The Regulation of Colicin Synthesis and Colicin Factor Transfer in *Escherichia coli* K12

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SUMMARY

Cells of *Escherichia coli*, newly infected with the colicin I factor (*colI*), showed an enhanced efficiency of transfer of this factor (HFC), and were also more likely to undergo lethal colicin synthesis, than were stably colicinogenic cells. Up to 20% of the cells of stably *colI*⁺ strains were induced to produce colicin by ultraviolet irradiation, and from such irradiated cultures transfer of the *colI* factor occurred more efficiently. To account for these results, it is proposed that the *colI* factor exists as an autonomous non-chromosomal genetic element which sets up its own system of self-regulation within cells of stably colicinogenic strains.

INTRODUCTION

Colicin production in stably *colI*⁺ strains of *Salmonella typhimurium* was demonstrated by Ozeki, Stocker & de Margerie (1959) who observed the release of colicin from single bacteria as minute clearings termed 'lacunae' in a lawn of colicin-sensitive cells. An enhancement in the number of these 'lacunae' following ultraviolet (u.v.) irradiation of colicinogenic strains was shown by Ozeki (1960) and ascribed to the induction of lethal colicin synthesis in an increased number of cells of a *col*⁺ population. In *Salmonella typhimurium*, cells newly infected with the *colI* factor are highly efficient in the further transfer of this factor (Stocker, Smith & Ozeki, 1963). It was suggested (Smith & Stocker, 1962; Stocker *et al.* 1963) that in stably colicinogenic cells, the *colI* factor exists chromosomally, but in a small minority of cells it may take up a cytoplasmic state, in which event it becomes capable of infectious transfer at low frequency (low-frequency colicinogeny transfer, LFC). Newly transferred *col* factors are suggested to persist for some time in this cytoplasmic state, during which time the efficiency of further *colI* transfer remains high, giving rise to a system of high-frequency colicinogeny transfer (HFC) before the proposed stable chromosomal condition is re-established. We have previously shown the existence of high-frequency colicinogeny transfer (HFC) in *Escherichia coli* K12 strains newly infected with *colI* (Monk & Clowes, 1964). In the present paper we report the incidence of colicin-producing cells ('lacunae') in both LFC and HFC cultures of *E. coli* K12, together with related studies on the induction of lacunae and *colI* transfer following u.v.-irradiation of stably *colI*⁺ *E. coli* K12 cultures. An alternative hypothesis to account for HFC and colicin induction based on a system of self-regulation of non-chromosomal *colI* factors is proposed.

METHODS

Media and bacterial strains. Those used in this study are described in Monk & Clowes (1964). To avoid re-adsorption of colicin, all the *colI*⁺ strains used were also *colI*^{-r}. In addition, derivatives of a non-lysogenic (λ^-) strain, w1655 (Lederberg & Lederberg, 1953) were used in u.v.-irradiation experiments to prevent lysis and death arising from induction of the λ prophage. Some w1655 derivatives were isolated from a strain cured of *F* by the method of Hirota (1960) by using acridine orange. One of these strains (59), an *F*⁻ derivative of w1655, made resistant to colicin I (*colI*^{-r}) and colicinogenic (*colI*⁺), was used in these studies.

Assay of colicin production by single cells (lacunae). To 2.5 ml. soft agar, seeded with 0.1 ml. of an overnight *col-s str-r* indicator strain, was added an inoculum of 10⁷ cells of a *colI*⁺ *str-s* strain, and the agar layered over a streptomycin nutrient agar plate. After incubation for 5–6 hr, small clearings (lacunae) in the confluent indicator lawn were visible. Alternatively using a *colI*⁺ strain which was *str-r*, several drops of chloroform were added to the broth culture, which was then incubated for 10 min. before adding diluted samples to the soft agar indicator mixture.

Ultraviolet (u.v.)-irradiation was achieved using a Hanovia 15 W. 'germicidal' lamp emitting 95 % of its radiation at 2537 Å. Cells were irradiated at 50 cm. from the lamp, the intensity at this distance being 5 ergs/mm.²/sec. Logarithmic-phase *colI*⁺ bacteria, grown in nutrient broth, were centrifuged at low speed (3000 rev./min.), and the supernatant fluid replaced with phosphate buffer (0.5 M; pH 7.5) to produce a cell concentration of 1 to 2 × 10⁸/ml. Five ml. samples were u.v. irradiated in 9 cm. Petri dishes for the desired time with gentle agitation. The irradiated cell suspension was then rapidly transferred to an equal volume of pre-warmed, double-strength nutrient broth and incubated with aeration in the dark.

Other colicin techniques were as described previously.

RESULTS

Lacunae production in K 12 colI⁺ strains

The small clearings (lacunae) produced by *colI*⁺ *str-s* Salmonella cells in a confluent lawn of *colI*⁻ *str-r* indicator bacteria on a streptomycin overlay plate were ascribed by Ozeki *et al.* (1959) to the release of colicin by single bacteria. Lacunae produced by *Escherichia coli* K 12 *colI*⁺ strains form just visible clearings in the indicator lawn and are difficult to score with precision, there being considerable variation in their size and clarity. Triplicate or quadruplicate platings were independently scored on at least two occasions, and differences were only regarded as significant when they exceeded tenfold. The frequency of lacunae observed in stably *colI*⁺ cultures is generally about 1/10⁵ cells (a variation from about 3 in 10⁵ to 3 in 10⁶ cells). These clearings were confirmed as lacunae since they were not produced with cell-free filtrates, and they occurred with a frequency directly proportional to the numbers of cells plated. Moreover, they were not transferred by subculture, and were thus non-infective, and they were not detected when a *colI*^{-r} strain was used as an indicator, nor when the *col-s* indicator was plated in the absence of a *colI*⁺ inoculum.

Induction of colicin I synthesis by ultraviolet-irradiation

Although induction of colicin E1, E2 and B by u.v.-irradiation has been reported in *Salmonella typhimurium* by Ozeki (1960), induction of colicin I was not achieved. A *colI*⁺ *colI*-*r* *str*-*s* strain of *Escherichia coli* κ 12, non-lysogenic for phage λ , was irradiated for various times and assayed for lacunae production after 100 min. of post-irradiation incubation. As the dose of u.v.-radiation was increased, the numbers of lacunae were increased, reaching a plateau after irradiation for 60 sec. when about 20 % of the cells formed lacunae. The time of 60 sec. was used in further experiments in which the number of lacunae arising after various times of post-irradiation incubation were assayed; the results are shown in Fig. 1. At this dose,

Fig. 1. Viable count and lacunae of a *colI*⁺ culture after u.v.-irradiation. A log-phase culture of *colI*⁺ *colI*-*r* *str*-*s* λ ⁻ bacteria (strain 59) was centrifuged and resuspended at 10⁸ cells/ml. in buffer. A 5 ml. sample was removed to 5 ml. double-strength nutrient broth to serve as a control unirradiated culture. A further 5 ml. was then u.v.-irradiated for 60 sec. at 50 cm. from the u.v. source, and then similarly diluted into 5 ml. double-strength nutrient broth. Both samples were then incubated at 37° with aeration and at various times further samples were removed and assayed on nutrient agar for viable count, and streptomycin nutrient agar with a *col*-*s* *str*-*r* indicator for the numbers of lacunae. $\blacktriangle$ --- $\blacktriangle$, Viable count of non-irradiated culture: $\triangle$ — $\triangle$, viable count of u.v.-irradiated culture: $\bullet$ --- $\bullet$, lacunae in non-irradiated culture: $\circ$ — $\circ$, lacunae in u.v.-irradiated culture.

the irradiated cells did not show any marked decrease in viable count, although cell-division was considerably delayed. The number of lacunae remained constant at the uninduced value until about 60 min. after irradiation, when a sharp increase occurred, reaching a maximum at 120 min. of about 10⁷ lacunae/ml., and corresponding to an induction of about 20 % of the total number of irradiated cells. At this time the amount of free colicin in the cell-free filtrate showed an increase of about 30-fold.

Enhanced transfer of colI from irradiated colI⁺ cultures

A culture of a stably *colI⁺* strain was u.v.-irradiated for various periods under the conditions optimal for induction, and samples of the irradiated culture mixed with equal numbers of log.-phase *colI-r str-r* recipient cells in double-strength broth, and re-incubated for 2 hr. The mixed culture was then diluted and the recipient cells assayed for acquisition of *colI*; the results are shown in Table 1. After irradiation for 10, 20 or 30 sec., a small but reproducible increase in *colI* transfer resulted.

Table 1. *Relative increase in colI transfer after u.v.-irradiation*

Log.-phase cultures of strain 59 were centrifuged and resuspended in buffer at 10^8 cells/ml. Samples (5 ml.) were u.v.-irradiated for the times shown, then 1 ml. samples mixed with 1 ml. log.-phase cells of a culture of 58-161 λ^+ F⁻ *colI-r str-r* (550) in double-strength nutrient broth at 10^8 cells/ml. Mixtures were incubated for 2 hr and *str-r* cells selected and tested for *colI⁺*. Three hundred to 1000 clones were scored at each time and expressed relative to the numbers of *colI⁺* found after contact with unirradiated colonies as 100. The absolute value ranged from 5 to 20 % transfer.

Exp.	Time of u.v.-irradiation (sec.)					
	0	10	20	30	40	60
	Relative no. of <i>colI</i> transfer					
1	100	180	230	220	200	170
2	100	—	180	—	160	110
3	100	310	360	—	110	50
4	100	180	220	210	150	110
5	100	140	150	130	100	80
6	100	200	200	170	120	100
7	100	150	150	180	150	100

This increase was from 1.3- to 3.6-fold, calculated on the numbers of *colI⁺* input cells. However, if the increase in infectivity is assumed to occur at the same time as the onset of induction, namely about 90 min., the non-irradiated control cells have multiplied by a factor of about 20. The relative transfer per viable cell might therefore be enhanced by as much as 30- to 70-fold as a result of u.v.-irradiation.

Enhanced lethal colicin synthesis in HFC cultures

When incubated overnight preparations of HFC were diluted 1/20 in broth to prepare standard HFC 'donor mixtures', it was found 15 min. later that the numbers of 'intermediate' cells which were recovered as viable colonies had decreased to about 50 %. Similarly, when a *colI-r* recipient strain was mixed with such a HFC donor mixture, only 50-70 % of the recipient cells survived as colony-formers when plated 15 min. after mixing. Since both populations which showed this decrease were non-adsorbing for colicin I, the observed lethal effects appear to be due to a transfer of the *colI* factor, rather than to any direct colicin action. The number of lacunae-forming cells and free colicin were therefore determined in a HFC donor mixture and compared with similar assays in a LFC donor culture; the results are shown in Table 2. It can be seen that under HFC conditions the numbers of lacunae increased more than 100-fold, with an accompanying 16-fold increase in the cell-free colicin.

Table 2. *Lethal colicin synthesis in HFC and LFC cultures*

HFC preparation: 5×10^4 cells of donor *colI*⁺ *str-s* (519), and 10^8 cells of intermediate *colI-r str-s* (547) were mixedly inoculated into 5 ml. nutrient broth, incubated overnight, diluted 1 in 20 and then incubated for 90 min. with aeration. For the control, the donor strain was mixed with the strain *colI*⁺ *colI-r str-s* (559) and cultured in the same way. Samples were removed and (i) diluted and plated with *colI-s str-r* indicator in 2.5 ml. soft agar on streptomycin nutrient agar for numbers of lacunae; (ii) centrifuged, and doubling dilutions of supernatant fluid spotted on a streptomycin nutrient agar indicator plate for colicin titre; (iii) mixed with log.-phase recipient *colI-r str-r* cells (550) at a ratio of 1:1 and incubated for 1 hr. The proportion of *colI*⁺ recipient cells was determined by triple overlay on streptomycin nutrient agar.

System	Viable cells/ml.	Lacunae/ml.	Limiting colicin titre	Transfer <i>colI</i> to recipient (%)
Newly infected <i>colI</i> ⁺ (HFC)	10^8	5×10^5	1/16	98
Stably <i>colI</i> ⁺ (LFC)	2×10^8	3×10^3	1	4.2

DISCUSSION

In *Escherichia coli*, cells newly infected with *colI* become highly efficient donors of this factor and transmission to a non-colicinogenic recipient is increased from about 1 % (LFC) to about 90 % (HFC) within the first hour (Monk & Clowes, 1964). The duration of this enhanced infectivity is about four to six generations, similar to the HFC condition first described in *Salmonella typhimurium* by Stocker *et al.* (1963). It has also been shown that an increase in the infectivity of a *colI*⁺ culture is accompanied by an increase in the numbers of *colI*⁺ cells which are able to form unions when mixed with non-colicinogenic recipient organisms. These unions may be observed by mixing *colI*⁺ cells, which have been stained by the uptake of a tetrazolium dye, with unstained *col*⁻ cells, whereupon pairing of *col*⁺ and *col*⁻ cells, or more frequently clumps including a number of both types of cells, may be seen. Such pairing or clumping is not seen in cultures of either strain independently. Stably *colI*⁺ cultures of *E. coli*, which can transfer *colI* to about 7–10 % of recipient cells in 2 hr, are able to form a small proportion of cell clumps (about 5 %), whereas in HFC *E. coli* mixtures, a much higher proportion of the cells (50–100 %) are seen in clumps (Monk & Clowes, 1964). *S. typhimurium* HFC cultures (transmitting 50 % *colI* in 1 hr) also form clumps, but no pairing or association of cells is seen under LFC conditions, when the transfer in the first 2 hr is less than 0.01 % (Ozeki, Stocker & Smith, 1962). Increased infectivity would thus seem to be due, in part at least, to an increase in the numbers of *colI*⁺ cells which can form stable contacts with *colI*⁻ cells. These contacts are likely to result from an increase in the numbers of cells which form a specific *col*-directed conjugating cell surface (possibly a *col*-conjugating antigen similar to that produced by the presence of the fertility factor *F*) or an increase in the amounts of such conjugating antigen produced by most cells. Increased *colI* infectivity might also be due to an increase in the numbers of *colI* factors per cell, increasing the probability of *colI* transfer per contact. Recent experiments by Dr H. Ozeki (personal communication) on the 'suicide' of ³²P-labelled *col* factors after transfer indicate that there is a rapid multiplication of these factors within the newly infected cells.

In a previous publication (Monk & Clowes, 1964) we concluded that *colI*, like other colicin factors such as *colE1* (see Clowes, 1964), appears to be transferred

extra-chromosomally, and no evidence was found to suggest a chromosomal location. The multiplication and activity of a stable cytoplasmic element or plasmid (Lederberg, 1952), which is also a potentially lethal agent, yet whose lethality is seldom expressed, must be regulated in some way. Such regulation in the case of non-chromosomal elements is likely to be self-imposed and may, by analogy with other regulated and potentially lethal systems such as temperate phage, be due to the presence of a cytoplasmic repressor. This picture of the *colI* factor as a self-regulating plasmid, also independently suggested by Clark & Adelberg (1962), appears to be more compatible with the data shown in the present paper than the alternative model proposed by Smith & Stocker (1962).

In stable *col*⁺ strains, the activities of the *colI* factor (which includes lethal synthesis of colicin, and the ability to form a conjugating cell surface) would be repressed in most cells. In a small proportion of *colI*⁺ cells, this repression breaks down and lethal synthesis results. In other cells, de-repression might lead to an enhancement of non-lethal activities, such as the synthesis of the *col*-directed surface 'antigen' (and possibly of the numbers of *col* factors existing in the cell), which would allow these cells to conjugate and transfer *col* factors, or in other words to become 'competent donors' of *colI*, either without lethal colicin synthesis, or before lethality prevented transfer. This repression would appear to be less stringent in *Escherichia coli* than in *Salmonella typhimurium*, resulting in about a hundred-fold greater number both of competent donors and of cells which proceed to lethal synthesis. (In *E. coli*, 'competent donors' are about 1 % and lethal synthesis is observed in about 1 in 10⁵ cells, whereas in *S. typhimurium*, competent donors are about 0.02 % and lethal synthesis is found in less than 1 in 10⁷ cells; Ozeki *et al.* 1962).

Transfer of the *colI* factor in the absence of its repressor would lead to a period of de-repression in newly infected cells. Such cells would themselves then have an increased probability of *colI* transfer, which could account for the epidemic spread of *colI*, giving rise to HFC donor mixtures under the appropriate conditions. De-repression in such newly infected cells would also be likely to have lethal consequences. This lethality is demonstrated when overnight HFC mixtures are subcultured, when extensive *colI* transfer to previously non-colicinogenic (intermediate) cells is occurring. This loss of viable cells is paralleled by the high incidence of lacunae-producing cells in such cultures (Table 2). When HFC donor mixtures are mixed with recipients to which *colI* is transferred to 80 % of the survivors after 30 min., this is also accompanied by lethal effects in the recipients, and 50–70 % do not survive 15 min. after mixing.

Such lethal effects following infection with *colI* are parallel to those subsequent to infection with temperate phage. Here infection may result in either lethal synthesis of phage, with consequent death of the cell (productive infection) or the establishment of a stably-lysogenic cell (reductive infection, Lwoff, 1953). Although in some instances, particularly the well documented phage λ system (Jacob, 1960), such lysogeny can be shown to be due to the integration of the phage genome as a prophage at a particular site on the bacterial genome, in other systems the phage genome may persist for some time in the cytoplasm without lethal effects (Luria, Fraser, Adams & Burrous, 1958). Moreover, even in the case of those phages in which prophage integration occurs, mutants may be isolated, such as phage λ b2,

which can no longer integrate, but retain the ability to set up an immune 'abortive' lysogenic state (Zichichi & Kellenberger, 1963). With other phages (particularly those capable of 'generalized' transduction, such as P1 or 363), it has not so far been possible to establish a chromosomal location (Jacob & Wollman, 1958), and they too may exist as plasmids, capable of establishing an immune, stably-lysogenic state without integration.

The destruction of a cytoplasmic repressor has been suggested to account for induction of prophage to lethal synthesis following u.v.-irradiation of a lysogenic strain (Jacob & Monod, 1961). In *colI*⁺ cultures of *Escherichia coli*, u.v.-irradiation also results in greater than a thousand-fold increase in the numbers of cells which proceed to lethal synthesis (Fig. 1). Such induction is accompanied by a small but reproducible increase in the infectivity of *colI*, both effects being ascribed to the destruction of a regulation system. Similar effects on enhanced transfer after u.v.-irradiation of strains which harbour RTF have been suggested (Watanabe & Fukasawa, 1961) to be due to a displacement of the RTF element from the chromosomal to the cytoplasmic state. De-repression of an autonomous plasmid is an alternative hypothesis that may be equally valid in this system.

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