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AD840846

TRANSLATION NO. 1975

DATE: 12 July 1967

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DEPARTMENT OF THE ARMY  
Fort Detrick  
Frederick, Maryland

IDENTIFICATION AND PURIFICATION OF A PEPTIDE  
SEGMENT OF THE BETA-GALACTOSIDASE OF  
ESCHERICHIA COLI BY COMPLE-  
MENTATION IN VITRO

Journal of Molecular Biology  
Vol 12, 1965, pp 918-923

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Several convergent series of observations have indicated that  $\beta$ -galactosidase of Escherichia coli is an oligomeric enzyme consisting of four identical sub-units or protomeres. Each has a molecular weight of approximately 130,000 (Cohn, 1957; Perrin, 1963; Zipser, 1963). It is not known whether the protomere with a molecular weight of approximately 130,000 consists of several different peptide chains. That this might be the case was suggested by different observations, but none was sufficiently convincing until now (Wallenfels, Sund, Weber, 1963; Zabin, 1963; Weber, Sund, Wallenfels, 1964; Craven, Steers, Anfinsen, 1964).

On the other hand, based on the frequencies of intragenetic recombinations, it was estimated that the gene structure of  $\beta$ -galactosidase ought to consist of about 3,500 nucleotides (Jacob and Monod, 1961a), and that its dimensions ought to be compatible with the molecular weight of the protomere. On that gene, different point mutations, when grouped in the trans position in diploid, heteromerozygotic bacteria, produced the phenomenon of complementation. Complementation was also observed in vitro in crude or purified mixtures of extracts from simple mutants (Jacob and Monod, 1961b). Utilizing these results, a complementation map was constructed and showed a complexity incompatible with the hypothesis that the observed activity could be attributed to the reassociation of different peptides, each

corresponding to a definite segment on the gene z. Expressed differently, it seemed that complementation among point mutations was more of the intracistronic type than of the inter-cistronic type (Perrin, 1963).

Meanwhile, in fact, quite recently, we were able to isolate from *E. coli* K-12 a series of deletions more or less located on gene z (Jacob, Ullmann, Monod, 1964). Assays of complementation occurring between these deletions and diverse point mutants have indicated that all deletions, not located beyond marker 242, gave a positive complementation with all point mutants located between marker 178 and the end of gene z (Fig 1, Table 1). Reciprocally, deletions near marker 178 did not form any complementation with any end mutations.

These observations suggested that gene z accommodated effectively several (at least two) distinctive segments, each determining the structure of a different peptide; each peptide being an essential constituent of the protomers of  $\beta$ -galactosidase. To verify that conclusion, we have tried to bring about the complementation *in vitro* in order to purify and identify the peptide produced by the end segment ( $\Omega$ ) of gene z.

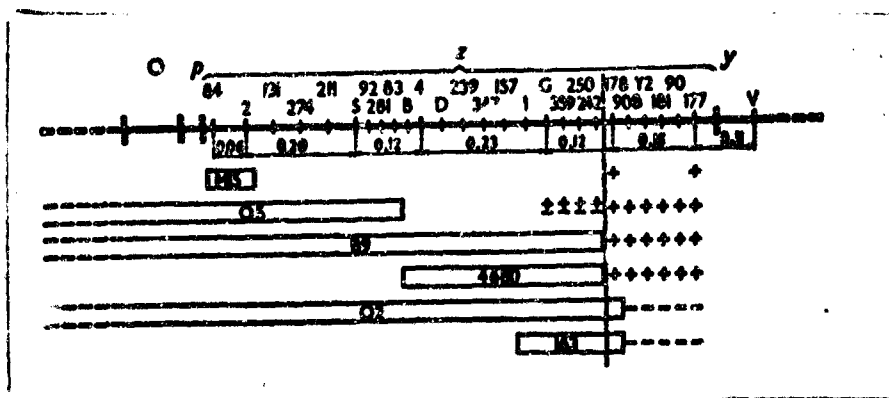


Fig 1. Schematic representation of the structural gene of  $\beta$ -galactosidase of *E. coli*: genetic and complementation map

The figures and letters placed above the thick line represent point mutations on gene z, the determinant of the structure of  $\beta$ -galactosidase. O = operator; p = promoter;

y = structural gene of  $\beta$ -galactoside permease. Figures below the thick line represent frequencies of observed recombinations in crosses between two point mutants. The rectangles in the lower portion of the graph represent the deletions: M15 isolated by Beckwith (1964), 4680 isolated by Cook and Lederberg (1962), 05, B9 and 02 isolated by Jacob et al (1964). + and - signs represent the results of complementations for  $\beta$ -galactosidase activity made in vivo with heterogenotes obtained by sexduction and scored on EMB-lactose agar. Each sign corresponds to an actual result with heterogenotes present. The deletion is shown on the same horizontal line, as well as the point mutation which is shown on the same vertical line. + indicates complementation efficiency, + weak complementation, - complementation not detectable.

Table 1

Production of  $\beta$ -galactosidase by heterozygous cultures diploid for gene z

Mutations on the chromosome	Mutations on the sexual episome				
	M15	05	B9	02	Haploid control
$\Delta_{200}$	*	155	< 1	*	< 1
$\Delta_{170}$	2500	871	1800	*	12.5
$\Delta_{200}$	1855	965	920	< 1	< 1
$\Delta_{101}$	*	1276	980	< 1	6
haploid control	< 1	< 1	< 1	< 1	

\* Not measured

Cultures were grown in a minimal medium with glycerol and  $10^{-4}$  M isopropylthiogalactoside. The figures represent enzyme units per mg of dry weight of bacteria. Under these conditions, a bacterium haploid for Lac<sup>+</sup> produced about 8,000 enzyme units. A unit is defined as that quantity of enzyme which in a solution of  $2.7 \times 10^{-3}$  M of orthonitrophenyl  $\beta$ -D-galactoside hydrolyses 1  $\mu$ mole per minute at 28°C in a 0.1 M sodium phosphate buffer, pH 7.0, containing  $10^{-3}$  M  $Mg^{2+}$  and 0.1 M  $\beta$ -mercaptoethanol.

When an extract of point mutant 908 was used as an "acceptor" and when increasing concentrations of an extract of deletion B9 were added,  $\beta$ -galactosidase activity gradually appeared. Maximum activity was obtained after 60 minutes at 28°C in the presence of a fixed quantity of mutant 908 extract. The activity remained stable and proportional to the concentration of extract B9 (Fig 2).

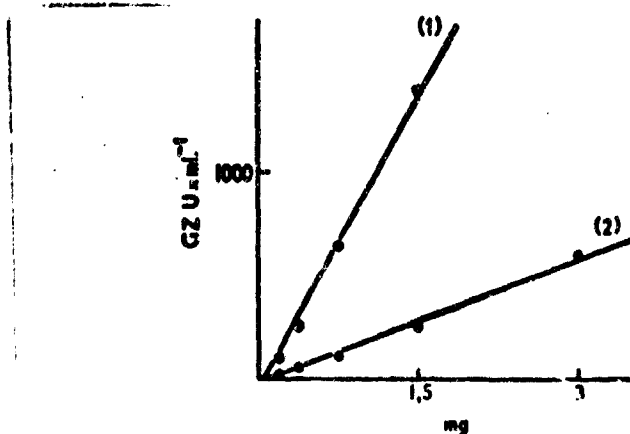


Fig 2. Dosage of the complementation fraction of culture B9. Increasing quantities of the purified fraction of peptide were added to a crude extract of bacterial culture S908

Curve 1: 6.2 mg of S908 proteins. Curve 2: 3.1 mg of S908 proteins. Final total volume was 0.2 ml. Dosage of  $\beta$ -galactosidase was measured after 60 minutes of incubation at 28°C under conditions described in Table 1.

Utilizing the above procedure, the peptide produced by culture B9 was fractionated by the following technique:

1) Bacteria were sonically disintegrated (1 gm wet weight per 1.5 ml) in a tris-buffer  $2 \times 10^{-2}$  M,  $\text{MgSO}_4$   $10^{-2}$  M, pH 7.2.

2) Spermine 0.2 M, pH 7.4, 0.1 volume, was added and the precipitate was discarded. The supernatant was treated with DNase and RNase (10  $\mu$ g/ml) and diluted in the above buffer solution to give 10 mg of protein per ml. The large amount of precipitate which formed was discarded.

3) Ammonium sulfate (neutralized) was added to the supernatant to 40% saturation. The precipitate was dissolved in buffer:  $\text{Na}_2\text{HPO}_4$   $2.5 \times 10^{-2}$  M,  $\text{MgSO}_4$   $10^{-3}$  M,  $\text{MnSO}_4$   $2 \times 10^{-4}$  M, Mg Titriplex  $2 \times 10^{-3}$  M,  $\beta$ -mercaptoethanol  $10^{-2}$  M, adjusted to pH 7.0 with HCl and dialyzed for 18 hours at 0°C against the same buffer solution.

4) Column chromatography was through Sephadex G 100 equilibrated with the same buffer solution at 18°C.

5) Fractions with maximum activity of complementation were combined and reprecipitated with ammonium sulfate, 55% saturation. The precipitate was dissolved in the buffer and again dialyzed as above. The precipitate which appeared after 18 hours was discarded.

In the presence of a crude extract of culture 908, containing 40 mg of protein, fractions thusly purified yielded 3,000 units of GZ per mg of protein or about 200 times more than prior to fractionation. However, it was not possible to define the specific activity of the preparation because the activity obtained remained a non-linear function of the concentration of the acceptor extract (908), as well as that of fraction "Q" (Fig 2). Complementation, ordinarily in a stable state, lead to an equilibrium reaction, and we must admit that only a portion of peptide "Q" present in the preparation participated effectively in the formation of active molecules. This could be explained, at least in part, by the fact that the specific activity obtained per mg of protein of the purified fraction "Q" was some 100 times weaker than that of the total peptide "Q" which participated in the formation of enzyme molecules whose activity was equal to that of normal enzyme molecules. The complementation fraction "Q" appeared to be homogeneous after centrifugation in a sucrose gradient which indicated (by comparison with appropriate samples) a sedimentation coefficient of 3.15. This coefficient of sedimentation suggested that the molecular weight of peptide "Q" might be of the order of 30,000 - 40,000.

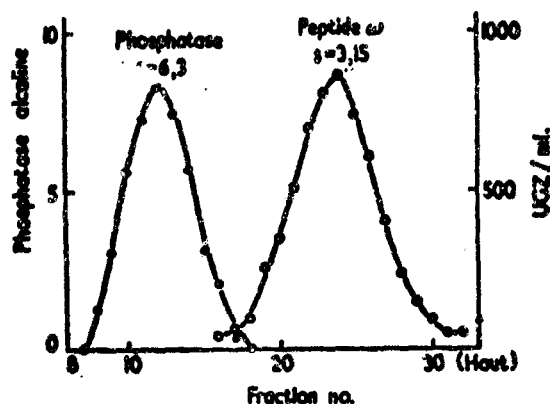


Fig 3. Sedimentation of peptide " $\Omega$ " in a sucrose gradient.

A solution containing 800  $\mu$ g of purified fraction " $\Omega$ " (See text) and 10  $\mu$ g of purified alkaline phosphatase were centrifuged in a linear sucrose gradient (5 ml of a solution of 5-20% sucrose in a SW 39 centrifuge for 17 hours at 39,000 rpm). The phosphatase activity was assayed by the hydrolysis of p-nitrophenylphosphate and the peptide " $\Omega$ " by complementation of an extract from culture S908 under conditions described in Fig 2.

The fact that a deletion, corresponding to about two-thirds of gene *z*, synthesized a protein with a molecular weight of one-third to one-fourth of that of the protomere of  $\beta$ -galactosidase, itself an inactive fraction, but capable of reactivating the protein produced by different point mutants, corresponding to the intact region of gene *z* in the deletion, does not necessarily prove that this peptide exists in the protein of the wild type. In fact, one may ask whether the formation of the peptide segment of  $\beta$ -galactosidase might not have been the result of the deletion itself and whether its



complementation activity might not be explained by the phenomenon of a repair reassociation, comparable to that observed with certain artificially detached peptides of ribonuclease.

If this was the case, one must expect that the complementation fraction produced by the extension deletions of the much weaker culture B9 would show characteristics (especially with respect to molecular weight) different from those of fraction "Q". We have subsequently repeated the same fractionation, using three different bacterial cultures. One culture was the deletion B9 already mentioned, while the other two were much weaker extension deletions (M15 isolated by Beckwith [1964] and O5 [see Fig 1]) which affected only a small portion of gene z. Figure 4 shows the distribution of complementation activity after passage through Sephadex G 100. It can be seen in the case of the large deletion almost all of the complementation activity was concentrated in a single, distinctive peak of the main proteins. On the contrary, in the two weak extension deletions, the complementary activity was distributed among three peaks, of which one corresponded to the peak given by the complementation extract of the large deletion, while another peak was associated with the main peak of the proteins and the third peak was intermediate. It must be emphasized that the results obtained from many repeated experiments were perfectly reproducible.

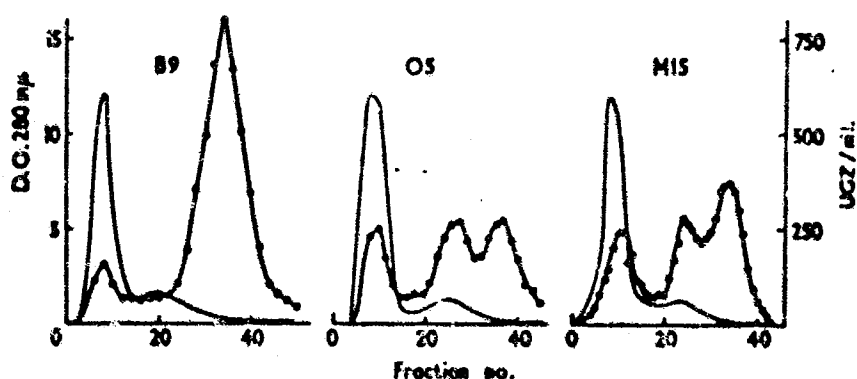


Fig 4. Distribution of the complementation activity found in extracts of different bacterial cultures after passage through Sephadex G100.

Ammonium sulfate was added to each extract to give 40% saturation. The precipitate obtained was dialyzed (see text) and passed through a column of Sephadex G100. The fractions were analyzed for optical density at 280 m $\mu$  (solid line) and for complementation activity by mixing with an extract from bacterial culture S908 (dotted line). Complementation activities were standardized against total activities of the initial extracts, using the extract of B9 as a standard.

In conclusion, it seemed peptide " $\Omega$ " was effectively present in the two small deletions, as well as in the large deletion, but that in the two cases, it existed at least partially associated with the other constituents, entirely corresponding to the intact segments of gene z which were present. Finally, a fraction possessing the properties of complementation and sedimentation of peptide " $\Omega$ " could have been obtained equally well from extracts of the point mutant z<sub>1</sub>. We must emphasize that in the case studied, the enzyme formed by complementation differed from the wild type enzyme by its sensitivity to temperature and urea. Conversely, the sedimentation constant (in the sucrose gradient) of the enzyme obtained by complementation was not significantly different from that obtained from the wild type enzyme (15.5). The sedimentation constant of the complementation fraction of the extract of culture 908 was about 13.

The observations we are about to summarize indicated gene z contained at least two distinctive segments which determined the structure of different peptides whose association constituted the protomere of  $\beta$ -galactosidase. Complementation studies in vivo have shown, among other things, that deletions with a feeble extension (M15 and 05) are weakly complementable with point mutants located between the markers G and 242. These observations suggested that the segment located between the operator and peptide " $\Omega$ " itself determined the structure of at least two distinctive peptides.

However, it is important to point out that several different mechanisms might account for the presence of several distinctive peptides in the protomere of  $\beta$ -galactosidase. One of these possible mechanisms, which cannot be excluded on account of actually available data, might be the intervention of cathepsin which, by cutting off a certain number of determinants, may give rise to a "poly-peptide precursor" which reflects the groupings on segment z.

Whatever the case, it must be admitted it was possible to recognize two levels of quaternary architecture in the protein. The first level corresponded to the association of dissociable units which then formed the protomere of molecular weight 130,000. The second level corresponded to the association of these protomeres among themselves to form the tetramere of molecular weight 520,000, which was the only active form of the protein. The complexity of the complementation maps, on the one hand, and the repartition of the complementation activity, extracted from a small or large deletion, on the other hand, suggested that the occurrence of the two levels of quaternary structure is normally sequential. In such a system, observed in vitro, complementation, according to the results obtained, could be based on two different mechanisms:

- 1) An exchange or substitution of sub-units for reconstituting a protomere of the wild type, at least, in its primary structure.
- 2) A repair reassociation between the protomeres which carry different point mutations (Brenner, 1959), according to the model proposed by Crick and Orgel (1964).

Certain mutants used in this investigation were isolated by Mr. Malamy, based on a newly developed technique of selection.

This investigation was aided by the National Science Foundation, National Institutes of Health, Jane Coffin Childs Memorial Fund, Atomic Energy Commission, and the General Committee for Scientific and Technical Research.

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Received 9 July 1964, and in revised form 20 April 1965.

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