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SHORT NOTES

The elimination of extrachromosomal elements in thymineless strains of *Escherichia coli* K12

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INTRODUCTION

The concept that the *F* sex factor of *Escherichia coli* K12 exists in an extrachromosomal state in F^+ cells, and in a chromosomally-integrated state in Hfr cells is supported by the demonstration that it may be eliminated from F^+, but not from Hfr males. This elimination of *F*, first reported in the presence of cobalt and nickel salts, was found to be accomplished more effectively by treatment with acridine dyes. Under optimal conditions virtually 100% of F^+ cells were 'cured' of *F* to become F^- (see Hirota 1960), whereas no curing was found in Hfr strains.

Since that time extrachromosomal elements (or plasmids) other than *F* have been extensively investigated, particularly in *E. coli* and related strains of Enterobacteriaceae. These elements include the *colicin factors* (or *col* factors) that control the maintenance of protein-like antibiotics or *colicins* in colicinogenic (col^+) strains. It has been suggested that although some of these *col* factors are infective and may be transferred in the absence of chromosomal characters, they may have a chromosomal location in the majority of the cells of a culture of a stable colicinogenic strain: (see Smith, Ozeki & Stocker, 1963).

A specific chromosomal site has in fact been claimed for *colE*1, but this conclusion does not appear to be supported by more extensive genetic experiments (see Clowes, 1964). Other genetic crosses suggest that other *col* factors are transferred as extrachromosomal elements and a concept of colicin factors as self-regulating extrachromosomal genetic elements has been proposed (see Monk & Clowes, 1964*a*, *b*) to account for their genetic features. However, in spite of their apparent non-chromosomal nature, the elimination or 'curing' of any *col* factor does not appear to have been accomplished (Ozeki, 1960).

This paper reports a method whereby elimination of *col* factors and of other extrachromosomal elements may be achieved from thymineless mutants under conditions of thymine limited growth.

MATERIALS AND METHODS

Bacterial Strains. The derivation and characteristics of the strains used in this study are listed in Table 1. The methods of isolation of colicin resistant and colicinogenic derivates have been described elsewhere (Monk & Clowes, 1964*a*).

Media. In addition to basic media described by Monk & Clowes (1964*a*), specific media

included: *Low phosphate medium*, the 'TG' medium of Echols, Garen, Garen & Torriani (1961) and *M9 medium*, made up as Na_2HPO_4 (anhydr.) 6 g., KH_2PO_4 (anhydr.) 3 g., NaCl 0·5 g., NH_4Cl 1·0 g., $MgSO_4$ 0·001 M, $CaCl_2$ 0·0001 M, Glucose 4 g., water to 1 l.

Techniques. Colicinogeny was tested by the triple overlay technique (see Monk & Clowes, 1964*a*). *ColE1*$^+$ colonies were tested in the presence of the colicin I factor by using a strain resistant to colicin I (510) as an indicator. Similarly, when testing for *colI*$^+$ colonies in the presence of the *colE1* factor, an E1-resistant indicator (511) was used.

F status of strains was checked by testing for resistance (F$^-$) or sensitivity (F$^+$ or Hfr) to the male-specific phage μ by a modified screening method (Monk & Clowes, 1964*a*).

Acridine orange treatment followed the method of Hirota (1960). A log phase culture at *c.* 10^8/ml. was inoculated to produce a cell density of about 10^4/ml. in broth at pH 7·6 containing acridine orange at concentrations from 40 to 100 μg./ml. The culture was incubated with aeration overnight, during which the cell density increased to about 10^8/ml. Dilutions were then plated for single colonies which were individually scored for colicinogeny by the triple overlay technique.

Isolation of thymineless mutants was achieved by a simplified version of the Okada, Yanagisawa & Ryan (1961) technique. A log phase culture (*c.* 2×10^8/ml.) was diluted to about 500 cells/ml. into M9 medium (supplemented with appropriate growth factors) containing thymine and aminopterin (both at 200 μg./ml.), and incubated for 48 hours at 37°. Samples were then plated on nutrient agar supplemented with thymine (200 μg./ml.) and clones of thymineless mutants were recognized by their inability to be transferred by replica plating to nutrient agar. In most instances, 50 to 100% of the surviving clones are found to be thymine requiring, but in rather high concentrations, 40 μg./ml. being required for optimal growth.

Growth on limiting thymine. 0·1 ml. samples of an overnight culture (10^9/ml.) of a thymineless mutant in M9 medium supplemented with appropriate growth factors and containing 40 μg./ml. thymine, were subcultured into a series of 5 ml. volumes of M9 medium, supplemented with the same growth factors and various sub-optimal concentrations of thymine (0, 2, 10, and 25 μg./ml.), and incubated for 24 hours with aeration. Appropriately diluted samples were then plated for single colonies in soft agar containing 50 μg./ml. thymine on nutrient agar plates similarly supplemented with thymine. Colonies which appeared after overnight incubation were tested for colicinogeny by the triple overlay method, and a random sample of colonies picked from the overlay were tested for maleness (*F*) with μ phage, and in some instances for colicinogeny by a stab technique (see Monk & Clowes, 1964*a*).

RESULTS

(A) *Acridine orange treatment*

The first experiments involved a strain (557) carrying the plasmids *F*, *colI* and *colE1* which after standard acridine orange treatment at 50 μg. and 75 μg. was plated for single colonies and tested for each of the three factors. Among 80 colonies tested at either AO concentration, all were found to have lost the F sex factor, but to have retained both *col* factors (Table 2, lines 1 and 2).

A further series of experiments involved strains which were first made resistant to colicin, before acquiring colicinogeny. In this way it was hoped that 'cured' cells would remain insensitive to the action of the free colicin liberated in the medium by the non-cured majority. With a strain both colicinogenic for, and resistant to colicin E1 (521), no colonies cured of *colE1* were found among 1604 tested, although every one of ten colonies tested for *F* were found to have lost this factor (Table 2, line 3). A further strain, both colicinogenic and resistant to colicins I and E1 (551) was treated with AO

Table 1. *Derivation and characters of bacterial strains*

(a) *Strains derived from* Escherichia coli *K*12, 58–161, met⁻

Strain No.	*Characters*	*Origin*	*Strain No.*	*Characters*	*Origin*
501	F⁺ (See Monk & Clowes, 1964*a*)		516	F⁺*colE1-r*	501
			521	F⁺(*colE1*)⁺*colE1-r*	516
502	F⁻*str-r*		546	F⁻(*colI*)⁺*colI-r*(*colE1*)⁺*colE1-r str-r*	512
518	F⁻		551	F⁺(*colI*)⁺*colI-r*(*colE1*)⁺*colE1-r str-r*	546/501
510*	F⁻*colI-r str-r*	502	528	F⁻(*colI*)⁺(*colE1*)⁺	518
511†	F⁻*colE1-r str-r*	502	557	F⁺(*colI*)⁺(*colE1*)⁺	528/501
512	F⁻*colI-r colE1-r str-r*	510	566	F⁺(*colI*)⁺(*colE1*)⁺*thy*⁻	557

(b) *Strains derived from other* E. coli *K12 strains*

Strain No.	*Characters*	*Origin*
779	A′Hfr(*colE1*)⁺*thy*⁻	732 (Monk & Clowes, 1964*a*)
770	W1655 *met*⁻*lac*⁺ (*Flac*⁺)	(Scaife & Gross, 1962)
790	*met*⁻*pro*⁻*lac*⁻*thy*⁻(*Flac*⁺)	A K12 recombinant made *thy*⁻ and infected with *Flac* from 770
533	W3747 *met*⁻*lac*⁻ (*Flac*⁺*ade*⁺)	The original strain carrying F13 = *Flac*⁺*ade*⁺ (Hirota & Sneath, 1961)
564	W3747 (*F13*) *thy*⁻	533

* Original source of *colI* and colicin I is *Shigella sonnei* (P9) (Monk & Clowes, 1964*a*).
† Original source of *colE1* and colicin E1 is *E. coli K30* (Clowes, 1964).

Table 2. *Elimination of plasmids with acridine orange*

Strain No.		AO concentration (μg./ml.)	*F* Examined	*F* % 'curing'	*colI* Examined	*colI* % 'curing'	*colE1* Examined	*colE1* % 'curing'
1.	557	50	40	100	2756	0	—	—
2.	557	75	40	100	3598	0	4027	0
3.	521	50	10	100	—	—	1604	0
4.	551	40	80	100	2580	0	2782	0
5.	551	50	80	100	1985	0	2155	0
6.	557	100*	40	100	1683	0	1307	0
7.	770	40	40	100	—	—	—	—
8.	533	40	40	0	—	—	—	—

* Grown in low phosphate medium (McFall, Pardee & Stent, 1958).

concentrations of 40 and 50 μg. Again although all tested colonies had apparently been cured of *F*, none of many thousands tested had lost either *col* factor (Table 2, lines 4 and 5).

It is known that under normal conditions of growth, K12 strains are multinucleate. Moreover, recent experiments (Ozeki, unpublished) suggest that replication of colicin factors occurs within a few minutes after transfer, thereby rendering heavily labelled (^{32}P) colicin factors stable to inactivation by ^{32}P 'suicide' unless the recipient cells are very rapidly frozen after infection by the colicin factor. In an attempt to limit the number of *col* factors and the number of nuclei per cell, the strains were grown on a low

phosphate medium, which has been shown to result in uninucleate cells (McFall, Pardee & Stent, 1958) prior to treatment with acridine orange at 100 μg./ml. Even under these conditions (Table 2, line 6) which produce 100% *F* 'curing', no (<0·075%) curing of either *col* factor occurred.

(B) *Thymine limitation*

Thymineless derivatives of various plasmid-infected K12 strains were grown for 24 hours under conditions of sub-optimal thymine supplementation, and the surviving colonies were tested for retention of plasmids, with the results shown in Table 3. Strain 566, a thymineless derivative of strain 557 (carrying *F*, *colI* and *colE1*) was grown in

Table 3. *Elimination of plasmids under thymine deprivation*

Strain No.	Concentration of thymine (μg./ml.)	*F*		*colI*		*colE1*	
		Examined	% 'curing'	Examined	% 'curing'	Examined	% 'curing'
1. 566	10	70	100	1311	0	2533	50·9
2. 566	2	40	100	1331	4·4	1350	99·2
3. 566	2	40	100	1232	4·0	831	99·9
4. 779	10	40	0	—	—	1529	79
5. 564	10	28	100	—	—	—	—
6. 564	10	40	100	—	—	—	—
7. 566	25	80	50	—	—	—	—
8. 564	25	80	52·5	—	—	—	—
9. 790	25	80	49	—	—	—	—

medium containing 10 μg./ml. thymine. There was 100% loss of *F*, and about 50% loss of *colE1*, but no loss of *colI*.

The effect of more severe deprivation of thymine (2 μg./ml.), and of its complete absence from the medium were tested. From an inoculum of 10^7 thymineless cells/ml., no survivors (<10 cells/ml.) were isolated after 24 hours' incubation in the absence of thymine. However at 2 μg./ml. thymine, the final cell concentration after 24 hours was *c.* 10^8 cells/ml. Among these cells, loss of all three plasmids was observed (Table 3, lines 2 and 3). All the examined clones were F^-, over 99% had lost *colE1*, and 4·0% were now 'cured' of *colI*.

The elimination of each colicin factor appeared to occur independently. Thus at 2 μg./ml. of thymine, less than 1% of the survivors retained *colE1*, nevertheless among those cured of *colI*, one out of fifty cells retained *colE1*. All the clones which had lost all three factors *F*, *colI* and *colE1* were found to remain *thy*$^-$, *met*$^-$ and *lac*$^+$.

The effects of thymine deprivation on an integrated *F* factor and a colicin factor were examined by subjecting an Hfr *colE1*$^+$ strain to this treatment. In such a strain (779), the expected elimination of *colE1* occurred but no elimination of *F* was found in 40 colonies examined.

(C) *Elimination of other plasmids by growth in limiting thymine*

The *F* factor of K12 has been shown to be capable of incorporating segments of chromosomal material, giving rise to '*F prime*' heterogenotic strains (see Driskell-Zamenhof, 1964). Some of these F prime strains, for example 770, a strain carrying an F prime factor incorporating a *lac*$^+$ marker (*Flac*$^+$) is completely cured of both *F* and

lac$^+$ after standard acridine orange treatment (Table 2, line 7). In contrast, another strain (533) with an F prime (*F13*) carrying a number of chromosomal genes (Hirota & Sneath, 1961) was resistant to curing by this method (Table 2, line 8). However, when a thymineless derivative of 533 (564) was grown in limiting (10 μg./ml.) thymine, all the isolated clones were found to have lost this plasmid as judged from inability to plate μ (Table 3, lines 5 and 6). Four of these presumed 'cured' strains were examined for their ability to transfer *lac*$^+$. All were now found to be unable to transfer this factor ($<1\%$) to an F$^-$*lac*$^-$ strain after 2 hours contact compared to a 100% transfer achieved by the parental 564.

When the three strains carrying *F*, *Flac* and *F13* respectively were grown under less severe thymine deprivation (25 μg./ml.) which did not cure every cell of *F* factors, these strains all showed the same sensitivity to thymine 'curing', the factor being lost from about 50% of the cells in each case (Table 3, lines 7, 8 and 9).

DISCUSSION

In confirmation of the results of Ozeki (1960), it was found that colicin factors, in this instance *colI* and *colE1*, were refractory to elimination by acridine orange treatment. No elimination of either *col* factor was found from strains under conditions which led to a complete elimination of the *F* sex factor.

In contrast, when thymineless strains were grown in limiting concentrations of thymine, both colicin factors were eliminated. The colicin E1 factor was lost in up to 99·9% of the population, whereas *colI* was lost in a smaller proportion, less than 5% of the surviving clones.

Since thymine deprivation is known to induce lethal phage synthesis in λ-lysogenic strains and lethal colicin synthesis in colicinogenic strains (Sicard & Devoret, 1962; Luzatti & Chevalier, 1964), it might be suggested that 'curing' of the colicin factors occurs as a result of the induction of a genetic element that was initially chromosomally integrated. The kinetics of exponential death of colicinogenic and non-colicinogenic strains under conditions producing thymineless death and colicin induction have been studied. Luzzati & Chevalier (1964), using resting cells, found no difference in these kinetics, whereas Sicard (1964) found that there is a more rapid loss of viability in cells of a colicinogenic strain under these conditions. Our experiments were also performed with resting cultures so it is possible that colicin induction is occurring only in these cells undergoing thymineless death, and the surviving clones have not arisen from cells that have undergone a non-lethal induction. In any event, no elimination of λ was found from strains in which successful *col* factor elimination had occurred, nor was there any elimination of chromosomally-integrated *F* under these conditions of *col* factor elimination.

It may be concluded, therefore, that factors integrated in the chromosome are not eliminated in cells surviving thymine deprivation, so that the elimination of *col* factors under these conditions may be adduced as evidence of their non-chromosomal nature. This conclusion is in agreement with observations on the genetic transfer of *colI* and *colE1*, and supports the suggestions that all colicin factors are extrachromosomal under all conditions so far investigated (see Clowes, 1964).

There is, however, a striking difference in the susceptibility to elimination shown by various extrachromosomal elements. Growth in the presence of acridine orange eliminates wild-type *F* and certain F prime factors with great efficiency, but is without effect on colicin factors, and on a particular F prime, (*F13* of Hirota & Sneath, 1961). In 10 μg./ml. thymine, there is complete elimination of *F13*, *c.* 50% elimination of *colE1*, and no elimination of *colI*. When thymine deprivation is more severe (2μg./ml.), *colE1* elimination is increased to 99% and a small proportion of cells now lose *colI*, but the

two *col* factors appear to be eliminated independently, since about 2% of the cells which lose *colI*, retain *colE1*.

Curiously, although *F13* is apparently refractory to curing by acridine orange in contrast to other *F* factors, they are all equally susceptible to elimination by thymine deprivation. At 25 μg./ml., approximately 50% elimination of *F* factors occurred from all strains, including that carrying *F13*.

Scaife & Pekhov (1964) suggest that in W3747, the strain in which the *F13* factor arose, the chromosomal genes carried by the F prime are deleted from the bacterial chromosome. This conclusion is difficult to reconcile with the ability to produce F^- clones (by the criteria of insensitivity to male specific phage and inability to transfer lac^+) by thymine deprivation of a thymineless mutant of W3747.

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