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1 of 18

The Genetic Control and Cytoplasmic Expression of "Inducibility" in the Synthesis of β-galactosidase by *E. Coli*†

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A number of extremely closely linked mutations have been found to affect the synthesis of β-galactosidase in *E. coli*. Some of these (*z* mutations) are expressed by loss of the capacity to synthesize active enzyme. Others (*i* mutations) allow the enzyme to be synthesized constitutively instead of inducibly as in the wild type. The study of galactosidase synthesis in heteromerozygotes of *E. coli* indicates that the *z* and *i* mutations belong to different cistrons. Moreover the constitutive allele of the *i* cistron is recessive over the inducible allele. The kinetics of expression of the i^+ (inducible) character suggest that the *i* gene controls the synthesis of a specific substance which represses the synthesis of β-galactosidase. The constitutive state results from loss of the capacity to synthesize active repressor.

1. Introduction

Any hypothesis on the mechanism of enzyme induction implies an interpretation of the difference between "inducible" and "constitutive" systems. Conversely, since specific, one-step mutations are known, in some cases, to convert a typical inducible into a fully constitutive system, an analysis of the genetic nature and of the biochemical effects of such a mutation should lead to an interpretation of the control mechanisms involved in induction. This is the subject of the present paper.

It should be recalled that the metabolism of lactose and other β-galactosides by intact *E. coli* requires the sequential participation of two distinct factors:

(1) The galactoside-permease, responsible for allowing the entrance of galactosides into the cell.

(2) The intracellular β-galactosidase, responsible for the hydrolysis of β-galactosides.

Both the permease and the hydrolase are inducible in wild type *E. coli*. Three main types of mutations have been found to affect this sequential system:

(1) $z^+ \rightarrow z^-$: loss of the capacity to synthesize β-galactosidase;

(2) $y^+ \rightarrow y^-$: loss of the capacity to synthesize galactoside-permease;

(3) $i^+ \rightarrow i^-$: conversion from the inducible (i^+) to the constitutive ~~(i^-)~~ state.

The $i^+ \rightarrow i^-$ mutation always affects *both* the permease and the hydrolase. All these mutations are extremely closely linked: so far all independent occurences of each of these types have turned out to be located in the "*Lac*" region of the *E. coli* K 12 chromosome. However, the mutations appear to be *independent* since all the different phenotypes resulting from combinations of the different alleles are observed (Rickenberg, Cohen, Buttin & Monod, 1955; Cohen & Monod, 1957; Cohn, 1957).

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It should also be recalled that conjugation in *E. coli* involves the injection of a chromosome from a ♂ (Hfr) into a ♀ (F^-) cell, and results generally in the formation of an incomplete zygote (merozygote) (Wollman, Jacob & Hayes, 1956). Recombination between ♂ and ♀ chromosome segments does not take place until about 60 to 90 min after injection; moreover segregation of recombinants from heteromerozygotes occurs only after several hours, thus allowing ample time for experimentation.

In order to study the interaction of these factors, their expression in the cytoplasm and their dominance relationships, we have developed a technique which allows one to determine the kinetics of β-galactosidase synthesis in merozygotes of *E. coli*, formed by conjugation of ♂ (Hfr) and ♀ (F^-) cells carrying different alleles of the factors *z*, *y* and *i* (Pardee, Jacob & Monod, 1958). Before discussing the results obtained with this technique, we shall summarize some preliminary observations on the genetic structure of the "*Lac*" region in *E. coli* K 12.

2. Materials and Methods

(a) *Bacterial strains*

A ♂ (Hfr) strain (no. 4,000) of *E. coli* K 12 was used in most experiments. It was derived from strain 58,161 F^+, and was selected for early injection of the "*Lac*" marker (Jacob & Wollman, 1957). This strain is streptomycin sensitive (S^s), requires methionine for growth and carries the phage λ. A second Hfr strain (no. 3,000), isolated by Hayes (1953), was used in some experiments. This strain is S^s, requires vitamin B_1, and does not carry λ prophage. Other Hfr strains carrying mutations for galactosidase (*z*), inducibility-constitutivity (*i*), and permease (*y*) were isolated from the Hayes strain after u.v. irradiation. These markers were also put into ♀ (F^-) strains, by appropriate matings and selection of the desired recombinants.

A synthetic medium (M 63) was commonly used. It contained per liter: 13·6 g KH_2PO_4 2·0 g $(NH_4)_2SO_4$, 0·2 g $MgSO_4 . 7H_2O$, 0·5 mg $FeSO_4 . 7 H_2O$, 2·0 g glycerol, and KOH to make pH 7·0. If amino-acids were required, they were added at a concentration of 10 mg/l. of the L-form. For mating experiments, the above stock medium was adjusted to pH 6·3 and vitamin B_1 (0·5 mg/l.) was added prior to use. Aspartate (0·1 mg/ml.) was generally added at the time of mating, according to Fisher (1957).

(b) *Mating experiments*

The desired volume of fresh medium was inoculated with an overnight culture (grown in the same medium) to an initial density of approximately 2×10^7 bacteria/ml. This culture was aerated by shaking at 37°C in a water bath. Turbidity was measured from time to time; and when the density reached 1 to 2×10^8 bacteria/ml., the experiment was started. Usually small volumes of ♂ and ♀ bacteria were mixed in a large Erlenmeyer flask, with the ♀ strain in excess (e.g. 3 ml. ♂ plus 7 ml. ♀ in a 300 ml. flask). The mixed bacteria were agitated very gently so that the motion of the liquid was barely perceptible. From time to time samples were removed for enzyme assay and plating on selective media, usually lactose-B_1-streptomycin agar, for measurement of recombinants. Under these conditions, in a mating of ♂ z^+Sm^s by ♀ z^-Sm^r, up to 20 % of the ♂ population formed z^+Sm^r recombinants (as tested by selection on lactose-streptomycin agar). More often 5 to 10 % recombinants were found.

Streptomycin (Sm)† was used in many mating experiments, to block enzyme synthesis by z^+Sm^s ♂ cells. Controls showed that the synthesis of β-galactosidase was blocked in these strains immediately upon addition of 1 mg/ml. of Sm. Incorporation of ^{35}S from $^{35}SO_4^=$ as well as increase of turbidity were also suppressed by this treatment. This concentration of Sm had no effect on Sm-resistant (Sm^r) mutants. In some experiments, virulent phage (T6) was used to kill the ♂ cells, thus preventing remating.

† The following abbreviations are used in this paper:

Sm = streptomycin
IPTG = *iso*propyl-thio-β-D-galactoside
ONPG = *o*-nitrophenyl-β-D-galactoside
TMG = methyl-thio-β-D-galactoside

It should be noted that if streptomycin was added initially, it significantly reduced the number of recombinants (e.g., 75 % fewer colonies were formed on lactose-B_1-streptomycin plates after 80 min mating in the presence of 1 mg/ml. streptomycin) relative to mating in the absence of streptomycin; but the antibiotic had little effect on enzyme formation by zygotes if added at the commencement of the experiment or after the z^+ locus had been injected.

When galactosidase synthesis had to be induced in zygotes, *iso*propyl-thio-β-D-galactoside (IPTG) was used at 10^{-3}M, a concentration at which this inducer is known to be active even in the absence of permease (Rickenberg *et al.*, 1956).

(c) *Recombination studies*

The blender technique of Wollman & Jacob (1955) was used to determine the times of penetration of markers into the zygotes. It should be noted that this treatment reduces enzyme-forming capacity in zygotes by 30 to 60 %. Recombinant colonies, selected on appropriate selective media, were restreaked on the selector medium and replica plating was used to determine unselected characters. Tests for galactosidase synthesis (with or without induction) were performed on maltose-synthetic agar plates with or without IPTG, using filter paper impregnated with ONPG, according to Cohen-Bazire & Jolit (1953).

Transductions were performed with phage 363, according to Jacob (1955).

(d) *β-galactosidase assay*

For this enzyme assay, 1 ml. aliquots of culture were pipeted into tubes containing 1 drop of toluene. The tubes were shaken vigorously and were incubated for 30 min at 37°C. They were then brought to 28°C; 0·2 ml. of a solution of M/75 *o*-nitrophenyl-β-D-galactoside in M/4 sodium phosphate (pH 7·0) was added, and the tubes were incubated a measured time, until the desired intensity of color had developed. The reaction was halted by addition of 0·5 ml. of 1 M-Na_2CO_3, and the optical density was measured at 420 mμ with the Beckman spectrophotometer. A correction for turbidity could be made by multiplying the optical density at 550 mμ by 1·65 and subtracting this value from the density at 420 mμ. One unit of enzyme is defined as producing 1 mμ-mole *o*-nitrophenol/minute at 28°C, pH 7·0. The units of enzyme in the sample can be calculated from the fact that 1 mμ-mole/ml. *o*-nitrophenol has an optical density of 0·0075 under the above conditions (using 10 mm light-path).

(e) *Chemicals*

o-nitrophenyl-β-D-galactoside (ONPG), methyl-thio-β-D-galactoside (TMG) and *iso*propyl-thio-β-D-galactoside (IPTG) were synthesized at the Institut Pasteur by Dr. D. Türk. Other chemicals were commercial products.

3. Genetic Structure of the "Lac" Region

Figure 1 presents the structure of the "*Lac*" region, as it can be sketched from the data available at present. This complex locus, as established long ago by Lederberg (1947) and confirmed by the blender experiments of Wollman & Jacob (1955), lies at about equal distances from the classical markers *TL* and *Gal*. The closest known markers are *Proline* (left) and *Adenine* (or *T6*) (right). As shown in the map, the several (about 10) occurrences of the y^- mutation all lie together probably at the left of the segment, while the different z^- mutations and the i^- mutant are packed together at the other end. No attempt has been made to establish the order of individual y^- mutations. The order of the z^- mutations relative to each other and to the i^- marker is unambiguously established, as shown, except for the z^-_U mutation, whose position is largely undetermined. Several independent occurrences of the i^- mutation have been isolated. They all appear to be closely linked to the i^-_3 marker, but they have not been mapped, for lack of adequate methods of selection i^+ recombinants. The evidence for this structure is briefly as follows:

(1) The frequency of recombination between z and y mutations is very low:

M

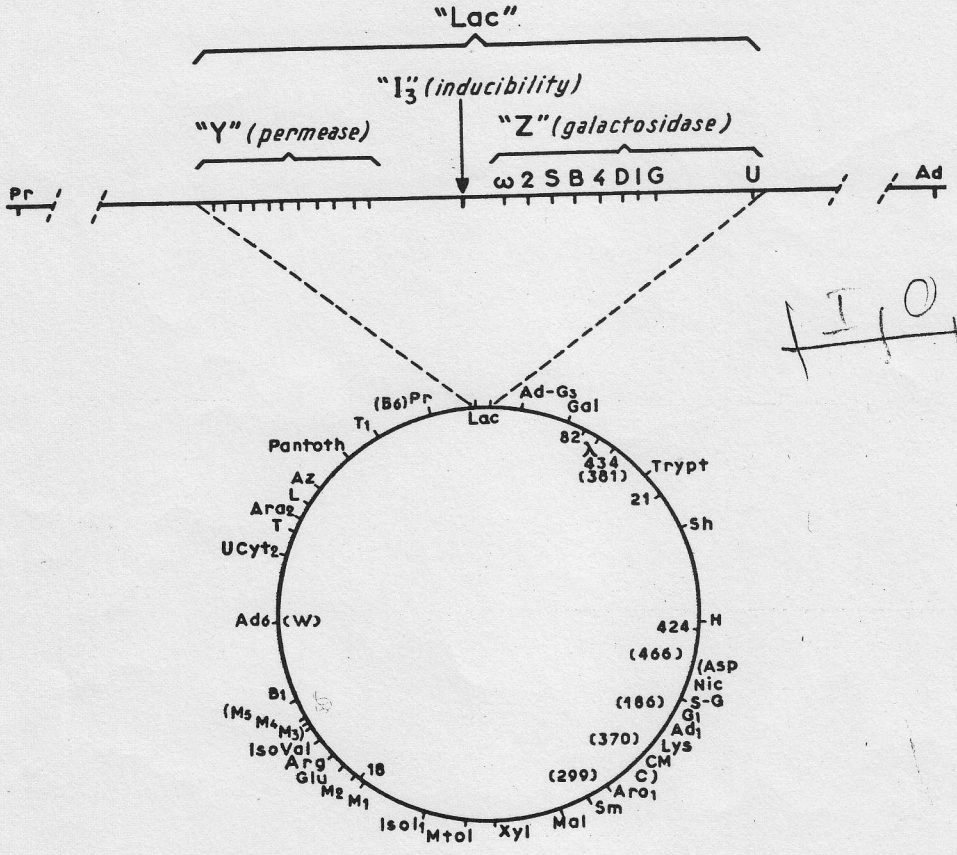


FIG. 1. Fine structure of the "*Lac*" segment.

The "*Lac*" segment is shown enlarged and positioned with respect to the rest of the *E. coli* K 12 linkage group for which the circular model (Jacob & Wollman, 1958) has been adopted.

roughly 1/100th of the frequency of recombination between *T1* and *Gal*. The frequency of recombination between individual *z* markers is about one order of magnitude lower.

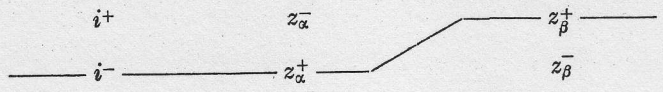
(2) When y^+z^+ recombinants are selected (by growth on lactose-agar) in crosses of the type:

$$y^+i^-z^+ \times y^-i^+z^+$$

the i^+ marker remains associated with z^+ 85 % of the time.

(3) The frequency of cotransduction of *i* with *z* (selecting for z^+ alone) is very high (> 90 %), while the frequency for *i* and *y* is also high, although definitely lower (about 70 %). (These data are somewhat ambiguous, because of the heterogeneity of the clones resulting from a transduction.)

(4) The selection of z^+ recombinants in crosses involving different z^- mutants, and *i* as unselected marker, invariably results in about 90 % of the progeny being either i^- or i^+, depending on the particular z^- mutants used. Assuming this result to be due to the position (left or right) of *i* with respect to the *z* group:



a linear order can be established, without contradictions, for the eight markers shown. This however leaves an ambiguity as to whether *i* lies *between* the *y* and the *z* groups, or outside.

Let us emphasize that this sketch of the *Lac* region is preliminary and very incomplete, and that the results concerning the relationships of certain markers are not understood. For instance, the z_U^- marker recombines rather freely with all the other mutants shown (both *y* and *z*) yet, by cotransduction tests, it is closely linked to *i* (25 % cotransduction). It should also be mentioned that certain of the z^- mutants (z_ω^- ; z_2^-; z_G^-) have apparently lost the capacity to synthesize *both* the galactosidase *and* the permease. Yet these mutations do not seem to be deletions. We shall not attempt, here, to interpret this finding, since we shall center our attention on the interaction between the *i* marker and the *z* region.†

A question which should now be considered is whether we may regard the *z* region as possessing the specific structural information concerning the galactosidase molecule. The fact that so far all the independent mutations resulting in loss of the capacity to synthesize galactosidase were located in this region might not constitute sufficient evidence‡. However, it has been found by Perrin, Bussard & Monod (1959, in preparation) that several of the z^- mutants synthesize, instead of active galactosidase, an antigenically identical, or closely allied, protein. Moreover several of these mutant proteins are different from one another by antigenic and other tests. These findings appear to prove that the *z* region indeed corresponds to the "structural" genetic unit for β-galactosidase.

4. β-Galactosidase Synthesis by Heteromerozygotes

(a) *Preliminary experiments*

The feasibility and significance of experiments on the expression and interaction of the *z*, *y* and *i* factors depended primarily on whether *E. coli* merozygotes are physiologically able to synthesize significant amounts of enzyme very soon after mating. It was equally important to determine whether the mating involved any cytoplasmic mixing. These questions were investigated in a series of preliminary experiments.

Since the physical separation of *E. coli* zygotes from unmated or exconjugant parent cells cannot be achieved at present, test conditions must be set up, such that the zygotes only, but not the parents, can synthesize the enzyme. This is obtained when the following mating:

$$♂\ z^+y^+i^+Sm^s \times ♀\ z_s^-y^+i^+Sm^r \qquad \text{(A)}$$

is performed in the presence of inducer (IPTG) and of 1 mg/ml. of streptomycin. The ♀ lack the z^+ factor; the ♂ are inhibited by streptomycin (cf. Methods); the zygotes are not, because they inherit their cytoplasm from the ♀ cells (see below

† Interaction of *i* with the *y* region is of course equally interesting, but since determinations of activity are much less sensitive with the galactoside-permease than with the galactosidase, we have used the latter almost exclusively.

‡ In addition to the mutants shown on Fig. 1, 20 other galactosidase-negative mutants, as yet unmapped, have been found to belong to the same segment by contransduction tests. None was found outside. Lederberg *et al.* (1951), however, have isolated some lactose-"non-fermenting" mutants (as tested on EMB-lactose agar) which are located at other points on the *E. coli* chromosome. In our hands, one of these mutants (Lac_3^-) formed normal amounts of both galactosidase and galactoside-permease (although it did form white colonies on EMB-lactose). Another one (Lac_7^-) formed reduced, but significant, amounts of both. A third (Lac_2^-) which is a galactosidase-negative, appears to belong to the "*Lac*" segment, by cotransduction tests.