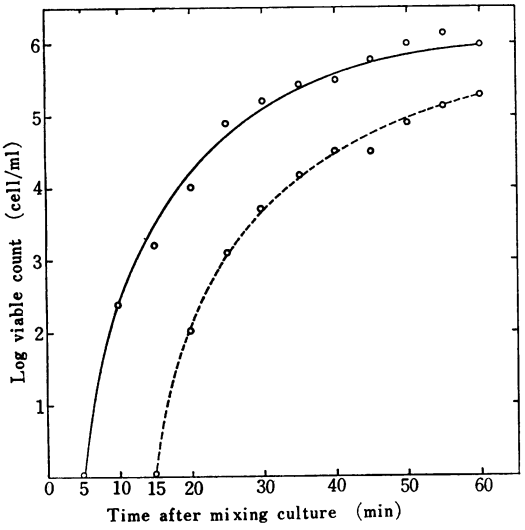

FIG. 1. *Transmission of R_{11} (TC.CM.SM.SA) factor isolated from a dysenteric patient. Shigella flexneri 3a R^+_{11} (TC.CM.SM.SA) was used as the donor of R factor. The recipient strain of R factor was Escherichia coli K-12 R^-. Number of bacteria used for mixed cultivation: 10^9/ml of S. flexneri 3a. $10^{8.7}$/ml of E. coli K-12 R^-. Selective medium contained CM (25 μg/ml) and TC (25 μg/ml). The ordinate indicates the number of bacteria which acquired (TC.CM.SM.SA) resistance.* O———O: *without blendor treatment before plating.* O- - - -O: *with blendor treatment before plating.*

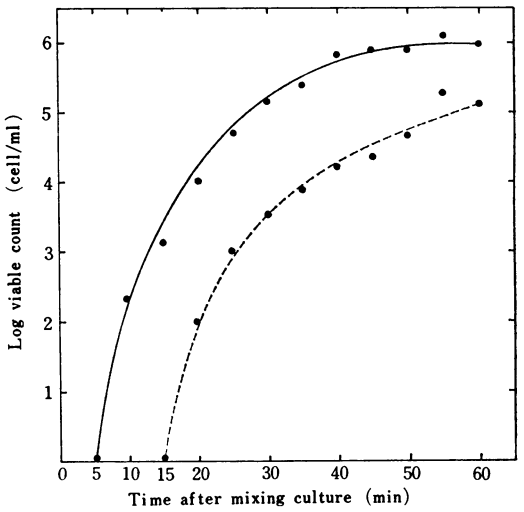

FIG 2. *Transmission of (TC.CM.SM.SA) resistance of Shigella flexneri 3a. S. flexneri 3a resistant to (TC.CM.SM.SA) was used as the donor of (TC.CM.SM.SA) resistance, which was obtained by superinfection with R_{17} (TC) and R_{12} (CM.SM.SA) factors. Escherichia coli K-12 R^- was the recipient. Number of bacteria used for mixed cultivation: $10^{9.1}$/ml of S. flexneri 3a resistant to (TC.CM.SM.SA), $10^{8.7}$/ml of E. coli K-12 R^-. Selective medium contained CM (25 μg/ml) and TC (25 μg/ml). The ordinate indicates the number of bacteria which acquired (TC.CM.SM.SA) resistance.* ●———●: *without blendor treatment before plating.* ●- - - -●: *with blendor treatment before plating.*

20 min after mixing and before plating on an appropriate selective medium. This indicates that the (TC.CM.SM.SA) resistance of *S. flexneri* 3a was transmitted to *E. coli* K12 R^- strain as one unit and in the same frequency as R_{11}(TC.CM.SM.SA) factor isolated from a dysenteric patient.

Transduction of (TC.CM.SM.SA) resistance of S. flexneri 3a obtained by superinfection with R_{17}(TC) and R_{12}(CM.SM.SA) factors. The donors of drug resistance in transduction with Plkc phage were *E. coli* K12 R_{11}^+ (TC.CM.SM.SA) strain and *E. coli* K12 strain resistant to (TC.CM.SM.SA), to which (TC.CM.SM.SA) resistance had been transmitted by conjugation from (TC.CM.SM.SA) resistant *S. flexneri* 3a obtained by superinfection.

In *E. coli* K12 R_{11}^+(TC.CM.SM.SA), 92.2% of the transductants were resistant to four drugs (TC, CM, SM, and SA); and 1.7% were resistant to CM, SM, and SA; and 6.0% were resistant to

TC alone. In *E. coli* K12 resistant to (TC.CM.SM.SA), obtained by superinfection with R_{17}(TC) and R_{12}(CM.SM.SA) factors, 86 to 89% of the transductants were resistant to TC, CM, SM, and SA; 1 to 2% were resistant to CM, SM, and SA; and 10 to 12% were resistant to TC alone (Table 2).

All of the transductants were able to transfer their drug resistance by conjugation. From these results, we shall refer to the newly formed R factor as R_{25}(TC.CM.SM.SA), which is able to transfer (TC.CM.SM.SA) resistance by conjugation and to be transduced into *E. coli* K12 with Plkc phage. The transduction rate of (TC.CM.SM.SA) resistance obtained by superinfection with R_{17}(TC) and R_{12}(CM.SM.SA) factors did not differ from that of the R_{11}(TC.CM.SM.SA) factor obtained from a dysenteric patient.

The fact that 86 to 89% of the transductants were resistant to TC, CM, SM, and SA indicates that R(TC.CM.SM.SA) factor was newly formed by combination of both factors in a host bacterium carrying both R_{17}(TC) and R_{12}(CM.SM.SA) factors and can be transduced by phage as one unit in the same fashion as R_{11}(TC.CM.SM.SA) factor found in a bacterium isolated independently from a dysenteric patient.

Transmission of R_{19}(CM) factor to R_{20}^+(TC) recipient strains or vice versa. Three types of drug resistance were obtained: (CM.TC), (CM), and (TC). The alternative (CM) or (TC) resistance of the recipient strains shows that either one or both R_{19}(CM) and R_{20}(TC) factors was separately lost after subculturing of several generations from the host strains carrying R_{19}(CM) and R_{20}(TC) factors by superinfection. Experiment 2 in Table 3 shows that the loss frequency of R_{20}(TC) factor was higher than that of R_{19}(CM) factor from the host strains carrying both R_{19}(CM) and R_{20}(TC) factors.

Complete loss of both R_{19}(CM) and R_{20}(TC) factors was found, and sensitive strains were obtained from the host resistant to CM and TC.

The (CM.TC) resistance of the host strains obtained by superinfection with both R_{19}(CM) and R_{20}(TC) factors. The (CM.TC) resistance ob-

TABLE 2. *Transduction of both R_{11}(TC.CM.SM.SA) factor and (TC.CM.SM.SA) resistance* of Escherichia coli K-12 with Plkc phage*

R factor	MOI†	Transduction rate	Drug-resistance patterns of transductants		
			(TC)	(CM.SM.SA)	(TC.CM.SM.SA)
			%	%	%
R_{11}(TC.CM.SM.SA)	13	10^{-7}	6.0	1.7	92.2
(TC.CM.SM.SA) resistance	12, 14	10^{-7}	10, 12‡	1, 2	86, 89

* The drug-resistance of 300 colonies of the transductants was determined. The (TC.CM.SM.SA) resistance of *E. coli* K-12 was transferred by conjugation from *S. flexneri* 3a resistant to (TC.CM.SM.SA), which was obtained by superinfection with R_{17}(TC) and R_{12}(CM.SM.SA) factors.

† MOI: multiplicity of infection.

‡ The results of two experiments are shown.

TABLE 3. *Substitution of R factor in a host bacterium after superinfection with another type of R factor**

Expt no.	Conjugation system		Selective drug	Recipient strain	Drug-resistance patterns of recipients		
	Donor	Recipient			(CM.TC)	(CM)	(TC)
					%	%	%
1	R_{19}(CM)	× R_{20}(TC)	CM	*E. coli* O-26G	31.7	68.2	0
2	R_{20}(TC)	× R_{19}(CM)	TC	*E. coli* O-26G	12.5	0	87.5

* The donor of R factors was *S. flexneri* 3a R^+_{19}(CM) or R^+_{20}(TC). The recipient strain was *E. coli* O-26G. The cultures of both donor and recipient strains were streaked on an appropriate selective medium containing CM (25 μg/ml) or TC (25 μg/ml). The recipient colonies were restreaked on the same selective medium for single-colony isolation, and the drug resistance of 200 colonies thus obtained was determined.

tained by superinfection with both R_{19}(CM) and R_{20}(TC) factors was lost completely or separately by subculturing on HI agar without drugs. A total of 1,000 colonies, on HI agar without drugs, was obtained from 100 *E. coli* K12 colonies, which were made resistant to (CM.TC) by superinfection with both R_{19}(CM) and R_{20}(TC) factors. Of the 1,000 colonies, 32.5% were resistant to (CM.TC), 40.6% resistant to (CM), 26.3% resistant to (TC) alone, and 0.6% sensitive.

By three successive selections of *E. coli* K12 carrying both R_{19}(CM) and R_{20}(TC) factors, six strains of (CM.TC) resistant *E. coli* K12 were obtained. They did not show separate loss of either CM or TC resistance by subculturing on free HI agar.

Transmission of (CM.TC) resistance of E. coli K12 to S. flexneri 3a by conjugation. All colonies of *S. flexneri* 3a obtained on an appropriate selective medium containing either CM (25 μg/ml) or TC (25 μg/ml) after mixed cultivation received (CM.TC) resistance by conjugation from six strains of *E. coli* K12 resistant to (CM.TC), which were obtained by superinfection with both R_{19}(CM) and R_{20}(TC) factors (Table 4).

Transduction of (CM.TC) resistance of E. coli K12 obtained by superinfection with R_{19}(CM) and R_{20}(TC) factors. The donors of drug resistance in transduction with Plkc phage were *E. coli* strain K12 R_{14}+(CM.TC) and a (CM.TC) resistant strain of *E. coli* K12, which was obtained by superinfection with R_{19}(CM) and R_{20}(TC) factors.

TABLE 4. *Transmission of (CM.TC) resistance of Escherichia coli K-12 obtained by superinfection with R_{19}(CM) and R_{20}(TC) factors**

Donor *E. coli* K-12 resistant to (CM.TC)	Selective drugs	
	CM	TC
2-0	104/104†	104/104†
2-2	104/104	104/104
2-3	104/104	104/104
97-1	104/104	104/104
97-2	104/104	104/104
97-3	104/104	104/104
R_{14}(CM.TC)‡	104/104	104/104

* The recipient strain was *S. flexneri* 3a.

† Number of (CM.TC) resistant colonies/number of tested colonies.

‡ R_{14}(CM.TC) factor was obtained from a dysenteric patient, and R_{19}(CM) and R_{20}(TC) factors were obtained by segregation of R_{14}(CM.TC) factor in transduction with Plkc phage.

TABLE 5. *Transduction of both R_{14}(CM.TC) factor and (CM.TC) resistance of Escherichia coli K-12**

R factor	MOI†	Transduction rate	Drug-resistance patterns of transductants		
			(TC)	(CM)	(CM.TC)
			%	%	%
R_{14}(CM.TC)..	12	10^{-7}	48.1	1.3	50.7
(CM.TC) resistance‡....	13	10^{-7}	45.5	0.3	54.4

* *E. coli* K-12 prototroph was used as a recipient strain. The drug resistance of 300 colonies of the transductants was determined.

† MOI: multiplicity of infection.

‡ The (CM.TC) resistance of *E. coli* K-12 was transferred by conjugation from *S. flexneri* 3a resistant to (CM.TC), which was obtained by superinfection with R_{19}(CM) and R_{20}(TC) factors.

In *E. coli* K12 R_{14}+(CM.TC) used as the donor of drug resistance, 50.7% of the transductants were resistant to (CM.TC), 48.1% were resistant to TC alone, and 1.3% were resistant to CM alone. In (CM.TC) resistant *E. coli* K12, obtained by superinfection with R_{19}(CM) and R_{20}(TC) factors, 54.3% of the transductants were resistant to (CM.TC), 45.4% were resistant to TC alone, and 2.3% were resistant to CM alone. From these results, we shall refer to the newly formed R factor as R_{31}(CM.TC), which is able to transfer (CM.TC) resistance by conjugation and to be transduced into *E. coli* K12 with phage Plkc (Table 5).

DISCUSSION

Polylysogenic subclones are found in most of the colonies of *E. coli* K12 surviving a multiple infection with genetically marked λ phages (Arber, 1960). If cells carrying a P2 prophage are superinfected with the heteroimmune phage P2 Hy *dis*, most of the infected cells lyse, but the cells still carying the old prophage are found among the remaining progeny (Cohen, 1959). The doubly lysogenic strains yield singly lysogenic or recombinant types (Arber, 1960). In the

field survey of drug-resistant *Shigella* and *Escherichia* strains, seven types of transmissible drug-resistance factor were obtained: R(TC.CM.SM.SA), R(CM.SM.SA), R(TC.SM.SA), R(CM.TC), R(SM.SA), R(SM), and R(TC). From the segregation of the R(CM.TC) factor, an R(CM) factor was obtained. In the previous paper, it was reported that the R^+(CM.SM.SA) cells became resistant to four drugs (CM.TC.SM.SA) when infected with R(TC) factor by mixed cultivation, and vice versa (Mitsuhashi et al., 1960*a*). The cells which thus acquired resistance to four drugs (CM.TC.SM.SA) were able to transfer their (TC.CM.SM.SA) resistance to recipient cells by mixed cultivation. In the host bacterium carrying two types of R factor by superinfection, the loss of either or both factors was found.

From (TC.CM.SM.SA) resistant *E. coli* K12 carrying R(TC) and R(CM.SM.SA) factors, *E. coli* K12 R^+(TC), *E. coli* K12 R^+(CM.SM.SA), and *E. coli* K12 R^- strains were found by subculturing on free HI agar. In the case of (CM.TC) resistant *E. coli* K12 carrying both R(TC) and R(CM) factors, *E. coli* K12 R^+(TC), *E. coli* K12 R^+(CM), and *E. coli* K12 R^- strains were found by subculturing on free HI agar. When the (TC.CM.SM.SA) resistance of *E. coli* K12 strain carrying doubly R(TC) and R(CM.SM.SA) factors was transferred to the recipient strains by conjugation, either (TC) resistant or (CM.SM.SA) resistant strains were obtained. By the transmission of (CM.TC) resistance of *E. coli* K12 strain carrying R(CM) and R(TC) factors, either (TC) resistant or (CM) resistant strains were obtained. These results suggest that two types of R factor, R(TC) and R(CM.SM.SA) or R(TC) and R(CM), can exist separately in a host bacterium. The stable or unstable existence of two types of R factor in a host bacterium will be described elsewhere (Hashimoto et al., *to be published*).

From the experiment described above, it can be concluded that a new type of R factor was formed by the combination of two R factors [R(TC) and R(CM.SM.SA), R(TC) and R(CM)] present doubly in a host bacterium. The R_{25}(TC.CM.SM.SA) factor newly formed by the combination of the R_{17}(TC) and R_{12}(CM.SM.SA) factors was able to transfer its resistance by conjugation and to be transduced as one unit into *E. coli* K12 by phage Plkc. The R_{31}(CM.TC) factor formed by the combination of R_{19}(CM) and R_{20}(TC) factors was also able to transfer its resistance by conjugation and to be transduced by phage Plkc just like the original R_{14}(CM.TC) factor from which R_{19}(CM) and R_{20}(TC) factors were obtained.

The formation of a new R factor in a host bacterium carrying two R factors may result from two possibilities: genetic recombination between two R factors present in a host bacterium, and combination of two R factors in a host bacterium by a mechanism which is not clearly understood.

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