EPISOME-MEDIATED TRANSFER OF DRUG RESISTANCE IN *ENTEROBACTERIACEAE*

II. Elimination of Resistance Factors with Acridine Dyes

TSUTOMU WATANABE AND TOSHIO FUKASAWA

Department of Bacteriology, Keio University School of Medicine, Tokyo, Japan

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We have reported in the preceding paper (Watanabe and Fukasawa, 1961) that multiple drug resistance (to streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), and sulfonamides (Su)) of Shigellae can be transferred to *Escherichia coli*, *Salmonella typhimurium*, and *Salmonella enteritidis* by conjugation. The responsible resistance factors were found to be transferred together and to replicate autonomously in the cytoplasm.

Ephrussi, Hottinguer, and Chimenenes (1949) found that cytoplasmic factors of yeast can be eliminated by acriflavine, converting the cells to "petite colonie" mutants. Hirota and Iijima (1957) reported that acriflavine converts F^+ cells of *E. coli* strain K-12 to F^- by eliminating the F factor. Hirota (1960) further found that the F factor in the integrated state (Hfr) cannot be eliminated by acridines.

If our resistance factors are of cytoplasmic nature, it might be possible to eliminate them with acridines and to convert resistant to sensitive cells. This possibility was examined in the present paper.

MATERIALS AND METHODS

Media and drugs. In addition to materials described in the preceding paper (Watanabe and Fukasawa, 1961), acriflavine hydrochloride and acridine orange (Tokyo Kasei Kogyo) were used.

Strains. Shigella flexneri 2b strain 222 (Sm, Cm, Tc, Su), strain CSH-2 (Sm, Cm, Tc, Su), which is a substrain of *E. coli* strain K-12, and *Salmonella typhimurium* strain LT-2 (Sm, Cm, Tc, Su) were used as resistant strains. The latter two strains had been made resistant by conjugation with 222 (Sm, Cm, Tc, Su). Substrains of K-12 were used as sensitive recipients.

Culture method, conjugation conditions, and selection of clones that received resistance factors. These were the same as those described in the preceding paper (Watanabe and Fukasawa, 1961).

Conditions of acridine treatment. Since Hirota (1960) reported that pH 7.6 is optimal for the elimination of F factor with acridines, Penassay broth adjusted to pH 7.6 with 1 N NaOH was used in the present experiments. Acridines were dissolved in distilled water at 1 mg/ml and autoclaved. From this stock solution a series of 2-fold dilutions were made with Penassay broth of pH 7.6. Each tube (containing 5 ml) was inoculated with 0.1 ml of culture containing about 10^4 cells of resistant strain. The inoculated media were incubated at 37 C for 24 hr without shaking. They were then diluted with saline and assayed for viable cells by plating 0.1 ml of each dilution on nutrient agar. From 50 to 100 of the resulting colonies were separately suspended in saline and streaked on nutrient agar containing 10 μg of Sm, 25 μg of Cm, or 25 μg of Tc, and on Mueller-Hinton agar containing 100 μg of Su per ml, to check their drug resistance.

Conditions of ultraviolet irradiation. Resistant strains were grown in Penassay broth with shaking and at the exponential phase of their growth 5 ml of each culture were transferred to a petri dish and irradiated with an ultraviolet lamp (National Company). The dose was that which gave approximately 1% survivors. The ultraviolet-irradiated cultures were incubated at 37 C for 1 hr and then used for experiments on acridine treatment and conjugation with sensitive recipients.

Penicillin screening of sensitive cells. Exponentially growing cultures of resistant strains were diluted with Penassay broth in 10-fold dilutions. Each dilution was mixed with Penassay broth containing penicillin and either Cm or Tc. The final concentrations of penicillin, Cm, and Tc were 250 units, 25 μg, and 25 μg per ml, respectively. The cultures with these antibiotics were gently shaken on a rotary shaker for 4 hr

at 37 C, then diluted with distilled water and plated on nutrient agar for viable cell assays. The colonies developed were separately suspended in saline and streaked on drug-containing agar plates to check their drug resistance. It was expected that Cm- or Tc-resistant cells would grow in the presence of either of these drugs and be killed by penicillin, whereas sensitive cells would survive.

RESULTS

Treatment of resistant cells with acridines. When strains 222 (Sm, Cm, Tc, Su) and CSH-2 (Sm, Cm, Tc, Su) were treated with acriflavine and acridine orange, it was found that resistant cells were converted to sensitive, although with low frequencies. The survival ratios and frequencies of loss of drug resistance of 222 (Sm, Cm, Tc, Su) are shown in Table 1. As is seen in this table, acridine orange was less toxic to both strains than acriflavine and was apparently less potent in converting the resistant cells to sensitive state. The frequencies of loss of resistance were rather low with both acridines. Essentially similar results were obtained with CSH-2 (Sm, Cm, Tc, Su). It should be pointed out that the sensitive cells obtained by acridine treatment were found to be sensitive to all drugs except in 222 (Sm, Cm, Tc, Su), where the sensitive cells obtained by acridine treatment were still resistant to Su alone.

Treatment with acridines of ultraviolet-irradiated resistant cells. Strains 222 (Sm, Cm, Tc, Su) and CSH-2 (Sm, Cm, Tc, Su) were irradiated with ultraviolet and, after intermediate incubation of the irradiated cultures for 1 hr, they were treated with acridines. As shown in Table 2, the frequencies of sensitive survivors were markedly increased by ultraviolet irradiation. Potency in sensitizing the resistant cells and toxicity were again higher in acriflavine than in acridine orange.

TABLE 1

Treatment of Shigella flexneri 2b strain 222 (Sm, Cm, Tc, Su) with acridine dyes

Acridine	Concn	Survivors	Frequency of Sensitive Cells
	μg/ml	cells/ml	%
Acriflavin	2.5	1.1×10^9	4.1
	5	0	
Acridine orange	2.5	1.1×10^9	0
	5	1.3×10^9	0
	10	8.4×10^6	?
	20	2.2×10^3	0
	40	0	0
Control	0	1.5×10^9	0

The cells were kept in Penassay broth (pH 7.6) containing the above concentrations of each acridine at 37 C for 24 hr before plating.

TABLE 2

Treatment of ultraviolet-irradiated Shigella flexneri 2b strain 222 (Sm, Cm, Tc, Su) with acridine dyes

Acridine	Concn	Survivors	Frequency of Sensitive Cells
	μg/ml	cells/ml	%
Acriflavin	1.25	7.0×10^8	4.2
	2.5	4.2×10^8	15.6
	5	8×10	100
Acridine orange	2.5	1.0×10^9	4.2
	5	8.1×10^8	2.1
	10	7.3×10^8	8.5
	20	2.5×10^8	0
	40	0	0
Control	0	1.3×10^9	0

The cells were ultraviolet-irradiated and incubated at 37 C for 1 hr. They were then kept at 37 C for 24 hr in Penassay broth (pH 7.6) containing the above concentrations of each acridine before plating.

Acridine sensitivity of sensitive cells obtained by treatment of resistant cells with acridines. Sensitive cells obtained by treatment of 222 (Sm, Cm, Tc, Su) and CSH-2 (Sm, Cm, Tc, Su) with acridines were found to have exactly the same levels of acridine sensitivity as the original resistant strains (K. Kinjo, *unpublished data*). Accordingly, the possibility of selection of sensitive cells by acridines can be excluded.

Mixed culture of sensitive recipient with sensitive cells obtained by acridine treatment. The sensitive cells converted by acridines from resistant cells were grown together with originally sensitive strains and plated on selective media containing each drug to see if they still retained the transmissible resistance factors in spite of their sensitive phenotype. In none of the experiments could drug resistance be successfully transferred to sensitive recipients from the converted sensi-

TABLE 3

Screening with chloramphenicol and penicillin of spontaneous chloramphenicol-sensitive segregants from Shigella flexneri 2b strain 222 (Sm, Cm, Tc, Su), CSH-2 (Sm, Cm, Tc, Su), a substrain of Escherichia coli strain K-12, and Salmonella typhimurium strain LT-2 (Sm, Cm, Tc, Su)

Resistant Strain	Inoculated Cells	Surviving Cells	Ratio of Sensitive Segregants among Survivors*	
			Sm, Cm, Tc, Su sensitive	Sm, Cm, Su sensitive and Tc resistant
	cells/ml	*cells/ml*	%	%
222 (Sm, Cm, Tc, Su)	6×10^6	2.8×10^2	84	3
CSH-2 (Sm, Cm, Tc, Su)	5.1×10^6	6.3×10^2	69	0
LT-2 (Sm, Cm, Tc, Su)	4.6×10^6	3.4×10^2	6	94

* The other survivors were resistant to all of the four drugs. The resistant cells were shaken at 37 C for 4 hr in Penassay broth containing 250 units of penicillin and 25 μg of chloramphenicol per ml.

tive cells. The 222 (Su) obtained by acridine treatment was also unable to transfer its Su resistance to CSH-2. Ultraviolet irradiation of 222 (Su) could not induce transfer of Su resistance by conjugation.

Effect of ultraviolet irradiation of resistant donor cells on frequency of transfer of resistance factors. Since the effect of ultraviolet irradiation of donor cells on the frequency of converting the resistant cells to a sensitive state by acridines was suspected to be due to some effect like ultraviolet induction of prophage (Lwoff, 1953), it was decided to determine whether ultraviolet irradiation also increased the frequency of transfer of resistance factors. In several independent experiments in which ultraviolet-irradiated 222 (Sm, Cm, Tc, Su) was grown together with CSH-2 for 1 hr at 37 C, the frequency of transfer of the resistance factors (per donor cell) was 10 to 100 times higher than in control experiments in which unirradiated cells were used.

Spontaneous loss of resistance factors. Spontaneous loss of resistance factors, either partly or wholly, was occasionally experienced by chance, although with very low frequencies. In case a part of the resistance factors was lost, either Tc alone or Sm, Cm, and Su together were lost without exception. Spontaneous loss of the resistance factors was studied systematically with a penicillin screening method suggested by K. Harada. The results of screening with a mixture of Cm and penicillin are shown in Table 3.

As is seen in this table, with two of the strains the survivors selected for Cm sensitivity were mostly sensitive to the other three drugs as well. With LT-2 only a few colonies were of this type, whereas most were sensitive to Cm, Sm, and Su, but not to Tc. No other type of segregation was found in any of these strains. When Tc and penicillin were used in the selection the loss of Tc resistance alone was noted as well as the simultaneous loss of all the resistance factors.

The transferability of resistance through conjugation by these sensitive segregants was tested and it was found again that the cells with drug-sensitive phenotype were not capable of converting CSH-2 to a resistant state. Su resistance of spontaneously arisen 222 (Su) could likewise not be transferred as in the above experiment.

Transferability of resistance factors by segregants with partial resistance factors. Spontaneous segregants with partial resistance factors of 222 (Sm, Cm, Tc, Su) were grown together with CSH-2 to determine whether the remaining resistance factors could be transferred. The segregants used were 222 (Sm, Cm, Su), 222 (Tc, Su), LT-2 (Sm, Cm, Su), and LT-2 (Tc). It was found that all of these segregants could transfer their resistance except that 222 (Tc, Su) could not transfer its Su resistance.

Acceptability of resistance factors through conjugation by sensitive segregants. Spontaneous segregant of CSH-2 (Sm, Cm, Tc, Su) with no resistance factors, and CSH-2 which received (Tc) or (Sm, Cm, Su) from spontaneous segregants 222 (Tc Su) or 222 (Sm, Cm, Su), were grown together with 222 (Sm, Cm, Tc, Su), 222 (Sm, Cm, Su), or 222 (Tc, Su). It was found that defective resistance factors could be complemented by conjugation, although the frequency of transfer of the defective resistance

factors was markedly lower than when the original sensitive strain was used as a recipient. It was further found that CSH-2 (Sm, Cm, Tc, Su) lines thus obtained were all able to transfer their resistance factors together by conjugation.

DISCUSSION

It is reported here that the resistance factors can be eliminated by treatment of resistant cells with acridine dyes, confirming the previously reached conclusion that the resistance factors are in an autonomous state (Watanabe and Fukasawa, 1961). The frequencies of elimination were rather low (at most 4.1%) as compared with the high frequencies of elimination of F factor reported by Hirota and Iijima (1957) and Hirota (1960). On the other hand, the resistance factors can be transduced in *E. coli* and *S. typhimurium*, as will be reported in a subsequent paper. The results of transduction with *S. typhimurium* suggest that the resistance factors are stably integrated onto host chromosomes. The low frequencies of elimination of resistance factors by acridine treatment might be due to the chromosomal integration of the resistance factors, because, as revealed by Hirota (1960), F factor in the integrated state cannot be eliminated by acridines. The remarkable increase by ultraviolet in the frequencies of elimination of resistance factors by acridines and in the frequencies of transfer of resistance factors might be due to conversion of the integrated state of the resistance factors to an autonomous state. The conversion by ultraviolet of the integrated state of temperate phage to an autonomous state is known as ultraviolet induction (Lwoff, 1953), but it is not known in F factor and colicinogenic factors. Ultraviolet induction of colicinogenic factors might occur but be masked by the induction of colicin production which is lethal to the cells.

The resistance factors seem to have rather close affinity to host chromosomes but possibly cannot be stably integrated, in contrast to the F factor. The integrated state and the autonomous state are possibly interchangeable in populations of resistant strains. This situation might resemble that of colicinogenic factors, which are known to be attached to host chromosomes and also to replicate autonomously (Frédéricq, 1957, 1958; Alfoldi et al., 1958). The failure of Hirota (1958) to eliminate the colicinogenic factor by acridines might be due to its close affinity to host chromosomes. It might be eliminated with low frequencies. F2, reported by Adelberg and Burns (1960), also seems to resemble the resistance factors in its behavior. It can be attached to host chromosomes, although at specific sites, and revert to the autonomous state. This hypothesis of interchange is convenient for explaining the low frequencies of transfer of resistance factors by unirradiated donor cells, and the effects of ultraviolet irradiation.

It seems rather important that all of the resistance factors are eliminated together by acridine treatment. This finding is compatible with the hypothesis that the resistance factors are carried by some episome, as proposed in the preceding papers (Watanabe and Fukasawa, 1960, 1961). The failure to eliminate Su resistance in 222 (Sm, Cm, Tc, Su) by acridine treatment, and the inability of 222 (Su) to transfer its Su resistance to sensitive recipient, are assumed to be due to the fact that 222 (Sm, Cm, Tc, Su) has double Su-resistance factors, one being chromosomal and another being episome-carried. This is understandable since the majority of Shigellae isolated at present from patients in Japan are resistant to Su. The treatment with acridines is thought to eliminate the episome-carried Su-resistance factor, together with other resistance factors, leaving only the chromosomal Su-resistance factor untouched. The transferability of Su resistance through conjugation by 222 (Sm, Cm, Tc, Su) can be taken as the evidence for the presence of episome-carried, Su-resistance factor.

In contrast to the results of Hirota (1960), in which acridine orange was more effective than acriflavine, our results indicated that acriflavine was more effective than acridine orange in our systems.

Spontaneous loss of resistance factors was found both by chance and by a penicillin screening method. All of the resistance factors were lost together in some instances but segregated loss of resistance factors was also noted in others. When the resistance factors were segregated, they were divided into Tc alone and Sm, Cm, Su, as a group. These segregants with partial resistance were all able to transfer their resistance by conjugation. It was further found that these sensitive segregants with partial resistance are able to acquire defective resistance factors by

conjugation with resistant donors, although with reduced frequencies (about 1:100). This reflects the presence of partial immunity. We do not yet know the mechanism of the spontaneous loss of the resistance factors or the mechanism of the observed segregation. It is known that other episomes are also spontaneously lost.

SUMMARY

The resistance factors of multiply drug-resistant strains of *Shigella flexneri* 2b strain 222 and *Escherichia coli* strain CSH-2 were found to be eliminated by treatment of the resistant cells with acridines. The resistance factors were eliminated together except in 222 (streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), and sulfonamides (Su)), where Su resistance alone could not be eliminated. 222 (Su) obtained by acridine treatment was unable to transfer its Su resistance by conjugation, whereas 222 (Sm, Cm, Tc, Su) could transfer its Su resistance. These results support the proposed episomal location of the four resistance factors in all the strains; strain 222 appears to have an additional chromosomal factor for Su resistance.

In contrast to the total elimination of resistance factors by acridine treatment, a part of the resistance factors can be lost spontaneously. In their spontaneous loss, either all of the four resistance factors, Tc alone, or Sm, Cm, Su together are lost. Spontaneous segregants with Tc resistance alone, or with Sm, Cm, and Su resistance, were all able to transfer their resistance factors by conjugation. The loss of resistance factors can be detected with a penicillin screening method.

Ultraviolet irradiation of the resistant cells increased the frequency of elimination of resistance factors with acridines and the frequency of transfer of resistance factors by conjugation.

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