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Mutants of *Escherichia coli* K12 $(\lambda)^+$ Non-Inducible by Thymine Deprivation

I. Method of Isolation and Classes of Mutants Obtained

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Summary. A selective enrichment method based upon differential killing by thymine deprivation of inducible as compared to non-inducible lysogens was employed to isolate mutants of *Escherichia coli* K12$(\lambda)^+$ deficient in lysogenic induction. The efficiency of the method is such that about 1% of the surviving colonies are resistant to thymineless induction.

About half of the mutants are *recA* recombination-deficient. Two other classes of non-inducible Rec$^+$ mutants can be distinguished. No temperature conditional bacterial mutations could be obtained.

Our results demonstrate that deficiency in the *recA* gene product is not the only bacterial factor which prevents lysogenic induction.

Introduction

Lysogenic induction, according to Jacob and Monod's theory (1961a, b), results from the action of an inducer on the repressor, which is a product of the cI gene; the inducer either stops the synthesis of the repressor or combines with it thus leading to repressor inactivation.

The results of subsequent research have demonstrated that thermal inactivation of the repressor destroys immunity and brings about the vegetative development of the prophage (Sussman and Jacob, 1962; Lieb, 1966). Recently, the λ phage repressor has been isolated (Ptashne, 1967). In contrast, attempts to identify an inducer have not succeeded yet (Goldthwait and Jacob, 1964).

The mechanism of induction would be clarified if the inducer could be characterized or if one knew what becomes of the repressor in the lysogens induced by UV light or thymine deprivation.

Two bacterial mutations modify the induction process: the T44 mutation (Goldthwait and Jacob, 1964; Kirby *et al.*, 1967) and the *recA* mutation (Brooks and Clark, 1967; Hertman and Luria, 1967). Since the *recA* mutation renders the λ lysogenic cells non-inducible by either UV light or thymine deprivation, the *recA* gene product might govern the activity of the inducer.

To comprehend better the role of the bacterial genes involved in lysogenic induction, we felt it important to know if there are bacterial mutations, other than *recA*, which can block the induction process. If *recA* was the sole bacterial mutation which abolished the induction response, the argument that the formation of the inducer is governed solely by the *recA* gene would be strengthened.

We also attempted to isolate temperature conditional bacterial mutants, inducible at 32° but not at 41°. This search has been unsuccessful so far.

In this paper, we describe a selective enrichment method for the isolation of non-inducible lysogenic bacteria. These included *recA* recombination-deficient mutants as well as *recA*$^+$. Our data demonstrate that *recA* deficiency is not the only mutation which can prevent lysogenic induction.

Materials and Methods

I. Bacterial Strains

The thymine auxotrophs described in this paper were all derived from GY158 (original strain: KMBL49, van de Putte *et al.*, 1964) whose genetic markers are, according to the nomenclature of Demerec *et al.* (1966), F$^-$ *thr-4 leu-8 thi-1 thyA34 dra-34 ura-49 supE lacY1 tonA101* $(\lambda)^+$; *dra* indicates a deficiency in deoxyriboaldolase (Ahmad and Pritchard, 1969).

Strain GY158$(\lambda)^+$ is totally induced by a 90 minute period of thymine deprivation or by 600 ergs $\times$ mm^{-2} 2,537 Å. Strains GY158$(\lambda$ *ind*$^-)^+$ and GY158$(\lambda 857)^+$ were used as controls.

The λ indicator strain was C600 (Appleyard, 1954); its lysogenic derivative C600 (λ cI857susR5t9b)$^+$ (Harris *et al.*, 1967), which is a temperature conditional mutant for cell lysis and release of λ phage, was also used as a control.

II. Phage Strains

Are listed in Table 1.

Table 1

Strain	Genetic markers	Origin
λ	wild type	Kaiser (1957), Dove (1969)
λ ind$^-$	non-inducible λcI	Jacob and Campbell (1959)
λ cI857	temperature conditional λcI	Sussman and Jacob (1962)
λ cI72	λ host range, λcI	Kaiser (1957)
λ cIL26	cI derivative of a hybrid phage, 434 host range, λ immunity	Kaiser and Jacob (1957)
λ b2imm434	434cI$^+$, λb2 deletion	gift of Eisen
λ pdbio10	plaque forming biotin transducing phage	Signer *et al.* (1969)

III. Cultures and Media

(a) Unless otherwise stated all cultures were grown and all plates were incubated at 41°.

(b) *YM9*:Na_2HPO_4, $7H_2O$:11 g; KH_2PO_4:3 g; NH_4Cl:1 g; NaCl:5 g; bidistilled water: 1 litre. This medium was used as a basic buffer medium and to prepare YM9C and YM9M.

YM9C: Casamino-acid vitamin free Difco decolorized with charcoal:10 g; glucose: 2 g; thymine: 40 mg; uracil: 40 mg; YM9: 1 litre.

YM9M: identical to YM9C but lacking thymine.

(c) *LB*:NaCl: 10 g; Bacto-Tryptone: 10 g; yeast extract: 5 g; demineralized water: 1 litre.

LA20:Biomar agar: 15 g; LB: 1 litre.

(d) *GT*:NaCl: 5 g; Bacto-tryptone: 5 g; Peptone UCLAF: 8 g; Biomar agar: 15 g; demineralized water: 1 litre.

(e) *EMBO*:NaCl: 5 g; Bacto EMB broth base Difco: 12.5 g; yeast extract: 1 g; Biomar agar: 13.5 g; demineralized water: 1 litre.

(f) *Soft Agar*: 7.5 g Difco agar per litre demineralized water. In some experiments we used GMS in which 10 g/l NaCl was added to the soft agar.

IV. Mutagenic Treatment

GY158$(\lambda)^+$ is grown in YM9C from 10^7 cells/ml up to about 8×10^8 cells/ml in the presence of 500 μg/ml 2-aminopurine (2-AP). A 0.1 ml sample is diluted into 10 ml fresh YM9C before

being subjected to the enrichment treatment. 2-AP was employed because it does not induce prophage λ. Furthermore, temperature conditional mutations have been obtained with this mutagen (Hill and Holland, 1967).

V. UV and X Ray Irradiations

The sources of UV light and X rays as well as their calibration were described in Devoret and George (1967).

Overnight cultures were streaked on LA20 plates, covered with a piece of cardboard and placed under the UV source. Then the cardboard was shifted to expose to 300 ergs × mm^{-2} a band corresponding to one fourth of the plate area. A second and third shifting permitted irradiation of a second and third band of equal width with the same dose. The fourth band of the plate was never exposed. Cumulative doses of 900, 600, 300 and 0 ergs × mm^{-2}, respectively, were given in this way.

The same technique was used for X ray irradiation except that the piece of cardboard was replaced by a plaque of lead and the cumulative doses were respectively 45, 30, 15 and 0 kiloroentgens.

Results

I. Principle of an Enrichment Method for the Isolation of Non-Inducible Lysogens

To isolate non-inducible bacterial mutants we made use of the fact that thymine deprivation kills Thy^- λ lysogens at a faster rate than non-lysogens or lysogens carrying the non-inducible prophage λ *ind*$^-$ (Sicard and Devoret, 1962). In other words: *non-inducible bacteria are more resistant to thymine deprivation than λ inducible ones.* This statement suggests that thymine deprivation can provide a selective enrichment method permitting to increase the relative frequency of non-inducible mutants in a population of inducible Thy^- bacteria as a function of the period of thymine deprivation.

II. Evaluation of the Enrichment Factor

Under identical conditions strains GY 158 $(\lambda)^+$ and GY158 $(\lambda\ ind^-)^+$ were deprived of thymine. The rates of thymine-less death of the two strains are shown in Fig. 1.

After 150 minutes of thymine deprivation, there are about 100 times more viable cells in GY 158 $(\lambda\ ind^-)^+$ than in GY 158 $(\lambda)^+$. Thus, if one assumes a hundredfold enrichment of non-inducible lysogens over λ inducible ones, then three rounds of cell growth followed by thymine deprivation for 150 minutes should increase the ratio of prospective mutants by about 10^6.

III. Observed Increase of Cells Resistant to Thymineless Induction

In order to prove that thymine deprivation of a culture of a λ lysogen leads to a marked increase of resistant cells, the following experiment was carried out:

After exposure to 2-aminopurine, a culture of GY158 $(\lambda)^+$ was subjected to a treatment comprising three successive cycles. Each cycle involved three steps:

1. Cell Growth: the culture was grown up to 10^8 cells/ml in YM9C.

2. Lysogenic Induction by Thymine Deprivation: the cells were centrifuged, washed with YM9, resuspended and incubated for 150 minutes in YM9M. Thymine starvation was always stopped at 150 minutes for two reasons: a) to avoid reaching a stage at which a fraction of the cell population can be resistant to thymineless

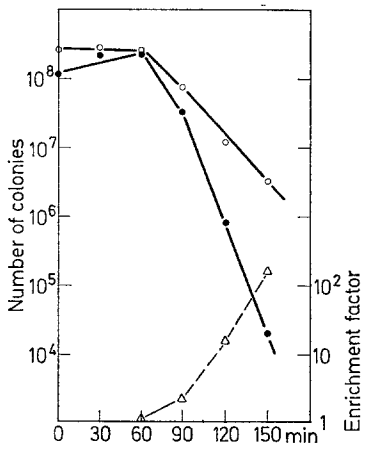

Fig. 1. Thymineless death of GY158(λ *ind*$^-$)$^+$ compared to GY158(λ)$^+$. Cells grown in YM9C up to 10^8 cells/ml were centrifuged, washed in YM9 and resuspended in YM9M. The cells were then incubated and diluted samples were plated at regular times on LA20 plates. Colonies of GY158(λ)$^+$ (•) and of GY158(λ *ind*$^-$)$^+$ (○) were counted after overnight incubation. The enrichment factor (△) is the survival ratio of GY158(λ *ind*$^-$)$^+$ over GY158(λ)$^+$

death for physiological reasons (Maaløe and Hanawalt, 1961); b) to prevent accumulation of mutations caused by thymine starvation itself (Coughlin and Adelberg, 1956).

3. Cell Lysis Produced by Readdition of Thymine: thymine was then added back (40 μg/ml) to the culture which was incubated for an additional 120 minutes. The culture was subsequently placed in a refrigerator overnight, centrifuged the following morning and the pellet resuspended in fresh YM9C.

The data concerning the viability of the cells during the first, second and third thymine deprivation step are shown in Fig. 2. It is apparent that after 3 cycles of thymine starvation the number of viable cells in culture no. 3 is 10^3 times more than that of culture no. 1.

In addition, examination of the cells of culture no. 3 with a microscope revealed very few filamenting cells.

IV. Screening of Mutants

About 18,000 colonies formed by cells which had survived the 3 cycles of the enrichment treatment were examined for their capacity to produce λ phage spontaneously. A total of 250 plates with 70 to 80 colonies per plate were used as master plates; each master plate was replica plated onto 2 GT plates containing the λ indicator strain C600, one of the replica plates was incubated overnight at 32°, the other at 41°.

Colonies forming plaques at 32° but not at 41° (criterion no. 1) or colonies *not* forming any plaque at both temperatures (criterion no. 2) were picked and studied further in the same way. First, they were placed as small patches arranged in geometrical arrays on a second master plate called "palette" with control colonies of strains C600 (λ cI857susRt9b)$^+$, GY158(λ)$^+$, GY158(λ *ind*$^-$)$^+$ and GY158

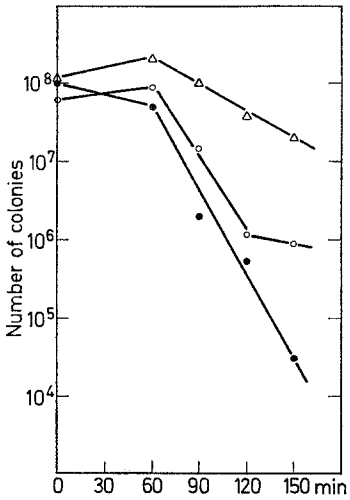

Fig. 2. Selection of bacteria resistant to thymineless induction. Diluted samples were plated at regular times on LA20 plates during each step of incubation for 150 minutes in YM9M. Colonies surviving the first (•), the second (○) and the third (△) cycle were counted after overnight incubation

$\lambda 857)^+$. Second, in order to check again spontaneous phage production and also UV induced plaque formation, these palettes were replica plated onto 4 plates containing a layer of C600 bacteria. The replica plates were submitted to 0 and 400 ergs $\times$ mm^{-2} 2,537 Å and incubated overnight at 32° and 41°. Third, to test the colonies for λ immunity according to Gottesman and Yarmolinsky (1968), the palettes were replica plated onto 2 EMBO plates, one containing λ cI72, the other λ cIL26.

V. Determination of the Mutant Yield, Efficiency of the Enrichment Method

In a series of experiments in which criterion 1 was used 7 mutants were isolated out of about 16,400 screened colonies; 6 of them gave very few plaques at 32°, one mutant, however, produced a normal level of plaques at 32° and no plaque at 41°. Unfortunately, this mutant, which possessed the looked-for phenotype, carried a mutation in the λ prophage and not in the bacterial chromosome. The failure of detecting true temperature conditional bacterial mutations prompted us to end the search for temperature conditional mutants. Therefore, in another experiment we used both criteria 1 and 2 to evaluate the overall efficiency of the enrichment technique. Twentyfour first master plates containing 1,644 colonies yielded 68 colonies unable to form plaques on the λ indicator strain. Placed on palettes, further tests revealed that only 25 out of 68 colonies could be retained as prospective mutants. Checked for immunity to λ phage, only 14 colonies were still immune and consequently true non-inducible mutants.

One can estimate that the frequency of λ non-inducible mutants in a population of cells submitted 3 times to thymine starvation is of the order of 1 per cent.

VI. Phenotypic Classes of the Selected Mutants

Out of about 18,000 colonies tested, a total of 21 mutants have been isolated which are immune to λ phage and display a low level of spontaneous (as well as UV-induced) production of phage as compared to the wild type lysogen.

To determine whether each phenotype was due to a bacterial or a prophage mutation all the mutants were eventually cured of the prophage they carried by hetero-immune superinfection with the hybrid phage λb2 *imm*434 (Signer and Beckwith, 1966). The cured mutants were all relysogenised with wild type λ and then retested for spontaneous and UV-induced production of λ phage. Moreover, strain C600 was lysogenised with the phage (if any) released from each mutant. This was done when it appeared that some of the mutants could not be cured. These tests showed that 2 mutants carried a defective prophage (see Table 2).

Since it was important to know which, among the bacterial mutations, were of the *recA* deficient type, each mutant was exposed to UV light and to X rays, since *recA* deficient mutants display a very high sensitivity to both radiations (Table 2) (Howard-Flanders and Boyce, 1966). In addition, all of the cured mutants were infected by phage λ *pdbio10* which has an extremely low efficiency of plating

Table 2. *Phenotype of mutants. Radiation resistance is scored as follows: the number of + signs corresponds to colony growth as a function of cumulative UV or X ray doses which are respectively a multiple of 300 ergs×mm^{-2} and of 15 kiloroentgens, e.g. ++− in column 3 means no colony growth above 600 ergs×mm^{-2}; −−− in column 4 means no colony growth even at 15 kiloroentgens. WT indicates that C600 lysogenised by the phage λ$_i$ released by the corresponding mutant behaves as C600(λ)$^+$*

Mutants	Isolation criterion	UV resistance	X resistance	Curing	λdbio growth	C600(λ_i)$^+$	Mutant class
GY6003—GY6007	1	+++	+++	+	+	WT	I
GY6015	1	++−	+++	+	+	WT	I
GY6004—GY6019	1	++−	+++	−		WT	II
GY6006	1	−−−	−−−	+	−	WT	*recA*
GY6018	1	+++	+++	+	+	mutant	temperature conditional mutation in the prophage
GY6010—GY6016	1+2	+++	+++	+	+	WT	I
GY6011	1+2	++−	+++	+	+	WT	I
GY6009	1+2	++−	+++	−		WT	II
GY6012—GY6023 GY6024—GY6025 GY6026—GY6027 GY6028—GY6031	1+2	−−−	−−−	+	−	WT	*recA*
GY6029	1+2	+++	+++	+	+	mutant	absolute defective prophage
GY6030	1+2	+−−	+−−	+	+	WT	I
GY158(λ)$^+$	—	+++	+++	+	+	WT	control

on the *recA* deficient bacteria (Signer *et al.*, 1969). Those mutants which were UV-sensitive, X-ray sensitive and did no support $\lambda pdbio$ growth were considered as *recA* recombination-deficient. Furthermore, some conjugation experiments with a few of these mutants used as F^- recipient cells proved definitely the recombination deficiency (J. George, personal communication). It is inferred from the several characteristics described above, that these mutants are *recA* recombination-deficient, although they have not yet been mapped.

Table 2 summarizes the phenotypic traits of the mutants. Nineteen mutants appear to carry a mutation in the bacterial chromosome. Nine of them are *recA* recombination-deficient mutants. They were isolated mostly when criterion no. 2 was used for the selection.

The ten remaining mutants which also have a low level of spontaneous λ phage production can be divided into two main classes defined by the ability to be cured of the resident prophage by hetero-immune superinfection. The seven mutants which were easily cured were assigned to class I. Three mutants which could not be cured were assigned to class II. These mutants are killed upon hetero-immune superinfection at high multiplicity of phage per cell. However, at low multiplicity, infection with phages of immunity 434, 21 or 82 does not give rise to the formation of plaques. In contrast, phage T2 forms plaques at all multiplicities of infection of these mutants. The properties of the mutants of class I and II will be detailed in subsequent publications.

Discussion

Our data demonstrate the feasibility of selecting lysogenic non-inducible mutants with a technique based upon the use of thymine deprivation.

In our experiments, as many as 1% of the colonies surviving thymine deprivation had lost immunity to λ phage. This curing could arise in the following ways:

a) The enrichment method might select the non-lysogenic segregants which are believed to be present at a very low frequency in any lysogenic culture (Bertani, 1958).

b) Thymine deprivation might have a similar curing effect for λ phage as it does for episomes (Clowes, Moody and Pritchard, 1965).

c) The enrichment treatment and the screening method might select strongly for λ mutants resistant to lysogenic induction by thymine starvation. For example, mutations occurring in some "early" genes of λ phage such as O and P confer on the UV irradiated lysogens a selective advantage over the wild type since with increasing UV doses more and more cells are cured of the defective resident prophage (Eisen *et al.*, 1966). One can imagine that mutagenic treatment with 2-aminopurine and also the known mutagenic action of thymine starvation might produce mutations in the "early" λ genes. This could lead to some subsequent curing brought about by thymineless induction.

The final 1% frequency of cured cells among the survivors is not excessive if one takes into account that each cycle of thymine deprivation gives an enrichment factor of about one hundred. Therefore, there is not a vast discrepancy between the expected low initial frequency of cured cells (a) and cells carrying mutant prophages (c), and the final yield of cured cells.

The low proportion of λ prophage mutations to the bacterial mutations which were obtained — only two mutants out of 21 — can be explained by the low probability of occurrence of a prophage mutation which would not cause any cell death upon induction by thymine deprivation. The target responsible for an *ind*$^-$ mutation in the cI gene is very small (Lieb, 1966). The same is true if one compares the size of the x, O, P operon to the whole length of the genome. Defective mutations located elsewhere do not prevent induction of the prophage and therefore cell death (Campbell, 1961; Eisen *et al.*, 1966).

Selection for lack of induction of phage λ by thymine deprivation led to the isolation of *recA* deficient mutants. This could be expected as λ lysogenic *recA* deficient strains are not inducible (Brooks and Clark, 1967) and are no more sensitive to thymine deprivation than the wild type (Cummings and Taylor, 1966). Although the *recA* deficient mutants are almost half of those which were collected, this is not the only bacterial mutation which prevents induction by thymine deprivation.

With our new technique we have found at least two new classes of mutation of the bacterial host which affect lysogenic induction by thymine deprivation which are distinct from the *recA* class. These mutants suggest that there are several steps involved in the biochemical pathway leading to the formation of an inducer as a result of thymine starvation. They should help us to elucidate these steps.

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