INFECTIVE HEREDITY OF MULTIPLE DRUG RESISTANCE IN BACTERIA

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INTRODUCTION 87
DRUG-RESISTANT SHIGELLAE IN JAPAN 88
DEVELOPMENT OF MULTIPLE DRUG RESISTANT SHIGELLAE AND *ESCHERICHIA COLI* 88
EPIDEMIOLOGY OF MULTIPLE DRUG RESISTANT SHIGELLAE AND *ESCHERICHIA COLI* 89
GENETICS OF MULTIPLE DRUG RESISTANCE 89
Transfer of Multiple Drug Resistance by Conjugation 89
Elimination of Resistance Factors with Acridines 92
Transduction of Multiple Drug Resistance 93
Concept of Resistance Transfer Factor (RTF) and R Factors 94
Concept of Episomes 95
Spontaneous Segregation of Resistance Factors 95
Recombination of R Factors 97
Circular Models of R Factors 97
Interactions between RTF and F Factor in Escherichia coli K-12 98
Interactions between RTF Type 1 and Colicinogenic Factors in Escherichia coli K-12 100
Evidence for Chromosomal Attachment of RTF 101
High Frequency Resistance Transfer System (HFRT) 101
Biological Functions and Mutations of RTF 102
Comparison of RTF with Other Episomes 104
Origin of Resistance Factors Carried by RTF 105
Molecular Basis of R Factors 106
BIOCHEMICAL MECHANISMS OF MULTIPLE DRUG RESISTANCE 107
SUMMARY 108
LITERATURE CITED 109

INTRODUCTION

In civilized countries, bacillary dysentery is no longer an important disease but in areas where public health is underdeveloped it still presents a serious problem. It seems rather paradoxical that in Japan, where sanitary conditions are considered to be fairly good, bacillary dysentery is still one of the most important infectious diseases. This is apparently due to the development of bacterial strains highly resistant to drugs. Shortly after World War II, a high incidence of sulfonamide-resistant shigellae appeared, and, since 1957, shigella strains with multiple drug resistance have been isolated with increasing frequency each year. This multiple drug resistance involves streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), and sulfonamide (Su). A small proportion of these *Shigella* strains are resistant to only some of the drugs but the majority are resistant to all. Since these drugs are our most powerful chemotherapeutics against dysentery, the phenomenon of multiple drug resistance creates a serious problem in the therapy of this disease.

In 1959, it was found by Japanese investigators (5, 6, 89) that multiple drug resistance can be easily transferred between shigellae and *Escherichia coli* by mixed cultivation. This discovery led many Japanese workers to the genetic study of multiple drug resistance. We have found (115, 123, 124, 125, 131) that the multiple drug resistance factors are carried and transferred by an episome (17, 47, 50). Multiple drug resistance is, therefore, an example of "infective heredity" (Zinder, 147; Lederberg and Lederberg, 61). Investigations of the biochemical mechanisms of multiple drug resistance indicate that, at least with Su, Cm, and Tc, the resistance is due to reduced permeability of the cells to the drugs.

Initially, the problem of multiple drug resistance received attention because of its medical importance, but more recently much effort has been devoted to genetic studies from which the episomal nature of the responsible factors is

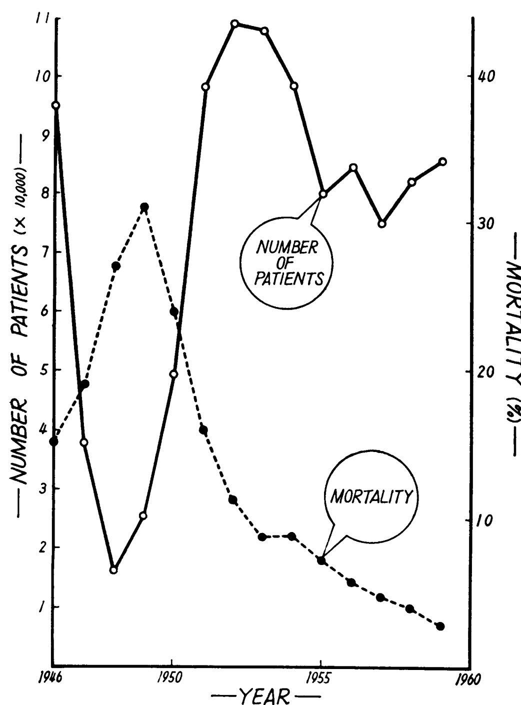

FIG. 1. *Statistics of bacillary dysentery in Japan between 1946 and 1959 (72).*

emerging as one of the most interesting problems. The reader is referred to two excellent reviews on episomes, Jacob, Schaeffer, and Wollman (47) and Campbell (17), which are relevant to the problem of multiple drug resistance. The present review will place particular emphasis on genetic studies of multiple drug resistance. Because many of the papers cited in the present review are written in Japanese, I will refer to them in more detail than is usual in reviews.

Drug-Resistant Shigellae in Japan

At the end of World War II, various derivatives of Su were introduced in Japan for the treatment of dysentery and proved to be very effective for the first several years, reducing the incidence considerably (Fig. 1). However, after about 1949, the incidence of dysentery again increased, despite extensive use of Su, and most of the *Shigella* strains isolated from those cases were found to be resistant to the drug. The peak incidence of dysentery occurred in 1952. Subsequently, newer antibiotics, such as Sm, Cm, and Tc, were employed for the treatment of Su-resistant shigellae, with initially excellent therapeutic effects. The number of dysentery patients was again reduced. However, within only 4 years *Shigella* strains resistant to these antibiotics emerged, and the number of dysentery patients has increased markedly since 1957. On the other hand, the mortality rate has continued to decline.

Development of Multiple Drug Resistant Shigellae and *Escherichia coli*

Strains of *Shigella* resistant to either Sm or Tc were isolated as early as 1953 but strains with multiple resistance were not found until 1955 (Table 1). The most frequently encountered type of resistance is for Sm, Cm, and Tc. The second most frequent type is for Sm and Cm. Strains resistant to either Tc or Sm alone are also fairly common. However, relatively few strains are resistant to Sm and Tc, or to Cm and Tc, and no strains resistant to Cm alone have been found. Many of the strains listed in Table 1 are resistant to Su as well. The first isolation of shigellae with multiple drug resistance was reported in a dys-

TABLE 1. *Statistics of antibiotic-resistant shigellae in Japan**

Year	No. of strains tested	No. of strains resistant to						
		Sm	Tc	Cm	Sm and Cm	Sm and Tc	Cm and Tc	Sm, Cm, and Tc
1953	4,900	5	2	0	0	0	0	0
1954	4,876	11	0	0	0	0	0	0
1955	5,327	4	0	0	0	0	0	1
1956	4,399	8	4	0	0	0	1	0
1957	4,873	13	45	0	2	2	0	37
1958	6,563	18	20	0	7	2	0	193
1959	4,071	16	32	0	71	0	0	74
1960	3,396	29	36	0	61	9	7	308
Total	38,405	104	139	0	141	13	8	613

* Only one strain from each epidemic is included in this table. The statistics from 1953 to 1958 are derived from several independent reports (Mitsuhashi et al., 76). The statistics for 1959 and 1960 were reported by the Ministry of Health and Welfare, Japan. Abbreviations: Sm = streptomycin; Tc = tetracycline; Cm = chloramphenicol; Su = sulfonamide.

entery patient who had just returned from Hong Kong in 1955 (Kitamoto et al., 52). After this report, many epidemics of multiple drug resistant shigellae were observed by many workers throughout Japan. In one of those epidemics, Matsuyama et al. (68) isolated multiple drug resistant *E. coli* strains from dysentery patients. Little attention was paid to this report until epidemiological studies of multiple drug resistant shigellae disclosed several unexpected features, which will be described below.

Epidemiology of Multiple Drug Resistant Shigellae and *Escherichia coli*

In epidemiological studies of dysentery, several workers repeatedly found that completely sensitive *Shigella* strains can be isolated from some patients, whereas the strains isolated from other patients in the same epidemic, and belonging to the same serological type, show multiple drug resistance (4, 68, 86). Some patients were even found to excrete both sensitive and multiple drug resistant *Shigella* strains of the same serological type. On the other hand, the administration of a single drug, for example Cm, to patients harboring sensitive *Shigella* strains sometimes caused the excretion of multiple drug resistant *Shigella* strains (51, 53, 88). This observation cannot be attributed to mutations, as will be discussed below. No acceptable explanation of these curious findings was forthcoming until Akiba (2) suggested that multiple drug resistance may be transferred from multiple drug resistant *E. coli* to shigellae in the intestinal tracts of the patients. This transfer was demonstrated independently by Ochiai et al. (89) and Akiba et al. (5, 6) in vitro. Judged by the severity of clinical symptoms, the multiple drug resistant *Shigella* strains did not differ from the sensitive strains in their pathogenicity to human beings (Ochiai, 86). The reduction in mortality seen in Fig. 1 is presumably due to improved methods for treatment of dysentery.

Genetics of Multiple Drug Resistance

Transfer of Multiple Drug Resistance by Conjugation

After Ochiai et al. (89) and Akiba et al. (5, 6) succeeded in discovering the transfer of multiple drug resistance in vitro, they attempted unsuccessfully to transfer the resistance with cell-free filtrates of resistant donor cultures. Accordingly, they concluded that cell-to-cell contact (or conjugation) is essential for the transfer. Their results indicated also that neither the serological nor the biochemical markers, which are studied routinely in diagnostic bacteriology, are changed by the transfer of multiple drug resistance. They suggested that multiple drug resistance may be transferred by mating (recombination after conjugation). Although this explanation proved to be incorrect, the previously mentioned unexpected epidemiological observations were clearly explained by the successful transfer of multiple drug resistance in vitro. Subsequently, Kagiwada et al. (51) and Akida et al. (7) proved with human volunteers that multiple drug resistance can be transferred from multiple drug resistant *E. coli* to shigellae in the intestinal tract. The transfer of multiple drug resistance in vivo was also shown in dogs (Mitsuhashi, Harada, and Hashimoto, 73) and mice (Akiba et al., 7).

Mitsuhashi et al. (80) reported that multiple drug resistance could be transferred among the substrains of *E. coli* K-12 irrespective of their sexuality (presence or absence of F factor), indicating that the F factor of K-12 is not required for the transfer of multiple drug resistance. Watanabe and Fukasawa (116, 123) found that the responsible resistance factors are transferred by mixed cultivation independently of the host chromosome, using F^- strains of *E. coli* K-12 with various chromosomal markers. We can, therefore, regard multiple drug resistance as a kind of "infective heredity" (Zinder, 147; Lederberg and Lederberg, 61). Mitsuhashi (72) reported that he was unable to isolate multiple drug resistant mutants by exposing sensitive *Shigella* and *E. coli* strains to single drugs in vitro, and that the single drug resistance thus obtained could not be transferred to other sensitive strains by mixed cultivation. Mutants with multiple drug resistance were obtained in vitro as multistep mutants by exposing sensitive strains to single drugs, one after another. Multiple drug resistance of these mutants, however, could not be transferred by mixed cultivation. Watanabe and Fukasawa (116, 123) reported that the transfer of multiple drug resistance does not reduce the growth rate of cells. These organisms, therefore, differ from the usual slow-growing drug-resistant mutants (114, 137, 138).

In addition to the multiple drug resistance, various combinations of resistance factors from naturally occurring drug-resistant *Shigella* and *E. coli* strains were found to be transmissible in mixed cultivation by Ochiai et al. (89), Akiba et al. (5, 6), and Mitsuhashi et al. (76, 77, 81). Those are (Su, Sm, Cm), (Su, Sm, Tc), (Cm, Tc), (Su, Sm), (Tc), (Sm), and (Cm). The drugs indicated in the parentheses represent the combinations of resistance factors that can be transferred together by conjugation. (Cm) was isolated as a spontaneous segregant (see below) from (Cm, Tc) in vitro and has never been isolated from natural specimens. Transmissible (Su) has never been obtained either from natural specimens or experimentally. Many of the studies on transmissible drug resistance have been concerned with the (Su, Sm, Cm, Tc) complex because of its medical and genetic importance.

Transfer of multiple drug resistance to genera other than Shigella and Escherichia. Nakaya, Nakamura, and Murata (84) and Harada et al. (32, 33) extended the findings of Ochiai et al. (89) and Akiba et al. (5, 6) and found that multiple drug resistance can be transferred to almost every genus of the family *Enterobacteriaceae*. Ochiai (87) reported that multiple drug resistance cannot be transferred to staphylococci and *Vibrio comma*. It was recently reported, however, that multiple drug resistance can be transferred to *Serratia marcescens* (Falkow et al., 22) and also to *V. comma* (Baron and Falkow, 15) by mixed cultivation.

The frequencies of transfer of multiple drug resistance differ considerably from donor to donor and also from recipient to recipient. F^- strains of *E. coli* K-12 are the best recipients so far encountered (Watanabe and Fukasawa, 122), the frequencies of transfer in 1 hr ranging from about 10^{-2} to less than 10^{-7} per donor cell, depending on the donor strains employed. Some of the donor strains were found to produce colicins and phages, which reduce the frequency of transfer by killing the recipient cells. Similarly, donors may be killed by colicins and phages produced by recipients. When various recipients were used for the same donor, *Salmonella* strains were found to develop multiple drug resistance only with very low frequencies, namely, less than 10^{-7} per donor cell even when good donors were used (Watanabe and Fukasawa, 116, 123). It is not known why salmonellae are poor recipients.

Effects of physiological conditions on the transfer of multiple drug resistance. Various effects of environmental conditions on the transfer of multiple drug resistance have been reported but some of them lack clear-cut interpretations and require further elucidation. Koyama and Akiba (55) reported that the addition of sodium deoxycholate to the media inhibits the transfer of multiple drug resistance from *E. coli* to shigellae. Since this compound did not inhibit the transfer of multiple drug resistance from *Shigella* to *Shigella*, and since it is known that *E. coli* is more sensitive to deoxycholate than shigellae, a reasonable interpretation is that sodium deoxycholate, even in sublethal concentrations, exerts drastic physiological effects on *E. coli* cells.

Yokota and Akiba (145) reported that sodium dodecylsulfate, an anionic detergent, suppresses the transfer of multiple drug resistance in mixed cultivation. Two other anionic detergents, caproic acid and xylenesulfonic acid, did not show this inhibitory effect. They also reported that mitomycin C, which specifically inhibits the synthesis of deoxyribonucleic acid (DNA; Sekiguchi and Takagi, 102), also inhibits the transfer of multiple drug resistance.

Akiba et al. (7) found that the transfer of multiple drug resistance does not take place in the intestinal tract as easily as it does in vitro. Kato (*personal communication*) showed that the addition of fecal filtrates to the culture media reduces the frequency of transfer. By fractionation, the active substances in the fecal filtrates were identified as fatty acids and deoxycholate. However, the concentration of deoxycholate found was too low to account for the observed effect.

Iijima (40) reported that treatment of resistant donor cells with sodium periodate transiently deprives them of the ability to transfer multiple drug resistance by conjugation. He assumed that this inhibition may result from the destruction of "mating substance" of polysaccharide nature. Sneath and Lederberg (106), in a similar experiment, found that treatment of F^+ and Hfr cells with sodium periodate temporarily devirilizes the male bacteria.

Yokota and Akiba (142) reported that multiple drug resistance can be transferred in the combinations of penicillin-spheroplast donor and intact recipient, intact donor and penicillin-spheroplast recipient, and also of penicillin-spheroplasts of both donor and recipient. These

findings are comparable to those of Lederberg and St. Clair (62) and Hagiwara (26) on the mating of *E. coli* K-12, and of Iijima (39) on the transfer of colicinogenic factor E1, indicating that the intact cell walls of donor and recipient cells are not essential for the transfer. On the other hand, if the "mating substances" are essential for the transfer of multiple drug resistance, F factor, and host chromosome, they must be retained by the penicillin spheroplasts.

Watanabe and Fukasawa (129) and Iwahara and Akiba (43) found that cultures in the stationary phase provide somewhat more competent donors than those in the logarithmic phase. The reason for this difference is not known. In addition, Watanabe and Fukasawa (129) observed that cells which have just received the multiple drug resistance factor can transfer it with exceedingly high frequencies. This finding will be discussed later.

Akiba and Iwahara (3) reported that the optimal pH and temperature for the growth of donor and recipient strains are also optimal for the transfer of multiple drug resistance. Similar findings have been obtained by Hayes (35) in the mating of *E. coli* K-12.

Egawa et al. (19) reported that the transfer of multiple drug resistance does not take place in saline, or in a synthetic medium lacking a carbon source, or under anaerobic conditions in a complete medium. They observed also that monoiodoacetate, sodium fluoride, and fluoroacetate have no significant effect on the transfer of multiple drug resistance, whereas potassium cyanide, malonate, sodium arsenite, and 2,4-dinitrophenol are inhibitory. They concluded that the energy provided by oxidative phosphorylation is essential for the transfer. A similar conclusion had been reached already by Fisher (23, 24) in studies on zygote formation of *E. coli* K-12. Furthermore, Fisher found that the energy is required uniquely by the Hfr parent, and only during the period of chromosomal transfer.

As indicated above, many reports have dealt with the effects of various chemical and physical conditions on the transfer of multiple drug resistance, but few have attempted to differentiate between effects on donors and recipients. This fact makes interpretation of the findings difficult.

Phenotypic expression of resistance factors. The resistance factors received by the recipient cells are rather rapidly expressed phenotypically. In the substrains of *E. coli* K-12, resistance to Cm and Tc is completely expressed within 10 min after donor and recipient are mixed (Watanabe and Fukasawa, 126). This is in contrast to the phenotypic expression of the usual (chromosomal) Sm resistance, which is considered to be recessive to its sensitive allele (Lederberg, 57; Watanabe and Watanabe, 137). Although phenotypic expression of this recessive Sm resistance marker requires segregation from its sensitive alleles, most of the factors involved in multiple drug resistance do not require cell division for their phenotypic expression. Only the Sm resistance factor apparently requires longer incubation for its phenotypic expression. However, with certain recipient cells, Sm resistance is expressed phenotypically before the first cell division, suggesting that it is not recessive. The difference between the Sm resistance factor and the other factors involved in multiple drug resistance is possibly due to the rapid bactericidal action of Sm. The other drugs are bacteriostatic and act rather slowly. Furthermore, as will be discussed later, the strains with multiple drug resistance were found to decompose Cm (Miyamura, 82; Yokota and Akiba, 144) and possibly Tc (Watanabe et al., *unpublished data*). When the mixed culture of donor and recipient is plated on selective media containing Cm or Tc, without eliminating donor cells, the donor cells decompose the drugs and reduce their active concentrations. Then the recipient cells, which have been unable to divide, start dividing slowly. In this manner, the transfer and phenotypic expression of multiple drug resistance takes place on selective media which initially contained high enough concentrations of Cm or Tc to inhibit the recipient.

In media containing Su, the potency of Su is presumably reduced by the production of antagonists by the donor cells. Therefore, to determine accurately the frequency of transfer of multiple drug resistance, it is essential to eliminate the donor cells before plating the mixed culture on the selective media. High concentrations of Sm (1,000 μg per ml) and high titers of phage T6, to which donor cells are sensitive and recipient cells are resistant, have been successfully used for eliminating donor cells (Watanabe and Fukasawa, 126). The Sm resistance factor included in the multiple drug resistance endows *E. coli* with only a low level of Sm resistance (between 10 and 25 μg per ml), as will be described subsequently.

The levels of resistance differ considerably among strains manifesting multiple drug resistance (Watanabe and Fukasawa, 116, 123). Sm resistance is very high in shigellae (more than 1,000 μg per ml) but rather low in *E. coli* (between 10 and 25 μg per ml). Tc resistance is low in *Salmonella* strains (about 10 μg per ml) but higher in shigellae and *E. coli* (between 100 and 250 μg per ml). Even among the substrains of *E. coli* K-12 with multiple drug resistance, the levels of Cm and Su resistance may differ. Thus multiple drug resistance provides an interesting technique for studying "nucleocytoplasmic" relationships.

Transferred multiple drug resistance can be further transferred to other sensitive strains indefinitely. There is no evidence that the resistance factors are modified by host cells during their transfer from strain to strain.

Kinetics of transfer of multiple drug resistance. It was found by Watanabe and Fukasawa (119, 123) that the transfer of multiple drug resistance can be interrupted by treatment with a blender, indicating that the transfer is actually caused by conjugation. By diluting the mixture of donor and recipient at various times to reduce the chances of conjugation, they showed further that the conjugation starts taking place almost instantaneously after donor and recipient are mixed. By interrupting the conjugation with phage T6 (Watanabe and Fukasawa, 126), it was found that the transfer of multiple drug resistance takes place within 1 min after mixing of donor and recipient. No segregational transfer of multiple drug resistance could be found, even when the conjugating mixture was treated with a blender or with phage T6 at an early stage.

Incubation of a small number of multiple drug resistant cells with a large number of sensitive cells, under conditions which preclude chromosomal transfer, caused a majority of the sensitive cells to acquire multiple drug resistance rather quickly, indicating that the resistance factors responsible for the transfer replicate faster than the host chromosome (Watanabe and Fukasawa, 116, 123). This finding, together with the wide host range for transfer of multiple drug resistance and the independence from host chromosomal markers, suggests that the resistance factors replicate in the cytoplasm independently of the host chromosome (17, 47). It should be emphasized here that all four resistance factors are transferred together as a unit, suggesting close linkage between them.

Elimination of Resistance Factors with Acridines

Ephrussi, Hottinguer, and Chimenenes (20) found that cytoplasmic factors of yeast can be eliminated by acriflavine, converting the cells to "petite colonie" mutants. Hirota and Iijima (37) and Hirota (36) reported that acriflavine converts F^+ cells of *E. coli* K-12 to F^- by eliminating the F factor. Watanabe and Fukasawa (118, 124) found that treatment of multiple drug resistant shigellae and *E. coli* with acriflavine and acridine orange converts the cells to drug sensitivity, although with low frequencies, indicating that the resistance factors are in an autonomous state. In the elimination of resistance factors with acridines, no segregated elimination was noted. Furthermore, acridine orange was less potent than acriflavine in eliminating the resistance factors, unlike the situation with the F factor reported by Hirota (36). Elimination of multiple drug resistance with acriflavine was also demonstrated by Mitsuhashi, Harada, and Kameda (78, 79).

We have no clear-cut explanation for this low frequency of elimination at the present time. It might result from prevalence of integrated (or chromosome-attached) factors of multiple drug resistance, as will be discussed below. Hirota (36) found that the F factor in Hfr strains, which is considered to be integrated, cannot be eliminated with acridines. The fact that treatment of ultraviolet-irradiated resistant cells with acridines results in more efficient elimination of multiple drug resistance (Watanabe and Fukasawa, 118, 124) might be interpreted as promoting "induction" of integrated resistance factors by ultraviolet, resembling the ultraviolet-induction of prophages (Lwoff, 66).

Cells, which have lost the resistance factors by treatment with acridines, were found to accept subsequent transfer of resistance factors about as readily as did the originally sensitive recipient. As will be discussed subsequently, recipients already possessing part of the resistance factors can only receive the other resistance factors with a reduced frequency. Hence, the sensitive cells obtained by treatment with acridines are assumed to have no memory of the multiple drug resistance, which might confer "immunity" upon them against reinfection.

Watanabe and Fukasawa (118, 124) reported a strain of *Shigella* with multiple drug resistance which gives rise to Su-resistant cells after treatment with acridines. Because this Su resistance is not transmissible, and because the original strain can transfer multiple drug resistance including Su resistance, they concluded that the original multiple drug resistant *Shigella* possessed two Su resistance factors, one cytoplasmic and the other chromosomal.

Transduction of Multiple Drug Resistance

Ochiai et al. (89), Akiba et al. (5, 6), Mitsuhashi, Harada, and Hashimoto (74), and Harada et al. (30) did not believe that transduction or transformation were involved in the transfer of multiple drug resistance, because cell-free filtrates of the resistant cultures were incapable of converting the sensitive recipient cells to resistance. Their conclusions were correct as far as their systems were concerned. However, Nakaya, Nakamura, and Murata (84) and Watanabe and Fukasawa (117, 125) later succeeded in transducing multiple drug resistance in *E. coli* K-12 with phage P1kc, and Watanabe and Fukasawa (117, 125) also effected transduction of multiple drug resistance in *Salmonella typhimurium* LT-2 with phage P-22. Nakaya, Nakamura, and Murata (84) did not investigate the transduction of Su resistance, although they employed a donor resistant to four drugs. Watanabe and Fukasawa (117, 125) studied the transduction of the four resistance factors involved and also the transferability of the transduced resistance factors by conjugation. The results are quite different in these two systems. (i) With P1kc, Su, Sm, Cm, and Tc resistance factors are transduced together most frequently; segregated transductions are rather rare, the patterns of segregation so far observed being the Tc-resistant type and the Sm, Cm, Tc-resistant type. (ii) All these transductants with P1kc can transfer their resistance factor(s) by conjugation. (iii) With P-22, the resistance factors are invariably segregated, the patterns of segregation being Su, Sm, Cm resistance, Tc resistance, and, very rarely, Su, Sm resistance. (iv) Only a minority of the multiple-resistant and the Tc-resistant transductants with P-22 can transfer their resistance factor(s) by conjugation. Ultraviolet irradiation of the other transductants did not induce such ability to transfer. By using spontaneous segregants (see below) derived from a multiple drug resistant strain, Tc resistance was transferred to an Su, Sm, Cm-resistant transductant of LT-2, and Su, Sm, Cm resistance was transferred to a Tc-resistant transductant of LT-2 with no ability to transfer the Tc resistance by conjugation. The four-drug resistant strains thus obtained, however, could transfer by conjugation only the resistance factor(s) received by conjugation and none of those factors initially received by transduction.

From these results, Watanabe and Fukasawa (120, 125) presented a hypothesis of episome-mediated transfer, according to which the drug resistance factors are carried and transferred by an episome, which they designated as "resistance transfer factor" (RTF). They postulated the participation of an episome, because the resistance factors in the transductants of LT-2, which cannot be transferred by conjugation, were suspected to be in an integrated state. They considered that unless the resistance factors are integrated, those segregated from the episome should be unable to replicate autonomously and would assume the state of abortive transduction (Stocker, 108; Lederberg, 59; Ozeki, 94). Such abortive transductants should fail to form colonies on drug-containing media. In fact, however, the resistant transductants of LT-2 inherit their drug resistance quite stably.

Harada et al. (34) succeeded in reproducing the results of transduction in *S. typhimurium* LT-2 with P-22 obtained by Watanabe and Fukasawa (117, 125), using instead *S. anatum* with phage ϵ^{15} (Iseki and Sakai, 41, 42; Uetake, Nakagawa, and Akiba, 113). The characteristics of the resistant transductants were quite similar to those of LT-2 with P-22. The transduced resistance factors could also be transduced by another phage, ϵ^{34} (Harada, 28, 29; Uetake, Luria, and Burrous, 112). In this second-round transduction by ϵ^{34}, Su, Sm, and Cm resistance factors were again transduced together.

Harada et al. (31) recently found that the transfer of F factor to a TC-resistant transductant of *S. anatum* induces the transfer of the resistance factor by conjugation to other strains independently of the host chromosomes. The recipient cells, which received both F factor and the resistance factor, were able in turn to transfer the resistance factor, but those which received only the resistance factor could no longer transfer it by conjugation. These results suggest that the resistance factor in the TC-resistant trans-

ductant of salmonellae may be in an autonomous state but cannot be transferred by conjugation, owing to some defect in the resistance factor. The resistance factors transduced to *Salmonella* strains are quite stably inherited, unlike those received by conjugation, in which state they frequently undergo spontaneous segregation (see below). The mechanism by which transduced resistance factors in salmonellae cells replicate autonomously but cannot be transferred by conjugation is not known at present. It is possible that the genetic determinants involved in the transmissible resistance factors, and responsible for their transfer by conjugation, cannot be picked up together with the resistance factors by transducing phage. On the other hand, it is also possible that the resistance factors segregated by transducing phage, from the genetic determinants responsible for their autonomous replication and transfer by conjugation, are able to replicate autonomously, being supported by the genome of the transducing phage. In fact, a similar situation was reported by Luria, Adams, and Ting (65) for P1d1 elements (defective P1 phage, which incorporated the lactose region of the host genome).

The exceptional type of Tc-resistant transductant can transfer its Tc resistance factor by conjugation as indicated above. The Tc resistance factor of these transductants is also spontaneously segregated. The factor which stabilizes the autonomously replicating nontransmissible resistance factors in the transductants is not known. Thus, the previously discussed hypothesis of Watanabe and Fukasawa (119, 124), according to which the transduced resistance factors in salmonellae may be integrated, may require reinvestigation.

It was difficult to understand why no Su, Sm, Cm-resistant type of transductant could be found in K-12 with P1kc in the experiments of Watanabe and Fukasawa (117, 125). Subsequent studies of transduction with *E. coli* K-12 and P1kc by Kondo, Harada, and Mitsuhashi (54), using a different four-drug resistant donor, enabled them to find this type of transductant, although with low frequencies.

The kinetics of phenotypic expression of transduced resistance factors was studied by Watanabe and Fukasawa (117, 125). They found that Su, Cm, and Tc resistance is phenotypically expressed rapidly, as in the transfer of resistance factors by conjugation, whereas the phenotypic expression of Sm resistance requires a rather long incubation period.

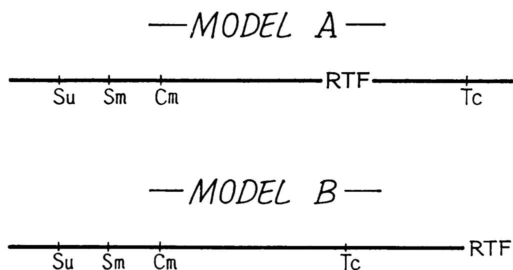

FIG. 2. *Linear models of an R factor (Su, Sm, Cm, Tc) proposed by Watanabe and Fukasawa (125).*

Concept of Resistance Transfer Factor (RTF) and R Factors

Watanabe and Fukasawa (125) presented two linear models of the resistance factors carried by RTF on the basis of their transductional studies (Fig. 2). They assumed that the resistance factors may have originated from a chromosome of some bacteria. This assumption necessitates the possibility of attachment of RTF to host chromosome when RTF picks up the chromosomal genes. In transduction, RTF without any resistance factors may well be produced, but there is no way at present to select for such transductants.

Before the genetic structure and episomality of the multiple drug resistance factors and other infective resistance factors were disclosed, Iseki proposed the term "R-factors" for the resistance factors which are easily transferred from cell to cell by conjugation. This is a simple and convenient term and has been used by Mitsuhashi (72) and Sugino and Hirota (109). Nakaya, Nakamura, and Murata (84) proposed the term "resistance transfer agents" (Rta) for the same factors. The term R factors has already been employed by several groups of workers, and it seems unwise to confuse the terminology further by using the term Rta. Furthermore, Rta can be confused with RTF. I will, therefore, use the term R factors in the present review. I wish to emphasize, however, that the term R factors should be used in a stricter sense than heretofore. As indicated subsequently, Watanabe and Lyang (134, 135) recently isolated two unusual strains of multiple drug resistance from a common type of four-drug resistant parent of *S. typhimurium* LT-2. Although the resistance factors of these

unusual strains are in an autonomous state, they cannot be transferred by conjugation. It is assumed that RTF in these strains has undergone a "defective" mutation. I believe that the term R factors should be used to denote agents which are composed of RTF and resistance factors, irrespective of their transmissibility by conjugation.

Watanabe and Fukasawa (115, 116, 123, 129) tested *Shigella* and *E. coli* strains with R factors for the production of colicins. Some strains were found to be colicinogenic, but the R factors and colicinogenic factors always segregated from each other in their transfer by conjugation, indicating that there are two independent factors. Strains of *E. coli* K-12 which received R factors were tested for colicinogeny, using more than 150 strains of *E. coli* and shigellae as indicators. These included *E. coli* K-12 and ϕ and *Shigella sonnei* E-90, which are known to be sensitive to many colicins. Colicinogeny was not detected. Watanabe and Fukasawa (115, 116, 123, 129) also studied the possibility that the R factors may make F^- strains of *E. coli* K-12 fertile, but failed to prove fertility, using various chromosomal genes as selective markers. Sugino and Hirota (109), however, recently reported that R factors endow F^- strains of K-12 with fertility; this will be discussed later.

It was shown by Ochiai et al. (89), Akiba et al. (5, 6), Mitsuhashi, Harada, and Hashimoto (74), and Harada et al. (30) that cell-free filtrates of donor cultures are ineffective in transferring multiple drug resistance. It is, therefore, evident that phages are not involved in the usual RTF systems, except in the rather exceptional instances where transduction occurs. Therefore, it is certain that RTF is not a phage.

R factors are the first clear-cut examples of episome-mediated transfer of drug resistance. Sager (101) reported on non-Mendelian inheritance of Sm resistance in *Chlamydomonas reinhardi*, but the chromosomal attachment of the responsible factor has not been demonstrated.

Concept of Episomes

The term episome was first used by Thompson (110), and only later by Jacob and Wollman (50), in a different sense. Episomes cannot be regarded as essential constituents of cells, for cells without them have no difficulty in surviving under normal conditions. When present in cells, episomes may exist in two alternative states, autonomous or integrated. In the autonomous state, they replicate independently of the host chromosome, and usually at a faster pace. The integrated state refers to the state of attachment of episomes to the host chromosome; in this state, the episomes replicate at the same pace as the host chromosome. It has generally been observed that autonomous replication of episomes is inhibited by the presence of the integrated state of the same episomes. Jacob, Schaeffer, and Wollman (47) included temperate phages, sex factors of *E. coli*, and colicinogenic factors among episomes. The "sporogenic factor" of *Bacillus* species was also considered to be a possible episome. These investigators pointed out a close relationship among the episomes, and assumed that each may be able to mutate to other types of episomes. Luria, Adams, and Ting (65) suggested that episomes other than temperate phages may be produced as the result of incorporation of fragments of host genome by temperate phages.

Campbell (17) proposed to include among episomes other hereditary cytoplasmic factors, whose integrated state has not yet been proved. For example, "lac-episome" (13, 14, 15, 22), "sporulation factor" in *Bacillus* species (47), mycelial factor in *Aspergillus* (100), and "controlling elements" in maize (69) would be regarded as episomes, in addition to temperate phages, F agents, colicinogenic factors, and resistance transfer factor. According to Campbell, these episomes could be divided into two classes, namely, temperate phages (or, more strictly, the genetic material thereof), and transfer factors. Transfer factors are the episomes which can pass from cell to cell during conjugation, independently of the bulk of the host genome. Some of the transfer factors play a causative role in the conjugation process itself.

Subsequent to the suggestion of Watanabe and Fukasawa (115, 120, 125) that RTF is a kind of episome, much work has been devoted to proving the episomal nature of RTF and R factors and studying its relationship to other known episomes. The autonomous state of R factors was proved (see above), and the integrated state of RTF was demonstrated (see below). We can, therefore, classify both RTF and R factors as episomes.

Spontaneous Segregation of Resistance Factors

Many investigators working on R factors have noted occasionally the spontaneous loss of part

or all of the resistance factors. It is our impression that the spontaneous segregation of resistance factors occurs more readily in aged cultures kept at room temperature than in fresh cultures. The reason for this is unknown. Watanabe and Fukasawa (118, 124) studied the spontaneous segregation of resistance factors with a penicillin screening method, modified from the original method of Davis (18). The cells with multiple drug resistance were incubated in broth containing penicillin and either Cm or Tc. The spontaneous segregants, lacking resistance factors to the latter drugs, cannot divide in broth containing these drugs, and selectively survive the lethal action of penicillin. The spontaneous segregants were found to be resistant either to Su, Sm, and Cm, or to Tc. Complete loss of the resistance factors was also noted. The frequency of spontaneous segregation of resistance factors was highest in salmonellae and lowest in *E. coli*. With shigellae, intermediate frequencies of spontaneous segregation were observed.

It should be emphasized that all the spontaneous segregants which possess any of the resistance factors can transfer these resistance factors by conjugation (Watanabe and Fukasawa, 115, 118, 124). Yamanaka (141) assumed that some of the resistance factors are mechanically cut off and cast off. This suggests that each resistance factor controls its transfer by conjugation. Such an explanation is unlikely in view of transduction experiments (Watanabe and Fukasawa, 117, 125), which showed that an episome RTF occupies a particular position on R factors. There is other evidence against the hypothesis that each resistance factor controls its transfer by conjugation. For example, Watanabe and Fukasawa (128) found that all types of R factors, including the spontaneous segregant types, suppress the acceptance of R factors and also inhibit the functions of F factor of *E. coli* K-12 (described in Section V-I). These functions of R factors were found not to be specific for any particular resistance factor, and may be ascribed to RTF, which is common to all R factors.

Watanabe and Fukasawa (115, 125) and Watanabe and Lyang (134) suggested that spontaneous segregation of resistance factors is brought about by genetic exchange between RTF-carried resistance factors and host genome (Fig. 3). They accounted for differences in the frequencies of spontaneous segregation of resistance factors among various bacterial species by the degrees of genetic homology between the R factors and the host genome.

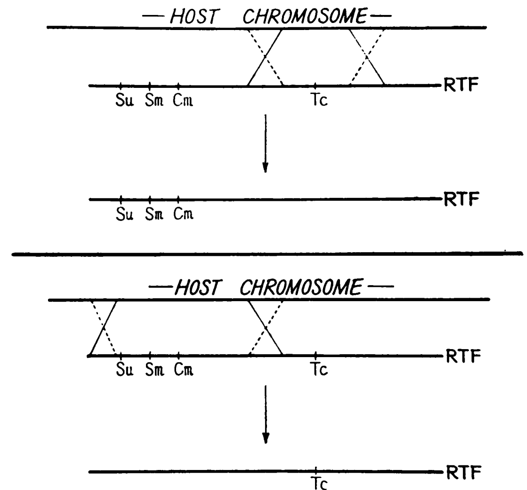

FIG. 3. *Mechanism of spontaneous segregation of resistance factors. Hypothesis of genetic exchange between R factor and host genome.*

Watanabe and Lyang (134) found that the frequency of spontaneous segregation of resistance factors is so high in *S. typhimurium* that clonal analysis of segregation is possible with the replica plating technique (Lederberg and Lederberg, 60). When approximately 500 cells of *S. typhimurium* with an R factor (Su, Sm, Cm, Tc) were inoculated into 5 ml of broth and incubated at 37 C for 24 hr, the resulting culture consisted of about 75% four-drug resistant cells and 25% cells lacking some or all of the resistance factors. The combination of Su, Sm, Cm resistance factors was lost most frequently. It was assumed that the genetic exchange takes place when the R factors are in the integrated state, thus preventing their autonomous replication in cytoplasm. Otherwise, genetic exchange should not easily give rise to segregant clones because of the autonomously replicating R factors. These investigators did not succeed in isolating drug-resistant clones with no RTF, which would be direct evidence for genetic exchange between RTF-carried resistance factors and host genome. They did isolate an Su, Sm, Cm resistant clone and a four-drug resistant clone, which were unable to transfer their resistance factors by conjugation. These clones were found to have "defective" RTF, as will be discussed later.

In addition to the possibility of genetic ex-

change between RTF-carried resistance factors and host genome, the following mechanism may be involved also. If replication of the R factor starts at one end and for some unknown reason stops somewhere between the two ends, segregant types of R factors should be produced. Circular models of R factors (see below) may be more useful in explaining the development of various combinations of resistance factors in R factors.

Epidemiological studies of drug-resistant shigellae clearly indicate that the four-drug resistant strains developed early. There is no evidence that the original multiple-drug resistance was produced in nature by the successive acquisition of single resistance factors. On the other hand, Ochiai et al. (89), Akiba et al. (5, 6), and Mitsuhashi et al. (76, 77, 81) found various types of R factors with various combinations of the resistance factors. Furthermore, it was found that R factors with multiple resistance factors can be "synthesized" by adding the missing resistance factors by conjugational transfer of R factors (see below). It is possible that multiple drug resistance develops in nature via this mechanism, between the spontaneous segregants of multiple-drug-resistant strains. Rare types of R factors such as (Su, Sm, Tc), (Cm, Tc), (Su, Sm), (Sm), and (Cm) could not be found as spontaneous segregants of (Su, Sm, Cm, Tc) by Watanabe and Fukasawa (115, 118, 124) and Watanabe and Lyang (134), but might be expected to develop on the basis of the two hypotheses proposed.

Recombination of R Factors

Mitsuhashi, Harada, and Hashimoto (74), Nakaya, Nakamura, and Murata (84), Yamanaka (140), and Ochiai, Yamanaka, and Kimura (90) found that additional R factors could be transferred to recipient strains possessing other types of R factor. However, according to Mitsuhashi (72), transfer of R factors to recipients possessing other types of R factor is only about 0.01 times as frequent as with sensitive recipients. Watanabe and Fukasawa (115, 120, 131) independently carried out similar experiments with an R factor (Su, Sm, Cm, Tc) and its spontaneous segregants, (Su, Sm, Cm) and (Tc). They also observed that the frequencies of transfer of R factors are reduced to about 10^{-2} by the presence of R factors in recipients, and they concluded that this suppression of acceptance of R factors is determined by RTF and not by resistance factors. It was assumed that this phenomenon may be related to the "immunity" or "para-immunity" of temperate phages (Lwoff, 66; Zinder, 148). Watanabe and Lyang (135) and Mitsuhashi, Harada, and Hashimoto (75) found that some of the four-drug resistant strains thus obtained are quite unstable genetically, segregating clones with either of the original two types of R factors with high frequencies. These findings indicate two possible explanations: (i) that R factors are frequently integrated and suppress the autonomous replication of any types of R factors; and (ii) that the number of particles of R factors in a cell is so limited that segregation takes place occasionally as the cells divide. We have no evidence at present for discriminating between these two possibilities. Neither is unreasonable. Integration of RTF has been shown (see below). Also, the number of F factors in a cell (and these resemble RTF in many respects) is assumed to be only about three (Jacob and Monod, 46). Furthermore, F factor of F^+ strains is assumed to be lost spontaneously with rather high frequencies (Skaar, Richter, and Lederberg, 105). Cells which have lost F factor are apparently easily reinfected with F factor by other cells in the culture that carry F factor. In contrast, cells which have lost R factors are apparently not so easily reinfected, judging from the low frequencies of transfer of R factors.

Some of the clones receiving two types of R factor develop quite stable four-drug resistant clones (Watanabe and Lyang, 135). The four-drug resistance thus established behaves in the same way as the original four-drug resistance and seldom segregates spontaneously. Also, in transduction with P1kc the patterns of linkage of resistance factors and RTF resemble the results obtained when the original four-drug resistant strain is used as a donor (Watanabe and Lyang, 135). In other words, the four-drug resistance factors are transduced together in most instances. Watanabe and Lyang (135) assumed that a stable R factor (Su, Sm, Cm, Tc) is formed by recombination of the two R factors (Su, Sm, Cm) and (Tc) (Fig. 4).

Circular Models of R Factors

After formulation of a circular chromosome model for a virulent phage by Streisinger (*personal communication* to Campbell, 17), Campbell (17) proposed a similar circular model for phage λ, F factor, colicinogenic factor, and F′. He sug-

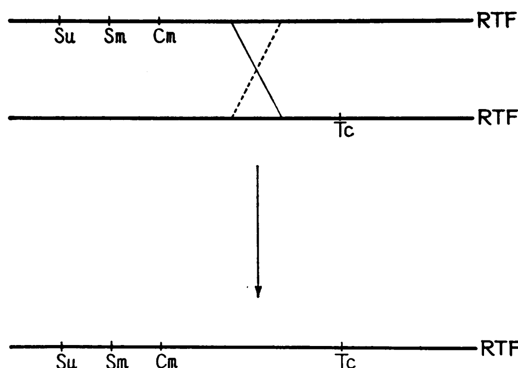

FIG. 4. *Models of recombination of R factors. In model A, crossovers anywhere between Cm and Tc should give rise to an R factor (Su, Sm, Cm, Tc).*

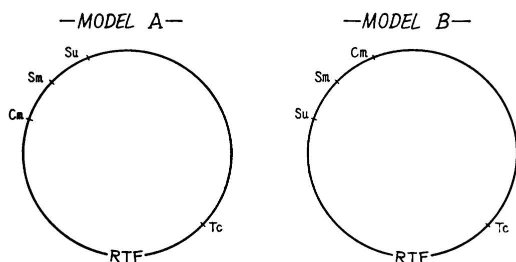

FIG. 5. *Circular models of an R factor (Su, Sm, Cm, Tc).*

gested that this circular model may be applicable to episomes in general. If such a circular model were applied to R factors, the two linear models indicated in Fig. 2 would be modified as in Fig. 5. All of the available data can be understood without the use of circular models. On the other hand, circular models are not incompatible with those results.

Using a circular model, Campbell (17) was able to explain the curious difference in linkage of chromosomal markers of phage λ in the vegetative state as compared with the prophage state, reported by Calef and Licciardello (16). The formation of λ *dg* in the absence of genetic homology between the genome of phage λ and *gal* genes, and the mechanism of integration and transition from integrated to autonomous state of phage λ, could be clearly understood in terms of the circular model. The mechanism of spontaneous segregation of resistance factors also may be clarified by using circular models. It may be that when R factors are integrated, the circular structures are stretched to linear forms, and when they revert to the autonomous state circular structures are again formed. Circles may be formed with only a portion of the original structures of R factors, leaving the remainder on the host chromosome. Thus, the segregant R factors, containing only part of the resistance factors, may form smaller circles than the original ones. Alternatively, the segregant R factors may pick up a part of the host genome as phage λ does. With this hypothesis, the concept of genetic homology between the RTF-carried resistance factors and host genome is not essential. Watanabe and Takano (136) recently succeeded in isolating strains of *E. coli* K-12 with stably integrated R factors. The validity of circular models for R factors can now be investigated experimentally, by comparing the linkage of resistance factors in transduction of integrated and autonomous R factors.

Interactions between RTF and F Factor in Escherichia coli K-12

Unique interactions were found between R factors and F factor in *E. coli* K-12, especially by Watanabe and Fukasawa (121, 122, 127, 131). The most characteristic interactions are the suppressions of the F factor functions by R factors. As will be discussed later, the inhibitory effects of R factors upon F factor were ascribed to RTF. Very recently, a class of R factors was found which did not suppress the functions of F factor when they are in male strains of *E. coli* K-12 (Watanabe and Takano, 136; Watanabe, Fukasawa, and Takano, 133). These authors proposed that the type of RTF which suppresses the functions of F factor be designated as RTF type 1; and the type which does not, RTF type 2. Among the prevalent strains of *Shigella* and *E. coli* with R factors, those with RTF type 1 are apparently predominant. Discussion in this section will be limited to RTF type 1. RTF type 2 will be discussed in the section on Biological Functions and Mutations of RTF.

Frequency of transfer of R factors to F^- and F^+ strains of E. coli K-12. Watanabe and Fukasawa (122, 131) found that the presence of F factor in recipients slightly suppresses the acceptance of R factors (reduced to only about one-half the frequency of transfer of R factors to F^- recipients). Since an R factor (Su, Sm, Cm, Tc) and

its segregants (Su, Sm, Cm) and (Tc) all exhibited the same effect, it was concluded that the acceptance of RTF is suppressed by F factor in the recipients.

Frequency of transfer of F factor, F′, and host chromosome to recipients with R factors. Watanabe and Fukasawa (127, 131) found that the presence of R factors in recipients suppresses slightly the acceptance of F factor, F′, and host chromosome. The transfer of F factor to the recipients with R factors cannot be easily detected, because, as will be indicated below, the presence of RTF in F^+ cells deprives them of their ability to transfer F factor and host chromosome to other cells. In contrast, the transfer of F′ and host chromosome can be accurately determined by utilizing donor cells with proper markers, carried by F factor and host chromosome, and recipient strains with proper markers.

Effects of the presence of R factors on F^+ and F'^+ strains. Watanabe and Fukasawa (121, 127, 128, 131) found that the F^+ and F'^+ strains with an R factor (Su, Sm, Cm, Tc) or its spontaneous segregants (Su, Sm, Cm) and (Tc) are unable to transfer their F factor, F′, and host chromosome. It was concluded that the inhibitory effects of R factors are mediated by RTF. The frequency of transfer of host chromosome by F^+ and F'^+ strains with R factors was less than 10^{-9} per donor cell. This fact suggests that F^+ strains with R factors also cannot transfer F factor. (The frequency of transfer of F factor cannot be determined accurately for technical reasons; i.e., the fertility of each recipient colony arising from the mixed culture of F^+ strains with R factors and F^- recipients must be determined.) The suppression of transfer of F factor, F′, and host chromosome is not due to the elimination or irreversible inactivation of F factor by RTF. This is indicated by the fact that spontaneous segregants without R factors, obtained by the penicillin screening method (see above), all behaved as F^+ and F'^+ strains.

The results of Watanabe and Fukasawa (121, 128, 131) on the suppression of recombination by R factors in F^+ strains were confirmed by Yoshikawa and Akiba (146), using an R factor (Su, Sm, Cm, Tc) of different origin. They reported, however, that small numbers of recombinants were developed in the crosses between F^+ strains with an R factor and F^- recipients, suggesting that the suppression of recombination by R factors present in F^+ donors is incomplete. We prefer to assume that the few recombinants they found may have been produced by the segregants of F^+ cells, which lost the R factor spontaneously. Alternatively, they might have been produced by R mating (Sugino and Hirota, 109), or the strains may have contained R factors with RTF type 2 (see below).

Watanabe and Fukasawa (127, 131) excluded the possibility that F factor might become closely associated with R factors by conducting an experiment in which F'^+ strains with R factors were treated with acridine orange. This caused F′ to be eliminated with high frequencies, and R factors with much lower frequencies. Their finding that F'^+ strains with R factors transfer the R factors but not F′ is also evidence that the two factors are not closely associated.

Effects of the presence of R factors on Hfr strains of E. coli K-12. Nakaya, Nakamura, and Murata (84) found that an Hfr strain of *E. coli* K-12 with an R factor (Su, Sm, Cm, Tc) can recombine with an F^- recipient only with reduced frequencies (about 10^{-2} of the frequencies of recombination of the original Hfr strain with no R factor). Their finding was extended by Watanabe and Fukasawa (121, 131) with three Hfr strains showing different orders of chromosomal transfer (Jacob and Wollman, 48, 49). The frequencies of recombination by all the Hfr strains with an R factor (Su, Sm, Cm, Tc) were reduced to about 10^{-2}. Similar degrees of suppression of recombination were brought about also by segregant types of R factors (Su, Sm, Cm) and (Tc) in Hfr strains. These results again suggest that the suppression of recombination of Hfr strains is due to RTF. The integrated F factor of Hfr strains was not eliminated or irreversibly inactivated by the infection of these strains with R factors, as evidenced by the fact that the drug-sensitive segregants with no R factors all behaved like the original Hfr strains. It was further shown that the origins and sequences of chromosomal transfer are not altered by R factors present in these strains.

Watanabe and Fukasawa (128, 131) found that Hfr strains with R factors can transfer the R factors with frequencies about equal to those of the F^-, F^+, and F'^+ strains with R factors. By use of a replica plating technique, the recipient

cells which received R factors from Hfr strains with R factors were found to be largely nonrecombinants. On the other hand, some of the recombinants from the Hfr strains with R factors had R factors. Although these findings will be discussed again in the section Evidence for Chromosomal Attachment of RTF, it should be emphasized here that the host chromosome and R factors can be transferred together in some instances.

Suppression by R factors of the f phage-sensitive mechanism in E. coli K-12 conferred by F factor. A group of phages (f phages), which specifically attack the male strains of *E. coli* K-12, were isolated by Loeb (63) and Loeb and Zinder (64). Some of these were shown to contain ribonucleic acid and no deoxyribonucleic acid. Since these phages were not adsorbed by F^- strains, the authors assumed that F factor induces the synthesis of a receptor substance for the phages. Watanabe, Fukasawa, and Takano (132, 133) observed that infection of F^+, F'^+, and Hfr strains with various types of R factors induces resistance to f phages. In addition, they reported that strains with both F factor and R factors do not adsorb f phages. Because the sensitivity of these strains to the phages of the T series is not changed by R factors, the development of resistance to f phages is considered to be specific. Watanabe and Fukasawa (127, 131) have shown that the F factor is not eliminated or irreversibly inactivated by RTF. Therefore, the inability of the above strains with R factors to adsorb f phages cannot be attributed to the absence of F factor. It seems that RTF somehow inhibits the synthesis of the f phage-specific receptor substance. It is also possible that some additional grouping is added to the end of the receptor substance. A similar situation was found by Uetake and Hagiwara (111) in somatic antigens of salmonellae. Phage ϵ^{34} induces the synthesis of antigen O 34 only in the cells with antigen 15. It is believed that phage ϵ^{34} modifies antigen 15 by adding glucose to the free end of antigen 15 (Robbins and Uchida, 99).

F^- strains with R factors do not adsorb f phages, indicating that RTF does not induce the synthesis of the f phage-specific receptor substance.

Sneath and Lederberg (106) found that treatment of F^+ and Hfr cells of *E. coli* K-12 with sodium periodate induces temporary infertility, and postulated the presence of a "mating substance" of polysaccharide nature on the cell surface, the synthesis of which is induced by F factor. They further postulated that this substance is essential for mating. Recently, Iijima (40) found that cells with R factors also lose their ability to transfer R factors after treatment with sodium periodate, suggesting the presence of a "mating substance" which is synthesized in the presence of R factors. The mating substance synthesized by cells with R factors must be different from that synthesized by cells with F factor, because F^+ and F'^+ strains with R factors can transfer R factors, but not F factor, F′, and host chromosome. On the other hand, Ørskov and Ørskov (91) discovered a new somatic antigen (f^+) in the male strains of *E. coli* K-12. Although it is not known at present whether the receptor substance for f phages, the mating substance, and antigen f^+ are identical, we can safely assume that they are at least closely related and possibly identical, for the synthesis of these substances is induced by a single agent, F factor.

On the basis of the above findings, Watanabe and Fukasawa (131) and Watanabe, Fukasawa, and Takano (132, 133) suggested that the genetic determinant of RTF, controlling its transfer, is epistatic to the comparable genetic determinant of F factor. According to this hypothesis, in the presence of both F factor and RTF, the polysaccharide specific for F factor is not synthesized but a polysaccharide specific for RTF is synthesized. We are now attempting to determine whether RTF induces the synthesis of some new somatic antigen. The relation of epistasis between RTF and F factor suggests a close similarity between these two factors.

Interactions between RTF Type 1 and Colicinogenic Factors in Escherichia coli K-12

Watanabe and Fukasawa (121, 131) found that the frequency of transfer of colicinogenic factor E_1 was less than 10^{-2} per donor cell when RTF was present in the same cells, whereas the frequency of transfer by F^+ strains without RTF was about 40%. This colicinogenic factor requires the presence of F factor in the same cells for its transfer (Fredericq, 25). Iijima (40) reported that F^- (col. E_1^+) cells with R factors can transfer the colicinogenic factor E_1, but only with low frequencies (about 10^{-2} per donor cell).

Watanabe and Fukasawa (131) also studied

the effect of RTF on the transfer of colicinogenic factor I, which does not require the presence of F factor for its transfer (95, 96). It was found that RTF does not suppress the transfer of this colicinogenic factor. Hence, the suppression of transfer of colicinogenic factor E_1 by RTF may well be mediated by the epistasis of RTF to F factor.

Evidence for Chromosomal Attachment of RTF

Although Campbell (17) classified as episomes several factors for which chromosomal attachment has not yet been proved, it is essential to demonstrate an integrated state for the autonomously replicating elements to permit their inclusion in the category of episomes as originally defined (Jacob and Wollman, 50; Jacob, Schaeffer, and Wollman, 47). Alfoldi et al. (10) have already demonstrated the integration of colicinogenic factor E_1, by employing several different types of Hfr strains carrying this factor and by studying the kinetics of its transfer. In an attempt to establish the chromosomal attachment of R factors, Watanabe and Fukasawa (130) carried out kinetic studies on the transfer of host chromosomal markers and R factors by Hfr strains with R factors. With selection for transfer of R factors, the results obtained were not clear-cut, probably because the autonomously replicating R factors are transferred much more frequently than the integrated R factors. With selection for transfer of host chromosomal markers, it was found that only the recombinants which received the segment of host chromosome between the B_1 and mannitol loci have R factors. It was concluded, therefore, that R factors are integrated at a specific site between B_1 and mannitol loci, and are transferred to recipient cells along with the host chromosome. The fact that some of the recombinants which received this chromosomal segment do not have R factors was interpreted as follows. Either these recombinants were produced by sensitive segregants with no R factors, or R factors could also be integrated at some other site(s) beyond the mannitol locus. These two possibilities were subsequently confirmed experimentally. Watanabe and Takano (136) succeeded in isolating clones with stably integrated R factors from Hfr strains with R factors by employing a replica plating technique for selecting clones with high frequencies of recombination. As indicated above, Hfr strains with R factors recombine only with reduced frequencies. The clones thus isolated were of two types, with and without R factors. Integrated R factors were found in the first type. The site of integration has not yet been determined accurately in these clones, but it is believed to be near the site of chromosomal attachment of F factor in one clone derived from Hfr strain W-2252. This suggests that integration of R factors need not be limited to the site between B_1 and mannitol loci. The integrated state of R factors appeared to be quite unstable in some of the clones but fairly stable in others, judging from the frequencies of segregation of clones which recombine only with low frequencies.

Segregant types of R factors, (Su, Sm, Cm) and (Tc), as well as (Su, Sm, Cm, Tc), were found to be frequently integrated between B_1 and mannitol loci, suggesting that, in *E. coli* K-12, integration is caused by RTF and not by resistance factors. Studies on transduction of R factors in *E. coli* K-12 with phage P1kc also support the following assumption. Resistant transductants with no RTF seem to develop the state of abortive transduction in K-12, because of the low genetic homology between the host genome and the RTF-carried resistance factors.

High Frequency Resistance Transfer System (HFRT)

Ozeki and Stocker (95) and Ozeki, Howarth, and Clowes (96) found that colicinogenic factor I can be transferred with very high frequency by the cells of *S. typhimurium* that have just received it, whereas cells carrying this colicinogenic factor are normally only poor donors. They succeeded in obtaining a population which contained a high ratio of competent donor cells, by growing a small number of colicinogenic cells with a large number of sensitive cells overnight without shaking, diluting the mixed culture 1:10 in fresh broth, and then incubating for 2 hr without shaking. A similar procedure was employed by Watanabe and Fukasawa (129) with R factors, and the final populations thus obtained were found to transfer R factors with extremely high frequencies. This system was referred to as HFRT, because of its analogy to the HFCT system of Ozeki and Stocker (95). The frequency of transfer of R factors was as high as 1.5 to 7.6 per resistant cell, when the mixture of recipient and HFRT population was incubated at 37 C for

1 hr. These surprisingly high values indicate that the exconjugants of the recipient, which received R factors during this incubation period, probably serve as competent donors for further transfer of R factors. It is also possible that each donor cell in the HFRT population conjugates with and transfers R factors to more than one recipient cell. In addition, recipient cells that received R factors require incubation at 37 C for about 8 min for the phenotypic expression of these factors (Watanabe and Fukasawa, 126). Thus, the phenotypically drug-resistant cells in the population of HFRT do not necessarily reflect the actual numbers of cells with R factors. Some of the cells with R factors may not be detected as resistant cells, owing to incomplete phenotypic expression of the drug resistance.

Ozeki (*personal communication*) proposed the following explanation for the mechanism of HFCT. Colicinogenic factor I is easily integrated, and the integrated factor is not normally transferred. It enters the autonomous state rarely, and is seldom transferred to the recipient cells by conjugation. The competent donor cells, thus developed, lose their competence, if the colicinogenic factor is integrated. This explanation is predicated on the assumption that the colicinogenic factor I is in an autonomous state in many cells of the HFCT. Ozeki's interpretation may not be able to account for the mechanism of HFRT, because clones containing R factors but unable to transfer them, or able to transfer them only with low frequencies because of their integration, are rather uncommon. It is possible that the integrated state of RTF is usually unstable. I prefer to assume that the autonomous state of R factors in the competent donor cells of HFRT is rather unusual in that these factors are transferred more easily by these cells than by cells with autonomously replicating R factors ("stable autonomous state"). The "unstable autonomous state" of R factors in the competent donor cells of HFRT may result from the unusually vigorous replication of R factors.

Mating of *E. coli* K-12 was not detected with HFRT, although Ozeki and Stocker (95) and Ozeki, Howarth, and Clowes (96) found mating of *S. typhimurium* with HFCT.

Biological Functions and Mutations of RTF

Watanabe and Fukasawa (115, 116, 123, 129) were unable to detect the specific production of colicins by R factors, as indicated above. They concluded, therefore, that RTF may not be a colicinogenic factor.

In an effort to determine whether R factors are related to temperate phages, Ochiai et al. (89), Akiba et al. (5, 6), Mitsuhashi (71), and Mitsuhashi, Harada, and Hashimoto (74) studied the effects of cell-free filtrates of strains with R factors but could not demonstrate the ability of the filtrates to transfer R factors. The usual transfer of RTF, therefore, does not involve transduction. However, as mentioned previously, R factors have been transduced in several systems of bacteria and phages. I believe, however, that transduction of R factors is rather exceptional under natural conditions, and that, in vivo, conjugation is probably the most common mode of transfer.

In the transduction experiments of Watanabe and Fukasawa (117, 125) with *S. typhimurium* LT-2 and phage P-22, RTF was shown to assume the causative role in conjugation and transfer of R factors. The fact that F factor and other transfer factors are not required for the transfer of R factors (80, 116) also indicates that RTF itself effects the conjugation and the transfer of R factors. The formation of a conjugation bridge (or canal), such as that revealed in the mating of *E. coli* K-12 (11, 58), has not yet been demonstrated during transfer of R factors. However, there is evidence to support the assumption that a conjugation bridge develops. First, the transfer of R factors can be interrupted by subjecting the mixture of donor and recipient to the action of a blender (Watanabe and Fukasawa, 119, 123), after the procedure employed to demonstrate conjugation bridges in the mating of *E. coli* K-12 (Wollman, Jacob, and Hayes, 139). Furthermore, transfer of R factors is also interrupted by addition of phage, to which the donor cells are sensitive and the recipient cells are resistant (Watanabe and Fukasawa, 126), an experiment similar to that performed by Hayes (35), who interrupted the conjugation caused by F factor with phage T6. Finally, the observation of Iijima (40) that colicinogenic factor E_1 can be transferred by F^- (col. E_1^+) cells with R factors, but not by F^- (col. E_1^+) cells without R factors, also suggests the formation, in the presence of R factors, of a conjugation bridge which cannot be formed by colicinogenic factor E_1 alone.

The finding of Watanabe and Fukasawa (121, 127, 131) and Watanabe, Fukasawa, and Takano (132, 133) that the functions of F factor are suppressed by RTF was ascribed by them to the epistasis of RTF to F factor in establishing their transfer systems. For elucidating the mechanism of this epistasis, the report of Watanabe, Fukasawa, and Takano (132, 133) that the receptor for f phages is synthesized in the presence of F factor, but not in the presence of both F factor and R factors, seems to be of fundamental importance. As we have already pointed out, it is not known whether the receptor substance for f phages, f^+ antigen (Ørskov and Ørskov, 91), and mating substance (Sneath and Lederberg, 106) are identical. If mating substance is identical to the receptor substance for f phages, failure of the cells to synthesize this receptor substance should result in loss of the ability to transfer F factor and host chromosome.

Although Watanabe and Fukasawa (115, 116) could not obtain evidence that R factors make F^- strains of *E. coli* K-12 fertile, Sugino and Hirota (109) subsequently reported such an effect. They referred to the mating caused by R factors as R mating. The frequencies of R mating differed for various R factors, and ranged from 10^{-6} to 10^{-8} per donor cell. R mating occurred with high frequencies (10^{-4} to 10^{-6} per donor cell) in an F^- derivative of W-3876 of an Hfr strain (Richter, 98). The order of chromosomal transfer by W-3876 with R factors was found to be identical to that with W-3876 and F factor. The high frequencies of R mating in an F^- derivative of an Hfr strain may indicate the difference between the original F^- strains and the F^- strains derived from Hfr strains. Jacob and Adelberg (45) and Adelberg and Burns (1) discovered what they called "souvenir" (or memory) as a result of genetic exchange between F factor and host genome. RTF might have some genetic homology to F factor, promoting the integration of RTF to the site of memory which F factor left on the host chromosome. Watanabe and Fukasawa (131) later found that R factors, which do not suppress the functions of F factor, give fertility to the F^- strains of K-12. If R mating were a general phenomenon for R factors, RTF should be considered to be a kind of F factor. The discrepancy in the results of Watanabe and Fukasawa (115, 116) and Sugino and Hirota (109) may be attributable to variations in the conditions of mating employed. Watanabe and Fukasawa (115, 116, 131) carried out their experiments in broth, Sugino and Hirota (109) on selective plates.

Egawa (*personal communication*) recently found a mutant of R factor (Su, Sm, Cm, Tc) which does not suppress the recombination of Hfr strains. Watanabe, Fukasawa, and Takano (133) and Watanabe and Takano (136) also found several such R factors. In addition, they observed that these R factors do not suppress the recombination of F^+ and F'^+ strains and the transfer of F factor and F' by F^+ and F'^+ strains. Another important distinguishing characteristic of these R factors is that they do not inhibit the synthesis by F factor of the receptor substance for f phages. Hence, F^+, F'^+, and Hfr strains with these R factors are susceptible to f phages. The type of RTF associated with these R factors is referred to as RTF type 2; and the more common RTF, as RTF type 1. Egawa (*personal communication*) found that types 1 and 2 can mutate to each other. A correlation between the inhibition of the synthesis of the receptor substance for f phages and the inhibition of mating in male bacteria with R factors is now established. These studies provide further evidence that a substance essential for mating is identical to the receptor substance for f phages.

Although Hfr strains with RTF type 1 cannot synthesize the receptor substance for f phages, they are still capable of transferring their host chromosomes, but with reduced frequencies. This fact indicates that the "transfer system" of host chromosome is switched over to that of R factors in these strains. It is considered that R factors in the autonomous state inhibit the transfer of host chromosome; therefore, chromosomal transfer by Hfr strains with R factors may be effected by cells with integrated R factors.

Attempts to isolate possible mutants of RTF have been continued but have not been very successful. This is probably due to the technical difficulty of selecting cells with mutant RTF. This difficulty is easily understood, if the problems encountered with F factor and other transfer factors where no mutants have been isolated to date are recalled. For example, the F2 variant of the F factor is considered to arise as a result of genetic exchange and not of mutation (Adelberg and Burns, 1). In contrast to the transfer factors, mutant types of temperate phages are rather

easily isolated. Perhaps the nature of the transfer process makes the isolation of mutant types of transfer factors difficult. Thus, transfer factors cannot be transferred in their integrated state unless host chromosome is transferred. On the other hand, they can be transferred when they are in the autonomous state. In this state, the numbers of particles of transfer factors are considered to be usually multiple and, even if one of them mutates, it cannot be detected easily unless it is segregated from the wild-type transfer factors.

A definite mutant of RTF, differing from RTF type 2, was discovered by chance by Watanabe and Lyang (134, 135), when they were studying the spontaneous segregation of resistance factors in *S. typhimurium* LT-2 carrying an R factor (Su, Sm, Cm, Tc). Two unusual clones were found; one had Su, Sm, Cm resistance, and the other was resistant to the four drugs. However, neither clone was able to transfer its resistance factors by mixed cultivation with sensitive recipients. These strains were found to segregate their resistance factors spontaneously with frequencies as high as the strains with normal R factors (Su, Sm, Cm, Tc) and (Su, Sm, Cm), indicating that their R factors might be in an autonomous state. Furthermore, the transfer of a segregant type of R factor (Tc) to the strain with ≪Su, Sm, Cm≫ (mutant R factor which cannot be transferred by conjugation) gave four-drug resistant clones, some of which were found to be genetically stable and able to transfer all four resistance factors by conjugation as a unit. Others were quite unstable and transferred only (Tc) by conjugation. The development of the stable four-drug resistant strains was assumed to be due to recombination of the two R factors. The RTF of the R factors, which cannot be transferred by conjugation, is assumed to be a mutant which is comparable to the defective mutants of temperate phages (Jacob, 44). It is not known yet which step or steps of the transfer of R factors are blocked by this mutation.

If various strains of shigellae and *E. coli* with R factors, which have been isolated from human specimens are studied, some of them are found to transfer their R factors by conjugation very easily, others with difficulty, and still others not at all. The reasons for the difficulty or failure to transfer resistance factors are multiple. Some donors and recipients produce colicins and phages which kill their partner cells. Some donor strains are only able to transfer resistance factors with low frequencies or not at all, because they contain defective mutants of RTF, or manifest stable integration of R factors, or possess only chromosomal resistance factors and no R factors. Watanabe and Fukasawa (*unpublished data*) found that the R factors of the donor strains with low frequencies of transfer sometimes acquire high transferability when they are transferred to other strains. The low transferability of R factors of the original strains must, therefore, be ascribed to some specific character of the host cells. Almost nothing is known about the role of the host cells in determining the frequencies of transfer of R factors by conjugation.

Watanabe and Fukasawa (116, 123) found that *Salmonella* strains are poor recipients for R factors. Baron, Carey, and Spilman (13, 14) also reported that salmonellae are not good recipients in the mating of Hfr strains of *E. coli* K-12. They were able to isolate good recipient clones from the original *Salmonella* strains and assumed that the cells had mutated from a state F^0 to some other state in which they functioned as good recipients. Watanabe and Fukasawa (123) originally postulated that the cells of salmonellae might contain a factor closely related to RTF (possibly RTF itself), which confers immunity against infection by R factors. They isolated spontaneous segregants with no resistance factors (and possibly with no RTF) from *S. typhimurium* LT-2 with an R factor (Su, Sm, Cm, Tc), using a penicillin screening method. These segregants, however, were found to receive R factors with frequencies as low as the original sensitive strain LT-2. The reason for low frequencies of transfer of R factors to salmonellae is not known at present.

Comparison of RTF with Other Episomes

The comparison of RTF with other episomic elements is summarized in Table 2. The term episome applies not only to the genetic element responsible for episomal behavior, but also to all associated material. However, when various episomes are compared, it is more rational and convenient to exclude the nonessential and definitely accessory material carried by the original types of episomes, as Campbell (17) suggested. Otherwise, each episome must be accompanied by numerous relatives. Using F factor as an exam-

TABLE 2. *Comparison of resistance transfer factor with other representative episomes*

Kind of episome	Autonomous replication	Integration	Cell conjugation	Transfer of host chromosome	Pick-up of host chromosomal genes	Killing of cells by autonomous replication	Colicin production	Production of extracellular infectious particles
Temperate phages	+	+	−	−	+	+	−	+
Temperate phage τ	+	+	+ (?)	+ (?)	− (?)	+	−	+
F factors	+	+	+	+	+	−	−	−
Colicinogenic factors	+	+	− or +	−	− (?)	−	+	−
Colicinogenic factor 1	+	+	+	+	− (?)	−	+	−
Resistance transfer factor	+	+	+	+ (?)	+	−	−	−

ple, we would have to place F′-*lac*, F′-*gal* and many other kinds of F′ would be placed on the same level as F factor, colicinogenic factor, and temperate phage. RTF without any resistance factors has not been observed as yet, but a variety of evidence suggests that the resistance factors are not essential for the episomality of the R factors.

This problem may be related to that of phage τ (tau). This is a unique temperate phage, which was isolated by Hakura and Hirota (27) and claimed to give fertility to F^- strains of *E. coli* K-12 in which it becomes a prophage. It infects also F^+ cells but cannot either lyse or lysogenize them.

Origin of Resistance Factors Carried by RTF

The origin of RTF, like that of other episomes, is obscure. Watanabe and Fukasawa (117, 125) presented a hypothesis that the multiple drug resistance factors carried on RTF were acquired by RTF in a single step from the host chromosome of some unknown bacteria. They postulated that R factors are comparable to F′ (Jacob and Adelberg, 45; Hirota and Sneath, 38), and that the transfer of R factors by conjugation is a phenomenon comparable to F-duction (sexduction; Jacob, Schaeffer, and Wollman, 47). It is difficult to understand why only the resistance factors and no other detectable markers should be located in close linkage on the host chromosome. It is quite possible that other markers are present among the resistance factors, but have not been detected. Further studies are awaited. Watanabe and Fukasawa (125) also suggested that RTF may pick up other chromosomal genes in addition to resistance factors. However, only the RTF with resistance factors would have a selective advantage in areas such as Japan where chemotherapy has been administered extensively.

If the results of epidemiological studies of drug-resistant shigellae in Japan (Table 1) are examined, it is evident that the four-drug resistant strains appeared very early. If RTF had acquired resistance factors separately, strains resistant to one, two, or three drugs should have been isolated more frequently and prior to the development of the four-drug resistant strains. Japanese workers in the field of public health have extensively studied, on a nation-wide scale, the drug resistance of the isolated *Shigella* strains; if any singly, dually, or triply resistant strains were developed, they should have been detected.

As mentioned earlier, multiple drug resistant R factor can be "synthesized" experimentally by combining various R factors, and it is likely that the same mechanism operates in the human intestinal tract. This should not be taken, however, as evidence for the successive acquisition of resistance factors in the development of the first multiple drug resistant strains in the natural environment.

Watanabe and Fukasawa (116, 117, 123, 131) demonstrated that the levels of drug resistance expressed by RTF-carried resistance factors can differ considerably from host to host. For example, *Shigella* strains, which have acquired the Sm resistance factor carried by RTF, withstand Sm concentrations of more than 1,000 μg per ml, whereas the Sm resistance of *E. coli* with this factor is as low as 10 μg per ml. The level of Tc resistance of *Shigella* and *E. coli* strains having RTF-carried Tc resistance factor is higher than 100 μg per ml but in corresponding *Salmonella* strains it is as low as 10 μg per ml. Even in the various substrains of *E. coli* K-12, the phenotypic expression of the Cm resistance factor may differ considerably. If these facts are

taken into consideration, failure to isolate from the human intestinal tract strains with high resistance to the four drugs unrelated to RTF does not necessarily exclude the possibility that the resistance factors came from bacterial chromosomal genes. In the human intestinal tract *Pseudomonas* species with rather high resistance to Sm, Cm, and Tc, and rather low resistance to Su, are fairly prevalent. Attempts to transfer these resistance factors to *E. coli* by mixed cultivation have been unsuccessful so far (*unpublished data*). However, it should not be too surprising if future experiments reveal that the resistance factors of R factors came from *Pseudomonas* or some other genus which does not belong to Kauffmann's *Enterobacteriaceae*. For example, R factors can be transferred to *S. marcescens* (Falkow et al., 22) and *V. comma* (Baron and Falkow, 15). Even though the origin of the resistance factors of R factors is still obscure, the hypothesis that they derived from some bacterial chromosome helps explain many experimental findings.

On the other hand, it is also possible that the R factors now prevalent in Japan are all descendants of the R factor (Su, Sm, Cm, Tc), which was found in a *Shigella* strain isolated by Kitamoto et al. (52) from a patient who returned from Hong Kong in 1955. This assumption may seem fruitless, because it again leads to a question. How was this original R factor produced? However, inasmuch as it is not known whether the "gene pick-up" (Campbell, 17) by RTF is now taking place in Japan, this possibility cannot be neglected.

Molecular Basis of R Factors

Since R factors replicate autonomously, it is most likely that they are composed of nucleic acids. As mentioned above, the resistance factors carried by RTF, in all probability, originated from a bacterial chromosome, and therefore may be composed of DNA. The episome RTF is also possibly DNA, judging from its affinity for host chromosome.

The effects of ultraviolet light and mitomycin C on R factors were studied by Watanabe and Fukasawa (118, 124) and Watanabe and Takano (136). It was found that some of the donor cells inactivated by ultraviolet light or mitomycin C were still able to transfer R factors by conjugation, thus increasing the frequency of transfer per surviving donor cell. When Hfr strains with stably integrated R factors were treated with ultraviolet light or mitomycin C, not only the frequencies of transfer of R factors per surviving donor cell but also the absolute frequencies of transfer were significantly increased (Watanabe and Takano, 136). This situation is probably comparable to the induction of prophages by ultraviolet light (Lwoff, 66) and by mitomycin C (Otsuji et al., 93).

Nakaya, Nakamura, and Murata (85) irradiated phage P1kc grown on *E. coli* K-12 containing an R factor (Su, Sm, Cm, Tc) with ultraviolet light and studied the dose-response curves of plaque-forming ability and transducibility of host chromosomal markers and R factors. Their results are essentially the same as those of Arber (12), who carried out similar experiments with F factor. The plaque-forming ability and transducibility of R factors were much more sensitive to ultraviolet light than the transducibility of the host chromosomal markers. (As mentioned earlier P1kc usually transduces the entire R factor.) They selected the resistant transductants on media containing individual drugs or a mixture of the drugs. The dose-response curves of transducibility of R factors were essentially the same with these media. These results indicate the inactivation of RTF by ultraviolet light rather than inactivation of the individual resistance factors.

Watanabe and Takano (*unpublished data*) studied the effect of decay of incorporated P^{32} on R factors in *E. coli* K-12 with the technique used by Lavallé and Jacob (56) in F′-*lac*, F′-*gal*, and colicinogenic factor E_1. Their results indicate that RTF is composed of DNA with a molecular weight close to those of F′ and phage λ (Stent, Fuerst, and Jacob, 107). They found also that inactivation of RTF results in inactivation of the entire R factor, even if the resistance factors are intact.

S. marcescens, which accepts R factors (Falkow et al., 22), has DNA of a base composition different from that of enteric bacteria. Fractionation of R factors by the density gradient centrifugation technique (Meselson and Stahl, 70), which was successful in fractionating F′-*lac* (Marmur et al., 67), will undoubtedly provide important information on the chemical nature, and perhaps the origin, of R factors.

BIOCHEMICAL MECHANISMS OF MULTIPLE DRUG RESISTANCE

Because multiple drug resistance factors can be transferred as a unit by conjugation, some workers assumed that multiple drug resistance may be controlled by a single genetic determinant. Hence, the biochemical mechanism of multiple drug resistance was ascribed to nonspecific reduction of cell permeability (Mizuno, 83). However, it has now been established that the R factors involve multiple genetic determinants. A simple unitary explanation for the biochemical mechanism of multiple drug resistance is, therefore, unlikely.

Miyamura (82) reported that *Shigella* and *E. coli* strains with Cm resistance factor enzymatically decompose Cm. Elaboration of the enzyme, chloramphenicolase, by the cells was therefore assumed to be a mechanism of Cm resistance. However, Yokota and Akiba (144) later found that the activity of this enzyme is about equal in Cm-sensitive and Cm-resistant strains. The Cm-resistant strains decompose more Cm only because they can grow in the presence of Cm. Thus, the enzyme which decomposes Cm is not essential for the mechanism of Cm resistance.

Yokota and Akiba (143) and Akiba and Yokota (8) studied the accumulation of folic acid precursors by two strains of *E. coli* resistant to Su alone (this Su resistance factor is not carried by RTF) and also by a strain of *E. coli* with an R factor (Su, Sm, Cm, Tc). One of the strains with Su resistance alone was isolated from a natural source, and the other was obtained as a multistep mutant in vitro from a sensitive parent. Prior to these studies, Sevag and Yokota (103, 104) reported that Su-resistant mutants of *E. coli* isolated in vitro synthesize increased amounts of the intermediates between pteridines and tetrahydrofolic acids, which counteract the action of Su. (Su inhibits folic acid formation by blocking the coupling of pteridine and *p*-aminobenzoic acid.) Yokota and Akiba (143) also found that the intracellular levels of pteridines, folic acids, and tetrahydrofolic acids were three to four times higher in the strains with simple Su resistance than in the sensitive strain. The multiple drug resistant *E. coli* did not show this metabolic change.

Using Su-resistant and multiple drug resistant strains of *E. coli*, Akiba and Yokota (8) studied the effect of Su on the enzyme, folicase, which catalyzes the combination of pteridine with *p*-aminobenzoic acid. It was found that this enzyme was not inhibited by Su in intact cells of Su-resistant or of multiple drug resistant strains, whereas it was inhibited in Su-sensitive cells. In cell-free homogenates, however, the folicase of the multiple drug resistant strain was inhibited to approximately the same degree as the enzyme of the sensitive strain. The folicase in a cell-free homogenate of the Su-resistant strains was not inhibited by Su. Whether this resulted from altered sensitivity of the enzyme to Su or from increased accumulation of the pterines which antagonize Su is not certain. They found also that S^{35}-sulfathiazole readily enters sensitive and Su-resistant strains but not the multiple drug resistant strain. These findings indicate that the Su resistance involved in multiple drug resistance is due to reduced permeability of the cells to Su.

Yokota and Akiba (144) then studied Cm resistance in a strain of *E. coli*, which had acquired Cm resistance in vitro as a result of multistep mutations from a sensitive parent, and also in a strain of *E. coli* with an R factor (Su, Sm, Cm, Tc). They determined the effect of Cm on the synthesis of β-galactosidase by osmotic lysates of penicillin-spheroplasts and lysozyme-protoplasts in the system of Reiner (97). The synthesis of this enzyme was found to be inhibited by Cm in the lysates of both resistant strains, whereas its synthesis, as well as the net synthesis of protein, by intact cells, penicillin spheroplasts, and lysozyme protoplasts was not inhibited by Cm. Accordingly, it was concluded that Cm resistance results from reduced permeability of cells to Cm in both types of resistant strains. The fact that both spheroplasts and protoplasts manifest Cm resistance suggests that the permeability barrier to Cm lies in the cytoplasmic membrane rather than in the cell wall. Similar studies were undertaken by Okamoto and Mizuno (92) on the effect of Cm on the incorporation of labeled amino acids into proteins, using sonically disrupted cell fractions. The same conclusion was reached.

Akiba and Yokota (9) studied the effects of Cm and Tc on the incorporation of labeled amino acids into proteins by isolated ribosome preparations from sensitive and multiple drug re-

sistant strains of *E. coli*. At concentrations as low as 5 μg per ml, Cm completely inhibited the incorporation of amino acids into proteins in both preparations. On the other hand, even at 25 μg per ml, Tc produced only partial inhibition in preparations from both strains. Since Tc acts as a chelating agent and combines with Mg ion, which is essential for the integrity of ribosomes, the results are not clear-cut. However, they suggest that the Tc resistance involved in multiple drug resistance may also be caused by reduced permeability of cells to Tc.

Watanabe and Takano (*unpublished data*) transferred an R factor (Su, Sm, Cm, Tc) to Sm-dependent mutants of *E. coli*. The recipients which received the R factor were still dependent on Sm. Since the biochemical mechanism of Sm dependence is not yet known, it is difficult to draw significant conclusions from this result.

Except for Sm resistance, about which very little is known, the results discussed above indicate that multiple drug resistance must be ascribed to reduced permeability of cells to the drugs. It should be emphasized again that the reduced permeability is specifically controlled by each resistance factor.

Summary

I will now summarize briefly this outline of the studies of transmissible (or, more accurately, infective) drug resistance which have been carried out almost exclusively by Japanese investigators. Several groups of workers are continuing work on this problem. Although I have tried to include the results of most of the pertinent publications, a number of new facts will have emerged by the time the present review appears in print.

The problem of the infective heredity of drug resistance aroused interest initially because of its medical importance. Only later did it develop into an area of considerable genetic interest. A great deal of data have already been accumulated by many workers on the subject of bacterial drug resistance but the infective drug resistance described in this review is the first case of episome-mediated transfer of drug resistance. It is interesting to note that the discovery of transfer of multiple drug resistance resulted from careful epidemiological observations of multiple drug resistant shigellae, and that this led subsequently to the discovery of a new episome, the resistance transfer factor (RTF).

The medical importance of infective drug resistance, especially multiple drug resistance, is apparently limited to Japan at present, but it could become a serious and world-wide problem in the future. The fact that R factors can be transferred to every genus of *Enterobacteriaceae* and to other genera, including *V. comma*, by nonpathogenic bacteria such as *E. coli* constitutes a serious public health problem. In individuals who ingest *E. coli* which contain R factors, the *E. coli* and other bacterial strains already present in the intestinal tract can be converted to drug resistance. Such an individual will become a carrier of drug-resistant intestinal flora. If infection with pathogenic bacteria such as salmonellae, shigellae, and *V. comma* occurs, these pathogenic organisms could easily acquire R factors from the intestinal flora. Although the transfer of R factors normally occurs less readily in the intestinal tract than in vitro, because of the presence of fatty acids and other inhibitory agents, it may be accelerated in the intestinal tract under the selective pressure of chemotherapeutics.

The history of the discovery of R factors, and subsequently RTF, is quite unusual in that R factors, which presumably resulted from gene pick-up, were discovered before RTF was recognized. Examples of episome-mediated transfer have been reported in other episomes, but in most instances the original episomes were discovered first because of their essential, discrete functions. *Lac*-episome (Falkow et al., 22; Baron and Falkow, 15) is a possible exception. *Lac*-episome behaves as an episome, although its integrated state has not been shown. It is assumed that the *lac* genes of *lac*-episome derived from the chromosome of *E. coli*. Baron, Carey, and Spilman (13), however, failed to differentiate the *lac* genes and an episome which presumably carries them in the experiment of interrupted conjugation. The episome RTF was recognized only because of the resistance factors it carried. It is assumed that RTF without any resistance factors should exist, but would not be identified because of its inability to manifest a discrete function, such as lysis of bacteria by phages, conversion to fertility by F factors, or production of colicins by colicinogenic factors. The only marker of RTF (RTF type 1) which

has been found suitable for selecting cells with RTF is suppression of F factor functions in *E. coli* K-12. Even this is not a good marker for selection.

RTF was added as a new member to the category of episomes, and it was anticipated that comparative studies of RTF with other known episomes will increase our understanding of episomes in general. Temperate phages have proved to be very valuable subjects for genetic analysis, and have been studied in great detail. In contrast, genetic studies on the other episomal "transfer factors" are extremely difficult, primarily because these factors cannot be transferred extracellularly. We may succeed in isolating cell-free particles of a transfer factor but at present there is no way to test for their biological activities. The observation that RTF interacts with F factor and the chance isolation of mutant types of RTF offer some hope for the study of the genetics of RTF and other transfer factors.

The most essential features of episomes are autonomous replication and integration. Many beautiful results have been obtained on these points with temperate phages (Jacob, 44; Campbell, 17). Another important characteristic of certain episomes, the transfer factors, is their transfer by conjugation, caused by the episomes themselves. The synthesis of the "mating substance," which was first implicated by Sneath and Lederberg (106) as essential for mating, was found to be inhibited by RTF type 1, which suppresses fertility due to F factor, but not to be inhibited by RTF type 2, which does not affect fertility due to F factor. Inhibition by RTF type 1 of synthesis of mating substance is considered to be the mechanism for suppression of the functions of F factor. Until this observation, genetic studies on R factors and RTF had to be conducted along lines successful with other episomes, especially temperate phages and F factors. The discovery of the interactions between RTF and F factor has opened a new field of research. It is expected that future studies of R factors and RTF will make fundamental contributions to our understanding of episomes in general.

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