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The Elimination of a Colicinogenic Factor by a Drug-resistance Transferring Factor in *Escherichia coli* K_{235}

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SUMMARY

When various potent R-factors were transferred to doubly colicinogenic *E. coli* K235 K^+X^+ and the recipient cells with acquired drug resistance were screened, X-cinogen or K-cinogen was found to have been lost in a fraction of the progeny. A similar X-non-colicinogenic mutant was also isolated by the replica method without contact with the drug. The elimination of the cinogen had no relationship to unselected suger fermentation markers.

When the R-factor of such noncolicionogenic mutants was eliminated by treating cells with an acridine dye, all the resulting drug-sensitive mutants were still noncolicinogenic and very sensitive to the colicine produced by the parent culture.

INTRODUCTION

It was reported by Miyama, Ozaki and Amano (1961) that about 40 per cent of Sm-resistant (independent) mutants isolated from the K^+X^+ strain of *E. coli* K235 L^+O had no X-cinogen. A similar phenomenon was also described by Lederberg and Lederberg (1952) for λ-lysogeny and Sm-resistance. They found that Sm independent mutants of some strains of *E. coli* K12 (λ) had no λ-prophage and stated that the λ-sensitive and Sm-resistant characters were indistinguishable from those of mutants previously isolated in a single step and that no explanation for this remarkable association could be offered. As the present authors could also give no explanation for their own curious observations, further direct analyses of the phenomenon were abandoned, and studies were made of the fate of colicinogenicity after introduction of an episomic drug resistance

* The following abbreviations are used: tetracycline, Tc; chloramphenicol, Cm; dihydrostreptomycin sulfate, Sm; sulfonamide, Sa; The abbreviations of drugs in parentheses indicate resistance to these drugs; tryptophan, Try; lactose, Lac: galactose, Gal; T_1, T_4 and P_1 phage, T_1, T_4 and P_1; carrying λ-prophage, Lp^+; sex factor, F; colicinogenic factor, cinogen.

factor (R-factor). During the two years, since the original independent findings of Ochiai *et al.* (1959) and Akiba *et al.* (1960), the mechanism of episomic transfer of drug resistance (Tc, Sm, Cm and Sa) from resistant to sensitive enteric bacteria has been elucidated in this country (Akiba *et al.*, 1960; Mitsuhashi *et al.*, 1960; Watanabe *et al.* 1960). The episomic nature of the drug resistance transferring factor was first shown by Watanabe *et al.* (1960, 1961) and the factor was variously termed the Rtf, Rta or R-factor by different groups. Very recently it has been agreed to call it the R-factor. The present paper reports the effect of transfer of various R-factors on the cinogens (colicinogenic factors) of *E. coli* K235.

MATERIALS AND METHODS

1. *Bacterial strains*

1) *Colicinogenic strains: E. coli K235 $L^+OK^+X^+$ and E. coli K235 $L^-OK^-X^+$:* The former strain was kindly given by Dr. Walther F. Goebel of the Rockefeller Institute and it was later shown to be doubly colicinogenic (K^+X^+) (Miyama, Ozaki and Amano, 1961). The latter strain was isolated from *E. coli* K235 $L^-OK^+X^+$ (also given by Dr. Goebel) while studying the double colicinogenicity (Miyama, Ozaki and Amano, 1961).

2) *R-factor transferring strains:* The bacteria carrying R-factor used in this study were isolated in routine bacterial examinations in the Osaka Municipal Hygiene Laboratory and identified as type 2a (2a-30, 2a-185 and 2a-50) and type 3a (3a-172 and 3a -1484) of *Shigella dysenteriae* Group B (Flexner). Some characteristics of these strains are shown in Table 1 together with those of the colicinogenic strains used.

Table 1. Characteristics of Strains Used

Strain	Drug resistance* (μg)				Sensitivity to Phage			Sensitivity to Colicine		Fermentation				
	Tc	Cm	Sm	Sa	T_1	T_4	P_1	K	X	Gal****	Mal	Lac	Xyl	Sorb
2a-30	100	100	1000	1000	+	+	+	—	+	+	—	—	—	—
2a-185	100	—	—	1000	+	+	+	+	—	+	—	—	—	—
2a-50	—	—	125	1000**	+	+	+	+	—	+	+	—	—	—
3a-172	100	—	—	—	+	+	+	+	—	+	+	—	—	+
3a-1484	—	100	1000	—	+	+	+	+	—	+	+	—	—	+
E. coli K235 $L^-OK^-X^+$	—	—	—	>100***	—	+	—	+	—	+	+	+	+	+
E. coli K235 $L^+OK^+X^+$	—	—	—	>100***	—	+	—	—	—	+	+	+	+	+

* all drug resistance markers were incorporated into the R-factor
** nontransmissible
*** spontaneous resistance (nontransmissible)
**** Gal: galactose Mal: maltose Lac: lactose Xyl: xylose Sorb: sorbitol

3) *Other E. coli strains: E. coli B K^SX^R and E. coli B K^RX^S:* These strains were isolated from *E. coli* B while studying the double colicinogenicity of *E. coli* K235. The former was sensitive to colicine K and resistant to colicine X, and the latter had the reverse sensitivity and resistance.

E. coli K-12 4627: This strain was kindly given by Dr. Iijima of the Department of Genetics, Osaka University Medical School. Its genetic character was Try^-, Lac^-, Gal^-, T_1^R, L_P^+, F^-

and it was resistant to phage A3, to which strains of *E. coli K*235 were sensitive. This strain was used as a recipient strain of the R-factor.

2. *Phages: T_1 phage:*

This stock was obtained by growing it on 2a-30 *Shigella dysenteriae* Group B. The stock contained 1×10^{10} particles per ml. All R-factor carrying strains used in this study were sensitive to T_1 phage.

A_3 phage: This virulent phage was isolated from Osaka City sewer water and grown on *E. coli* K235 $L^-OK^-X^-$. The phage stock contained 1×10^{10} particles per ml. This phage could attack all the strains of *E. coli* K235 used in this study.

3 *Assay of colicinogenicity and immunity*

The method of Fredericq (1958) was used. A nutrient plate was seeded with the test organism by sticking the plate with a needle at a point. After 40 hours incubation at 37°C, the surface of the plate was sterilized by chloroform vapor and carefully covered with 5 ml of melted soft agar containing 1×10^8 cells of a colicine indicator strain. After solidification, the plate was again incubated at 37°C for 6 hours. If the organism tested was colicinogenic and the indicator strain was sensitive to the colicine, a zone of inhibition was detected surrounding the dead colonies of the test organism.

To assay immunity, the colicine indicator starin was replaced by the strain, whose immunity was to be tested. If the strain was immune, no inhibition zone could be detected.

4. *Agglutination reaction*

The aggutination reaction was used as a criterion that the isolated mutants were derivatives of *E. coli* K235. The antiserum used was the colicine K antiserum, the agglutinins of which had been shown to be O antibodies (Amano, Goebel and Smith, 1958). To test for L^-O variants, intact bacterial cells were used, whereas, when testing for L^+ O variants, boiled suspensions were used.

RESULTS

1. *The effect of transfer of R-factors on the colicinogenicity of E. coli K235.*

To see whether Sm-resistance is related to loss of X-colicinogenicity even in the episomic transfer of Sm-resistance, *E. coli* K235 $L^-OK^-X^+$ was grown with each of the R-factor carrying strains of *Shigella flexneri*. As these strains were sensitive to colicine K, and colicine is produced in the L-broth (Lennox, 1955) by K^+ strains, K^-X^+ strain was used in this experiment. Colicine X was not produced in shaking cultures in L-broth.

Aliquots of 0.25 ml of actively growing *E. coli* K235 $L^-OK^-X^+$ cultures in L-broth containing 1×10^5 cells per ml were introduced into five tubes containing the same volume at the same population density of cultures of *Shigella* strains, 2a-30, 2a-50, 2a-185, 3a-1484 and 3a-172. Tubes were shaken at 37°C for 3 hours and 0.5 ml aliquots of T_1 phage suspension in broth were added. After further incubation at 37°C for 1 hour, 0.1 ml of a 1:10 dilution of each mixture was spread on BTB-L-agar, containing 1.0 per cent lactose in place of 0.1 per cent glucose. After over-night incubation, replica platings were made on the same BTB-L-agar containing 25μg of Sm or Tc. Replica plates were incubated at 37°C for 24 or 48 hours. Sixty to eighty yellow colonies were selected, suspended in L-broth, incubated at 37°C for 3 hours, and streaked on plates of BTB-lactose-L-agar containing drug. After over-night incubation, one yellow colony was selected at random from one plate and tested for X-colicinogenicity using *E. coli* B K^RX^S as the indicator. There was no inhibition zone with colicine X. The immunity of the strain to the parent *E. coli* K235 $L^-OK^-X^+$ was tested and, in addition, the agglutination test was carried out to identify it as having been derived

from the parent. When still X-colicinogenic, the least X-colicinogenic colonies were suspended in L-broth and spread on drug containing BTB-lactose-L-agar plates. Thus the procedure was repeated. The results are presented in Tables 2 and 3.

Table 2 shows the details of isolation of K^-X^- (Tc, Sa) mutants of the parent K^-X^+ strain after transfer of R (Tc, Sa)-factor from 2a-185 (Tc, Sa). It can be seen that many K^-X^- (Tc, Sa) mutants were obtained from slightly X-producing clones. These slightly X-producing clones were fairly sensitive to colicine X, and thus such clones could be regarded to be mixtures of bacteria which were still X-positive and bacteria cured of X-cinogen.

Table 2. Elimination of X-cinogen in *E. coli* K235 $L^-OK^-X^-$ (Tc, Sa)***

Number of Platings	R (Tc, Sa)		R (Tc, Sa)			Control	
1	*X^+**	32/32§	X^+	0/32	0/16	X^-	80/80
	$X^=$	0/32	$X^=$	31/32	15/16	$X^=$	0/80
	X^-	0/32	X^-	1/32	1/16	X^-	0/80
2	X^+	32/32	X^+	0/16	0/16	X^-	80/80
	$X^=$	0/32	$X^=$	1/16	14/16	$X^=$	0/80
	X^-	0/32	X^-	15/16	2/16	X^-	0/80
3						X^-	80/80
						$X^=$	0/80
						X^-	0/80

§ : Denominator, number of colonies tested.
Numerator, number of colonies of colicinogenicity indicated.
* X^+, $X^=$, X^- These were indicated as colicine X producing activity.
X^+: very marked inhibition zone. $X^=$: inhibition zone of 1-2 mm. X^-: no inhibition zone.
** one colony was selected, resuspended in L-broth and spread on a new agar plate.
*** Tc-and Sa-resistance were transferred from 2a-185 (Tc, Sa).

Table 3 shows the difference in effect of the R-factors transferred. Some colonies with drug-resistance acquired by transfer of the R-factor from 2a-30, 2a-185 and 3a-172 had lost X-colicinogenicity. In the control experiment 160 parent colonies were examined for loss of X-colicinogenicity but no colony was isolated which had lost this character. 2a-30 carrying R (Tc, Cm, Sm, Sa), 2a-185 carrying R (Tc, Sa) and 3a-172 carrying R (Tc) could eliminate X-cinogen, whereas 2a-50, which transmitted R (Sm), and 3a-1484, which transferred R (Sm, Cm), colud not. In these experiments no close relaitonship between Sm-resistance and loss of X-colicinogenicity could be demonstrated, but a new phenomenon was seen. Although the above results indicate that Tc-resistance and loss of X-cinogen are closely related, this cannot be assumed, because the R-factor from 2a-50 could eliminate X-cinogen when *E. coli* K235 $L^+OK^+X^+$ was used as a recipient for the R-factor which could transfer only R(Sm). Thus, when strains 2a-185 and 2a-50 were used as donors the efficiency of elimination of X-cinogen was much

Table 3. Effects of Transferred R-factors on X-cinogen of *E. coli* K235 $L^-OK^-X^+$

R-factor	Tc, Cm, Sm, Sa		Sm		Tc, Sa		Tc		Cm, Sm	
Donor strain	2a-30		2a-50		2a-185		3a-172		3a-1484	
Selected drug marker	Tc	Sm	Sm		Tc		Tc		Sm	
X^+	75/80 §	72/80	80/80	80/80	69/80	70/80	75/80	72/80	64/64	64/64
$X^\pm$	4/80	4/80	0/80	0/80	4/80	4/80	4/80	6/80	0/64	0/64
X^-	1/80	4/80	0/80	0/80	7/80	6/80	1/80	2/80	0/64	0/64

§ : as in Table 2.

higher than with the $L^-OK^-X^+$ variant when the R-factor was transferred from 2a-185, as shown in Table 4. The transfer of R(Sm) from 2a-50 could eliminate the X-cinogen in this case.

Table 4. Elimination of X-cinogen in *E. coli* K235 $L^-OK^-X^+$ by R-factors

R-factor		Tc, Sa		Sm	
Donor strain		2a-185		2a-50	Control
1st plate	X^+	116/160 §		45/48	160/160
	$X^\pm$	** 44/160		3/48	0/160
	X^-	0/160		0/48	0/160
2nd plate	X^+	0/16	0/16	0/16	160/160
	$X^\pm$	15/16	16/16	11/16	0/160
	X^-	1/16	0/16	5/16	0/160
3rd plate	X^+	0/16	0/16	0/16	160/160
	$X^\pm$	0/16	1/16	10/16	0/160
	X^-	16/16	15/16	6/16	0/160

§, ** : as in Table 2.

2. *The relationship between the elimination of X-cinogen and of other unselected markers*

This experiment was to study the relationship between the elimination of X-cinogen of *E. coil* K235 $L^-OK^-X^+$ by the R-factor from 3a-185, which transmits R(Tc, Sa), and the mutation of other unselected markers. As unselected markers, sugar fermentating abilities (lactose, sorbitol, xylose) were adopted. The experimental procedures were the same as for the previous experiment. However in this experiment BTB-L-agar containing 25μg per ml Tc and 1 per cent of one

of the three sugars was used for isolation of colonies. The colonies at the last step of isolation were replicated to other plates of BTB-L-agar containing 25μg per ml of Tc and the other two kinds of sugar.

As seen from Table 5, the elimination of the X-cinogen had no relationship to the mutation of the unselected markers.

Table 5. Effect on Unselected Sugar Fermentation Marker of Transferred R-factor in *E. coli* K235 $L^-OK^-X^-$

Selected sugar marker		Lactose	Sorbitol	Xylose
Distribution of X-colicinogenicity among colonies	X^+	76/80§	79/80	78/80
	X^-	4/80	1/80	2/80
Lactose		—	80/80	80/80
Sorbitol		80/80!	—	80/80
Xylose		80/80	80/80	—

§ : as in Table 2.
! : Demonimator, number of colonies tested
Numerator, number of colonies positive in fermentation on BTB-sugar plates

3. *The indirect selection of the K^+X^-(Tc, Sa) mutant of E. coli K235 $L^+OK^+X^+$ after transfer of the R-factor*

In the above experiments it can not be concluded that some R-factor could eliminate X-cinogen, because the colonies with acquired drug resistance were always selected on drug containing media and such drugs might inhibit the replication of X-cinogen and hence the X-cinogen might have been lost in the progeny. To test this possibility, indirect selection of the drug resistant colonies from the T_1 treated culture of *E. coli* K235 $L^+OK^+X^+$ and 2a-185 (Tc, Sa) was made using the replica method. Among the colonies on replica plates containing 25μg per ml of Tc, K^+X^- (Tc, Sa) colonies were looked for and then K^+X^- (Tc, Sa) was concentrated from the original plate. After much effort only one pure culture of the mutant could be isolated. From this fact it can be concluded that the transmission of the R-factor was essential for the loss of X-cinogen.

4. *The elimination of K-colicinogenicity from K235 $L^+OK^+X^+$*

In the above experiment, only the X-cinogen seemed to be excluded on introduction of R-factors. Further attempts were made to isolate K^-X^+ mutants. The experimental procedures used were the same as those of the first experiment of this paper, except that the donor strain of the R-factor was 3a-172 (Tc, Sa), *E. coli* K235 $L^+OK^+X^+$ was used as a recipient and colonies producing less colicine K were first screened on Tc-containing replica plates. Then plating was repeated selecting single colonies produce little colicine K at random from each plate to

obtain colonies producing much less colicine K. Finally $K^{-}X^{+}$ (Tc, Sa) mutants were obtained in about 15 per cent of the clones from the colonies grown on Tc-containing replica plates. These cultures were very sensitive to the colicine K of the parent. The results are shown in Table 6.

Table 6. Elimination of K-cinogen in *E. coli* K235 $L^{+}OK^{+}X^{+}$ (Tc, Sa)

Donor strain		2a-185 (Tc, Sa)			Control
1st plate	K^{+}			137/160§	160/160
	$K^{=}$			23/160**	0/160
	K^{-}			0/160	0/160
2nd plate	K^{+}	0/16	0/16	0/16	160/160
	$K^{=}$	6/16	4/16	13/16	0/160
	K^{-}	10/16	12/16	3/16	0/160
3rd plate	K^{+}	0/16			160/160
	$K^{=}$	11/16			0/160
	K^{-}	5/16			0/160

§, **: as in Table 2

It was found in the course of screening, that, when less colicine K (but definitely some) was produced by a clone, the clone was more sensitive to colicine K. These clones may not have been homogeneous in K-colicinogenicity and have been segregated $K^{-}X^{+}$ (Tc, Sa) mutants. In addition, no doubly cured $K^{-}X^{-}$ (Tc, Sa) mutant was obtained in these experiments. It is obvious from these facts that when the R-factor is introduced it interferes with the autonomous replication of some cinogens in some cells of colicinogenic strains.

5. *The further transmissibility of the R-factor from E. coli K235 $L^{+}OK^{+}X^{-}$ (Tc, Sa) and $L^{+}OK^{-}X^{+}$ (Tc, Sa)*

The above experiments show that the R-factor caused some change in recipient cells. However it was conceivable that the R-factor itself might also be changed in character in the recipient cells after elimination of X-cinogen. As to its character, the expression of drug resistance was intact, however the further transmissibility of the R-factor may have been modified. To examine this possibility, the following experiment was carried out.

Actively growing cultures of *E. coli* K235 $L^{+}OK^{+}X^{-}$ (Tc, Sa) or of E. *coli K235* $L^{+}OK^{-}X^{+}$ (Tc, Sa) were mixed with *E. coli* K-12 4627. After 3 hours incubation, the former bacteria were killed by A3 phage and then the culture was plated on BTB-L-agar containing 1 per cent lactose. After the second plating the colonies were replicated on BTB-L-agar containing 25μg per ml of Tc and 1 per cent lactose, and lactose negative colonies were isolated.

The incidence of Tc-resistant colonies in the replica was about 10^{-3} and the efficiency of transmission of the R-factor seemed to be somewhat higher than that

of the original strain 2a-185. Therefore, the R-factor was completely intact in *E. coli* K235 $L^+OK^+X^-$ (Tc, Sa) and *E. coli* K235 $L^+OK^-X^+$ (Tc, Sa).

6. *The effect of elimination of the R-factor from E. coli K235 $L^+OK^+X^-$ (Tc, Sa) and $L^+OK^-X^+$ (Tc, Sa) by acriflavin*

In the examination of X-cinogen by R-factor it was conceivable that the R-factor transferred might inhibit the production of colicine X and also the expression of immunity to colicine X. To exclude this possibility the R-factor was eliminated from *E. coli* K235 $L^+OK^+X^-$ (Tc, Sa) and from E. coli K235 $L^+OK^-X^+$ (Tc, Sa) by acriflavin.

1×10^6 cells were seeded into L-broth containing 5μg per ml of acriflavin, After over-night incubation, the culture was spread on nutrient agar plates, and Tc sensitive colonies were selected. From 2.5 to 5 per cent of the colonies became sensitive to Tc. Fifteen sensitive colonies from each parent were tested for X- and K-colicinogenicity and immunity to colicine X and K. All were still X- or K-nonproducing and no immunity to colicine X or K had been recovered. In control experiments, the effect of acriflavin on *E. coli* K235 $L^+OK^+X^+$ was studied and no change in either of the colicinogenicities was demonstrated.

7. *The fate of X-cinogen in stepwise increased tetracyclin resistant mutants of E. coli K235 $L^+OK^+X^+$*

In the above experiments, R-factor carrying only Tc-resistance could eliminate the X-cinogen of the recipient cells. This suggested that Tc-resistance eliminates X-cinogen, so 13 Tc-resistant mutants were isolated from *E. coli* K235 $L^+OK^+X^+$ by streaking them on Tc agar plates of stepwise increasing concentrations. Thirteen clones resistant to about 125μg per ml of Tc were obtained and their X-cinogen was examined. All were still X-colicinogenic. This suggests that Tc-resistance itself has nothing to do with the mechanism of elimination of X-cinogen and that some potent R-factor must be transferred to the recipient for the elimination.

DISCUSSION

No close relationship between Sm-resistance and the elimination of X-cinogen could be demonstrated in the case of the epsomic transfer of drug resistance by R-factors. In the experiment shown in Table 3 there was apparently a close relationship in the transfer of Tc-resistance. However, this was not always so and X-cinogen was eliminated by an R-factor carrying nothing but Sm-resistance, as shown in Table 4. There was further evidence against the concept that the transfer of Tc-resistance eliminates X-cinogen. Thus, in the last experiment, 13 Tc-resistant mutants were isolated from *E. coli* K235 $L^+OK^+X^+$ and these mutants were all X-colicinogenic. Considering these results, the authors are inclined to assume that the elimination of cinogens is evoked by the introduction of some

R-factor itself and not of a drug resistance marker. The ability of donor strains to eliminate X-cinogen seems to be in accordance with their ability to transfer R-factors, as shown in Table 3.

When a potent R-factor was transferred, no all the recipient cells lost their colicinogenicity on the first plate, and the clones producing little colicine gave many noncolicinogenic mutants and were partially susceptible to the colicine produced by the parent strain. The phenomenon is not merely a segregation in the strict sense, but it could be caused by interference of replication of the cinogen by the R-factor introduced. Even when a potent R-factor was introduced, some recipient cells which were resistant to the drug, were still invariably colicinogenic. If interference is the mechanism underlying this elimination, such clones must have a tendency to loose R-factor and to revert to the same type as the parent. Experiments on this have not yet been performed.

Noncolicinogenic mutants were obtained by the replica method without coming in contact with the drug, which partially exclude, the role of the drug in mutant formation. The possibility of whether the drug used in the screening enhances the interfering effect of an introduced R-factor has not yet been studied.

It is conceivable that cinogen was not really lost in the "cured" mutants and the expression of colicine production as well as of the immunity mechanism may have been inhibited by the introduction of a potent R-factor. If this is the case, the gradual loss of colicine production and the immunity mechanism after transfer of the R-factor cannot be explained. Furthermore this objection can be ruled out since R-factor was eliminated from non-colicinogenic mutants. The R-factor was lost on acridine treatment. However, no colicine producing ability or immunity to the colicine produced by the parent was recovered by the mutants.

The elimination of K-cinogen on introduction of the R (Tc, Sa) factor was an unexpected result. At first the authors were inclined to assumed that the transmissible cinogen (including K-cinogen) has a tendency to replicate very rapidly in the cells and this capacity is hardly affected by the R-factor while the non-transmissible cinogen was affected by the R-factor. Since K-cinogen was also eliminated, such an assumption seemed no longer tenable. However, it is still probable that the transmissible cinogen is not replicating rapidly in all the population and there must be a fraction of the population in which the cinogen is not rapidly replicating and that in such a state it can be affected by the R-factor. This might be the reason for the difficulty in demonstrating the elimination of K-cinogen. The failure to obtain doubly cured mutants (K^-X^-) can be very easily explained by the low incidence of loss of each cinogen in the bacterial population and also by the transfer of K-cinogen to the K^- mutants.

The authors are unable to provide a hypothesis explaining both the above results and the observation described in the previously paper that X-cinogen is lost in Sm-resistant (independent) mutants. Further studies will be made on this problem.

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