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Double Colicinogenicity of Escherichia Coli K_{235}

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SUMMARY

The double colicinogenicity of colicine K producing *E. coli* K_{235} L^-OC^- (K^-X^-) was demonstrated and two mutants producing colicine K and X respectively were isolated. Another colicine found was termed colicine X.

E. coli B was sensitive to both colicines and two mutants resistant to colicine K and X respectively were also obtained.

Colicine K antiserum could neutralize colicine K but not colicine X.

Very little colicine X was produced in a normal liquid medium but a small but definite amount of it could be produced in a special medium containing lactate and potassium phosphate buffer. It was non-dialyzable and resistant to heat treatment at 100°C for 10 min. but was labile when the culture was aerated or foamed.

About 40 per cent of the streptomycin resistant (independent) mutants isolated from *E. coli* K 235 $L^+OK^+X^+$ lacked X-colicinogenicity and the remaining 60 per cent were doubly colicinogenic. Two types of resistant mutants were also isolated by the replica method without contact with streptomycin.

INTRODUCTION

According to Fredericq (1958) it is a general rule that a noncolicinogenic mutant which has recovered from a colicinogenic *E. coli* loses its immunity to the colicine. However, this was not true of a noncolicinogenic mutant, *E. coli* K_{235} L^-OC^-, obtained from a colicine K producing strain, *E. coli* K_{235} L^-OC^+, by one of the present authors (Amano, Goebel and Miller Smidth, 1958). This strain was completely resistant to purified colicine K even when it was tested in soft agar by spotting a loopful of the saturated solution (10 mg per ml) onto the agar surface. Under the same conditions the growth of the best indicator of colicine K, *E. coli* B, was completely inhibited by spotting a loopful of it containing 1.0μg per ml onto the agar. On the other hand, the growth of the noncolicinogenic mutant was inhibited when it was tested against the colicine produced by a single colony of the parent strain on an agar plate and a somewhat turbid inhibition zone of 7 - 8 mm in radius was observed around the colony of the parent. This inhibition zone could not be explained as due to the action of colicine K alone, and so the present studies were made.

MATERIALS AND METHODS

1. *Bacterial strains:* *E. coli* K_{235} L^-OC^- and *E. coli* K_{235} L^-OC^+: These colicinogenic strains (kindly given by Dr. Walther F. Goebel of the Rockefeller Institute) were known as colicine K producers and shown to be doubly colicinogenic (K^+X^+) in this study. Their characteristics were described in detail by Goebel, Barry and Shedlowsky (1956) and by Amano, Goebel and Miller Smidth (1958). The antigenic structure of the former strain was reported to be K 1 : O 1 by Barry (1959).

E. coli K_{235} L^-OC^-: This noncolicinogenic mutant from *E. coli* K_{235} L^-OC^+ was originally isolated by one of the present authors in the Rockefeller Institute (Amano, Goebel and Miller Smidth, 1958).

E. coli $K8C^-$: This strain is identical with strain W1177 described by Lederberg *et al.* (1951), except that it is a colicine K-resistant (mutually T6-resistant) mutant of strain W1177 obtained by successively transferring the organism in nutrient broth containing increasing concentrations of colicine K.

E. coli $K8C^+$: K-colicinogenicity was transferred to *E. coli* $K8C^-$ according to the procedures of Fredericq (1954). The altered colicinogenic strain was termed *E. coli* $K8C^+$. The nature of this strain has been described by Amano, Goebel and Miller Smidth (1958).

E. coli B: This strain was used as a colicine K-indicator.

2. *Purified colicine K*

This was also kindly sent to us by Dr. Walther F. Goebel. The purification procedures were as described by Goebel and Barry (1958).

3. *Antisera*

Colicine K antiserum: Antiserum of purified colicine K was obtained by injecting rabbits with a sterile solution of colicine K as previously described by Goebel and Barry (1958).

Antiserum of *E. coli* K_{235} L^-OC^+: The immunization schedule for rabbits was as described by Amano, Goebel and Miller Smidth (1958).

4. *Assay method for colicine in solution*

The assay method used in this study was method B as described by Goebel, Barry and Schedlowsky (1956).

5. *Assay method for colicinogenicity*

The method used in this study was that of Fredericq (1958). A nutrient plate was seeded with the organism to be tested by sticking the plate at a point. After overnight incubation at 37°C, the surface of the plate was sterilized by chloroform vapour 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 colony of the organism tested.

6. *Colicine neutralization test*

Neutralization of colicine in solution: The procedures used in this report was the same as that described by Amano, Goebel and Miller Smidth (1958).

Neutralization of colicine produced by a colony on a nutrient plate: The procedures were identical with that of the assay method for colicinogenicity, except that to 5 ml of soft agar containing 1×10^8 cells of a colicine-indicator an appropriate amount of an antiserum was added.

7. *Lysozyme spheroplasting*

The procedure for formation of spheroplasts by lysozyme/EDTA was as described by Fraser *et*

al. (1957). The preparation of the cell wall digest and of the cytoplasmic lysate were as described by Miyama, Ichikawa and Amano (1959).

RESULTS

1. *Colicines of the colicine K producing E. coli* K_{235} L^-OC^+

When a colony of *E. coli* K_{235} L^-OC^+ on a nutrient plate was examined for colicinogenicity using the noncolicinogenic mutant, *E. coli* K_{235} L^-OC^-, as a colicine-indicator, a somewhat turbid inhibition zone could be detected as shown in Fig. 1B, although this mutant was completely resistant to the purified colicine K prepared by Goebel and Barry (1957). Phages were first suspected as the causative agents of the inhibition zone. However, no phages were found in the inhibition zone when *E. coli* K_{235} L^-OC^- was used as a phage-indicator. Thus, it seemed more likely that the parent strain could produce another colicine on the agar surface as well as colicine K and that the noncolicinogenic mutant, *E. coli* K_{235} L^-OC^-, was sensitive to the other colicine but not to colicine K.

Support was found for this in the following experiment. The colicinogenicity of *E. coli* K_{235} L^-OC^+ was transferred to *E. coli* $K8C^-$ according to the method of Fredericq (1954) and a colicinogenic *E. coli* $K8C^+$ was isolated. This transferred strain also was insensitive even to a saturated solution of purified colicine K, but it was sensitive to a colicine produced by colonies of *E. coli* K_{235} L^-OC^+ on an agar surface, as shown in Fig. 1C. However, *E. coli* K_{235} L^-OC^- was insensitive to the colicine produced by colonies of *E. coli* $K8C^+$. Thus it can be assumed that only K-colicinogenicity was transfererd to *E. coli* $K8C^+$ and that the parent strain, *E. coli* K_{235} L^-OC^+, could produce another colicine "X" in addition to colicine K.

2. *The isolation of an X-colicinogenic mutant from E. coli* K_{235} L^-OC^+ (K^+X^+)

To prove the above idea, attempts were made to isolate a mutant producing only colicine X (K^-X^+) from the doubly colicinogenic parent *E. coli* K_{235} L^-OC^+ (K^+X^+). The replica method was employed in the same way as for isolation of *E. coli* K_{235} L^-OC^-(K^-X^-) (Amano, Goebel and Miller Smidth, 1958), except that colicine K antiserum was not added to the original agar plates.

For the isolation of a K^-X^+ mutant, colonies of the parent on a nutrient plate were replicated upon two nutrient plates which had been pre-seeded with about 1×10^8 cells of *E. coli* B and *E. coli* K_{235} L^-OC^-(K^-X^-) respectively. It was expected, that on the plate, pre-seeded with the former microorganism, the inhibition zone would be formed by both colicines and that on the other plate, preseeded with the latter microorganism, the zone would be formed by colicine X only. However, no inhibition zone was detected around replicated colonies upon the latter plate. Thus it was assumed that the growth of *E. coli* B pre-seeded in the replica plates was inhibited by colicine K but not by X, although *E. coli*

B was thought to be sensitive to both colicines. Therefore *E. coli* B was used as an indicator of colicine K to isolate the mutant K^-X^+.

A culture of *E. coli* K_{235} $L^-OK^-X^-$ was grown overnight in nutrient broth. The bacteria were collected by centrifugation, washed once and suspended in saline. 2.5 ml of the suspension were placed in a sterile Petri dish and irradiated with ultraviolet light (19 watt Toshiba germicidal lamp) at a distance of 50 cm for 100 seconds. 0.1 ml aliquots of the irradiated suspension were spread on nutrient plates. After incubating the cultures overnight the colonies which appeared (100 to 200 per plate) were replicated upon nutrient plates which had been pre-seeded with *E. coli B* in 10 ml of melted 2.0 per cent nutrient agar. Both the original plate and the replica plates were incubated for 6 hours at 37°C. Those colonies which showed no inhibition zones on the replica plates were subcultured from the original plate upon slants. The colicinogenicity of these strains was tested using *E. coli* B as an indicator and looking for a mutant displaying a somewhat turbid inhibition zone. At the same time the sensitivity of these strains was tested against the colicines of the parent *E. coli* K_{235} $L^-OK^+X^+$.

It was found that nearly all the colonies which originally had no inhibition on the replica plates were still doubly colicinogenic and insensitive to the colicines of the parent strain. Finally a mutant capable of producing only colicine X was isolated. The mutant had been derived from the parent, because it was agglutinated to almost the same extent as the parent by the antiserum of the parent strain and by colicine K antiserum, as shown in Table 1.

Table 1. Agglutination of E. coli K_{235} $L^-OK^+X^+$ and the mutants, K^-X^+ and K^+X^-, in antibacterial serum and in colicine K antiserum

Antiserum	Antigens tested	Final dilution of antiserum							
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800
E. coli K_{235} $L^-OK^+X^+$ antibacterial	$L^-OK^+X^+$	3	3	3	3	2	1	1	1/2
	$L^-OK^-X^-$	3	3	3	3	2	2	1	1/2
	$L^-OK^+X^-$	3	3	3	2	2	1	1/2	0
Colicine K antiserum	$L^-OK^+X^+$	3	3	3	3	3	2	1	1/2
	$L^-OK^-X^-$	3	3	3	3	3	1	1/2	0
	$L^-OK^+X^-$	3	3	3	2	1	1/2	0	0

3: complete agglutination, clear supernatant
1/2: end point.
0: no agglutination.

The characters of the mutant capable of producing only colicine X are demonstrated in Fig. 1A, B, C. The inhibition zone of the colicine X produced by surface colonies of this mutant was compared with that of the parent strain and of *E. coli* $K8C^+(K^+X^-)$ using *E. coli* B, *E. coli* K_{235} $L^-OK^-X^-$ and *E. coli* $K8K^+X^-$ as indicator strains. The inhibition zone of this mutant against *E. coli* B was somewhat turbid and quite different in appearance from that of the parent strain or of *E. coli* $K8K^+X^-$. In addition, *E. coli* K_{235} $L^-OK^-X^-$ and *E. coli* $K8K^+X^-$ were sensitive to the colicine of this mutant (Fig. 1B and C) and conversely this mutant

was sensitive to the colicine K of the parent as well as to a purified colicine K preparation, as shown in Table 2. From these facts this mutant should be termed *E. coli* K_{235} $L^-OK^-X^+$.

Table 2. Susceptibility of various strains to colicine K and X

Indicator strains	Colicinogenic strains				Colicine K preparation
	E. coli K_{235} L^-O			E. coli K8	
	K^-X^+	K^-X^+	K^+X^-	K^+X^-	
E. coli B	+	+	+	+	+
E. coli K_{235} $L^-OK^-X^-$	+	+	—	—	—
E. coli K8 K^+X^-	+	+	—	—	—
E. coli K_{235} $L^-OK^-X^+$	+	—	+	+	+
E. coli K_{235} $L^-OK^+X^-$	+	+	—	—	—
E. coli B K^RX^S	+	+	—	—	—
E. coli B K^SX^R	+	—	+	+	+
E. coli B K^RX^R	—	—	—	—	—

+ : indicates the presence of inhibition zone.
— : no inhibition.

When a plate with the inhibition zone of this mutant against *E. coli* B was further incubated for two days, several resistant colonies of *E. coli* B appeared and a pure culture was obtained. This in turn was tested for sensitivity against mutant K^-X^+ and it was found to be completely resistant to colicine X. But like the parent *E. coli* B, it was sensitive to purified colicine K and also to the colicine K of *E. coli* $K8K^+X^-$ and of *E. coli* K_{235} K^+X^+. This strain was termed *E. coli* B K^SX^R. These facts also show that the colicinogenic mutant isolated must be *E. coli* K_{235} $L^-OK^-X^+$.

3. *The isolation from the same parent of a mutant producing only colicine K*

For the isolation from *E. coli* K_{235} $L^-OK^+X^+$ of mutant producing only colicine K, a mutant of *E. coli* B sensitive to colicine X and resistant to colicine K was required. Such a mutant was isolated from the inhibition zone of *E. coli* $K8K^+X^-$ against *E. coli* B. Finally, a single resistant colony was isolated and a pure culture was obtained. This was in turn tested for its resistance to purified colicine K and to colicine K produced by colonies of *E. coli* $K8K^+X^-$. It was completely resistant to colicine K. On the other hand it was sensitive to the colicine X of *E. coli* K_{235} K^-X^+, as shown in Fig. 2 and hence was called *E. coli* B K^RX^S.

As two mutants of *E. coli* B K^SX^R and K^RX^S were available, attempts were made to isolate a mutant, *E. coli* K_{235} $L^-OK^+X^-$, from the parent.

The experimental procedure was almost the same as that employed in the isolation of *E. coli* K_{235} $L^-OK^-X^+$ from the same parent. The colonies (100 - 200) grown on the original plates were replicated onto nutrient plates, which were then incubated at 37°C for 12 hours. The replicated colonies were treated with chloroform vapour and then carefully covered with 5 ml of melted soft agar con-

taining 1×10^8 cells of *E. coli* B K^RX^S. The plates were again incubated at 37°C for 12 hours to detect colonies without inhibition zones. Colonies on the original plates were also replicated on nutrient plates pre-seeded with 1×10^8 cells of *E. coli* B K^SX^R.

The colonies, which showed no inhibition zones against *E. coli* B K^RX^S and clear inhibition zones against *E. coli* B K^SX^R, were isolated and their pure cultures were examined for colicinogenicity. Of about 800 cultures, only one was a mutant producing only colicine K, as shown in Fig. 3. It was shown immunologically to be derived from the parent, because it could be agglutinated by colicine K antiserum and by antiserum of the parent, as shown in Table 1. The character of the exclusively K-colicinogenic mutant was shown by its loss of immunity to colicine K, as shown in Table 2. Therefore it was called *E. coli* K_{235} K^+X^-.

4. *Neutralization of colicine K and X by colicine K antiserum*

As no appreciable amount of colicine X could be produced in liquid media, neutralization experiments were performed on colicines produced on agar plates.

An agar plate was sticked at four points with four strains: the parent K^+X^+, the mutant K^+X^-, the mutant K^-X^+ and *E. coli* $K8K^+X^-$. After overnight incubation at 37°C the plate was sterilized with chloroform vapour and covered with 5 ml of melted soft agar containing 0.5 ml of colicine K antiserum together with 1×10^8 cells of *E. coli* B. The plate was incubated at 37°C for 6 hours and the diameters of the resulting inhibition zones were measured.

As can be seen in Table 3, the inhibition zones of *E. coli* K_{235} $L^-OK^+X^-$ and *E. coli* $K8K^+X^-$ were completely neutralized by the antiserum, whereas those of *E. coli* K_{235} $L^-OK^+X^+$ and *E. coli* K_{235} $L^-OK^-X^+$ were not. The inhibition zone of *E. coli* K_{235} $L^-OK^+X^+$ after addition of colicine K antiserum was somewhat turbid and identical in appearance to that which the mutant K^-X^+ formed against the same colicine-indicator, *E. coli* B, without the antiserum. It is obvious from these results that colicine K antiserum contains no neutralizing antibodies to colicine X and that the purified colicine K preparation is not contaminated by inactive but antigenically potent colicine X.

Table 3 Neutralization of colicine(s) on agar plates by colicine K antiserum

Colicine (s) from	Diameter⁺ of inhibition zone (mm)	
	with added antiserum*	without added antiserum
E. coli K_{235} $L^-OK^+X^-$	12	25
E. coli K_{235} $L^-OK^+X^-$	0	25
E. coli K_{235} $L^-OK^-X^-$	20	25
E. coli K_8 K^+X^-	0	22

* Colicine K antiserum employed in these experiments completely neutralized the activity of 100 μg of colicine K with 0.5 ml of 1 : 30 diluted antiserum.

⁺ The diameters of colonies were about 4-5 mm.

It was not yet known, however, whether the colicine X of the parent and that of the mutant K^-X^+ were identical, because the colicine X of the latter might be

a mutation product from colicine K evoked by ultraviolet irradiation. To investigate this, neutralization experiments were performed on the colicines of the parent, using *E. coli* B K^RX^S and K^SX^R as colicine-indicators. When *E. coli* B K^RX^S was tested against the colicines of the parent in the presence of the antiserum, the inhibition zone was the same as that in a control experiment without the antiserum. When *E. coli* B K^SX^R was tested in a similar manner by the addition of colicine K antiserum, the inhibition zone was completely neutralized. The result of the last experiment indicates that the colicine X of the parent strain and of the mutant K^-X^+ were identical, because *E. coli* B K^SX^R, which had been isolated from the inhibition zone of K^-X^+ against *E. coli* B, was completely resistant to the colicine X of the parent on addition of colicine K antiserum. Further evidence of the identity of the colicine X of the two strains was gained by isolating a mutant, *E. coli* B K^RX^R, from an inhibition zone of *E. coli* K_{235} $L^-OK^-X^+$ against *E. coli* B K^RX^S. The isolated doubly resistant mutant was resistant to the colicines produced by *E. coli* K_{235} $L^-OK^+X^+$. These results show that the colicine X of the two strains are identical and that the colicine X was not a mutation product from colicine K evoked by ultraviolet light irradiation.

5. *The production of colicine X in liquid media*

As colicine X was shown to differ from colicine K, it was assayed in the supernatant of the culture of *E. coli* K_{235} $L^-OK^+X^+$ in nutrient broth enriched with yeast extract using *E. coli* B K^RX^S as an indicator. No colicine X was detected. The bacterial cells from the culture were also tested for colicine X by protoplasting the cells with lysozyme/EDTA. However, colicine X could not be detected in the cell wall digest or in the cytoplasmic lyzate. Further attempts were made to demonstrate colicine X in a liquid medium. After many unsuccessful trials, a detectable amount was found by the following experiment.

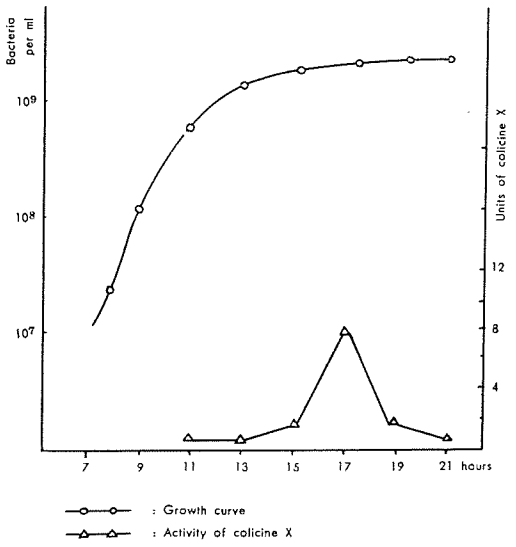
The composition of the medium was as follows: K_2HPO_4 (3.0 gr) KH_2PO_4 (1.5 gr), $MgSO_4$ (0.2 gr), NaCl (2.0 gr), lactic acid (10.0 ml), Takeda Polypeptone (10 gr) and water (1 L). After the solution had been autoclaved, the pH was adjusted to 7.2. 5.0 gr of yeast extract dissolved in 20 ml of water and sterilized by filter were added to the medium. The medium was seeded with *E. coli* K 235 K^-X^+ to give an initial population of 2×10^4 per ml and was gently stirred with a magnetic stirrer taking care that no foam was formed.

The growth curve and activity of colicine X in the centrifuged supernatants are presented in Fig. 4. Colicine X could be detected in the stationary phase. However, it could not be found in the bacterial cells. It was not inactivated by heating at 100°C for 10 min. at between pH 3.0 and 9.5 and was nondialyzable through a cellophane membrane.

6. *Streptomycin resistant mutants of E. coli* K_{235} $L^+OK^+X^+$

As a streptomycin resistant (independent) one step mutant of *E. coli* K_{235} L^+OC^+, which was also demonstrated to be K^+X^+, was occasionally required, two mutants were first isolated. The colicinogenicity of K and X was examined to

Fig. 4 The growth curve and the activity of colicine X



see that they were derived from the parent, and the X-colicinogenicity was found to have been lost. To reconfirm this, bacterial cells from each of 20 agar slants were suspended in 1.0 ml of saline and poured onto nutrient agar plates containing 200 μg of streptomycin per ml. The plates were incubated overnight at 37°C. Pure cultures of the resistant colonies were made and examined for K- and X-colicinogenicity. Assuming that when colonies grown on the same plate had the same character they had been derived from a single clone, 14 clones were found. Among these 8 clones were K- and X-colicinogenic and 6 were only K-producers and was called *E. coli* K_{235} $L^+OK^+X^-S_m{}^R$.

Apparently *E. coli* K_{235} $L^+OK^+X^-S_m{}^R$ must be regarded to be a double mutant. However, it cannot be a double mutant, because the chances of isolating the two kinds of streptomycin resistant mutants were almost equal and the two mutation rates were roughly between 10^{-10} and 10^{-11} like those of other normal *E. coli* strains. To find a more probable mechanism, two possibilities were considered; that is, whether X-colicinogenicity was eliminated from one kind of streptomycin resistant mutant by contact with streptomycin, or whether streptomycin sensitivity and X-colicinogenicity might have a metabolic process in common and this be lost in the apparent double mutant. To study this, attempts were made to isolate the two kinds of streptomycin resistant (independent) mutants without contact with streptomycin using the replica method described by Lederberg and Lederberg (1952). After several steps of concentrating those mutants, two kinds of streptomycin resistant (independent) mutants were finally isolated. One was

doubly colicinogenic and the other produced only colicine K. Thus it is obvious that the apparent double mutant had existed before contact with streptomycin. The detailed mechanism is still under study in this laboratory.

DISCUSSION

It was hard to understand that a noncolicinogenic mutant *E. coli* K_{235} L^-OC^- was insensitive to purified colicine K but sensitive to the colicine of a colony of the parent, *E. coli* K $_{235}L^-OC^+$, grown on a nutrient plate. This peculiar phenomenon could easily be explained by demonstrating the double colicinogenicity of the parent. According to Fredericq (1958) to state that a strain is doubly colicinogenic, the following two prerequisites should be satisfied: (1) both singly colicinogenic mutants should be isolated and each should be sensitive to the second colicine of the parent, and (2) the two mutants resistant to the respective colicines of the doubly colicinogenic strain should be isolated from an indicator strain sensitive to both colicines. These prerequisites were satisfied in this study as follows: (1) two singly colicinogenic mutants, *E. coli* K_{235} $L^-OK^-X^+$ and K^+X^-, were isolated and each strain was found to be sensitive to the colicine of the parent which they did not produce, and (2) *E. coli* B K^SX^R and K^RX^S were isolated from *E. coli* B which was an indicator for both colicines K and X. Thus, the double colicinogenicity of *E. coli* K_{235} $L^-OC^+(K^+X^+)$ was proved and the noncolicinogenic mutant was termed *E. coli* K_{235} $L^-OK^-X^-$. According to Fredericq, it should be sensitive to both colicines, but actually it was sensitive to colicine X and not to colicine K. Perhaps, insensitivity to colicine K would result from mutation by ultraviolet light irradiation from a colicine K- and X-sensitive noncolicinogenic double mutant. Therefore it seemed to be a triple mutant fortunately isolated from the parent by one of the present authors (Amano, Goebel and Miller Smidth, 1958).

It was also hard to understand that the inhibition zone of *E. coli* K_{235} L^-OC^+ (K^+X^+) against *E. coli* B could not be neutralized by colicine K antiserum and that the inhibition zone became turbid without decrease in radius, despite the fact that colicine K antiserum was very potent in the neutralization test with purified colicine K (Amano, Goebel and Miller Smidth, 1958). Such a peculiar phenomenon could also be explained by the double colicinogenicity of the strain and by the lack of colicine X neutralizing antibodies in colicine K antiserum.

The identity of colicine X of *E. coli* K_{235} K^+X^+ with that of the K^-X^+ mutant was shown by a neutralization experiment. There was a possibility that the colicine X of the K^-X^+ mutant might have been a mutation product from colicine K evoked by ultraviolet light irradiation and that the colicine of the parent other than colicine K might have been lost in the mutant. However, such a possibility was ruled out by a neutralization experiment on the colicines of the parent, using colicine K antiserum and *E. coli* B K^SX^R, which was isolated from one of the resistant colonies grown in the inhibition zone of the K^-X^+ mutant against *E. coli* B. *E. coli*

B K^SX^R was resistant to the colicine X of the parent K^+X^+, because no inhibition zone could be detected in this experiment on addition of colicine K antiserum and a clear inhibition zone was found when colicine K neutralizing antiserum was omitted. From these results it can be stated that the colicine X of the parent was identical with that of the mutant K^-X^+. This was confirmed since *E. coli* B K^RX^R could be isolated from the inhibition zone of the mutant K^-X^+ against *E. coli* B K^RX^S and was not inhibited by the colicines of the parent. Further attempts were made to demonstrate the X-colicinogenicity of the parent to a non-colicinogenic *E. coli* by transferring it. But the transfer of X-colicinogenicity has so far been unsuccessful, despite the fact that the K-colicinogenicity was easily transferable.

Though colicine X could be easily demonstrated around agar surface colonies, it could not be detected either in the supernatant of broth cultures or in cell wall digests or in cytoplasmic lyzates when the cells were converted to spheroplasts by lysozyme/EDTA. After several unsuccessful attempts, colicine X with a very faint activity was extracted from a nutrient plate, which had been seeded with the K^-X^+ mutant at about fifty points and treated with chloroform vapour and extracted with broth. Ultraviolet light induction was also examined without success. Colicine X of 8 - 16 units per ml could be produced in the liquid medium described above. Aeration and foaming the culture decreased the yield of colicine X. It is interesting that colicine X, like the colicine K-O antigen complex, was very stable to heat treatment at 100°C for 10 minutes. However, the mode of production of each colicine differed. Colicine X was found in highest concentration in culture supernatants from stationary phase cultures, whereas colicine K was produced at the end of log phase.

It was surprising that about 40 per cent of streptomycin resistant (independent) mutants isolated from *E. coli* K_{235} $L^+OK^+X^+$ on streptomycin agar plates lacked X-colicinogenicity. Others retained both colicinogenicity. The former were apparently double mutants, but such an explanation was not tenable, because the mutation rates of the former were almost the same as those of the latter and of normal *E. coli* strains. To study this, streptomycin resistant (independent) mutants were isolated by the replica method as described by Lederberg and Lederberg (1952) and two kinds of the mutants were obtained. The one was K^+X^- and the other was K^+X^+. In the former, X-colicinogenicity had already been lost before contact with streptomycin. Similar results were reported by Lederberg and Lederberg (1953). They isolated streptomycin resistant and λ-nonlysogenic mutants from two strains of *E. coli* K12 (λ) on a streptomycin containing medium. They were also able to obtain streptomycin resistant mutants by the replica method without contact with streptomycin and to demonstrate the λ-sensitivity of the mutant. They further stated that "the λ-sensitive and streptomycin resistant characters were not distinguishable from mutants previously isolated in single steps" and "no explanation for this remarkable association can be offered". The present authors can also offer

no explanation for their curious phenomenon.

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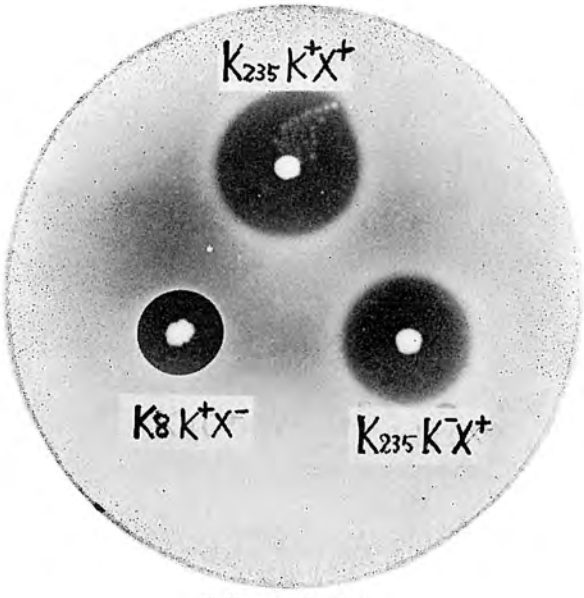
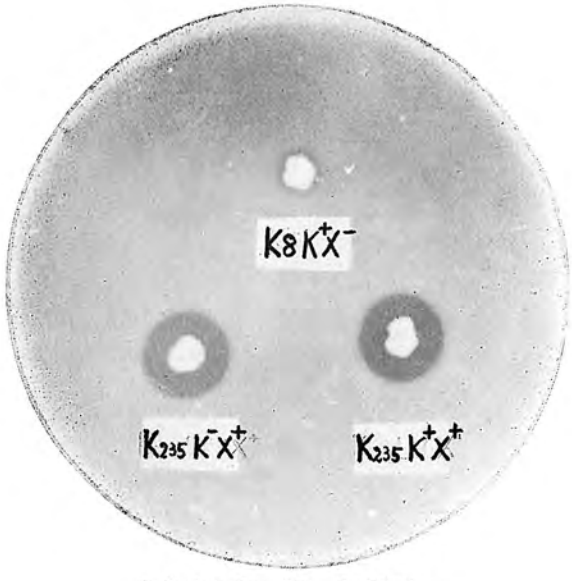
Fig. 1A



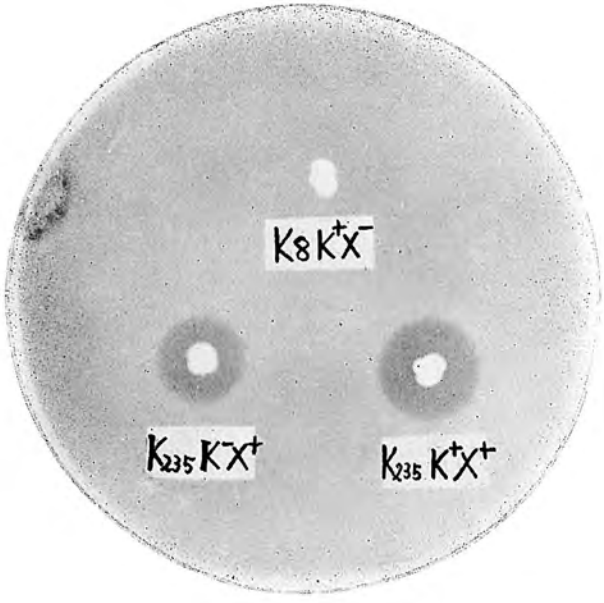
Indicator : E. coli B

Fig. 1B



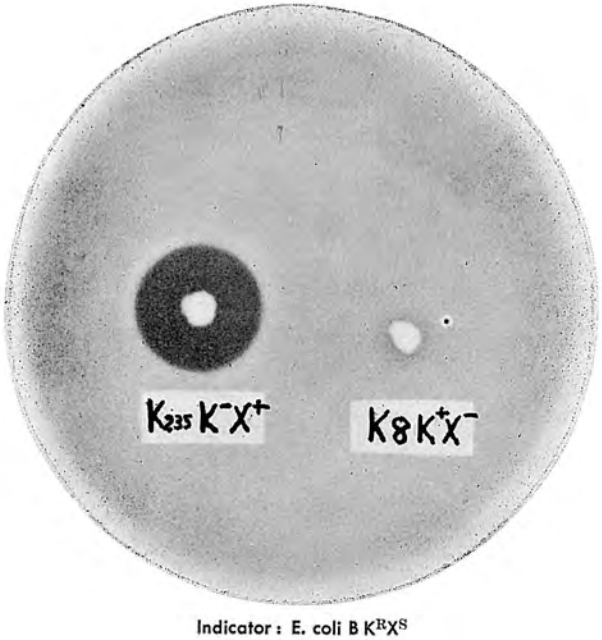
Indicator : E. coli K_{235} $L^-OK^-X^-$

Fig. 1C



Indicator : E. coli $K_8K^+X^-$

Fig. 2



Indicator : E. coli B K^RX^S

Fig. 3A

Indicator : E. coli B $K^{R}X^{S}$

Fig. 3B

Indicator : E. coli B $K^{S}X^{R}$