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EPISOME-MEDIATED TRANSFER OF DRUG RESISTANCE IN ENTEROBACTERIACEAE

VII. TWO TYPES OF NATURALLY OCCURRING R FACTORS

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ABSTRACT

WATANABE, TSUTOMU (Keio University, Tokyo, Japan), HIROSHI NISHIDA, CHIZUKO OGATA, TOSHIHIKO ARAI, AND SACHIKO SATO. Episome-mediated transfer of drug resistance in Enterobacteriaceae. VII. Two types of naturally occurring R factors. J. Bacteriol. **88**:716-726. 1964.—Naturally occurring R factors are classified into two types, *fi*$^+$ and *fi*$^-$, depending on their *fi* characters. The term *fi* is an abbreviation of fertility inhibition and *fi*$^+$ and *fi*$^-$ mean, respectively, the presence and absence of suppression of the functions of the sex factor F of *Escherichia coli* K-12. It was found that *fi*$^-$ R factors reduce the efficiency of plating of phages λ and T_1 in K-12; *fi*$^+$ R factors did not have this inhibitory action. One of the *fi*$^-$ R factors reduced the efficiency of plating of phage T_7 as well. Phages λ and T_1 underwent host-induced modifications in the host carrying some *fi*$^-$ R factors. At least two types of *fi*$^-$ R factors were recognized by the types of their restriction and host-induced modification of these phages. $CaCl_2$ exhibited antagonistic actions against the restrictions of phages λ and T_1 by *fi*$^-$ R factors. Transduction of the ability to ferment galactose with HFT lysates of λ was reduced by *fi*$^-$ R factors. Ultraviolet induction of λ was not affected by any R factors. Furthermore, adsorption of phages λ and T_1 was not altered by the presence of any R factors. From these results, we concluded that the suppression of progeny formation of these phages by *fi*$^-$ R factors is due to some step(s) after adsorption of the phages to the bacteria. Superinfection immunity and mutual exclusion were found between two different *fi*$^+$ R factors but not between *fi*$^+$ and *fi*$^-$ R factors. The two different *fi*$^+$ R factors were frequently genetically recombined, but *fi*$^+$ and *fi*$^-$ R factors were not genetically recombined, as indicated by findings of independent transfer of these R factors by conjugation and by transduction from the donors having these two R factors. It was assumed from these findings that *fi*$^+$ and *fi*$^-$ R factors are considerably different episomes having different resistance-transfer factors.

Since the discovery of multiple-drug resistance R factors (or episomic drug-resistance factors), which confer upon host bacteria resistance to sulfonamide (Su), streptomycin (Sm), chloramphenicol (Cm), and tetracycline (Tc), many types of R factors carrying various combinations of these drug-resistance markers have been found in Japan (*see* Watanabe, 1963*a*). R factors have also been isolated in England by Datta (1962) and in West Germany by Lebek (1963*a*, *b*). The multiple-drug-resistant *Shigella* strains, which were isolated in Israel by Marberg, Altmann, and Eshkol-Bruck (1958), were recently reported to have R factors (Nakaya, *personal communication*). Accordingly, the geographical distribution of R factors is now not restricted to Japan but is apparently a world-wide problem. The R factor isolated by Lebek (1963*a*) was found to give to host cells kanamycin and neomycin resistance in addition to resistance to the above four drugs (Watanabe, Ogata, and Sato, 1964). Thus, R factors can be classified by their drug-resistance markers. On the other hand, it was found by Watanabe and Fukasawa (1962*b*) and Watanabe, Fukasawa, and Takano (1962) that some of the R factors suppress the functions of the sex factor F of *Escherichia coli* K-12 whereas others do not. The R factors which suppress the functions of F were formerly called type 1 (Watanabe et al., 1962) and recently were designated as *fi*$^+$ (Watanabe, 1963*b*). The term *fi* is an abbreviation of fertility inhibition. The second type, formerly called type 2, is now called *fi*$^-$. All of the naturally occurring *fi*$^-$ R factors which we have studied have been found to lack the Cm-resistance marker. The *fi* character of R

factors was ascribed to the RTF (resistance transfer factor), which occupies a certain position on the structure of R factors and which controls the episomality of R factors (Watanabe, 1963*a*).

Sugino and Hirota (1962) reported that both *fi*$^+$ and *fi*$^-$ R factors cause mating in F$^-$ strains of *E. coli* K-12, although Watanabe and Fukasawa (1962*b*) succeeded in mating of F$^-$ strains only with *fi*$^-$ R factors. Arber (*personal communication*) recently found with two of our R factors that an *fi*$^-$ R factor reduced the efficiency of plating (EOP) of phage λ in K-12, although an *fi*$^+$ R factor did not possess this inhibitory action. We have studied Arber's findings using more R factors of independent origins, and we have not only confirmed his results but also have found that phages T_1 and T_7 are also inhibited in their progeny formation by *fi*$^-$ R factors but not by *fi*$^+$ R factors. Yoshikawa and Akiba (1962), in studies independent of Arber's and of ours, found the reduction of EOP of phages λ and P1 by *fi*$^-$ R factors. Furthermore, they showed that phage λ undergoes host-induced modifications in the hosts carrying *fi*$^-$ R factors. We have studied host-induced modifications of phages λ, T_1, and T_7 by various *fi*$^-$ R factors and have obtained some interesting results. Watanabe and Fukasawa (1962*b*) found that R factors in recipients suppress the transfer of the other R factors to about 1% of that found with recipients not having R factor. Since they employed only *fi*$^+$ R factors in their experiments, we have repeated similar experiments using both *fi*$^+$ and *fi*$^-$ R factors. Our results are striking in that suppression of transfer was not found between *fi*$^+$ and *fi*$^-$ R factors, whereas it occurred between two different *fi*$^+$ R factors as well as between two different *fi*$^-$ R factors. It was shown by Watanabe and Lyang (1962) that bacteria carrying two *fi*$^+$ R factors with different drug-resistance markers are genetically very unstable under such conditions. One of the two R factors is segregated, or genetic recombination of the two types of R factors occurs. Mitsuhashi et al. (1962) also reported on similar findings with naturally occurring R factors. We have reinvestigated this point, employing more R factors of both *fi*$^+$ and *fi*$^-$ types and of independent origins. We have found that "mutual exclusion" does not take place between *fi*$^+$ and *fi*$^-$ R factors, whereas it occurs between two different *fi*$^+$ R factors. Furthermore, genetic recombination did not occur between *fi*$^+$ and *fi*$^-$ R factors. These results will be reported in detail in this paper, and the differences between *fi*$^+$ and *fi*$^-$ R factors will be discussed.

MATERIALS AND METHODS

Media. Liquid cultures were prepared in Penassay Broth (Difco) or in Lennox (1955) broth. Plating media were nutrient agar (Difco), bromothymol blue-lactose or -galactose nutrient agar (containing 2% lactose or galactose), and Lennox (1955) agar. Agar media were used either with or without enrichment with various concentrations of $CaCl_2$. (In some experiments, $MgCl_2$ or a mixture of $CaCl_2$ and $MgCl_2$ was added.) Semisolid media of nutrient agar or Lennox agar containing 0.7% agar were employed for phage titrations. Comparable results were obtained with the two media.

Drugs. Drugs used were those described in a previous paper (Watanabe and Fukasawa, 1961*a*).

Bacterial strains. Strains CSH-2 (methionine-requiring), W3102 (galactose-negative and λ$^-$), and W677/Pro$^-$T$_6^r$Sm^r (threonine-, leucine-, proline-, and vitamin B_1-requiring; mannitol-, xylose-, maltose-, galactose-, and lactose-negative; and resistant to phage T_6 and to high concentrations of Sm), all of which are F$^-$ substrains of *E. coli* K-12, were used.

Phage strains. Phage λ wild type (λ^{wt}), a clear mutant of λ (λ^{cI}), a virulent mutant of λ (λ^{vi}; *see* Jacob, 1960), the phages of T series, and phage P1kc were employed. The mutants of λ and the phages of T series were kindly supplied by Junichi Tomizawa.

R factors. The R factors employed are listed in Table 1. These R factors had been carried by *Shigella* strains isolated from dysentery patients of independent epidemics.

General phage techniques. General phage techniques followed were those described by Adams (1950). For determining the EOP of phages, aerated cultures in the exponential phase were used throughout the present study.

Method of transduction of ability to ferment galactose. HFT lysates of λ were prepared by the procedure of Morse, Lederberg, and Lederberg (1956), and transduction of ability to ferment galactose (*gal*$^+$) was also carried out by their

procedure, with W3102 as a recipient. *Gal*$^+$ transductants were scored on bromothymol blue-galactose nutrient agar.

Method of transduction of R factors. The method of transduction of R factors with phage P1kc in *E. coli* K-12 was described previously (Watanabe and Fukasawa, 1962*a*).

Method of elimination of R factors with acridines. The method of elimination of R factors with acridine orange and acriflavine was the same as that reported in a previous paper (Watanabe and Fukasawa, 1961*b*).

Conditions of ultraviolet induction of phage λ in CSH-2. CSH-2 strains with and without R factors were irradiated in Penassay Broth or in Lennox broth with a National germicidal lamp for various periods of time at room temperature, and were then aerated in a water bath (37 C) until the reduction in turbidity reached a minimum. Precautions were taken to avoid photoreactivation. When dose-response curves were to be determined, samples were taken at various time points from the ultraviolet-irradiated samples and assayed for free phage.

TABLE 1. *R factors employed in the present study*

Strain no. of R factor	Drug-resistance markers[a]	*f*[b]
N-1	Su, Sm, Tc	−
N-3	Su, Sm, Tc	−
N-6	Su, Sm, Tc	+
N-9	Su, Sm, Tc	+
R-15	Su, Sm	−
222	Su, Sm, Cm, Tc	+
K	Su, Sm, Cm, Tc	+
222-R$_3$[c]	Su, Sm, Cm	+
222-Tc[c]	Tc	+
K-R$_3$[d]	Su, Sm, Cm	+
K-Tc[d]	Tc	+

[a] Su, Sm, Cm, and Tc are the abbreviations of sulfonamide, streptomycin, chloramphenicol, and tetracycline, respectively.

[b] Presence (+) and absence (−) of suppression of the functions of F of *Escherichia coli* K-12.

[c] Spontaneous segregants from 222.

[d] Spontaneous segregants from K.

RESULTS

Efficiencies of plating of phage λ and its mutants on W3102 carrying various R factors. Phage λ^{wt} used was obtained by ultraviolet induction of CSH-2. Phages λ^{cI} and λ^{vi} were propagated on W3102. The EOP of these phages on W3102 carrying various R factors was determined on nutrient agar and Lennox agar. No visible plaques were formed by these phages on the indicators carrying R factor N-1 (Table 2). The EOP on W3102 (N-3) and W3102 (R-15) was also considerably reduced. However, when the agar media containing 0.0025 M $CaCl_2$ were used for plating, visible plaques of λ^{cI} and λ^{vi} were formed on W3102 (N-1), although they were smaller than the plaques developed on the other indicater strains (Table 3). On the agar media containing 0.0025 M $CaCl_2$, λ^{wt} still did not form visible plaques on W3102 (N-1) but, when the concentration of $CaCl_2$ was increased to 0.025 M, very turbid, small plaques of λ^{wt} were formed on this indicator strain, although the EOP was reduced to about 10^{-1}. Various concentrations of $MgCl_2$ (0.0025 M ~ 0.025 M) were added to the plating media instead of $CaCl_2$, but there was no detectable effect on the EOP and plaque morphology of the above phages. When a mixture of $CaCl_2$ and $MgCl_2$ was added to the plating media, the effect of $CaCl_2$ was reversed by $MgCl_2$.

TABLE 2. *Efficiency of plating of phage λ and its mutants on Escherichia coli W3102* with R factors*

Indicator	Efficiency of plating† of		
	λ^{wt}	λ^{cI}	λ^{vi}
W3102	10^0	10^0	10^0
W3102 (N-1)	$<3.0 \times 10^{-8}$	$<3.0 \times 10^{-8}$	$<3.0 \times 10^{-8}$
W3102 (N-3)	5.0×10^{-3}	1.5×10^{-2}	3.9×10^{-2}
W3102 (N-6)	10^0	10^0	10^0
W3102 (N-9)	10^0	10^0	10^0
W3102 (R-15)	$<3.2 \times 10^{-3}$	$<2.8 \times 10^{-2}$	$<5.5 \times 10^{-2}$
W3102 (222)	10^0	10^0	10^0

* W3102 is a galactose-negative λ$^-$F$^-$ substrain of *E. coli* K-12.

† Efficiencies of plating were determined on semisolid nutrient agar.

TABLE 3. *Effect of $CaCl_2$ on the efficiency of plating of phage λ and its mutants on Escherichia coli W3102* with R factor* N-1

Indicator	Concn of $CaCl_2$ added to plating medium	Efficiency of plating† of		
		λ^{wt}	λ^{cl}	λ^{vi}
	M			
W3102	0	10^0	10^0	10^0
W3102	0.0025	10^0	10^0	10^0
W3102	0.025	10^0	10^0	10^0
W3102 (N-1)	0	$<5.1 \times 10^{-9}$	$<4.4 \times 10^{-7}$	$<3.2 \times 10^{-10}$
W3102 (N-1)	0.0025	$<5.1 \times 10^{-9}$	8.3×10^{-3}	9.1×10^{-3}
W3102 (N-1)	0.025	1.5×10^{-1}	1.2×10^{-1}	7.1×10^{-2}

* W3102 is a galactose-negative λ^-F^- substrain of *E. coli* K-12.

† Efficiencies of plating were determined on semisolid Lennox agar. The plaques which developed on $CaCl_2$-free medium were smaller than those on media containing 0.0025 and 0.025 M $CaCl_2$.

Comparable results in the EOP were obtained with nutrient agar and Lennox agar in the above experiments as well as in the following experiments.

Transduction of gal+ by HFT lysates of λ into W3102 with and without various R factors. The frequency of *gal+* transduction was reduced by the presence of *fi−* R factors, but not by the presence of *fi+* R factors, in the recipient (Table 4). It is interesting to note that R factor N-1 did not reduce the transduction of *gal+* so severely as the EOP of λ phages.

Ultraviolet inducibility of phage λ in CSH-2 strains with and without various R factors. Phage λ of CSH-2 with and without various R factors was ultraviolet-induced, and dose-response curves were obtained. No significant difference could be found in the ultraviolet inducibility of phage λ in these strains. Spontaneous liberation of λ was also unaffected by any R factors.

Attempt to lysogenize with phages λ^{cl} and λ^{vi} W3102 carrying fi− R factors. The reduced EOP of phage λ on the indicators with *fi−* R factors was suspected to be due to increased lysogenization by the presence of *fi−* R factors. Since the EOP of phages λ^{cl} and λ^{vi}, which lack the capacity of lysogenization partially or completely, are also reduced by *fi−* R factors, we attempted to lysogenize W3102 carrying *fi−* R factors by use of these mutants of λ. All of the clones which survived λ^{cl} and λ^{vi} were still sensitive to λ; thus, the above possibility was excluded.

Efficiency of plating of phage T_1 on CSH-2 carrying various R factors. Phage T_1 was propagated on W3102. The EOP of phage T_1 on CSH-2 carrying various R factors is shown in Table 5. It can be seen that *fi−* R factors and not *fi+* R factors again reduced the EOP of phage T_1. In addition, it was noted that the plaques of T_1 which developed on the indicators other than CSH-2 (N-1) were as large and as clear as those on the indicator without an R factor. The plaques which developed on CSH-2 (N-1) were small and turbid (Fig. 1). When 0.0025 M $CaCl_2$ was added to the plating media, the EOP of T_1 on CSH-2 (N-1) was specifically increased five to ten times; the size of the plaques of T_1 on this indicator strain was also slightly increased. Similar results were obtained when W3102 was used instead of CSH-2.

TABLE 4. *Frequency of transduction of gal+ by HFT lysates of phage λ* into Escherichia coli W3102† with and without R factors*

Recipient	Frequency of *gal+* transduction
W3102	10^0
W3102 (N-1)	3.0×10^{-3}
W3102 (N-3)	5.4×10^{-2}
W3102 (N-6)	10^0
W3102 (N-9)	10^0
W3102 (R-15)	3.1×10^{-2}
W3102 (222)	10^0

* HFT lysates of phage λ were obtained by the procedure of Morse et al. (1956); the procedure of transduction of *gal+* (galactose fermentability) was also that described by these authors.

† W3102 is a galactose-negative λ^-F^- substrain of *E. coli* K-12.

Efficiency of plating of phage T_7 on CSH-2 carrying various R factors. Phage T_7 used was propagated on W3102. The EOP of phage T_7 on CSH-2 carrying various R factors was studied. Only R factor N-1 slightly reduced the EOP of phage T_7.

TABLE 5. *Efficiencies of plating of phage T_1 on Escherichia coli CSH-2* with R factors*

Strain	Efficiency of plating†‡
CSH-2	10^0
CSH-2 (N-1)	8.7×10^{-3}‡
CSH-2 (N-3)	1.3×10^{-4}
CSH-2 (N-6)	10^0
CSH-2 (N-9)	10^0
CSH-2 (R-15)	7.1×10^{-4}
CSH-2 (222)	10^0

* CSH-2 is a methionine-requiring F^- substrain of *E. coli* K-12.

† Phage T_1 used was propagated on W3102, a galactose-negative λ^-F^- substrain of *E. coli* K-12. Efficiencies of plating were determined on semi-solid nutrient agar.

‡ The plaques which developed on CSH-2 (N-1) were much smaller than those on the other strains, as shown in Fig. 1.

The plaques of T_7 which developed on CSH-2 (N-1) were slightly smaller than those on the other indicators. Similar results were obtained when W3102 was used instead of CSH-2.

Efficiency of plating of the phages of T series other than T_1 and T_7 on CSH-2 carrying various R factors. The EOP and plaque size of phages T_2, T_3, T_4, T_5, and T_6 were not affected by any R factors employed.

Adsorption of phages λ^{wt}, T_1, and T_7 to W3102 carrying various R factors. A 9-ml amount, containing about 5×10^8 cells per ml, of bacteria grown in Penassay Broth at 37 C with aeration was mixed with 1 ml of each phage having a titer of 5×10^7 per ml and aerated at 37 C for 10 min. A 0.1-ml sample was diluted with 10 ml of ice-cold Penassay Broth and then centrifuged at 5,000 rev/min for 5 min in the cold. The supernatant liquid was treated with chloroform and assayed for free phage. There was no significant difference in the adsorption rates of these phages to W3102 carrying various R factors.

Host-induced modifications of phages λ^{c1}, T_1, and T_7 in the strains carrying various R factors. Phage λ^{c1} was employed in this experiment, W3102 (N-1) in agar media containing 0.0025 M $CaCl_2$. The plaques of phages λ^{c1}, T_1, and T_7 which developed on W3102 carrying various R factors in Lennox agar containing 0.0025 M $CaCl_2$ were scraped off and suspended in Penassay Broth and homogenized. The homogenates were centrifuged at 5,000 rev/min for 10 min, and the supernatant liquids were treated with chloroform and then plated for phage titrations on various indicator strains. Growth of phage T_1 on the strains carrying *fi*$^-$ R factors, N-3 or R-15, resulted in increased EOP on these same two strains to the EOP on the indicators without R factors and with *fi*$^+$ R factors (Table 6). Similar results were obtained with phage λ^{c1} (Table 7). It was further noted that none of these phages, including T_7, was modified by growth in W3102 (N-1).

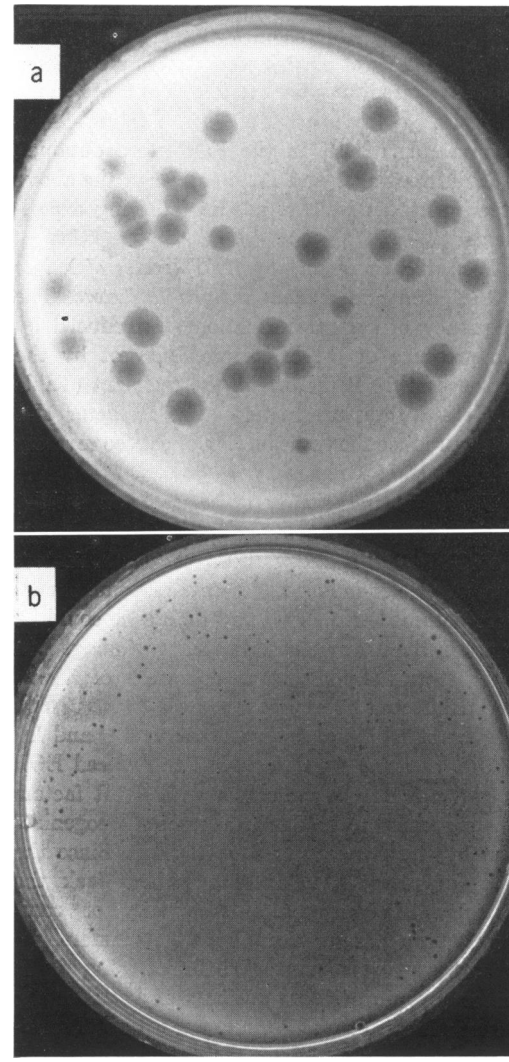

FIG. 1. *Plaques of phage T_1 on Escherichia coli K-12 with and without R factors. (a) Plaques on indicators without an R factor or with R factors other than N-1. (b) Plaques on indicators with an fi$^-$ R factor N-1.*

TABLE 6. *Efficiency of plating on Escherichia coli CSH-2* with R factors of phage T_1 grown on CSH-2 with R factors*

T_1 grown on	Efficiency of plating† on						
	CSH-2	CSH-2 (N-1)‡	CSH-2 (N-3)	CSH-2 (N-6)	CSH-2 (N-9)	CSH-2 (R-15)	CSH-2 (222)
CSH-2 (N-1)	10^0	2.3×10^{-2}	3.1×10^{-4}	10^0	10^0	1.2×10^{-3}	10^0
CSH-2 (N-3)	10^0	2.0×10^{-2}	10^0	10^0	10^0	10^0	10^0
CSH-2 (R-15)	10^0	5.0×10^{-2}	10^0	10^0	10^0	10^0	10^0
CSH-2 (N-3) and then on CSH-2	10^0	4.0×10^{-2}	1.5×10^{-3}	10^0	10^0	1.5×10^{-3}	10^0
CSH-2 (R-15) and then on CSH-2	10^0	3.1×10^{-2}	1.2×10^{-3}	10^0	10^0	1.3×10^{-3}	10^0

* CSH-2 is a methionine-requiring F^- substrain of *E. coli* K-12.

† Efficiencies of plating were determined on semisolid nutrient agar.

‡ The plaques on CSH-2 (N-1) were much smaller than those on the other indicators with any of the stocks of phage T_1 employed in this experiment (Fig. 1).

TABLE 7. *Efficiency of plating on Escherichia coli W3102* with R factors of phage λ^{cl} grown on W3102 with R factors*

λ^{cl} grown on†	Efficiency of plating‡ on						
	W3102	W3102 (N-1)	W3102 (N-3)	W3102 (N-6)	W3102 (N-9)	W3102 (R-15)	W3102 (222)
W3102 (N-1)	10^0	$<3.5 \times 10^{-6}$	8.6×10^{-2}	10^0	10^0	$<3.5 \times 10^{-6}$	10^0
W3102 (N-3)	10^0	$<3.3 \times 10^{-9}$	10^0	10^0	10^0	10^0	10^0
W3102 (R-15)	10^0	$<2.6 \times 10^{-9}$	10^0	10^0	10^0	10^0	10^0
W3102 (N-3) and then W3102	10^0	$<5.0 \times 10^{-10}$	1.7×10^{-2}	10^0	10^0	2.6×10^{-2}	10^0
W3102 (R-15) and then W3102	10^0	$<4.1 \times 10^{-9}$	2.5×10^{-2}	10^0	10^0	3.0×10^{-2}	10^0

* W3102 is a galactose-negative λ^-F^- substrain of *E. coli* K-12.

† The phage was grown in semisolid Lennox agar containing 0.0025 M $CaCl_2$; the developed plaques were scraped off, suspended in Penassay Broth, homogenized, centrifuged, treated with chloroform, and used for titrations.

‡ The efficiencies of plating were determined on semisolid Lennox agar without $CaCl_2$ enrichment.

Tables 6 and 7 also indicate that further propagation of the modified phages in W3102 without an R factor reduced their EOP on the indicators with *fi*$^-$ R factors. These results suggest that the observed phenomena are host-induced modifications of the phages (Bertani, 1953; Luria, 1953).

Effect of fi$^-$ *transductant R factors obtained from an fi*$^+$ *R factor upon the multiplication of phages* λ *and* T_1. Although a majority of drug-resistant transductants obtained with phage P-22 in *Salmonella typhimurium* LT-2 carrying a four-drug-resistance *fi*$^+$ R factor are unable to transfer their drug resistance by conjugation (Watanabe and Fukasawa, 1962*a*), a few transductant R factors resistant to Su, Sm, and Cm have recently been found to be transmissible by conjugation (Watanabe, 1963*b*). Some of them were found to be *fi*$^-$ upon their conjugational transfer to male bacteria of *E. coli* K-12. The effects of these *fi*$^-$ transductant R factors on the EOP of phages λ (λ^{wt}, λ^{cl}, and λ^{vi}) and T_1 on W3102 were investigated. It was found that these *fi*$^-$ transductant R factors, like the original *fi*$^+$ R factors but unlike the naturally occurring *fi*$^-$ R factors, do not exert any inhibitory effect against the progeny formation of phages λ and T_1.

Frequencies of conjugational transfer of R factors to recipients already having R factors. CSH-2 and W677/Pro$^-$$T_6^r$Sm^r were a donor and a recipient, respectively. The procedure for determining the frequency of conjugational transfer of R factors was the same as that described previously (Watanabe and Fukasawa, 1961*a*). A 0.5-ml amount of a Penassay Broth culture of a donor, containing about 5×10^8 cells per ml, was mixed

TABLE 8. *Frequency of conjugational transfer of R factors from Escherichia coli CSH-2* to E. coli W677/Pro⁻T₆ʳSmʳ† with and without R factors*

R factor in donor strain CSH-2		R factor in recipient strain W677/Pro⁻T₆ʳSmʳ		Frequency of transfer of R factor‡
Strain no.	*fi*	Strain no.	*fi*	
N-1	−	—		1.4×10^{-3}
N-1	−	R-15	−	1.2×10^{-4}
N-1	−	222-R_3	+	2.5×10^{-3}
N-1	−	222-Tc	+	2.0×10^{-3}
N-3	−	—		1.8×10^{-3}
N-3	−	R-15	−	1.5×10^{-4}
N-3	−	222-R_3	+	1.3×10^{-3}
N-3	−	222-Tc	+	1.2×10^{-4}
R-15	−	—		3.3×10^{-4}
R-15	−	222-Tc	+	1.2×10^{-4}
222	+	—		3.5×10^{-2}
222	+	N-1	−	3.7×10^{-2}
222	+	N-3	−	2.8×10^{-2}
222	+	R-15	−	5.6×10^{-2}
222	+	222-R_3	+	3.8×10^{-4}
222	+	222-Tc	+	4.5×10^{-4}
K	+	—		2.4×10^{-2}
K	+	N-1	−	3.7×10^{-2}
K	+	N-3	−	3.2×10^{-2}
K	+	R-15	−	3.2×10^{-2}
K	+	222-R_3	+	2.7×10^{-4}
K	+	222-Tc	+	5.3×10^{-4}

* CSH-2 is a methionine-requiring F⁻ substrain of *E. coli* K-12.

† W677/Pro⁻T₆ʳSmʳ is a threonine-, leucine-, proline-, and vitamin B -requiring; mannitol-, xylose-, maltose, galactose-, and lactose-negative; and phage T_6- and streptomycin-resistant F⁻ substrain of *E. coli* K-12.

‡ The frequencies of transfer of R factors were determined with the procedure described in the text and expressed as the values per introduced donor cell.

with 4.5 ml of a Penassay Broth culture of a recipient (about 5×10^8 cells per ml) in a 200-ml Erlenmeyer flask and incubated in a water bath (37 C) without aeration. A 0.1-ml sample was taken after 1 hr of incubation, and was added to 0.9 ml of phage T_6 (titer: about 5×10^{10} per ml). The phage-treated mixed culture was then incubated at 37 C for 10 min and plated on bromothymol blue-lactose-nutrient agar containing 1,000 μg/ml of Sm and a proper concentration of a drug to which the donor is resistant. Thus, the recipient cells which received the donor R factor were selected on this medium as lactose-negative colonies (Table 8). It can be seen that the frequency of transfer of *fi*⁺ R factors was reduced by a factor of about 10^2 when the recipients already possessed *fi*⁺ R factors. These data confirm previous reports (Watanabe and Fukasawa, 1962*b*; Mitsuhashi et al., 1962). Furthermore, the frequency of transfer of *fi*⁻ R factors was also reduced when the recipients already carried *fi*⁻ R factors, but this reduction was rather slight. In contrast, the transfer of *fi*⁺ R factors to recipients with *fi*⁻ R factors and the transfer of *fi*⁻ R factors to recipients with *fi*⁺ R factors occurred with frequencies about equal to those of transfer of the corresponding R factors to the recipients without R factors. The above mentioned *fi*⁻ transductant R factors derived from an *fi*⁺ R factor behaved in exactly the same way in their superinfection immunity pattern as did the original *fi*⁺ R factor.

Genetic stability of the strains with two different R factors. W677/Pro⁻T₆ʳSmʳ strains with two different R factors, which were obtained in the above superinfection experiment, were subcultured in drug-free Penassay Broth, and were studied for the genetic stability of their R factors by plating proper dilutions on drug-free nutrient agar and replicaplating the resultant colonies onto drug-containing nutrient agar (*see* Watanabe and Lyang, 1962). The strains with both an *fi*⁺ R factor and an *fi*⁻ R factor were found to be genetically quite stable, whereas the strains with two types of *fi*⁺ R factors were very unstable, segregating either one of the R factors. The two different types of *fi*⁺ R factors were sometimes stably retained by the host bacteria but, in this case, the two types of R factors are assumed to have been genetically recombined, producing a recombinant R factor as was reported by Watanabe and Lyang (1962) and as will be mentioned below.

Transfer by conjugation and by transduction of R factors from donors possessing two different R factors. Strains CSH-2 with two different R factors were used as donors of R factors in conjugation and transduction with phage P1kc, and the frequency of transfer of each R factor was studied. The results of conjugational transfer are shown in Table 9, and those of transduction, in Table 10. As can be seen in Table 9, the donors doubly infected with *fi*⁺ and *fi*⁻ R factors seemed to transfer the two R factors with frequencies slightly higher than those expected on the basis

of their independent transfer. However, the results of transduction of *fi*$^+$ and *fi*$^-$ R factors, which were present in the same strains, indicate that they were transduced separately (Table 10), suggesting that the *fi*$^+$ and *fi*$^-$ R factors existed in a cell as independent units. The results in Table 10 also indicate that the two different *fi*$^+$ R factors were transduced together as a unit by phage P1kc. This finding, together with the previous finding of simultaneous transfer of two *fi*$^+$ R factors by conjugation (Watanabe and Lyang, 1962), suggests that the two different *fi*$^+$ R factors in the genetically stable strain were genetically recombined.

Elimination of various R factors with acridine dyes. CSH-2 strains carrying various R factors were treated with acridine orange or acriflavine, and the frequency of elimination of R factors was determined. With all of the R factors studied, frequencies were equally as low as with R factor 222 previously reported by Watanabe and Fukasawa (1961*b*).

DISCUSSION

The results reported here indicate that *fi*$^+$ and *fi*$^-$ R factors differ considerably from each other, not only in their *fi* characters but also in some other important functions. First, *fi*$^-$ R factors reduced the EOP of phages λ and T_1, whereas *fi*$^+$ R factors did not produce this reduction. One of the *fi*$^-$ R factors, N-1, reduced the EOP of phage T_7 as well. The reductions in the EOP of these phages by *fi*$^-$ R factors must be due to some step(s) following the adsorption of the phages to the bacteria, because there was no significant difference in the adsorption rates of these phages to the bacteria carrying various R factors. Transduction of *gal*$^+$ with HFT lysates of phage λ was also reduced by *fi*$^-$ R factors in the recipients. The fact that N-1 did not reduce *gal*$^+$ transduction as severely as the formation of plaques of λ may suggest that the failure of λ to form plaques on the strain with N-1 is not due to the failure of nucleic acid injection of the phage. Nor is it probably due to increased lysogenization with λ by the presence of N-1, because the EOP of phages λ^{c1} and λ^{vi}, which lack the ability to lysogenize, were also reduced. The finding that ultraviolet inducibility of λ was not affected by *fi*$^-$ R factors is difficult to interpret, because ultraviolet treatment might inactivate the "repressor" for λ, if any, which might be produced by *fi*$^-$ R factors. We are now studying the nucleic acid injection of phages λ and T_1 into the bacteria carrying various R factors. The mechanism of the improving effect of $CaCl_2$ on the EOP of these phages on the strains with *fi*$^-$ R factors is also now under investigation.

TABLE 9. *Frequency of conjugational transfer of R factors from Escherichia coli CSH-2* having one or two R factors to W677/Pro$^-$T$_6^r$Sm^r†*

R factor in donor strain CSH-2		Frequency of transfer of R factor‡		
fi$^+$ R factor	*fi*$^-$ R factor	*fi*$^+$ R factor	*fi*$^-$ R factor	*fi*$^+$ and *fi*$^-$ R factors
—	N-1	—	1.0×10^{-3}	—
—	N-3	—	3.0×10^{-4}	—
—	R-15	—	3.0×10^{-3}	—
222-R_3	—	3.0×10^{-2}	—	—
K-R_3	—	1.0×10^{-2}	—	—
K-Tc	—	1.0×10^{-2}	—	—
222-R_3	N-1	1.2×10^{-2}	1.7×10^{-3}	1.4×10^{-3}
K-R_3	N-1	1.0×10^{-2}	6.0×10^{-3}	4.0×10^{-3}
222-R_3	N-3	6.0×10^{-2}	1.0×10^{-3}	4.0×10^{-4}
K-R_3	N-3	1.0×10^{-2}	1.2×10^{-4}	4.0×10^{-5}
K-Tc	R-15	1.0×10^{-2}	4.0×10^{-3}	1.7×10^{-4}

* CSH-2 is a methionine-requiring F$^-$ substrain of *E. coli* K-12.

† W677/Pro$^-$T$_6^r$Sm^r is a threonine-, leucine-, proline-, and vitamin B_1-requiring; mannitol-, xylose-, maltose-, galactose-, and lactose-negative; and phage T_6- and streptomycin-resistant F$^-$ substrain of *E. coli* K-12.

‡ The frequencies of transfer of R factors were determined with the procedure described in the text and expressed as the values per introduced donor cell.

TABLE 10. *Frequency of transduction of R factors of Escherichia coli CSH-2* carrying two different R factors to sensitive CSH-2*

R factor in donor strain having a drug-resistance marker		Frequency of transduction of R factor† selected by		
Cm	Tc	Cm	Tc	Cm + Tc
K-R_3	222-Tc	5.0×10^{-6}	1.5×10^{-5}	3.3×10^{-6}
222-R_3	K-Tc	1.8×10^{-5}	1.5×10^{-5}	1.2×10^{-5}
222-R_3	N-3	5.0×10^{-5}	1.5×10^{-6}	$< 5.3 \times 10^{-8}$
K-R_3	N-1	7.5×10^{-6}	3.1×10^{-7}	$< 3.1 \times 10^{-8}$

* CSH-2 is a methionine-requiring F$^-$ substrain of *E. coli* K-12.

† The frequencies of transduction of R factors were expressed as the values per infective phage particle.

Some of the *fi*$^-$ R factors were found to cause host-induced modifications of phages λ and T_1, but R factor N-1 did not cause modifications of phages λ, T_1, and T_7. The small plaques of these phages on the indicator strains with N-1 are assumed to be due to the absence of the host-induced modifications of the phages. Phages T_1 and λ modified by N-3 were found to be adapted also to the bacteria with R-15 and vice versa. It is obvious from these results that there are at least two types of *fi*$^-$ R factors, based on their restriction and modification of the phages. The *fi*$^-$ transductant R factors obtained from an *fi*$^+$ R factor, unlike the naturally occurring *fi*$^-$ R factors, were found not to affect the EOP of the phages, suggesting that the genetic determinant(s) of *fi*$^-$ character is possibly not directly related to the restrictions of the phages by *fi*$^-$ R factors.

Second, *fi*$^+$ and *fi*$^-$ R factors exhibited different patterns in their superinfection immunity, as revealed in the reduction of the frequency of conjugational transfer of R factors to the recipients with different R factors. In other words, there was no superinfection immunity between *fi*$^+$ and *fi*$^-$ R factors, although it was observed between homologous types of R factors with regard to *fi*. On the other hand, "mutual exclusion" was found between two different *fi*$^+$ R factors but not between *fi*$^+$ and *fi*$^-$ R factors. The possibility of mutual exclusion between two different *fi*$^-$ R factors could not be investigated, because *fi*$^-$ R factors with suitable drug-resistance markers were not available. The superinfection immunity and the mutual exclusion observed between two homologous types of R factors with regard to *fi* are possibly due to a common mechanism, as will be discussed below.

Third, it was shown that *fi*$^+$ and *fi*$^-$ R factors are not genetically recombined, although two different *fi*$^+$ R factors can be easily recombined. This fact was evidenced by the finding that *fi*$^+$ and *fi*$^-$ R factors are transferred independently of each other both by conjugation and by transduction with phage P1kc. Although an *fi*$^-$ R factor was transduced independently of an *fi*$^+$ R factor which was present in the same cell, the frequency of conjugational transfer of the *fi*$^-$ R factor together with the coexisting *fi*$^+$ R factor was higher than the theoretical value calculated on the basis of their independent transfer. A similar situation was encountered by Ozeki, Stocker, and Smith (1962) in colicinogenic factors. These results do not necessarily indicate that the two factors, which are transferred together, are physically associated with each other, but rather that one of the two factors merely helps the transfer of another factor.

Jacob, Brenner, and Cuzin (1963) recently presented a hypothesis that autonomous F may replicate on rather few specific sites, possibly one site for one chromosome, on the internal side of the cell surface. They assumed a similar mechanism for R factors. If this were the case, and if we postulate that the sites of attachment of autonomous R factors are different for *fi*$^+$ and *fi*$^-$ R factors, our findings reported in this paper are easily explained. In other words, this hypothesis can account for our findings in terms of competition between homologous types of R factors in occupying the few specific sites for replication. There may be other explanations, such as the inhibition of replication of an R factor by the "repressor" produced by another R factor or the metabolic competition between the two R factors.

If is of considerable interest that *fi*$^+$ and *fi*$^-$ R factors are different from each other not only in their *fi* characters but also in some other functions. The fact that *fi*$^-$ transductant R factors derived from an *fi*$^+$ R factor behaved differently from the naturally occurring *fi*$^-$ R factors and like the original *fi*$^+$ R factor, in their restriction of phages λ and T_1 and in their superinfection immunity, may suggest that the naturally occurring *fi*$^-$ R factors have possibly not developed in single mutations from *fi*$^+$ R factors. When we discuss the differences between *fi*$^+$ and *fi*$^-$ R factors, it is important to bear in mind the fact that all of the naturally occurring *fi*$^-$ R factors so far studied have been found to lack the Cm-resistance marker. It might be possible that the *fi* determinant is closely linked with the Cm-resistance marker, and that deletion of the Cm-resistance marker results in the loss of the *fi* determinant. However, the results reported in the present paper seem too complicated to be accounted for by this simple hypothesis of deletion. They might suggest that the RTF of *fi*$^+$ and *fi*$^-$ R factors are different from each other. The RTF of *fi*$^+$ and *fi*$^-$ R factors might have been different already before they incorporated the drug-resistance genes, or the *fi*$^-$ R factors might have developed as a result of interaction of *fi*$^+$ R factors with some other episomes. In fact, it is now known that stable associations take place

between R factors and some other episomes, such as phages P1 (Kondo and Mitsuhashi, 1963), P-22 (Watanabe, 1963*b*; Dubnau, *personal communication*), and F (Harada et al., 1962).

The immunity of heterologous phages, which is conferred by the presence of a prophage, has been called prophage interference (Bertani, 1953) or dysgonia (Lwoff, 1959). It was found that phage T_1 is not only restricted but also modified in P1-lysogenic hosts (Lederberg, 1957; Christensen, 1961). A similar fact was reported between phage λ and prophage P1, and was studied for its molecular nature (Arber and Dussoix, 1962). The results reported in the present paper suggest that they are possibly analogous to prophage interference or dysgonia in their mechanisms. Jacob, Schaeffer, and Wollman (1960) presented a hypothesis that phages and nonphage episomes may mutate to each other, for which no experimental evidence has yet been obtained. Our results may suggest a genetic similarity between *fi*$^-$ RTF and some phages.

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