REGULATION OF NEWLY EVOLVED ENZYMES. I. SELECTION OF A NOVEL LACTASE REGULATED BY LACTOSE IN ESCHERICHIA COLI

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ABSTRACT

Thirty-four lactose-utilizing strains of E. coli were selected from a lac Z deletion strain. In 31 of these, the synthesis of the newly evolved lactase is regulated by lactose. The lactase activity in all the strains is indistinguishable from the ebg+ activity identified by CAMPBELL, LENGYEL and LANGRIDGE (1973).

The study of the evolution of enzyme systems which permit microorganisms to grow on novel substrates has shown that a frequent evolutionary pathway is one which leads to the constitutive synthesis of an enzyme which has a low level of activity on the novel substrate, but which is repressed in the progenitor strains (see review by HEGEMAN and ROSENBERG 1970). By “novel substrates” we mean metabolites not utilized by the progenitor strains. CAMPBELL, LENGYEL and LANGRIDGE (1973) have recently reported that, in a strain of E. coli bearing a deletion of the lac Z gene, the gene for β-galactosidase, intensive selection for the ability to grow on lactose* as a sole carbon source yields strains which have evolved a new enzyme capable of hydrolyzing lactose (see also WARREN 1972). The new enzyme, called ebg, is synthesized constitutively. We decided to examine a large number of lactose-utilizing derivatives of a lac Z deletion strain in order to determine whether or not all such lactose-utilizing strains selected by the method of CAMPBELL, LENGYEL and LANGRIDGE are the same. They are not. In this paper we report the selection of lactose-utilizing strains in which synthesis of the newly evolved lactase is regulated by lactose.

MATERIALS AND METHODS

Bacterial strains

<table>
<thead>
<tr>
<th>E. coli K12 strains:</th>
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<tr>
<td>DS4680A: HfrC, lac Z- (del. W4680) Y+, spc-, ebg-</td>
</tr>
<tr>
<td>DLH11: F-, lac Z- (del. W4680) Y+, str-, tolC6+, ebg-</td>
</tr>
<tr>
<td>A-2: HfrC, spc-, ebg+ (Type I), lac Z Y+ derived from DS4680A</td>
</tr>
</tbody>
</table>

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* Abbreviations: α-lactose = 4-O-β-D-galactopyranosyl-D-glucopyranose; melibiose = 6-O-α-D-galactopyranosyl-D-glucopyranose; allolactose = 6-O-β-D-galactopyranosyl-D-glucopyranose; raffinose = α-D-galactopyranosyl p-(1→6)-α-D-glucosyl p-(1→2)-β-D-fructoside; ONPG = α-nitrophenyl-β-D-galactopyranoside; IPTG = isopropyl 1-thio-β-D-galactopyranoside.

A-4: HfrC, spc, ebg+ (Type II), lac Z-Y+ derived from DS4680A
K10Ca: HfrC, wild-type prototroph
A-44R: lac Y+ constitutive derivative of A-4 selected by growth on raffinose as described in
 Miller (1972)
S. typhimurium strain RC903 colicin E1-producer

Culture media and growth conditions
All cultures were grown at 37° with constant aeration.
L-broth (Miller 1972) was used as a rich medium for matings.
Minimal medium was P-buffer containing 0.2% appropriate sugar as a carbon source. As required: IPTG 2 x 10^-4 M, streptomycin 114 μg/ml, spectinomycin 100 μg/ml. P-buffer consists of 0.062 M potassium phosphate buffer pH 7.0 containing 0.042% sodium citrate, 0.01% MgSO4, 0.1% (NH4)2SO4, and 2 x 10^-4 M FeCl3.
Cell densities are reported as A260 in a 1 cm pathway.

Lactase assays.
In vivo assay: Cells grown under conditions permitting the synthesis of the lac Y+ gene product (lac permease) were resuspended in P-buffer containing 100 μg/ml chloramphenicol. A260 of the resuspended cells was recorded, and the sample was divided into three 0.5 ml aliquots. One ml substrate (3 mg/ml ONPG in P-buffer + chloramphenicol) at 37° was added to two of the tubes, and all three tubes were incubated at 37° until a noticeable yellow color appeared. One-half ml of 1 M Na2CO3 was added to each tube and mixed well to quench the reaction. One ml of substrate was then added to the third tube, which was used as a blank. The quenched samples were centrifuged fifteen minutes at 4600 RPM to pellet the cells, and the A260 of the supernatant from the two sample tubes was read versus the blank. One unit of activity equals the release of one nanomole of ONP per minute at 37°. Specific activities are in terms of units/ml/A260.

In vitro assay: Fifty microliters of cell extract was added to 1.0 ml of substrate (5 mM ONPG in 0.125 M KPO4 buffer pH 7.5 containing 5 mM MgSO4) at 37°. The change in A260 was monitored in a Gilford Model 2400S recording spectrophotometer in which the cuvette chamber was maintained at 37°. One unit = release of one nanomole of ONP per minute. Specific activities are reported in terms of units/mg protein.

Protein determinations
Total protein was estimated by the method of Lowry et al. (1951).

Isolation of lactose-fermenting derivatives of DS4680A
Strain DS4680A was streaked out on lactose-tetrazolium plates (prepared according to Miller 1972, p. 54) containing IPTG. The plates were sealed with parafilm and incubated at 25° and 70-80% relative humidity. All colonies appeared deep red, but within three weeks large white papillae had appeared on some of them. Papillae were picked and streaked out on MacConkey-lactose-IPTG plates. Single lactose-fermenting colonies were picked and stored for analysis. All such strains were checked for resistance to spectinomycin and for sensitivity to the male specific phage R17. This method is virtually identical to that of Campbell, Lengyel and Langridge (1973) except for the inclusion of IPTG in the selective plates.

Preparation of cell extracts
Cells were harvested by centrifugation and the pellet was mixed with twice its wet weight of alumina and ground gently. The broken cells were diluted with 0.05M KPO4 buffer, centrifuged ten minutes at 12,000 x g and the supernatant made 25 mg/ml in streptomycin sulfate. The precipitated nucleic acids were removed by centrifugation and the extract stored frozen at -18°.

RESULTS
Thirty-four lactose-utilizing derivatives of DS4680A were isolated as described in MATERIALS AND METHODS. Among these, we found two distinct types.
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Figure 1.—Growth of A-2 (Type I) and A-4 (Type II) strains in lactose + IPTG. Cultures were grown in glycerol minimal medium containing IPTG. After the glycerol was exhausted, lactose was added to a concentration of 0.2% and the cultures were shaken at 37°. Turbidity was monitored at 720 nm in a Spectronic 70 spectrophotometer. The first order growth rate constant \( \alpha = 0.449 \text{ hr}^{-1} \) for the Type I strain A-2, and the final growth rate for the Type II strain A-4 was \( \alpha = 0.112 \text{ hr}^{-1} \). Open circles: A-2; closed circles: A-4.

Type I strains: Type I strains are capable of growth in lactose minimal medium in the presence of IPTG, which is necessary to induce the synthesis of the lactose permease. Figure 1 shows a typical curve for the Type I strain designated A-2. The cells begin to grow immediately upon addition of lactose to the medium. There is no lag before growth on lactose commences, and the first order growth rate constant \( \alpha = 0.449 \text{ hr}^{-1} \) (doubling time = 95 minutes).

Synthesis of the lactase is apparently constitutive. Extracts of cells grown in glycerol in the presence or absence of IPTG have the same specific activities; thus the sole role of IPTG in permitting growth in lactose is to induce synthesis of the permease. Table 1 shows the level of lactase in Type I cells grown in lactose and in glycerol.

Type II strains: Type II strains also require IPTG for growth in lactose minimal medium. Figure 1 shows a typical growth curve for the Type II strain designated A-4. There is a lag of several hours after the addition of lactose before

| Table 1 |
|---|---|---|
| Differential rates of lactase synthesis in Type I and Type II strains |
| Growing in glycerol | Type I | 33.3 | Type II | 0.0 |
| Growing in lactose | 28.8 | 8.25 |

Activity was assayed in whole cells as described in Materials and Methods. The medium contained \( 2 \times 10^{-4} \text{ M IPTG} \).

It is possible that the level of lactase activity in Type I cells is underestimated because the rate-limiting step in the hydrolysis of ONPG may be the rate of entry into the cell. In the Type II cells it is clear that permeation is not the rate-limiting step, and these estimates are valid.

Lactase activities are in units/ml per \( A_{720} \).
growth begins. The length of the lag is variable, ranging from 200 minutes to as much as 2,000 minutes. The growth rate of Type II strains in lactose is much slower than that of Type I strains, these having doubling times on the order of 400 minutes. The growth rates show considerable day-to-day variation in any single strain.

Synthesis of lactase activity in Type II strains occurs only if lactose is present in the medium. This apparent regulation was first suggested by the long lag before growth begins in lactose. Assays of whole cells grown in glycerol plus IPTG failed to reveal any lactase activity even though Type II cells growing in lactose synthesize about one-third as much lactase activity as do Type I cells (Table 1).

While confining our detailed studies to the Type I strain A-2 and the Type II strain A-4 (and its lac permease constitutive derivative), we have screened thirty-four lactose-fermenting derivatives of DS4680A in order to classify them as Type I or Type II. Those strains which grow on lactose with little or no lag and double in less than 150 minutes ($\alpha \geq 0.289$ hr$^{-1}$) are classified as Type I. Those which lag at least 200 minutes before growing on lactose and have doubling times in excess of 350 minutes ($\alpha \leq 0.119$ hr$^{-1}$) are classified as Type II strains. Each strain could be classified unambiguously as being Type I or Type II, and no intermediates were found.

We obtained 3 Type I strains. The mean growth rate was $\alpha = 0.355 \pm 0.039$ hr$^{-1}$. The remaining 31 isolates were Type II with a mean growth rate $\alpha = 0.099 \pm 0.006$ hr$^{-1}$. These means correspond to 117 and 442 minute doubling times, respectively.

Is the Type I lactase different from the Type II lactase?

Since Type II cells differ from the Type I cells not only in regulation but in the level of lactase and growth rate, it was necessary to determine whether or not the lactase synthesized in the two types is the same protein. We have approached this problem biochemically, immunologically and genetically.

1. Biochemical: Extracts of A-2 cells grown in glycerol minimal medium, and of A-4 cells grown in lactose + IPTG, were prepared as described in MATERIALS AND METHODS. Table 2 shows the specific activities of these extracts. Figure 2 shows the double reciprocal plots of relative activity versus substrate concentration in the presence and absence of 5 mM Mg$^{++}$. Table 2 shows the results of these experiments and other data concerning properties of the lactases from the

<table>
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<th>Property</th>
<th>A-2 (Type I)</th>
<th>A-4 (Type II)</th>
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<tr>
<td>Specific activity of crude extract</td>
<td>148 units/mg</td>
<td>16 units/mg</td>
</tr>
<tr>
<td>Km, no Mg$^{++}$.125 M KPO$_4$</td>
<td>0.88 mM ONPG</td>
<td>0.89 mM ONPG</td>
</tr>
<tr>
<td>Km, 5 mM Mg$^{++}$.125 M KPO$_4$</td>
<td>2.51 mM ONPG</td>
<td>2.58 mM ONPG</td>
</tr>
<tr>
<td>Increase in relative maximal velocity</td>
<td>7.9 fold</td>
<td>5.0 fold</td>
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<td>in the presence of 5 mM Mg$^{++}$</td>
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two strains. It should be noted that, when assayed under the conditions of Campbell, Lengyel and Langridge (1973), the $K_m$'s of the lactases are both 1.5 mM, a value very close to the $K_m$ of 1.4 mM reported by Campbell, Lengyel and Langridge for the $ebg^+$ enzyme.

Since the enzymes in A-2 and A-4 are indistinguishable by $K_m$ under three sets of conditions, we consider it likely on biochemical grounds that the lactase activity in Type I and Type II strains is the same, and that this activity is the $ebg^+$ activity of Campbell, Lengyel and Langridge.

2. Immunological: Rabbit anti-$ebg^+$ antibody was obtained as a gift from John Campbell and Judy Arraj. The antibody forms precipitin lines with $ebg^+$ enzyme but not with lac-Z enzyme (Arraj 1973). The antibody was tested against extracts of A-2, A-4 and an extract of the wild-type K10C4 in which the Z gene product had been induced. Precipitin lines were formed in double diffusion plates with the A-2 and A-4 extracts but not with the K10C4 extract. From these observations, we conclude that the lactase formed in both Type I and Type II cells is $ebg^+$ enzyme, and that this enzyme is immunologically indistinguishable from the $ebg^+$ enzyme reported by Campbell, Lengyel and Langridge.
3. Genetic: Campbell, Lengyel and Langridge (1973) report that $ebg^+$ co-transduces at a frequency of 5% with metC, but does not cotransduce with serA, and is therefore probably rather close to tolC at 59 minutes on the E. coli map (Taylor and Trotter 1972). A-2 and A-4, both HfrC, were mated with DLH11 without interruption for 90 minutes. $TolC^+ str^-$ recombinants were selected and scored for the ability to ferment lactose. In three experiments, a total of 1,995 $tolC^+ str^-$ recombinants were obtained when A-2 (Type I strain) was the donor. Of these, 343 were lac-, giving a recombination frequency of 17.2%. Similarly, in three experiments in which A-4 (Type II) was the donor, 3,224 $tolC^+ str^-$ recombinants were obtained, of which 634 were lac-, giving a recombination frequency of 19.7% between tolC and the lactase gene. These recombination frequencies are not significantly different, as judged by the nonsignificance of the $F$ value in an analysis of variance of the recombination frequencies subject to an angular transformation.

The results of the biochemical, immunological and genetic studies strongly suggest that the lactase synthesized by Type I and Type II strains is the same, and that this lactase is the $ebg^+$ enzyme described by Campbell, Lengyel and Langridge (1973). We shall therefore refer to this enzyme as the $ebg^+$ enzyme from this point on.

Can $ebg^+$ produce an inducer of the lac operon?

Campbell, Lengyel and Langridge (1973) showed that $ebg^+$ lac $i^+ZY^+$ strains ferment lactose only in the presence of IPTG although the $ebg^+$ enzyme is synthesized constitutively, and our observations confirm this property of the $ebg^+$ enzyme. They suggested that either $ebg^+$ enzyme is unable to carry out the transgalactosidation reaction which converts lactose to allolactose, the inducer of the lac operon, or that $ebg^+$ carries out the reaction inefficiently. One possibility to consider is that the $ebg^+$ enzyme may be capable of carrying out the transfer reaction, but that the concentration of lactose inside an $ebg^+$ cell with an uninduced level of permease is below that required for the production of inducing levels of allolactose. This could happen because uninduced cells do not concentrate lactose, whereas induced cells may achieve internal concentrations of lactose as high as 100-fold greater than that in our medium (Kennedy 1970). This implies that an $ebg^+$ lac $i^+ZY^+$ cell might continue to grow in lactose in the absence of IPTG, provided that the permease had previously been induced with IPTG. This is not the case.

Figure 3 shows the results of an experiment with Type I strains in which cells grown in glycerol minimal medium + IPTG were washed and resuspended in lactose minimal medium without IPTG. Initial growth rates are those typical of the two types, and in each case growth ceases after a few doublings. Cells were diluted into fresh prewarmed medium upon reaching cell densities of $A_{720} = 0.45$ to avoid growth inhibition which occurs at high cell densities. The $A_{720}$ shown is that calculated from the observed absorbance times the appropriate dilution factor. Other experiments in this laboratory indicate that $ebg^+$ strains can be diluted and grown repeatedly in lactose + IPTG without significant change in growth rate.
From these experiments we conclude that even when the internal concentration of lactose is sufficient for growth, \( \text{ebg}^+ \text{ lac}^- \) cells are unable to achieve a concentration of allolactose sufficient to sustain the derepression of the \( \text{lac} \) operon.

**Is protein synthesis required for the appearance of \( \text{ebg}^+ \) activity in Type II cells?**

Although the lactases in Type I and Type II strains are indistinguishable, there remains the major difference that the synthesis of \( \text{ebg}^+ \) activity is regulated in Type II strains. The experiment shown in Figure 4 was designed to determine whether the \( \text{ebg}^+ \) activity can be induced in the absence of protein synthesis.

In all the data previously presented, the possibility remains open that IPTG is necessary but not sufficient for the induction of \( \text{ebg}^+ \) activity in Type II strains. To exclude this possibility, we selected strain A44R (see Materials and Methods) for use in the experiment described in Figure 4. Strain A44R synthesizes its \( \text{lac} \) permease constitutively.

Figure 4 shows that no activity was detected in either cells starved for a carbon source or in cells inhibited by chloramphenicol. However, cells in the presence of lactose began accumulating \( \text{ebg}^+ \) activity immediately upon the addition of lactose. In this experiment, growth did not begin until the level of activity had risen to about 2.5 units/ml/\( A_{720} \). A parallel experiment with strain A-2 (Type I) showed that cells incubated in lactose plus chloramphenicol retain 90% of...
**Figure 4.**—Induction of lactase in a Type II strain. A44R cells were grown exponentially in P-buffer containing limiting glycerol. When growth ceased for 20 minutes due to the exhaustion of glycerol, the culture was divided into three parts. To the first aliquot (squares) nothing was added, to the second (open circles) lactose and chloramphenicol were added, to the third (filled circles) lactose only was added. All cultures were shaken at 37°C. Samples were withdrawn at the times indicated, and the cells were assayed for lactase activity by the whole cell assay described in Materials and Methods.

Their $ebg^+$ activity over this same time period. Since the whole cell assay employed in this experiment requires the cells to transport ONPG, it is reasonable to assume that the A-4 (Type II) cells suspended in lactose plus chloramphenicol are able to transport lactose. Thus if lactose alone were sufficient to activate a cryptic enzyme, some activity would have been observed.

The requirement for protein synthesis in the presence of lactose in order to synthesize $ebg^+$ activity in strain A44R supports the hypothesis that either lactose or some derivative of lactose induces $ebg^+$ synthesis.

**Discussion**

We have obtained, as papillae growing from colonies on lactose-tetrazolium-IPTG plates, 34 derivatives of a $lac$ Z deletion-bearing strain which are capable of growth on lactose by virtue of having evolved a new lactase which appears to be the same enzyme obtained by Campbell, Lengyel and Langridge (1973) in similar experiments. Campbell, Lengyel and Langridge report that the enzyme they studied was obtained from a strain which had undergone four rounds of selection in addition to the one which gave rise to the papilla, and that each
round of selection improved the growth on lactose. The observation that a virtually identical enzyme can be obtained in strains derived from the papillae without further selection suggests that either our progenitor strain had a more "mature" ancestral gene, or that the improvements in growth rate upon further selection reported by CAMPBELL, LENGYEL and LANGRIDGE involved changes in genes other than the ebg gene.

Although our data suggest very strongly that the ebg+ enzyme is the same in Type I and Type II strains, we have noticed that the loss in ebg+ activity upon toluenizing Type II cells is about fivefold more severe than the loss upon toluenizing Type I cells.

We have shown that in Type II cells the synthesis of the ebg+ activity is induced by either lactose or some derivative of lactose. The in vivo inducer is not known. We have observed in strain A-4 (Type II) that the time lag before growth on lactose commences is consistently shorter in cells pregrown in melibiose + IPTG than in cells pregrown in glycerol + IPTG. This suggests that the α-galactoside melibiose might serve as an inducer, but no ebg+ activity could be detected in cells grown in melibiose + IPTG.

JOBE and BOURGEIOS (1972) have shown that the natural inducer of the lac operon is allolactose. We have shown that Type I strains with fully induced levels of lac permease are unable to undergo more than six doublings in lactose in the absence of IPTG. Since the ebg+ enzyme is synthesized constitutively in this strain, the data show that ebg+ strains are unable to maintain induction of the lac operon, and thus suggest that the ebg+ enzyme does not convert lactose to allolactose. We therefore think it unlikely that allolactose is the natural inducer of ebg+.

Inasmuch as no deliberate selection for the regulation of the ebg+ was made in these experiments, it may be that the protein which is the progenitor of ebg+ is itself induced by lactose or some structurally related molecule.

In any case, this system is exceptional among all those examined in the study of long-term molecular evolution (see HEGEMAN and ROSENBERG 1970). To our knowledge, this is the only instance in which the enzyme permitting growth on a novel substrate is regulated by the novel substrate.

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