JOURNAL OF BACTERIOLOGY
Vol. 88, No. 4, p. 922-928 October, 1964

EPISOME-MEDIATED TRANSFER OF DRUG RESISTANCE IN ENTEROBACTERIACEAE

VIII. SIX-DRUG-RESISTANCE R FACTOR

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Received for publication 8 May 1964

ABSTRACT

WATANABE, TSUTOMU (Keio University School of Medicine, Tokyo, Japan), CHIZUKO OGATA, AND SACHIKO SATO. Episome-mediated transfer of drug resistance in Enterobacteriaceae. VIII. Six-drug-resistance R factor. J. Bacteriol. **88**:922-928. 1964.—The multiple-drug-resistant *Escherichia coli* strain isolated by Lebek in 1963 was found to transfer resistance to sulfonamide, streptomycin, chloramphenicol, tetracycline, kanamycin, and neomycin together by conjugation, as well as by transduction with phage P1kc, suggesting that these drug-resistance markers are carried by a single R factor (R_6). The results of transductional and spontaneous segregations of the drug-resistance markers of R_6 have shown that R_6 has independent genetic determinants for sulfonamide, streptomycin, chloramphenicol, tetracycline, and kanamycin-neomycin resistance. Resistance to kanamycin and neomycin is probably controlled by a single gene, because no segregation was observed between these two. The resistance transfer factor of R_6 was found to be of the *fi*$^+$ type.

R factors (or episomic drug-resistance factors) have been found to carry various combinations of markers of resistance to sulfonamide (Su), streptomycin (Sm), chloramphenicol (Cm), and tetracycline (Tc; *see* Watanabe, 1963*a*). Lebek (1963) recently isolated a strain of *Escherichia coli* which transfers to other strains the resistance to Su, Sm, Cm, Tc, and kanamycin (Km) by mixed cultivation. Although this *E. coli* strain was found to be resistant to neomycin (Nm) as well, the possibility of transfer of Nm resistance was not investigated. This *E. coli* strain was supplied to us by G. Lebek, and we have found that this strain carries an R factor which gives to host bacteria resistance to Nm as well as to the above-mentioned five drugs. The results of genetic studies of this R factor will be presented here.

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 nutrient agar (containing 2% lactose), Lennox (1955) agar, and a minimal agar described by Davis and Mingioli (1950).

Drugs. Su, Sm, Cm, and Tc used were those described in a previous paper (Watanabe and Fukasawa, 1961*a*). Km employed was kanamycin sulfate (Meiji Seika Kaisha, Ltd., Tokyo, Japan), and Nm used was fradiomycin sulfate (Takeda Chemical Industries, Ltd., Osaka, Japan). In addition, the drug sensitivity of bacterial strains was studied with polymyxin B-sensitivity disks (Difco) and with colimycin-erythromycin-, and oleandomycin-sensitivity disks (Eiken).

Bacterial strains. In addition to the *E. coli* strain supplied by Lebek, we employed 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; W2252 (methionine-requiring and λ^-), an Hfr derivative of K-12; and *Salmonella typhimurium* LT-2.

Phage strains. Phage strains used were f2 (Loeb and Zinder, 1961), P1kc, P-22, λ, and T_1. Phage λ was obtained by ultraviolet induction (Lwoff, Siminovitch, and Kjeldgaard, 1950) of strain CSH-2.

R factors. An *fi*$^+$ R factor 222 (Su, Sm, Cm, Tc) and an *fi*$^-$ R factor N-3 (Su, Sm, Tc; *see* Watanabe et al., 1964) were used.

Transfer of drug resistance by mixed cultivation. The methods of transfer of drug resistance by mixed cultivation and of selection of recipient cells which acquired drug resistance were described in a previous paper (Watanabe and Fuka-

sawa, 1961*a*). For the quantitative determination of transfer of drug resistance, strains CSH-2 (R) and W677/Pro$^-$T$_6^r$Sm^r were separately grown in Penassay Broth at 37 C with gentle aeration to about 5×10^8 cells per ml. A 0.5-ml amount of the donor culture was mixed with 4.5 ml of the recipient culture in a 100-ml Erlenmeyer flask, and the flask was incubated in a water bath (37 C) without aeration. A sample (0.1 ml) was taken at each of various time points and added to 0.9 ml of phage T$_6$ (titer: 5×10^{10} per ml) to eliminate donor cells. The phage-infected mixed culture was incubated in a water bath (37 C) for 10 min for phenotypic expression of drug resistance of the recipient cells which received the R factor, and was then plated on bromothymol blue-lactose nutrient agar containing 1,000 μg/ml of Sm plus a proper concentration of another drug. The recipient cells which received the R factor could be detected as lactose-negative colonies on this selective medium.

Determination of levels of drug resistance of bacterial strains. Bacterial cultures grown at 37 C with aeration to about 5×10^8 cells per ml were diluted with saline to 10^{-4}, and a loopful of this dilution was streaked on nutrient agar containing various concentrations of each drug. Thus, the average drug resistance (Watanabe, 1959), which reflects the level of drug resistance of a majority of cells of the culture, was determined.

Transduction of R factor. The methods of transduction of R factor with phage P1kc in *E. coli* K-12 and with phage P-22 in *S. typhimurium* were the same as those described in a previous paper (Watanabe and Fukasawa, 1961*b*).

Study of spontaneous segregation of drug resistance of R factor. The method of study of spontaneous segregation of drug-resistance markers of R factor was the same as that described by Watanabe and Lyang (1962); bacterial strains carrying R factor were daily subcultured in drug-free Penassay Broth and plated on drug-free nutrient agar at each subculture. The resultant colonies were studied for their drug sensitivity by replica plating.

Study of fertility inhibition by R factor. R factors can be classified into *fi*$^+$ and *fi*$^-$ types, depending on their suppression of the functions of the sex factor F of *E. coli* K-12 (Watanabe et al., 1964). R factor was transferred to W2252 by mixed cultivation, and the sensitivity to phage f2 and the frequency of sexual recombination of this strain were studied by use of W2252 without R factor as a control (Watanabe, Fukasawa, and Takano, 1962; Watanabe and Fukasawa, 1962).

RESULTS

Average drug resistance of E. coli CSH-2 and S. typhimurium LT-2 which received drug resistance from Lebek's E. coli strain. Lebek's drug-resistant *E. coli* strain was grown together with *S. typhimurium* LT-2, and Cm-resistant LT-2 was isolated on minimal agar containing 25 μg/ml of Cm by use of the auxotrophic character of CSH-2 and the prototrophy of LT-2. Next, this LT-2 (R) was grown together with CSH-2, and lactose-positive CSH-2 (R) was selected on bromothymol blue-lactose nutrient agar containing 25 μg/ml of Cm. It is obvious from the results in Table 1 that Lebek's *E. coli* strain transfers to CSH-2 and LT-2 the resistance to Su, Sm, Cm, Tc, Km, and Nm. This *E. coli* strain of Lebek, however, did not give to either strain resistance to colistin, erythromycin, oleandomycin, and polymyxin B.

Kinetics of transfer of drug resistance from drug-resistant E. coli CSH-2 to E. coli W677/Pro$^-$T$_6^r$-Sm^r by conjugation. The culture of drug-resistant CSH-2 in Penassay Broth was centrifuged, and the supernatant fluid was sterilized with chloroform. Since this sterilized supernatant fluid could not confer drug resistance upon other sensitive strains and only the viable culture was capable of transferring drug resistance, it is apparent that the observed transfer of drug resistance is not transduction but is caused by conjugation.

TABLE 1. *Levels of drug resistance of Escherichia coli CSH-2* and Salmonella typhimurium LT-2 with and without R$_6$*

Drug	Strain†			
	CSH-2	CSH-2 (R$_6$)	LT-2	LT-2 (R$_6$)
	μg/ml	μg/ml	μg/ml	μg/ml
Su.....	5	1,000	50	1,000
Sm.....	1	50	10	50
Cm.....	10	250	10	250
Tc.....	10	250	25	250
Km.....	10	500	10	1,000
Nm.....	1	100	1	100

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

† The levels of drug resistance of each strain are averages which reflect the resistance of a majority of cells of the culture.

The kinetics of transfer of drug resistance was studied with the above-mentioned procedure. The kinetics of transfer of drug resistance resembled that of a previously studied R factor 222 (Watanabe and Fukasawa, 1961a). The colonies developed on Sm plus Tc and Sm plus Cm plates at each time point were replica-plated onto nutrient agar containing each of the other drugs. More than 3,000 colonies were studied, and all of them were found to be resistant to all of the six drugs. It is, therefore, almost certain that the resistance markers for the six drugs are all carried by a single R factor, which will be called R_6 from now on.

Transduction of R_6 with phage P1kc in E. coli CSH-2. Drug-resistant transductants were selected on calcium-free Lennox agar containing a single drug after incubating the phage-infected culture in calcium-free Lennox broth at 37 C for 60 min. As shown in Table 2, a majority of the drug-resistant transductants received resistance to the six drugs, together with the ability to transfer the resistance by conjugation. It can be seen also that each of the Su, Sm, Cm, and Tc markers segregated from other markers, although with low frequencies. Km and Nm resistance also segregated from other markers, but no segregation was observed between Km and Nm resistance. No drug-resistant transductants could be detected on selective medium containing Su, probably because of disturbance by the background growth.

Transduction of R_6 with phage P-22 in S. typhimurium LT-2. In the transduction of R_6 in *S. typhimurium* LT-2 with phage P-22, no transductants could be detected on selective medium containing either Su or Sm after incubation of the phage-infected culture at 37 C for 60 min, as was already experienced with a four-drug-resistance R factor 222 (Watanabe and Fukasawa, 1961*b*). In contrast to the system of phage P1kc and *E. coli*, the drug-resistant transductants of *S. typhimurium* LT-2 obtained with phage P-22 did not inherit all of the drug-resistance markers (Table 3); the Tc marker consistently segregated from other markers. Here again there was no segregation between Km and Nm. Another characteristic point in the drug-resistant transductants of LT-2 is that none of them was capable of transferring their drug resistance by mixed cultivation.

Spontaneous segregation of drug resistance in E. coli CSH-2 and S. typhimurium LT-2 with R_6

TABLE 2. *Types of drug-resistant transductants obtained in transduction of R_6 with phage P1kc in Escherichia coli K-12*

Selected by*	Drug-resistance markers	No.	Conjugational transferability of drug resistance
Sm (10 μg/ml)	Su, Sm, Cm, Tc, Km, Nm	52	All +
	Su, Sm, Tc, Km, Nm	1	+
	Sm, Cm, Tc, Km, Nm	1	+
	Sm	6	1 +, 5 −
Cm (25 μg/ml)	Su, Sm, Cm, Tc, Km, Nm	192	All +
	Su, Sm, Cm, Km, Nm	9	8 +, 1 −
	Sm, Cm, Tc, Km, Nm	3	All +
	Sm, Cm, Km, Nm	3	2 +, 1 −
Tc (25 μg/ml)	Su, Sm, Cm, Tc, Km, Nm	183	All +
	Sm, Cm, Tc, Km, Nm	3	All +
	Sm, Tc, Km, Nm	1	+
	Tc	10	All +
Km (50 μg/ml)	Su, Sm, Cm, Tc, Km, Nm	166	All +
	Su, Sm, Cm, Km, Nm	1	+
Nm (50 μg/ml)	Su, Sm, Cm, Tc, Km, Nm	123	All +
	Su, Sm, Cm, Km, Nm	3	All +

* No drug-resistant transductants could be detected on selective medium containing Su (500 μg/ml), probably because of disturbance by the background growth.

When CSH-2 (R_6) was subcultured daily in drug-free Penassay Broth for 10 days and more than 100 colonies were studied at each subculture, all of them were found to retain their resistance to the six drugs. In contrast, *S. typhimurium* LT-2 (R_6) cells frequently segregated their drug-resistance markers(Fig. 1). Figure 1 shows the results of two parallel experiments started from two colonies of LT-2 (R_6). Segregants which lost all drug-resistance markers other than Tc were most frequent. Segregants with no drug resistance were found as well. Other types of spontaneous segregants obtained were (Su, Sm, Tc, Km, Nm), (Su, Sm, Cm, Km, Nm), and (Cm, Tc). All of the segregants which had a portion of the drug-resistance markers were found able to transfer their drug resistance by mixed cultivation.

Fertility inhibition by R_6. R_6 was transferred to *E. coli* W2252 from *S. typhimurium* LT-2 by conjugation. W2252 (R_6) thus obtained was found resistant to phage f2, a male-specific phage, and to carry out mating with W677/Pro$^-$T$_6^r$Sm^r only in frequencies reduced by a factor of about 10^2. These results are in accordance with those of naturally occurring *fi*$^+$ R factors (Watanabe et al., 1962; Watanabe and Fukasawa, 1962).

Efficiencies of plating of phages λ and T_1 on E. coli W3102 with R_6. We reported that *fi*$^-$ R factors reduce the efficiency of plating of phages λ and T_1 in *E. coli* K-12, whereas *fi*$^+$ R factors do not possess this inhibitory action (Watanabe et al., 1964). The efficiency of plating of phages λ and T_1 was not reduced by R_6 in W3102.

Superinfection immunity pattern of R_6. Our previous studies (Watanabe and Fukasawa, 1962; Watanabe et al., 1964) showed that R factors of homologous type with regard to *fi* character exert immunity in their superinfections, whereas superinfection immunity is not found between *fi*$^+$ and *fi*$^-$ R factors. The transfer of R_6 from CSH-2 (R_6) to W677/Pro$^-$T$_6^r$Sm^r with an *fi*$^-$ R factor N-3 occurred with frequencies about equal to those to the recipient with no R factor. However, the frequencies of transfer of R_6 to the recipient with an *fi*$^+$ R factor 222 were reduced by a factor of about 10^2. These results indicate that R_6 shows a superinfection immunity pattern characteristic of *fi*$^+$ R factors.

TABLE 3. *Types of drug-resistant transductants obtained in transduction of R_6 with phage P-22 in Salmonella typhimurium LT-2*

Selected by*	Drug-resistance markers	No.	Conjugational transferability of drug resistance
Cm (25 μg/ml)	Su, Sm, Cm, Km, Nm	128	All −
	Cm	2	Both −
Tc (50 μg/ml)	Tc	121	All −
Km (50 μg/ml)	Su, Sm, Cm, Km, Nm	123	All −
	Su, Sm, Km, Nm	3	All −
	Km, Nm	4	All −
Nm (50 μg/ml)	Su, Sm, Cm, Km, Nm	98	All −
	Su, Sm, Km, Nm	2	Both −
	Cm, Km, Nm	1	−
	Km, Nm	4	All −

* No drug-resistant transductants could be detected on selective media containing either Su (500 μg/ml) or Sm (25, 50, and 100 μg/ml), probably because of disturbance by the background growth.

DISCUSSION

We have reported here that the multiple-drug-resistant *E. coli* strain isolated by Lebek (1963) transfers resistance to Su, Sm, Cm, Tc, Km, and Nm together by conjugation. The resistance to these drugs is also transferred together by transduction with phage P1kc in *E. coli* K-12. These results indicate that the resistance markers to these drugs are carried by a single R factor (R_6). The patterns of segregation of drug-resistance markers by transduction with phage P1kc in *E. coli* K-12 and with phage P-22 in *S. typhimurium* LT-2, and also the patterns of their spontaneous segregation, demonstrate that resistance to Su, Sm, Cm, Tc, and Km-Nm is controlled by independent specific genetic determinants located on the structure of the R factor. The fact that Km and Nm resistance did not segregate from each other suggests either that the genetic determinants for resistance to these drugs are very closely linked to each other or that the resistance to Km and Nm is controlled by a single genetic determinant. Since Kunin and Finland (1958) found cross-resistance between Km and Nm in presumably chromosomal gene mutations, it seems more

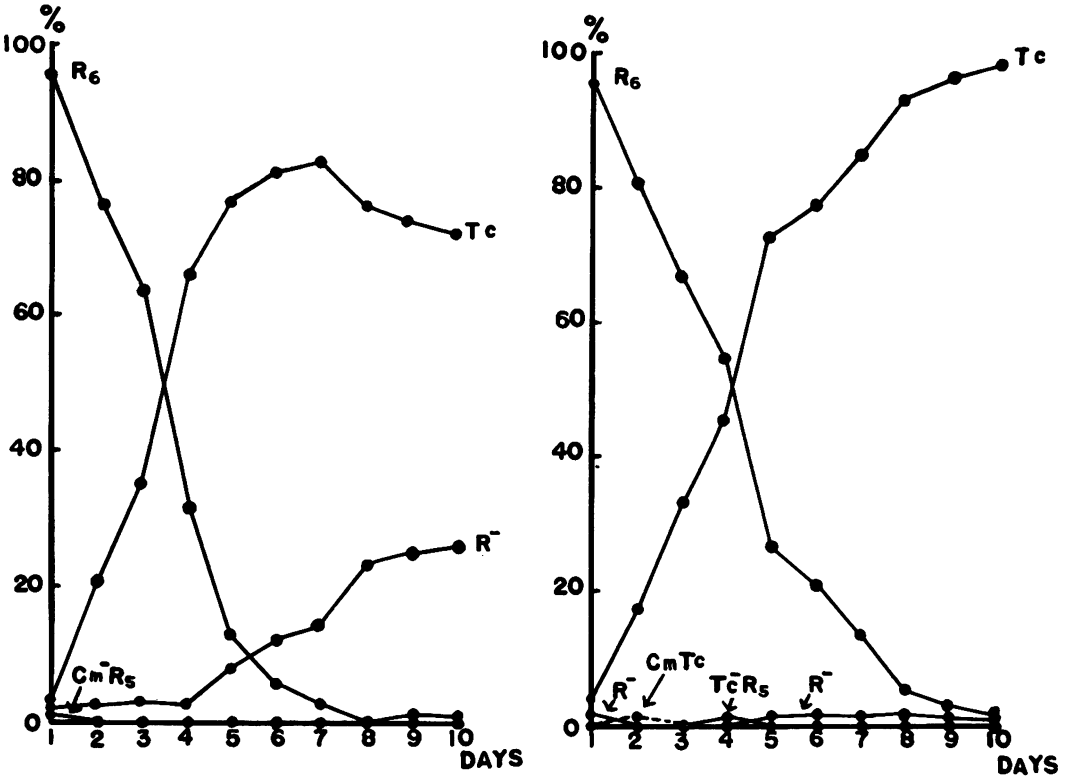

FIG. 1. *Spontaneous segregation of drug-resistance markers of R_6 in Salmonella typhimurium LT-2. Results of two parallel experiments started from two colonies of LT-2 (R_6) are shown. Each colony was inoculated into Penassay Broth and subcultured daily. At each subculture, more than 100 cells of the culture were studied for their drug sensitivity by replica plating. Abbreviations: R_6, cell with an R factor (Su, Sm, Cm, Tc, Km, Nm); Cm^-R_5, cell with an R factor (Su, Sm, Tc, Km, Nm); Tc^-R_5, cell with an R factor (Su, Sm, Cm, Km, Nm); CmTc, cell with an R factor (Cm, Tc); R^-, cell with no drug resistance.*

likely that the resistance to Km and Nm given by R_6 is also due to cross-resistance.

In West Germany, a majority of pathogenic *E. coli* strains have been found resistant to Sm, Tc, and Cm since 1958 (Linzenmeyer, Scheppe, and Schuster, 1962). Further, according to these authors, more and more Nm-resistant strains have been isolated since 1959. The frequencies of Nm-resistant strains isolated in 1959 and 1960 were 8.3 and 45.3%, respectively, although no Nm-resistant *E. coli* was found in 1958. However, Km sensitivity of these strains was not studied. On the basis of the rapid increase in the frequencies of the multiple-drug-resistant strains and of the results reported in the present paper, the multiple-drug-resistant *E. coli* strains which have become prevalent in West Germany are most likely to result from R factors, and they may well be resistant to Km also.

In contrast, very few Km-resistant enteric bacteria have been isolated in Japan, and most of these Km-resistant strains have been found unable to transmit their Km resistance by mixed cultivation despite rather extensive field investigations (Kimura et al., 1963). This might be because Km and Nm have not been used extensively in Japan, whereas in West Germany these drugs have been used rather extensively for treatment of enteric infections (G. Linzenmeyer, *personal communication*). Kimura et al. (1963) recently isolated a *Shigella* strain capable of transferring resistance to Su, Sm, Cm, and Km by mixed cultivation.

It seems difficult to reach definitive conclusions concerning the genetic structure of R_6 on the basis of our genetic studies. We could at least point out that the segregation patterns of drug-resistance markers and their conjugational trans-

ferability, if we do not take the Km-Nm resistance marker into consideration, fit quite well the linear and circular models of four-drug-resistance R factors which we presented previously (Watanabe and Fukasawa, 1961*b;* Watanabe and Lyang, 1962; Watanabe, 1963*b*). If we follow these models, we may be able to locate a Km-Nm resistance marker between Sm and Cm markers. These models, as well as some other possible models, apparently cannot account for all the results we have obtained; a few transductants do not fit these models. These "exceptional" types of transductants might be due to transducing phage particles which have developed as a result of multiple crossovers between the genomes of phage and R factor.

We do not know yet about the mechanism of development of R factors, although we have presented a hypothesis that R factors have developed as a result of "gene pick-up" by an episome RTF (resistance transfer factor; *see* Watanabe, 1963*a*), indicating that RTF is comparable to F, and R factors to F′ (Watanabe and Fukasawa, 1916*b;* Watanabe, 1963*a*).

Because of the discovery of a six-drug-resistance R factor, a new problem has emerged as to whether or not established R factors can pick up some additional drug-resistance genes from some as yet unknown bacteria. It is certain that naturally occurring Su, Sm, Cm, Tc-resistant R factors do not have sensitive alleles of the Km-Nm resistance marker, in view of the finding that we have been unable to isolate Km- and Nm-resistant mutants from the four-drug-resistance R factors even by ultraviolet treatment (*unpublished data*).

It is not known yet whether or not the ancestors of R factors are multiple; the R factors which are now distributed all over the world might have been derived from a common ancestor, or the R factors might be arising independently of each other in various places. Our comparative studies of the R factors from various parts of the world have shown that they are quite similar to those isolated in Japan (Watanabe, 1963*c*). The R_6 discovered in Lebek's strain is very particular in its Km and Nm resistance, but its RTF type was found by the present investigation to be identical to the fi^+ R factors isolated in Japan and some other countries. Furthermore, the genetic structure of R_6 seems to have much in common with the usual four-drug-resistance R factors.

In addition to the above genetic interest, the discovery of R_6 obviously bears a considerable medical importance. The increasingly wide administration of Km and Nm for treatment of enteric infections will undoubtedly give a selective advantage for Km- and Nm-resistant R factors. If R_6 developed as a result of gene pick-up by established R factors, it is possible that R factors will add even more drug resistance markers in the future, presenting a serious obstacle to chemotherapy.

ADDENDUM IN PROOF

After this paper was submitted for publication, we received a personal communication from G. Lebek. He has found that R_6 also transfers resistance to framycetine, paromomycin, and aminosidine, all of which are structurally related to kanamycin and neomycin. He assumes that resistance to all of these drugs is possibly controlled by a single gene on the R factor (*unpublished data*). We also learned from Lebek that he had studied the spontaneous segregation of R_6 and had assumed that the Km marker is closely linked to the Cm marker (Z. Hyg. Infektionskrankh. **149**:255, 1963).

ACKNOWLEDGMENTS

We are indebted to G. Lebek for supplying his *E. coli* strain. We also thank D. Ushiba for his encouragement and helpful discussions.

This investigation was supported by Public Health Service research grant E-4740 from the National Institute of Allergy and Infectious Diseases, by a research grant from the Ministry of Education, Japan, and by a research grant from the Waksman Foundation of Japan.

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