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Acridine treatment of F$^+$ and Hfr strains of *Escherichia coli* K12 carrying a neomycin-kanamycin resistance determinant

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SUMMARY

The F factor and the K resistance determinant form an R factor (KF) in *E. coli* K12. In this R factor the two plasmids are independent of each other and occupy different attachment sites in the host cell.

Treatment of an F$^+$(K) strain with acriflavine and acridine orange yielded the following classes: F$^+$K$^-$; F$^-$K$^+$; and F$^-$K$^-$. The K determinant was thus curable independently of F, which supports the hypothesis of independence of the two plasmids. In Hfr(K) strains, K remains curable with acridines while F is immune. Thus, K remains independent of F in both the F$^+$ and Hfr states.

The use of higher concentrations of acridine orange than those needed for cure of K in the F$^+$(K) strain resulted in no elimination of K, although the F factor was absent from all surviving lines tested. It is suggested that this is caused by equal inhibition of replication of both the chromosome and K, and greater inhibition of that of F.

1. INTRODUCTION

The transfer of a neomycin-kanamycin resistance determinant (K) by the F factor of *Escherichia coli* K12 (= K12) was described recently (Anderson, Mayhew & Grindley, 1969). The K determinant was originally identified in a strain of *Salmonella typhimurium* phage type 29 (Anderson, 1968; Anderson, Pitton & Mayhew, 1968). Its transfer was mediated by an *fi*$^+$ transfer factor with which it maintained a reversible association. It has been postulated that, in the class of R factors resulting from this type of association, the resistance determinant and the transfer factor occupy different attachment sites in the cell; that they are ordinarily in the dissociated state; and that the determinant is transferred only when it is associated with the transfer factor (Anderson & Lewis, 1965; Anderson, 1968; Anderson, 1969).

It was suggested by Anderson *et al.* (1969) that K and the F factor form an R factor of this class, and their association during transfer is independent of the association of F with the chromosome. This applied to both the extrachromosomal (F$^+$) and the integrated (Hfr) states of the F factor. The hypothesis of independence of K and F was supported by the results of conjugation experiments. The findings in acridine 'curing' experiments led to similar conclusions.

This article presents a detailed account of our observations on the acridine treatment of strains of K12 carrying both the K determinant and the F factor.

2. MATERIALS AND METHODS

Media

The medium routinely used for bacterial growth consisted of 20 g of Bacto Dehydrated Nutrient Broth (Difco Laboratories) and 8·5 g NaCl per litre of distilled water (= nutrient broth). For the preparation of solid medium (nutrient agar), New Zealand powdered agar was added in a concentration of 13 g/l.

Double strength nutrient broth, used in acridine curing experiments, contained 40 g of dehydrated nutrient broth per litre.

Kanamycin agar, which was used for the selection of kanamycin-resistant clones and for replica-plating experiments, contained 20 μg of kanamycin sulphate/ml of nutrient agar.

Bacterial strains

The strains used are listed in Table 1.

Strains 10R854 and 16R212 have been described previously (Anderson *et al.* 1969). Strain 18R149 is an Hfr strain isolated from 10R854 in this laboratory.

Table 1. *Strains used in acridine curing experiments*

ERL no.	Description
10R854	K12F$^+$(K)
16R212	K12HfrH(K)
18R149	K12HfrE(K)

Acridine treatment

Two acridines were used, acriflavine (AF) and acridine orange (AO). Solutions containing 80 μg/ml AF and 500 μg/ml AO respectively were freshly prepared in sterile distilled water for each experiment and checked for sterility before use.

For acridine treatment, 5 ml of double strength nutrient broth was diluted with distilled water and the amount of acridine solutions required to give the final concentration of the respective dye, the total volume being 10 ml. The dye concentrations chosen are indicated in Tables 3, 4 and 5. The pH values shown are those of the final broth–acridine mixtures.

The method used for treatment with acriflavine was similar to that of Watanabe & Fukasawa (1961). The test strains were cloned on kanamycin agar and single colony picks were grown in nutrient broth with agitation for about 7 h at 37 °C. One ml volumes of the acriflavine–broth mixtures at pH 7·6 were inoculated with about 10^3 organisms from these cultures. The tubes were incubated in the dark for 16 h without agitation and for a further 5 h with agitation. No attempt was made to exclude light during preparation of the bacterial suspensions in acridine broth or after the incubation period. Serial dilutions of the treated cultures were then plated on nutrient agar and, after overnight incubation, plates showing a suitable number of colonies were used as master plates for replication to kanamycin agar. This identified lines which had lost K.

The technique used for treatment with acridine orange was a modification of that

of Hirota (1960). In most respects it was similar to that used with acriflavine. The bacterial inoculum was about 10^4 organisms/ml.

A control culture in broth free from acridines was included in each experiment, in order to measure the spontaneous rate of loss of K. Loss of F was never observed in these controls.

Tests for loss of F

Colonies from master plates were grown in nutrient broth to an opacity of about 5×10^8 organisms/ml, and a standard loopful (*c.* 0·01 ml) was spread over an area of about 1 cm diameter on nutrient agar. The same volume of undiluted male-specific phage μ_2 (Dettori, Maccacaro & Piccinin, 1961), titre *c.* 10^{12} plaque-forming units (pfu) per ml, was then spotted on the centre of the inoculated area. Similar tests were carried out on each culture with the so-called female-specific phage ϕ_2 (Cuzin, 1965), which had an approximate titre of 10^9 pfu/ml.

Table 2. *Reactions of K12F⁺, K12Hfr and K12F⁻ strains with phages μ_2 and ϕ_2*

	Phages	
Strains	μ_2	ϕ_2
K12F⁺	OL	OL
K12Hfr	OL	OL
K12F⁺(K)	OL	—
K12Hfr(K)	OL	—
K12F⁻	—	CL
K12F⁻(K)	—	CL

Symbols: OL = opaque lysis, confluent background growth.
CL = confluent lysis, clear background.
— = no lysis.

F⁺ and Hfr strains of K12 tested in this way give 'opaque lysis' (OL), that is, lysis with confluent secondary growth, with phages μ_2 and ϕ_2. All F⁻ lines, whether or not they carry K, give no lysis with μ_2 and confluent lysis with ϕ_2. Lines carrying both K and F, whether the F factor is in the F⁺ or Hfr state, give OL with μ_2 and no lysis, or only slight inhibition of bacterial growth, with ϕ_2. When K is lost, both F⁺ and Hfr lines again give OL with both phages. Table 2 summarizes these reactions.

It was confirmed that K⁺ clones which have a negative reading with μ_2 were F⁻, by their inability to transfer K. Kanamycin-sensitive lines which were resistant to μ_2 were shown to have lost F because they were unable to mobilize the K determinant when used as donor strains in the triparental cross, devised to detect transfer factors in drug-sensitive lines (Anderson, 1965; Anderson & Lewis, 1965).

3. RESULTS

Experiments with acriflavine

The results obtained with acriflavine are shown in Table 3.

It is evident from this table that the K determinant could be cured with acriflavine in all three strains examined. As was expected, however, the F factor was curable in the F^+ but not the Hfr state. With $K12F^+(K)$, either the K determinant, or the F factor, or both, could be cured, that is, the following classes were obtained: F^+K^-; F^-K^+; and F^-K^-. For example, at an acriflavine concentration of 10 μg/ml (= AF10), 96 % of colonies had lost K, but only two out of 10 colonies tested had also lost F.

Table 3. *Treatment of $K12F^+(K)$ and $K12Hfr(K)$ with acriflavine*

Strain	AF concentration (μg/ml)	Final viable count (per ml)	Final bacterial count as percentage of control	Loss of K		Loss of F	
				No. of colonies tested	K loss (%)	In K^+ colonies	In K^- colonies
10R854	0*	$1{\cdot}4 \times 10^9$	100	518	0·2	0/7†	0/1
$K12F^+(K)$	9	$2{\cdot}0 \times 10^7$	1·4	399	95	2/10	9/10
	10	$3{\cdot}0 \times 10^7$	2·1	695	96	1/10	2/10
	12	$8{\cdot}5 \times 10^6$	0·6	343	3	2/10	3/9
	14	$6{\cdot}2 \times 10^6$	0·4	238	18	0/10	0/10
16R212	0	$2{\cdot}4 \times 10^9$	100	487	0·4	0/20	0/2
HfrH(K)	2	$1{\cdot}3 \times 10^8$	5·4	250	7·6	0/20	0/16
	4	$9{\cdot}1 \times 10^6$	0·38	265	0	0/8	—
18R149	0	$1{\cdot}3 \times 10^9$	100	476	0·2	0/10	0/5
HfrE(K) ex	6	$1{\cdot}6 \times 10^7$	1·2	311	26	0/5	0/5
10R854	8	$3{\cdot}4 \times 10^6$	0·3	231	10	0/5	0/5
	9	$3{\cdot}9 \times 10^6$	0·3	269	30	0/10	0/10
	10	$3{\cdot}3 \times 10^6$	0·3	324	5	0/5	0/15

* = control.

† = colonies which had lost F/number of colonies tested.

Earlier experiments had shown that HfrH(K) was more sensitive than $F^+(K)$ to acriflavine. The minimal inhibitory concentration of the dye for HfrH(K) was 6 μg/ml, while that for $F^+(K)$ was 16 μg/ml. It was therefore suspected that under our experimental conditions it might be difficult to reach dye concentrations sufficiently high to eliminate K without killing its host HfrH strain. In fact, in spite of many experiments, only one of which is summarized in Table 3, it proved impossible to obtain reproducible cure of K with acriflavine treatment of HfrH(K).

The K determinant had been introduced into HfrH by superinfection with the KF resistance factor (Anderson *et al.* 1969). As was expected, the superinfecting F factor was lost in this cross (Scaife & Gross, 1962), but the K determinant was retained (Anderson, 1966). The findings in the acridine treatment of $F^+(K)$ and Hfr(K) would clearly be more directly comparable if the strains were isogenic. An

Hfr(K) line was therefore isolated from 10R854 by the method of Berg & Curtiss (1967). This strain, 18R149, designated HfrE(K) (see Table 1), proved to be more sensitive to acriflavine than its F⁺(K) progenitor 10R854. In 18R149 the F factor is integrated between *his* and arg*G* (the precise point of insertion is under investigation), and chromosomal transfer is anticlockwise.

Acriflavine eliminated K from 18R149 with higher frequency and greater reproducibility than from 16R212, as Table 3 shows.

Experiments with acridine orange

The results obtained with acridine orange are summarized in Tables 4 and 5.

In this series the dye was used at concentrations from 25 to 75 μg/ml, and at a range of pH values between 7·2 and 8·0. At a given concentration acridine orange was more lethal at the higher pH levels. As Table 4 shows, its efficiency in eliminating K from F⁺(K) was also highest at pH 8·0. This table also establishes that the elimination of K is independent of that of F in F⁺(K). This is specially evident in this series, because of the high efficiency of AO in curing F.

Table 4. *Treatment of K12F⁺(K) with acridine orange*

pH	AO concentration (μg/ml)	Final viable count (per ml)	Final bacterial count as percentage of control	Loss of K		Total percentage loss of F	Loss of F	
				No. of colonies tested	K loss (%)		In K⁺ colonies	In K⁻ colonies
7·2	0	1×10^9	100	311	2·2	0	0/20	0/4
	50	$3{\cdot}1 \times 10^8$	31	398	6·0	54	11/20	8/20
	75	$2{\cdot}8 \times 10^8$	28	553	78	100	20/20	20/20
7·6	0	1×10^9	100	1238	1·0	0	0/40	0/12
	25	$6{\cdot}6 \times 10^8$	66	714	8·4	95	19/20	15/16
	50	$1{\cdot}2 \times 10^8$	12	1109	88	100	30/30	40/40
	75	1×10^5	0·01	355	1·1	100	20/20	4/4
8·0	0	1×10^9	100	720	0·5	0	0/20	0/3
	25	$2{\cdot}6 \times 10^8$	26	552	89	99	9/10	20/20
	50	7×10^5	0·07	140	0	100	10/10	—
	75	0	0	—	—	—	—	—

It was found that, when the AO concentration was such that the final bacterial count after incubation was less than 0·1 % of that of the control, there was often no elimination of K from the F⁺(K) strain. For example, in Table 4, although AO25 at pH 8·0 had eliminated K from 89 % of surviving cells, no K loss could be demonstrated with AO50 at the same pH. The bacterial count in the experiment concerned was 0·07 % of that of the control. At AO25 it was usually greater than 20 %. This phenomenon was observed on a number of occasions and it was evident that the frequency of K elimination under these conditions was usually below the rate of spontaneous loss of the plasmid. Indeed, in most experiments no K loss could be demonstrated at these higher AO concentrations.

The F factor in $F^+(K)$ did not show the same variability as K in relation to cure with AO. It was eliminated with high efficiency at all AO concentrations used, as Table 4 shows.

These observations establish that AO is more efficient at curing the F factor than the K determinant.

The loss of K from HfrE(K) treated with AO was usually lower than that from the $F^+(K)$ parent strain.

Table 5. *Treatment of K12Hfr(K) with acridine orange*

Strain	pH	AO concentration (μg/ml)	Final viable count (per ml)	Final bacterial count as percentage of control	Loss of K: No. of colonies tested	Loss of K: K loss (%)
18R149	7·6	0	$1{\cdot}0 \times 10^9$	100	307	1·3
HfrE(K) ex		25	$3{\cdot}3 \times 10^8$	33	550	59
10R854		50	$4{\cdot}0 \times 10^5$	0·04	189	96
		75	0	0	—	—
	8·0	0	$2{\cdot}4 \times 10^9$	100	635	1·4
		25	$5{\cdot}8 \times 10^8$	24	921	59
		50	0	0	—	—
		75	0	0	—	—
	7·6	0	$1{\cdot}6 \times 10^9$	100	416	0·7
		25	$2{\cdot}8 \times 10^8$	17·5	228	0·9
		50	$2{\cdot}0 \times 10^8$	12·5	308	22
		75	0	0	—	—
	8·0	0	$1{\cdot}1 \times 10^9$	100	410	0
		25	$9{\cdot}0 \times 10^6$	0·8	173	6·4
		50	$2{\cdot}0 \times 10^1$	(2×10^{-6})	4	0
		75	0	0	—	—
16R212	7·2	0	$8{\cdot}2 \times 10^8$	100	248	1·2
HfrH(K)		50	$1{\cdot}2 \times 10^8$	14·6	358	3·4
		75	$8{\cdot}8 \times 10^5$	0·1	181	5·0
	7·6	0	$3{\cdot}1 \times 10^8$	100	536	1·7
		50	$1{\cdot}2 \times 10^6$	0·4	206	7·2
		75	$7{\cdot}0 \times 10^3$	0·002	205	20
	8·0	0	$3{\cdot}6 \times 10^8$	100	447	2·2
		50	$2{\cdot}1 \times 10^4$	0·006	424	0·7
		75	$7{\cdot}0 \times 10^1$	$(1{\cdot}9 \times 10^{-5})$	15	60

There was no cure of F in Hfr strains in about 500 colonies tested.

HfrH(K) proved to be less satisfactory than HfrE(K) for experiments with AO. Nevertheless, cure of K was obtained, while F was unaffected, as can be seen from Table 5.

4. DISCUSSION

The KF resistance factor belongs to the class of R factors in which the resistance determinant and the transfer factor are independent of each other. In this class the

determinant and the transfer factor occupy different cellular attachment sites in the host cell. In a previous paper we showed that the K determinant was transferred to F⁻ strains at the same frequency by both F⁺ and Hfr strains of K12, and that, in HfrH(K), although K seemed to be associated with the proximal region of the chromosome in transfer, a specific locus could not be assigned to it (Anderson *et al.* 1969). It was therefore concluded that, during transfer from HfrH(K), the K determinant was associated with the anterior end of F, that is, the 'origin', but was not integrated into the chromosome. The association between K and F was postulated to be the same in both F⁺ and Hfr strains. It could thus be assumed that K occupied the same cellular attachment site in Hfr as in F⁺ strains, and that it was independent of F in the host cell. If this hypothesis is correct, the cure of the K determinant by acridines should be independent of the cure of F.

The experiments summarized in Tables 3, 4 and 5 not only confirm this prediction, but also indicate that acridine orange is more efficient in the elimination of the F factor than the K determinant. These findings strongly support the hypothesis that the two plasmids are independent of each other in the host cell.

The F factor is no longer curable with AO in Hfr(K) lines, but K remains curable, although it seems rather less susceptible to AO elimination than in F⁺(K) strains, and curing experiments are less reproducible with Hfr(K) than with F⁺(K) strains. It has already been suggested that this may be caused by attachment of K to the chromosomally integrated F factor during parts of its life cycle (Anderson *et al.* 1969). As the integrated F factor is immune to acridine elimination, it may extend its protection to K during this period.

The K determinant is spontaneously lost with relatively high frequency (*c.*1·0 %) from F⁺(K) and Hfr(K) strains (see control sections of Tables 3 and 4). When this occurred in F⁺(K) lines, it was never accompanied by loss of F. This gives further support to the postulate of independence of K and F in the host cell.

The absence of cure of K at higher concentrations of AO presents an interesting problem. It has been suggested that the elimination of plasmids by acridines is caused by selective inhibition of their replication while the chromosome can still replicate (Hirota, 1960; Jacob, Brenner & Cuzin, 1963; Stouthamer, de Haan & Bulten, 1963). However, if higher concentrations of AO retard replication of the chromosome itself to the same extent as that of K, the plasmid would be at no selective disadvantage in the surviving cells. This hypothesis is supported by the fact that in these experiments the rate of K loss at higher AO concentrations was often below its frequency of spontaneous loss. Indeed, K loss was usually so low under these conditions as to be undetectable, although the loss of F was such that all colonies tested were F⁻. If these concentrations of AO were more inhibitory for F than for K and the chromosome, F would be lost during cell growth while K would be retained.

If chromosomal replication in Hfr strains can be initiated at the F factor (Nagata, 1963; Vielmetter, Messer & Schütte, 1968), and if the acridines are selectively inhibitory for F even when it is in the integrated state, Hfr strains should appear to be more sensitive than F⁺ strains to acridines, as we observed.

Assuming that F is more sensitive than K to acridines, surviving cells in which inhibition of F-initiated chromosomal replication had occurred would not lose K, so that K would appear to be more difficult to cure reproducibly in Hfr than in F^+ strains, which is also in accordance with our findings. This hypothesis is an alternative to that of protection of K by its attachment to the integrated F factor, suggested above. The same effect could occur even if replication were not initiated at the integrated F factor, provided that F retained its higher sensitivity to acridines.

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