EPISOME-MEDIATED TRANSFER OF DRUG RESISTANCE IN *ENTEROBACTERIACEAE*

I. Transfer of Resistance Factors by Conjugation

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In recent years many Shigellae isolated from human cases of dysentery in Japan have been found to be resistant to more than two of the drugs streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), and sulfonamide (Su) (Ochiai, 1959). Indeed, approximately 10% of the Shigellae isolated in Japan in 1959 were found to be resistant to all the drugs listed. The strains resistant to one or two of these drugs are rather less frequent than those resistant to three or four drugs. Strains resistant to multiple drugs sometimes have been isolated from only a part of the patients in an epidemic in which strains isolated from other patients have been found to be sensitive to these drugs. Furthermore, frequently the administration of one of these drugs to patients harboring sensitive dysentery bacilli has caused the excretion of dysentery bacilli resistant to not only the administered drug but also other drugs as well.

From these findings, Ochiai et al. (1959) and Akiba et al. (1960) suspected that *Escherichia coli* strains resistant to multiple drugs may play an important role in producing drug-resistant Shigellae in human intestinal tracts; and they succeeded in transferring multiple drug resistance in vitro from resistant *E. coli* to Shigella, and also from resistant Shigella to *E. coli*. They mentioned that no other markers accompanied the transfer of drug resistance. They also reported that the mechanism of transfer of drug resistance is neither transduction nor transformation but conjugation, because cell-free filtrates of the resistant strains could not confer resistance upon sensitive strains. This finding was confirmed by Mitsuhashi et al. (1960*a*).

Harada et al. (1960) and Nakaya and Nakamura (1960) found that multiple drug resistance can be transferred to many other bacteria, including most genera of *Enterobacteriaceae*, by mixed culture. Mitsuhashi et al. (1960*b*) also found that F factor is not necessary for the transfer of drug resistance among the substrains of *E. coli* strain K-12. Nakaya and Nakamura (1960) then succeeded in transducing drug resistance in *E. coli* strain K-12 with phage P1kc.

We have conducted a series of studies on the genetics of the resistance factors concerned and have published our preliminary reports elsewhere (Watanabe and Fukasawa, 1960*a–h*). The results we have so far obtained may be summarized as follows:

1) In agreement with the work of others, resistance is transferred in ordinary cultures only by cell-to-cell contact.

2) Resistance can also be transferred by transduction in *Salmonella typhimurium* strain LT-2 with phage P-22, and in *E. coli* strain K-12 with phage P1kc.

3) A majority of the resistant transductants of LT-2 produced with P-22 cannot transfer their resistance to sensitive recipients by conjugation, whereas the transductants of K-12 with P1kc are capable of transferring their resistance by conjugation. It should be mentioned here that both K-12 and LT-2 cells which acquired resistance by conjugation are able to further transfer their resistance to sensitive recipients by conjugation.

4) Transfer of resistance factors by conjugation requires about 15 min but transferred resistance is phenotypically expressed rapidly by the recipients in both conjugation and transduction.

5) Resistance factors are transferred together in conjugation but may be segregated in transduction.

6) All of the resistance factors can be eliminated together by treatment with acridine dyes, although with low frequencies.

7) Frequency of transfer of resistance factors by conjugation may differ from recipient to recipient.

8) Expression of transferred resistance factors,

that is the level of resistance, may differ from recipient to recipient.

9) Resistance factors seem to replicate faster than host chromosomes.

10) The frequency of transfer of all the resistance factors by conjugation can be increased equally by ultraviolet irradiation of donor cells before conjugation.

11) The efficiency of elimination of resistance factors by acridine treatment can be increased by irradiating the cells with ultraviolet before treating them with acridine dyes.

12) A part or all of the resistance factors may be lost spontaneously. Sensitive segregants can be detected with a penicillin screening method by exposing resistant populations to penicillin and chloramphenicol or penicillin and tetracycline.

13) Resistance factors do not confer maleness or transferability of chromosome upon F^- strains of K-12.

14) In agreement with the work of Mitsuhashi et al. (1960*b*), resistance factors are transferred by conjugation apparently independently of the sex factor among the substrains of K-12. We have found, however, that the sex factor, when present in the recipient cells, slightly reduces the frequency of acceptance of resistance factors.

15) F^+ cells of K-12 which received resistance factors phenotypically resemble F^- cells. In other words, they are unable to recombine with F^- cells and to transfer F factor and colicinogenic factor to F^- cells. However, they recover the characteristics of F^+, if they lose resistance factors spontaneously.

16) Hfr cells of K-12 with resistance factors recombine with F^- cells with frequencies lower than the original Hfr strains (about 1:100) in accordance with the report of Nakaya and Nakamura (1960).

It is apparent from these findings that the factors responsible for the transmissible drug resistance we are dealing with exist as cytoplasmic elements; on the other hand, our transduction experiments with *S. typhimurium* suggest that resistance factors can be attached to host chromosomes, as will be reported in a later paper.

Jacob and Wollman (1958) proposed the term "episomes" for temperate phages, sex factors, and colicinogenic factors of bacteria. The characteristics of episomes are that they exist in cytoplasm in an autonomous state, replicating faster than the host chromosomes; that they may be attached to host chromosomes, becoming integrated; and that the episomes in the integrated state suppress the autonomous replication of themselves in cytoplasm. Jacob and Adelberg (1959) found that chromosomal genes can be carried and transferred by F factor and called this phenomenon "F-duction" or "sex-duction" (Jacob, Schaeffer, and Wollman, 1960). Comparable phenomena were observed in phage λ by Morse, Lederberg, and Lederberg (1956) and in colicinogenic factor by Ozeki and Stocker (1960).

We have proposed the term "resistance transfer factor" (RTF) for the genetic unit, because it is considered to be a new episome (Watanabe and Fukasawa, 1960*e*). The details of our results are presented in this and subsequent papers.

MATERIALS AND METHODS

Media. Liquid cultures were prepared in Penassay broth (Difco). Plating media were nutrient agar, bromthymol blue-lactose agar (containing 2% lactose), Mueller-Hinton agar (containing bromthymol blue and 2% lactose), and minimal agar described by Davis and Mingioli (1950). The minimal medium was enriched with appropriate nutrients when needed. For scoring drug-resistant cells, each drug was incorporated in the medium in proper concentrations.

Drugs. Dihydrostreptomycin sulfate (Meiji), chloramphenicol powder (Parke, Davis and Company), tetracycline hydrochloride (Lederle), and sulfathiazole (Takeda) were used.

Strains. Shigella flexneri 2b strains 222 (Sm, Cm, Tc, Su) (supplied by R. Nakaya) and Kato (Sm, Cm, Tc, Su) (supplied by K. Ochiai) were used as originally resistant strains. Since comparable results were obtained in preliminary experiments with both strains, strain 222 (Sm, Cm, Tc, Su) was mainly used. Both strains are resistant to Sm (streptomycin), Cm (chloramphenicol), Tc (tetracycline) and Su (sulfonamides). In addition, strain 222 (Sm, Cm, Tc, Su) was found by Nakaya to require nicotinic acid, tryptophan, and methionine for its growth. As sensitive recipients, substrains of *E. coli* strain K-12, *Salmonella typhimurium* strain LT-2, and *Salmonella enteritidis* strain no. 11 were used. The substrains of K-12 used are shown in Table 1.

TABLE 1

Substrains of Escherichia coli strain K-12 used

Strain No.	Auxotrophic Characters			Energy Source Utilization					Response to Phages					Response to 1,000 μg/Ml Streptomycin	Sex
	TL	thi	met	lac	gal	mal	xyl	mtl	T1	T2	T3	T5	T6		
58-161*	+	+	−	+	+	+	+	+	s	s	s	s	s	s	F^+
W2252	+	+	−	+	+	+	+	+	s	s	r	s	r	s	Hfr
W3753	+	+	+	+	−	+	+	+	s	s	r	s	r	s	Hfr
W3780	+	+	−	+	+	+	+	+	s	s	r	s	r	r	Hfr
CSH-2	+	+	−	+	+	+	+	+	s	s	s	s	s	s	F^-
W677	−	−	+	−	−	−	−	−	r	r	s	r	s	s	F^-
W1177	−	−	+	−	−	−	−	−	r	r	s	r	s	r	F^-

* In addition, 58-161 requires biotin.

Culture method. Each strain was grown in Penassay broth overnight without aeration. This culture was diluted with Penassay broth 1:50 and aerated by gentle rotary shaking at 37 C for 3 hr.

Conjugation conditions. Donor and recipient cultures were diluted with Penassay broth and equal volumes of varying dilutions of each culture were mixed and incubated at 37 C, with or without gentle shaking. Samples were withdrawn at various times.

Selection of clones that received resistance factors. When resistance was to be transferred from Shigella or Salmonella to lactose-fermenting strains of *E. coli* by conjugation, varying dilutions of the mixture of donor and recipient were plated on drug-containing bromthymol blue-lactose agar or minimal agar enriched with nutrients required by *E. coli* and deficient for Shigella or Salmonella. When resistance was to be transferred from Shigella or *E. coli* to prototrophic Salmonella, the mixture was plated on drug-containing minimal agar deficient for donors. Lactose fermentation and nutritional requirements were the selective markers used also in other combinations of donors and recipients. For a nutrient medium selecting Su-resistant clones Mueller-Hinton agar was used, since this medium contains little antagonist to Su. Inoculated nutrient agar plates were incubated for 24 hr before scoring, whereas minimal agar plates were incubated for 48 hr. The recipient cells which received resistance factors were detected as large, lactose-fermenting colonies on the non-fermenting background growth of donor culture on nutrient agar containing bromthymol blue, lactose, and drug. On the drug-containing media deficient for the donor culture only resistant recipient cells formed colonies.

Interruption of conjugation. Five-milliliter cultures of donor and recipient were mixed in a 200-ml Erlenmeyer flask and kept in a 37 C water bath. Samples of 0.2 ml were taken every 5 min and diluted 1:1,000 with chilled Penassay broth to prevent further conjugation. The diluent contained 0.01% silicone (Shinetsu Kagaku Company) to prevent foaming. Half this diluted sample was treated in a blender at 20,000 rev/min for 15 sec. Both blender-treated and untreated samples were incubated in a 37 C water bath for 1 hr for phenotypic expression, and 0.1 ml of each sample was plated on selective media containing each drug.

RESULTS

Drug sensitivity of the strains used. Approximately 100 to 500 cells of each strain were plated on various agar media containing varying concentrations of each drug and the drug concentration at which most cells formed visible colonies was taken as its average resistance (Watanabe, 1959). The results are shown in Table 2. As seen in this table, the values differ with the media used.

Inefficacy of sterile filtrate of donor culture in transferring resistance. The culture of strain 222 (Sm, Cm, Tc, Su) was filtered with a Seitz filter and the sterile filtrate was mixed with CSH-2. The mixture was incubated at 37 C overnight and samples were plated on agar media containing varying concentrations of each drug. Resistant cells were not increased in comparison with the

TABLE 2

Average resistance to streptomycin, chloramphenicol, tetracycline, and sulfonamides of Shigella flexneri 2b strain 222 (Sm, Cm, Tc, Su), Escherichia coli strain K-12, Salmonella typhimurium strain LT-2 and Salmonella enteritidis strain no. 11

Drug	Medium	Strain			
		222 (Cm, Tc, Sm, Su)	K-12	LT-2	No. 11
		μg/ml	*μg/ml*	*μg/ml*	*μg/ml*
Dihydrostreptomycin	BTB*-lactose agar	= >1,000	= >0.5-<1	= >5-<10	= >1-<5
	Minimal agar	= >1,000	= >1-<5	= >100-<500	= >5-<10
Chloramphenicol	BTB-lactose agar	= >100-<200	= >5-<10	= >5-<10	= >5-<10
	Minimal agar	= >50-<100	= >1-<5	= >1-<5	= >1-<5
Tetracycline	BTB-lactose agar	= >100-<200	= >5-<10	= >10-<50	= >10-<50
	Minimal agar	= >100-<200	= >0.5-<1	= >0.5-<1	= >0.5-<1
Sulfathiazole	Mueller-Hinton agar	= >1,000	= >1-<5	= >10-<50	= >10-<50
	Minimal agar	= >1,000	= >1-<5	= >5-<10	= >1-<5

Minimal agar for strain 222 (Sm, Cm, Tc, Su) was enriched with tryptophan, nicotinic acid, and methionine. Drug concentration at which most cells formed visible colonies was referred to as average resistance.

* BTB = bromthymol blue.

control culture. This result is in agreement with the results of Ochiai et al. (1959), Akiba et al. (1960), and Mitsuhashi et al. (1960*a*). The centrifuged supernatant treated with chloroform was also inactive in converting sensitive to resistant cells. Neither sample produced any plaque or inhibition zone on recipients.

Transfer of resistance from resistant Shigella to sensitive E. coli, S. typhimurium, and S. enteritidis. Three-hour cultures of donor and recipient were mixed and incubated overnight without shaking and 0.1 ml of varying dilutions was plated on agar media containing each drug. As shown in Table 3, the frequency of transfer differed considerably between *E. coli* and Salmonella. One hundred resistant colonies which developed on the medium containing each drug were tested for their resistance to other drugs and found resistant to all of them.

Further transfer of resistance from recipients made resistant by conjugation with resistant donor. CSH-2 (Sm, Cm, Tc, Su), which was made resistant by conjugation with 222 (Sm, Cm, Tc, Su), was grown together with substrains of K-12 or *S. typhimurium* or *S. enteritidis* and transfer of resistance was examined by utilizing the methionine requirement and lactose fermentation of CSH-2. It was found that resistance was further transferred from CSH-2 (Sm, Cm, Tc Su) to other sensitive strains.

Transfer of resistance factors from LT-2 (Sm, Cm, Tc, Su) and no. 11 (Sm, Cm, Tc, Su) to K-12 strains took place as frequently as from 222 (Sm, Cm, Tc, Su). These results indicate that poor recipients can be good donors in transferring resistance factors.

Expression of resistance by cells that received resistance factors by conjugation. Ten colonies of each recipient that were made resistant by conjugation with 222 (Sm, Cm, Tc, Su) were tested for their average resistance to each drug. The phenotypic expression of the transferred resistance factors was uniform in the same recipient but differed markedly from recipient to recipient (Table 4). It is seen in this table that the levels of Sm resistance are much lower in *S. typhimurium* and *E. coli* than in Shigella, and also that Tc resistance of *S. typhimurium* is lower than that of the other two strains.

Plating of the mixture of donor and recipient on varying concentrations at various times. The mixture of 222 (Sm, Cm, Tc, Su) and CSH-2 was gently shaken at 37 C and samples were taken, diluted, and plated on selective agar media containing varying concentrations of each drug. As is shown in Fig. 1, the transfer and

phenotypic expression of resistance factors seem to take place very rapidly and to increase sharply with time. In addition, at lower concentrations of the drugs a few resistant colonies were found to develop even at zero time. This tendency was marked in Cm, Tc, and Su resistance, but not in Sm resistance. Furthermore, it was found that more resistant colonies developed earlier on lower concentrations of any of these drugs. On any of the concentrations of drugs lower than the resistance levels of the recipients with resistance factors, all of the transferred resistance factors seemed to be phenotypically expressed earlier than the first cell division.

It should be pointed out here that donors and recipients used in the present studies were found to grow together with about the same speed and that neither significant killing nor suppression was noted in any combinations of donors and recipients.

Frequency of transfer of resistance factors in mixtures of various sizes of donor and recipient populations. Varying concentrations of 222 (Sm, Cm, Tc, Su) were mixed with varying concentrations of CSH-2 in Penassay broth and shaken gently at 37 C. After 1 hr, varying dilutions were plated on selective media containing each drug. The frequency of transfer was rather low in any of the combinations and was dependent on the numbers of donor cells introduced (about 10^{-3} per donor cell).

Mixed culture of a small number of donor cells and a large number of recipient cells. About 10^2 cells of 222 (Sm, Cm, Tc, Su) or CSH-2 (Sm, Cm, Tc, Su) were mixed with about 10^6 cells of sensitive CSH-2 and the mixture was incubated at 37 C for 16 hr. The mixture was then diluted and plated on selective media containing each drug. The results obtained indicated that almost half the total population was composed of cells resistant to all drugs. This experiment suggests not only that resistance factors are transmitted very easily but also that they replicate faster than host chromosomes.

Time required for conjugation. Equal volumes

TABLE 3

Transfer of resistance factors from Shigella flexneri 2b strain 222 (Sm, Cm, Tc, Su) to CSH-2, a substrain of Escherichia coli K-12, Salmonella typhimurium strain LT-2, and Salmonella enteritidis strain no. 11 by growing them together overnight

Recipient	Frequency of Transfer per Surviving Recipient Cells	Frequency of Transfer per Introduced Donor Cells
CSH-2	6/10	>1
LT-2	$1/3.6 \times 10^7$	$1/3.4 \times 10^6$
No. 11	$1/10^8$	$1/2 \times 10^8$

Equal volumes of donor and recipient cultures were mixed and incubated overnight without shaking. CSH-2 (Sm, Cm, Tc, Su) cells were detected by plating varying concentrations of the mixed culture on bromthymol blue-lactose agar containing (per ml): Sm, 10 μg; Cm, 25 μg; or Tc, 25 μg; and on Mueller-Hinton agar containing 100 μg/ml of sulfathiazole. LT-2 (Sm, Cm, Tc, Su) and no. 11 (Sm, Cm, Tc, Su) cells were selected on minimal agar containing (per ml): Sm, 50 μg; Cm, 25 μg; Tc, 25 μg; or Su, 500 μg.

TABLE 4

Levels of resistance expressed by various strains that carry resistance factors

Drug	Strain		
	Escherichia coli strain K-12 and its substrains	*Salmonella typhimurium* strain LT-2	*Shigella flexneri* 2b strain 222
	μg/ml	μg/ml	μg/ml
Dihydrostreptomycin	= >10-<25	= >25-<50	= >1,000
Chloramphenicol	= >100-<200	= >100-<200	= >100-<200
Tetracycline	= >100-<200	= >25-<50	= >100-<200
Sulfathiazole	= >1,000	= >1,000	= >1,000

Levels of resistance to dihydrostreptomycin, chloramphenicol, and tetracycline were expressed in average resistance on nutrient agar containing varying concentrations of drug. Drug concentration at which most cells formed visible colonies was referred to as average resistance. Average resistance to sulfathiazole was determined on Mueller-Hinton agar.

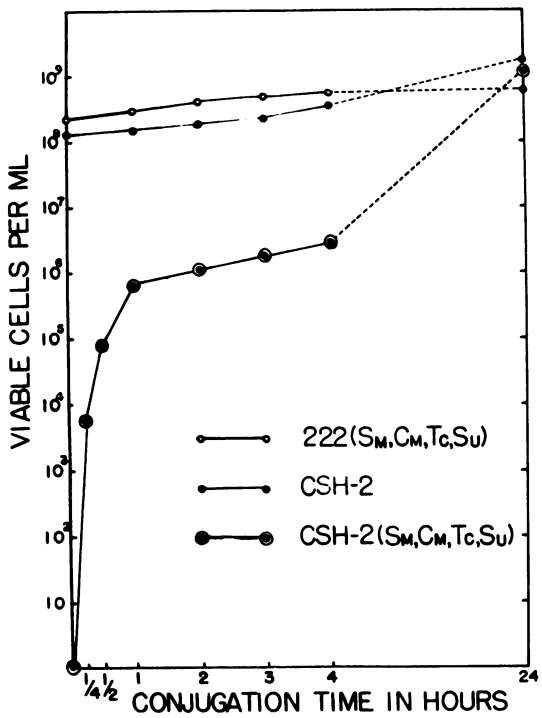

Fig. 1. Kinetics of the transfer of resistance factors by conjugation between *Shigella flexneri* 2b strain 222 (Sm, Cm, Tc, Su) and CSH-2, a substrain of *Escherichia coli* strain K-12. These cultures were mixed in Penassay broth and incubated in a 37 C water bath. Samples were taken at intervals and assayed for viable cells of 222 (Sm, Cm, Tc, Su) and CSH-2 on bromthymol blue-lactose agar. CSH-2 (Sm, Cm, Tc, Su) cells were assayed on bromthymol blue-lactose agar containing 10 μg of Sm, 25 μg of Cm, or 25 μg of Tc per ml, and also on bromthymol blue-lactose-Mueller-Hinton agar containing 100 μg of Su per ml.

TABLE 5

Time required for conjugation of Shigella flexneri 2b strain 222 (Sm, Cm, Tc, Su) with strain CSH-2, a substrain of Escherichia coli strain K-12

Time from Mixing Donor and Recipient to Dilution	Lac⁺ Colonies on		
	Sm, 10 μg/ml	Cm, 25 μg/ml	Tc, 25 μg/ml
min			
0	168	277	196
1	241	349	275
2	279	327	406
3	320	440	476
4	385		490
5	457	529	473
6	460	571	688
7	491	515	575
8	518	657	680
:	:	:	:
15	544	713	726

Equal volumes of 222 (Sm, Cm, Tc, Su) (1.8×10^9/ml) and CSH-2 (1.3×10^9/ml) were mixed, and after shaking the mixture at 37 C for varying durations, 0.1-ml samples were diluted in 100 ml Penassay broth. The diluted samples were incubated at 37 C for 1 hr and 0.1 ml was plated on bromthymol blue-lactose agar containing each drug.

Transfer of resistance could not be detected when the mixture of donor and recipient was plated on the above concentrations of drugs immediately after both strains were mixed.

In the control experiment where the mixture of 10^{-3} dilutions of donor and recipient were mixed and incubated at 37 C for 1 hr, 0.1 ml of the mixture produced 22 colonies on Sm 10 μg/ml, 42 on Cm 25 μg/ml, and 54 on Tc 25 μg/ml.

of undiluted cultures of 222 (Sm, Cm, Tc, Su) and CSH-2 were mixed and gently shaken at 37 C. Samples of 0.1 ml were withdrawn at 1-min intervals and gently transferred to 100 ml Penassay broth, prewarmed to 37 C, to prevent further conjugation. The inoculated broth was incubated at 37 C for 1 hr to allow the mated recipients to receive the resistance factors and express their phenotype. One-tenth milliliter of this culture was then plated on selective media containing each drug. As shown in Table 5, conjugation between donor and recipient cells begins almost instantly after their mixing and then proceeds to increase with time. In a control experiment, in which 10^{-3} dilutions of donor and recipient were mixed and incubated at 37 C for 1 hr, 0.1 ml of the mixture contained, on the average, 33 drug-resistant, Lac⁺ cells. It is seen in the table that the colony counts on Sm are consistently less than those on Cm and Tc.

Interrupted conjugation. The conjugation of 222 (Sm, Cm, Tc, Su) and CSH-2 was interrupted with a blender. The results are shown in Fig. 2. As shown in this figure, about 15 min incubation is required for resistance factors to be irreversibly transferred from donor to recipient. In several other experiments, not shown here, transfer of resistance factors could not be detected when the conjugating samples were treated in a blender within 15 min after mixing donor and recipient.

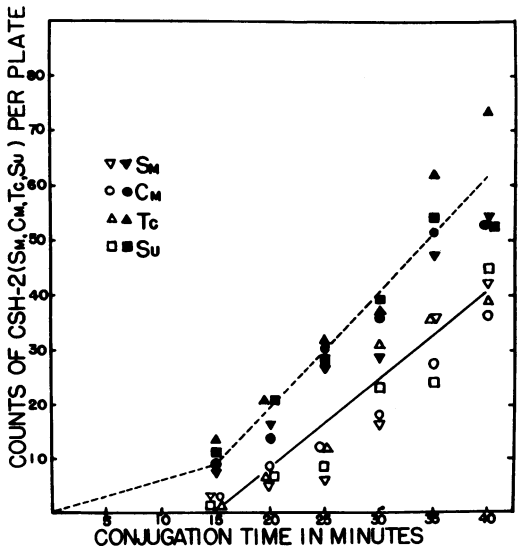

Fig. 2. Kinetics of the transfer of resistance factors in interrupted conjugation between *Shigella flexneri* 2b strain 222 (Sm, Cm, Tc, Su) and CSH-2, a substrain of *Escherichia coli* strain K-12. Equal volumes of 222 (Sm, Cm, Tc, Su) (2.1×10^8/ml) and CSH-2 (1.2×10^8/ml) were mixed and incubated in a 37 C water bath. Samples were taken at intervals and diluted 1:1,000 with chilled Penassay broth. A half of this dilution was treated in a blender at 20,000 rev/min for 15 sec. Both blender-treated and untreated samples were incubated at 37 C for 1 hr and 0.1 ml of each sample was plated on bromthymol blue-lactose agar containing 10 μg of Sm, 25 μg of Cm, or 25 μg of Tc per ml, and also on bromthymol blue-lactose-Mueller-Hinton agar containing 100 μg of Su per ml. *Solid line* represents the resistant cells in blender-treated samples and *dotted line* the controls of untreated samples.

Transfer of resistance factors from resistant Shigella to various substrains of E. coli strain K-12. When the mixture of donor and recipient was incubated for 1 hr and plated on selective media containing each drug, the frequency of transfer of resistance factors was found to be about 10^{-3} to 10^{-4} per donor cell. In contrast to the finding of Mitsuhashi et al. (1960*b*), it was found that the frequency of transfer of resistance factors is slightly affected by the presence of F factor in the recipients. The frequency was the highest in F^- and the lowest in F^+. The frequency with Hfr was between F^- and F^+. The details will be reported in a later paper. All of the substrains of K-12 which acquired resistance factors were found to transfer their resistance factors to other sensitive recipients by conjugation.

DISCUSSION

The prevalence in Japan of Shigellae resistant to multiple drugs is quite a serious problem from the standpoint of epidemiology and public health. Furthermore, resistance is transferred to pathogenic enteric organisms including Salmonellae both directly and by way of non-pathogenic enteric bacteria such as *E. coli*. The problem of multiple drug resistance, therefore, is not limited to Shigellae but is also a problem of all *Enterobacteriaceae*. There is little doubt that treatment of shigellosis and salmonellosis in Japan will encounter serious difficulty in the future since multiple drug resistance includes the most powerful chemotherapeutic agents available against these diseases at the present time.

Apart from this medical importance, multiple drug resistance offers interesting material for bacterial genetics. As mentioned in the introduction, the "resistance transfer factor" seems to be a new episome.

As shown in the present paper, and in accordance with the results of Mitsuhashi et al. (1960*b*), resistance factors can be transferred from resistant donors to sensitive recipients in systems in which chromosomes are not transferred. Examples include transfer from Shigella to *E. coli* (Shigellae were found by Luria and Burrous (1957) to be F^-) and, in *E. coli* strain K-12, from F^- to F^-, F^+, or Hfr, although we have found some interaction between the present resistance factors and F factor. These results indicate that the resistance factors are possibly not of chromosomal nature. They can be transferred from strain to strain indefinitely, suggesting that they are some transmissible factors like F factor and colicinogenic factor.

They are further assumed to replicate faster than host chromosomes in view of the fact that a small number of resistant cells introduced into a large population of sensitive cells converts the whole population to resistance faster than the multiplication of the recipient cells. It is now evident from these findings that the resistance factors proliferate in cytoplasm autonomously. It was further found that the resistance factors can be transduced, as will be reported in a later paper. Transduction has been generally believed to specifically carry the chromosomal elements or chromosome-attached episomes until recently.

Arber (1960) found that F factor in autonomous state is also transduced. Transducibility of resistance factors, therefore, cannot be taken as evidence for their chromosomal integration. The results of transduction with *S. typhimurium*, however, indicate that the resistance factors are stably integrated onto host chromosomes, suggesting that the resistance factors have affinity for host chromosomal markers, possibly because of some genetic homology. We can assume, therefore, that the resistance factors had possibly originated from bacterial chromosomes. Sager (1954) reported non-mendelian inheritance of streptomycin resistance in *Chlamydomonas reinhardi*, but the responsible factor is not known to be attached to host chromosomes.

It should be emphasized that all the resistance factors are transferred together by conjugation and usually do not segregate from each other in their transfer. They could be considered to be controlled by a single transmissible element but were found to segregate in transduction, as will be reported in a later paper. The most reasonable hypothesis at the present moment is that the resistance factors are carried by an episome. Experimental findings in support of this hypothesis will be presented in subsequent papers.

The resistance factors transferred by conjugation seem to be phenotypically expressed very rapidly, sometimes without apparent lag. This is quite in contrast to the expression of transduced streptomycin resistance as studied by Watanabe and Watanabe (1959), and this point was investigated in detail. Our kinetic studies on the transfer of resistance factors by interrupting conjugation with a blender revealed that the irreversible transfer of resistance factors requires about 15 min of incubation of conjugated cells under optimal conditions. Conjugation, on the other hand, takes place almost instantly after donor and recipient are mixed. The apparent absence of lag for the phenotypic expression of transferred resistance factors, when the mixture of donor and recipient cells is plated on drug-containing media, is probably because the cells are allowed to express their phenotype to some extent in the presence of low but inhibitory concentrations of drug. This tendency was more marked in Cm, Tc, and Su than in Sm. This difference is possibly due to bactericidal action of Sm and bacteriostatic actions of the other drugs.

It should also be pointed out here that the resistance factors are apparently phenotypically expressed before the first cell division takes place, whereas chromosomal streptomycin resistance is not phenotypically expressed until the first cell division is completed, segregating its sensitive alleles, because streptomycin resistance is recessive to sensitivity (Lederberg, 1951; Watanabe and Watanabe, 1959). We do not know yet, however, whether the sensitive recipients are equipped with sensitive alleles to the genetic determinants of drug resistance in the present multiple drug resistance.

The remarkable difference in the frequency of transfer of resistance factors between *E. coli* and Salmonella as recipients seems to be worth investigating. We do not know yet whether it is due to immunity, as in lysogenic bacteria, or to other factors.

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SUMMARY

Transfer of resistance from naturally occurring multiply drug-resistant Shigellae to *Escherichia coli*, *Salmonella typhimurium*, and *Salmonella enteritidis* was studied. All of the resistance markers (streptomycin, chloramphenicol, tetracycline, and sulfonamide) were found to be transferred regardless of the transfer of host chromosomes. They were further transferred indefinitely to sensitive recipients and assumed to replicate autonomously in cytoplasm. Transfer of resistance factors required about 15 min under optimal conditions, and the transferred resistance factors were found to be phenotypically expressed apparently before the first cell division. The frequency of transfer of resistance factors, and the levels of resistance of the cells which received resistance factors, were found to differ markedly among recipient strains.

ADDENDUM

Our recent studies on the kinetics of transfer of resistance factors with interrupted conjugation using phage T6 (Hayes, 1957) have shown that they are transferred much earlier than 15 min.

Half a milliliter of T6-sensitive CSH-2 (Sm, Cm, Tc, Su) was mixed with 4.5 ml of W-677/T6^{r}S^r and kept at 37 C. Samples of 0.1 ml were withdrawn at 1-min intervals and added to 0.9 ml T6 (titer: 3.5×10^{10}/ml). The multiplicity of phage input was about 10,000 per donor cell. The mixture of phage and bacteria was incubated at 37 C for 30 min and then plated on nutrient agar containing Sm (1,000 μg/ml) and either Cm or Tc (25 μg/ml), and on Mueller-Hinton agar containing Sm (1,000 μg/ml) and Su (100 μg/ml). An early sample mixed with T6 was incubated at 37 C for varying durations before plating on drug media to study the kinetics of phenotypic expression of drug resistance by the recipient cells that received resistance factors. It was found that resistance factors can be transferred within 1 min and that phenotypic expression of resistance to the above drug concentrations is completed within 10 min, indicating that the incubation for 30 min is quite enough. Similar experiments with 222 (Sm, Cm, Tc, Su) revealed that the frequency of transfer is about $\frac{1}{10}$ of CSH-2 (Sm, Cm, Tc, Su) at 1-hr-mixed culture with W-677/T6^{r}S^r. Furthermore, possibly because of this low frequency of transfer, the transfer of resistance factors by 222 (Sm, Cm, Tc, Su) was detected only after 8 min of mixed cultivation. The recipient cells selected on either Cm, Tc, or Su were all found to be resistant to all four drugs, even when they were selected from the early samples.

In view of these findings, the result obtained with blender interruption is assumed to be an artifact due to the inefficient method of detecting the transfer of resistance factors and the employment of a poor donor, 222 (Sm, Cm, Tc, Su).

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