# REGULATION OF NEWLY EVOLVED ENZYMES. IV. DIRECTED EVOLUTION OF THE *EBG* REPRESSOR

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ABSTRACT

In *Escherichia coli*, the wild-type repressor of ebg (evolved β-galactosidase) enzyme synthesis, specified by the *ebgR*+ gene, responds very weakly to lactulose (fructose-β-D-galactopyranoside). Selection for a functional repressor that responds strongly to lactulose as an inducer reveals the existence of *ebgR*+$^{L}$ mutants, which occur spontaneously at a frequency of about $2 \times 10^{-10}$. *ebgR*+$^{L}$ mutants are pleiotropic in that they specify ebg repressor with a greatly increased response to lactulose, lactose, galactose-arabinoside and methyl-galactoside as inducers. Selection of *ebgR*+$^{L}$ mutants is discussed within the framework of directed evolution of a regulatory function.

MOST of the work in the area of experimental evolution has been directed toward the selection of microorganisms that are able to utilize novel metabolites or to exhibit improved functions under marginal conditions. The evolutionary changes uncovered in these experiments fall into two broad categories: (1) mutations in structural genes that lead to enzymes with improved activity, and (2) mutations in regulatory genes that lead to increased amounts of enzyme synthesis. These studies have been reviewed by HEGEMAN and ROSENBERG (1970) and, more recently, by CLARKE (1974). One of the most frequently observed ways in which microorganisms evolve new metabolic functions is by the constitutive production of a previously inducible enzyme or permease that already possesses an activity toward the novel substrate. LERNER, WU and LIN (1964) isolated a mutant of *Aerobacter aerogenes* that was able to utilize xylitol, due to constitutive synthesis of ribitol dehydrogenase. A second mutation led to an improved activity of the ribitol dehydrogenase toward xylitol, but the third mutation for faster growth on xylitol was attributable to constitutive synthesis of a D-arabitol transport system that could also transport xylitol (WU, LIN and TANAKA 1968). Similarly, mutants of *Klebsiella aerogenes* that could utilize D-arabinose were shown to be constitutive for the synthesis of L-fucose isomerase (CAMYRE and MORTLOCK 1965). CLARKE and her co-workers have obtained similar results employing the amidase system of *Pseudomonas aeruginosa*. Neither formamide nor butryamide is an inducer of amidase synthesis in wild-type strains, but both are poor substrates of the amidase. Selection for strains that could use formamide or butryamide as a nitrogen source resulted in mutants that

expressed the amidase gene *amiE* constitutively (BRAMMAR, CLARKE and SKINNER 1967; BROWN and CLARKE 1970). In all of these examples, evolution of a new cellular function (utilization of a novel metabolite) has occurred *via* the loss of a molecular function (regulation of enzyme synthesis). Such a loss of regulation resulting in constitutive synthesis of an enzyme must be energentically very costly if the enzyme's substrate is present only occasionally in the cellular environment (SAVAGEAU 1974, 1977). It thus seems likely that genetic changes which lead to more prudent utilization of energy resources would often be selectively advantageous; hence, constitutive enzyme synthesis should probably be viewed as an intermediate stage in the evolution of a new metabolic capability.

One system in which constitutive enzyme synthesis is the exception, rather than the rule, is the *ebg* β-galactosidase system of *E. coli*. This laboratory has been studying the directed evolution of the *ebg* β-galactosidase in strains of *E. coli* K12 that carry a large deletion in the *lacZ* gene. The evolution of lactose utilization in such strains requires: (1) a mutation in the *ebgA* gene that leads to an altered *ebg* enzyme with greatly increased activity toward lactose and, (2) a mutation in the regulatory gene *ebgR* that permits an increased level of gene expression (HALL and HARTL 1974; HALL 1976a, 1977; HALL and CLARKE 1977). It must be emphasized that mutations in both *ebgA* and *ebgR* are required for lactose utilization; neither alone is sufficient (HALL and CLARKE 1977). The regulatory mutations are of two kinds. One, $ebgR^-$, permits constitutive expression of the *ebgA* gene and is attributable to the loss of *ebg* repressor function (HALL and HARTL 1975). The other, $ebgR^{+U}$, results in a repressor that permits four-fold more *ebg* enzyme synthesis during lactose induction than does the wild-type repressor, but does not alter the basal (uninduced) level of enzyme synthesis (HALL and CLARKE 1977). The surprising property of this system is that 90% of more than 100 lactose-utilizing strains isolated are not constitutive, but instead carry the $ebgR^{+U}$ mutation. This was interpreted as suggesting that there is strong selection for the retention of regulation of enzyme synthesis (HALL and CLARKE 1977).

We have been able to direct the evolution of the *ebg* enzyme itself toward a variety of new substrate specificities, much as CLARKE directed the evolution of the *P. aeruginosa* amidase to a variety of new substrate specificities (see CLARKE 1974, for a summary of those studies). Single point mutations can lead to a specificity for lactose (glucose-β-galactoside) (HALL 1976a, 1977), for methyl-β-galactoside (HALL 1976b), or for lactulose (fructose-β-galactoside) (HALL 1977, 1978). A specificity for galactose-arabinoside requires two mutations in the *ebgA* gene, and a specificity for lactobionate (gluconic acid-β-galactoside) requires three (HALL 1978). Of those substrates, only lactose is an effective inducer of *ebg* enzyme synthesis, while methyl-galactoside, lactulose and galactose-arabinoside are weak inducers and lactobionate is a noninducer (HALL and CLARKE 1977, and unpublished data).

Many studies have focused upon the directed evolution of new enzyme functions, and it has become clear that new enzyme specificities can easily be selected in the laboratory. Because of the importance of regulation in coordinating cellular

metabolism, the present study was undertaken to determine whether evolution of a regulatory gene could be directed in a manner similar to the directed evolution of enzyme activities. The regulatory locus chosen was the *ebgR* gene in *E. coli*.

## MATERIALS AND METHODS

*Bacterial Strains and culture conditions*: All strains are *Escherichia coli* K12, and carry *lacZ* deletion W4680. Strain DS4680A is *HfrC*, *ebgA*$^0$, *ebgR*+, *spc* (HALL and HARTL 1974). Strain 5A1 is an *ebgA51*+ mutant of Strain DS4680A (HALL and CLARKE 1977). Strain SJ-20 is *F*$^-$, *strA*, *ebgA*$^0$, *ebgR1*, *tolC*. The derivation of other strains is described in the text.

Cells were grown in minimal-salts medium (HALL and HARTL 1974) containing either 1.0% (w/v) sodium succinate or 0.2% (v/v) glycerol as a carbon source. Lactulose-minimal plates consisted of minimal salts with 0.1% lactulose as a carbon source and $2 \times 10^{-4}$ M IPTG (isopropyl-$\beta$-D-thiogalactopyranoside) to induce the *lac* permease. MacConkey-lactulose agar was prepared according to instructions for MacConkey Agar Base (Difco) and contained 1.0% lactulose and $2 \times 10^{-4}$ M IPTG. XGAL plates were glycerol-minimal medium containing $2 \times 10^{-4}$ M IPTG and 40 $\mu$g/ml of XGAL (5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside). All cultures are grown at 37°.

*Enzyme activities*: Cell extracts were prepared by grinding with alumina (HALL and HARTL 1975) or by the following procedure. Cell suspensions were prepared by harvesting 50 ml cultures at mid-exponential phase (*ca.* $5 \times 10^8$ cells/ml), concentrating the cells by centrifugation and resuspending the pellet in 0.2 ml chilled 0.0125 M potassium phosphate buffer, pH 7.5. 10 $\mu$g of lysozyme were added to the cell suspension, and the mixture held in ice for 30 min. The lysozyme treated suspension was subjected to five cycles of freeze-thawing in dry ice-ethanol, and 5 mg of streptomycin sulfate were added to precipitate nucleic acids. The solution was centrifuged to precipitate cell debris and nucleic acids, and the supernate was retained. The resulting extracts typically contained 3 to 5 mg of protein per ml.

Ebg enzyme activity was assayed by measuring the hydrolysis of ONPG (O-nitrophenyl-$\beta$-D-galactoside), according to HALL and HARTL (1975). One unit is the hydrolysis of one nM of ONPG per min at 37°. Protein concentrations were determined from the $A_{225}$, as described by HALL and HARTL (1975). Specific activity is units of activity per mg of protein.

Whole cell activity was determined as follows: cultures were grown in minimal medium (with or without lactulose) containing $2 \times 10^{-4}$ M IPTG and 0.01% glycerol. This concentration of glycerol limits cell densities to $10^8$ ml$^{-1}$. Five ml overnight cultures were filtered and resuspended in minimal-salts medium lacking a carbon source, but containing 5 mM ONPG, and incubated at 37°. After two hr, the tubes were examined and those showing an intense yellow color were scored as positive. Those tubes showing no yellow color were scored as negative, indicating a lack of enzyme activity. No intermediate values were detected in this test.

## RESULTS

Several *ebgA* alleles, among them *ebgA51*+, specify ebg enzyme, which is active on both lactose and lactulose (HALL 1978). In strain 5A1, *ebgA51*+ is under control of the wild-type repressor specified by *ebgR*+ (HALL and CLARKE 1977). Thus, for strain 5A1, lactulose is a novel metabolite that is a substrate of the existing ebg enzyme, but is an ineffective inducer of enzyme synthesis.

The wild-type repressor has been extensively characterized in terms of its sensitivity to a wide variety of $\beta$-galactoside inducers, including lactose, lactulose, methyl-galactoside, galactose-arabinoside, and IPTG. Lactulose induces ebg enzyme to ten-fold the basal level, while lactose induces to 100-fold the basal

level (HALL and CLARKE 1977, and Table 3). In neither case is the amount of enzyme produced sufficient to permit growth on the sugar as a sole carbon and energy source (HALL and CLARKE 1977). The strategy, therefore, was to select derivatives of strain 5A1 that were able to grow on lactulose as a sole carbon source, but were not constitutive for ebg enzyme synthesis.

It was previously shown that there is a threshold level of about 5.8 units $mg^{-1}$ of *in vivo* lactase activity required for growth on lactose (HALL and CLARKE 1977). Based upon the assumption that the energy yield of lactulose is about the same as that of lactose, and the observation that ebg enzyme in strain 5A1 is about 40% more active on lactulose than on lactose *in vivo* (HALL 1978), it was estimated that a 12-fold increase in *ebg* enzyme synthesis during lactulose induction would be required to achieve the threshold level of lactulase activity required for growth. In effect, this would mean that the evolved repressor must respond to lactulose better than the wild type repressor responds to lactose.

*Selection of lactulose-inducible mutants*

Strain 5A1 was grown in glycerol-minimal medium containing IPTG to induce the *lac* permease, washed twice in minimal-salts buffer, and spread onto lactulose-minimal plates at a density of $7.9 \times 10^9$ cells per plate. After four days of incubation at 37°, there was an average of 342 colonies per plate, based upon counting three plates. Thus, the frequency of mutations that permitted lactulose utilization was about $4.3 \times 10^{-8}$. Ten plates were replicated to XGAL plates. The vast majority of the lactulose-utilizing colonies were blue on XGAL plates, indicating that the cells synthesized ebg enzyme constitutively. A total of nine white colonies were detected and isolated from the XGAL plates. These colonies were presumed to be inducible by lactulose, and were purified and saved. Colonies of the parental strain 5A1 are white on MacConkey-lactulose agar, indicative of a failure to ferment lactulose. The nine isolates, designated 5A101 to 5A109, formed red colonies on MacConkey-lactulose agar, showing that they did ferment lactulose. Two tests were applied to be sure that none of the isolates was a contaminant: (1) the parental strain is resistant to spectinomycin. All nine isolates were likewise resistant to spectinomycin. As spectinomycin resistance is a rare mutation, it is unlikely that any contaminants would be spectinomycin resistant (2) strains that depend upon ebg enzyme for the hydrolysis of lactose or lactulose have the peculiar property of exhibiting a negative phenotype if IPTG is absent from the medium. Other organisms, including wild-type *E. coli*, do not require the presence of IPTG in order to ferment lactose or lactulose. All nine strains formed white colonies on MacConkey-lactulose medium lacking IPTG (negative phenotype) and red colonies on MacConkey-lactulose medium containing IPTG. Taken together, these tests make it extremely likely that the lactulose-fermenting isolates are descended from strain 5A1.

Extracts were prepared from cultures of strains 5A101 to 5A109 grown in succinate-minimal medium with and without added lactulose + IPTG. Table 1 shows the ebg enzyme activity in these extracts. None of the nine strains was constitutive; all were inducible by lactulose.

TABLE 1

*Induction of Ebg enzyme by lactulose: Specific activities of crude extracts in units per mg*

| Strain | Uninduced | Induced by lactulose |
|---|---|---|
| DS4680A* | 0.28 | 3 |
| 5A1 | 0.41 | 2 |
| 5A101 | 0.08 | 91 |
| 5A102 | 0.14 | 85 |
| 5A103 | 0.54 | 215 |
| 5A104 | 0.48 | 128 |
| 5A105 | 0.33 | 90 |
| 5A106 | 0.87 | 81 |
| 5A107 | 0.36 | 137 |
| 5A108 | 0.17 | 148 |
| 5A109 | 0.66 | 99 |

* DS4680A values are from HALL and CLARKE 1977.

Because of the difficulty in detecting rare white colonies among several hundred blue colonies, it is possible that several more lactulose-inducible colonies were present on the XGAL plates. I am confident, however, that the number of undetected colonies did not exceed the number detected. Thus, I estimate that the frequency of spontaneous mutations to lactulose inducibility is not more than $2 \times 10^{-10}$ (*i.e.*, 18 (maximum) lactulose-inducible colonies divided by $7.9 \times 10^9$ cells per plate $\times$ 10 plates screened). This frequency is close to the estimated spontaneous mutation rate per base pair per replication in *E. coli*, suggesting that there is a paucity of sites in the *ebgR* gene that can be mutated to this phenotype.

Three of the nine strains were chosen for further study.

*Mapping studies*

It is conceivable that the ability to be induced by lactulose might arise as a consequence of mutations in loci other than *ebgR*. For instance, a mutation in *ebgA* might well result in a form of ebg enzyme that could convert lactulose to an extremely effective inducer. To be sure that the observed mutations were in the *ebgR* gene, the evolved phenotype was mapped by conjugation. Each of the three chosen strains, 5A101, 5A103, and 5A108, was mated with the $F^-$ strain SJ-20 (*ebgA*$^0$ *ebgR*$^-$ *tolC strA*), and the mating mixture was plated onto MacConkey-lactulose agar containing 300 μg/ml streptomycin sulfate as a counter-selective agent. MacConkey medium kills *tolC*$^-$ cells; thus, this medium selects the *tolC*$^+$ marker, which is transferred shortly after the *ebg* region in these crosses (HALL and HARTL 1975). MacConkey medium is a fermentation indicator, as well as a selective medium. Since the *ebgA*$^0$ allele specifies an enzyme that is inactive toward lactulose (HALL 1978), recombinants between *ebgA* and *tolC* are white on this medium. All of the white colonies (*ebgA*$^0$ *tolC*$^+$) were tested on XGAL

medium to determine which *ebgR* allele was present. *ebgR*$^-$ strains are blue on XGAL, while *ebgR*$^+$ strains are white. The map of this region is:

*ebgA* ——— (1.6%) ——— *ebgR* ——— (29%) ——— *tolC*

with distances given in recombination frequencies (HALL and HARTL 1975). The colonies that were white on MacConkey-lactulose medium must have undergone recombination between *ebgA* and *tolC*, and it would be predicted that in $5.2\% = 1.6/(1.6 + 29.0)$ of these, the recombination would be between *ebgA* and *ebgR*. Such recombinants would be *ebgA0 ebgR*$^{+L}$ *tolC*$^+$, with *ebgR*$^{+L}$ indicating a lactulose-inducible repressor allele.

Table 2 shows that the frequency of colonies that are white on both MacConkey-lactulose medium and on XGAL medium is very close to the predicted 5.2%. If the phenotype "lactulose inducibility" is due to a mutation in the *ebgR* gene, all of those colonies should be lactulose inducible; whereas, if it is due to a mutation in the *ebgA* gene, none should be lactulose inducible. Each of those recombinants was tested for lactulose inducibility by the whole-cell assay described in MATERIALS AND METHODS, and was shown to be lactulose inducible (Table 2). This demonstrates that "lactulose-inducibility" does not arise as a consequence of a mutation in *ebgA*, or in another locus to the left of *ebgA* on the above map. The data do not rule out the possibility of mutations that permit some other gene (between *ebgR* and *tolC*) to specify a product that converts lactulose to inducer. However, the most reasonable interpretation is that the evolution of lactulose-inducibility occurred *via* mutations in the *ebgR* gene, so that it is proper to designate such strains *ebgR*$^{+L}$.

*Regulation of ebg enzyme synthesis in* ebgR$^{+L}$ *strains*

Table 3 shows the level of enzyme synthesis induced by various galactosides. Clearly the basal level of enzyme synthesis is unaffected by *ebgR*$^{+L}$ alleles. The *ebgR*$^{+L}$ alleles are 20 to 40 times more inducible by lactulose than is *ebgR*$^+$. This increased sensitivity to lactulose exceeds by several fold the minimum increase predicted.

When one new function of ebg enzyme is selected, the mutations often generate additional unselected new functions. Thus, ebg enzyme selected to hydrolyze lactose shows a three- to four-fold increase in activity toward β-methylgalactoside (HALL 1976b). In some cases, selection for lactose utilization results

TABLE 2

*Analysis of recombinants between donors and strain SJ-20*

| Donor | (A) Total *tolC*$^+$-*strA* recombinants | (B) White on MacConkey lactulose agar | (B) as % of (A) | (C) Colonies from B that are white on XGAL | (C) as % of (B) | (D) % of colonies from C which are inducible by lactulose |
|---|---|---|---|---|---|---|
| 5A101 | 448 | 121 | 24.3 | 7 | 5.8 | 100 |
| 5A103 | 428 | 103 | 24.1 | 5 | 4.9 | 100 |
| 5A108 | 590 | 142 | 24.1 | 8 | 5.6 | 100 |

TABLE 3

*Induction of Ebg enzyme by various β-galactosides: Mean specific activities of crude extracts in units per mg*

| Strain | *ebgR* allele | Galactoside added: None | Lactulose* | IPTG | Galactose* arabinoside | Methyl-galactoside | Lactose* |
|---|---|---|---|---|---|---|---|
| DS4680A | *ebgR+* | 0.28† | 3† | 0.30† | 2.3 (4)‡ | 1.4† | 29† |
| 5A1 | *ebgR+* | 0.23 (3) | 4.1 (3) | 0.26 (1) | 1.2 (2) | 0.8 (1) | 23 (4) |
| 5A101 | *ebgR103+$^L$* | 0.24 (3) | 58 (4) | 0.47 (1) | 64 (3) | 14 (4) | 75 (4) |
| 5A103 | *ebgR105+$^L$* | 0.27 (3) | 126 (4) | 0.48 (1) | 211 (3) | 55 (4) | 230 (4) |
| 5A108 | *ebgR110+$^L$* | 0.30 (3) | 84 (4) | 0.45 (1) | 215 (3) | 51 (3) | 199 (4) |

* $2 \times 10^{-4}$ M IPTG was also present to induce the *lac* permease.
† Value from HALL and CLARKE 1977.
‡ The number in parentheses is the number of independent cultures assayed in this experiment.

in an enzyme with more activity toward lactulose than toward lactose (HALL 1977, 1978). The *ebgA51+* allele arose *via* such selection. Selection for lactulose utilization always results in ebg enzyme with 10- to 14-fold increased activity toward lactose (HALL 1978). If evolution of the repressor is similar to evolution of the ebg enzyme, it might be expected that *ebgR+$^L$* repressors would be sensitive to other galactosides as inducers. Strains 5A101, 5A103, and 5A108 were therefore tested for sensitivity to IPTG (a powerful inducer of the *lac* operon but a noninducer of *ebgR+*), lactose, methyl-galactoside, and galactose-arabinoside as inducers. Table 3 shows the results of these tests. There is little, if any, increase in the response of these strains to IPTG. All of the *ebgR+$^L$* strains, however, exhibit a greatly increased response to both galactose-arabinoside and to methyl-galactoside as inducers. The response of 5A103 and 5A108 to galactose-arabinoside is particularly surprising, in that the level of synthesis induced by galactose-arabinoside in these strains approaches half of the maximum (constitutive) synthesis permitted by the *ebgR-* allele (525 to 580 units per mg, HALL and CLARKE 1977). Likewise, lactose is a much more effective inducer of *ebgR+$^L$* repressors than of *ebgR+* repressors. Single cultures of each of the nine *ebgR+$^L$* isolates were tested for sensitivity to lactose, and all showed an increased sensitivity to lactose as an inducer. One *ebgR+$^L$ ebgA$^0$* recombinant was tested and shown to be strongly induced by lactose, methyl-galactoside, and galactose-arabinoside, demonstrating that the complete phenotype is a property of the *ebgR+$^L$* allele. Thus, evolution of the ebg repressor is similar to evolution of the ebg enzyme, in that selection for one new function results in indirect selection for additional new functions.

## DISCUSSION

We have pointed out that evolution of the ability to utilize a new metabolite often occurs *via* loss of a regulatory function. The results presented here show that it is also possible to evolve the ability to utilize a new metabolite by gaining a regulatory function: increased sensitivity to the metabolite as an inducer. This

finding is not without precedent. LEBLANC and MORTLOCK (1971) obtained mutants of *E. coli* that were able to utilize D-arabinose, due to mutations that allowed D-arabinose to function as an inducer of L-fucose pathway enzymes. Similarly, BRAMMAR, CLARKE and SKINNER (1967) obtained mutants of *P. aeruginosa* that were able to utilize formamide as a nitrogen source, due to mutations that led to formamide becoming 10- to 30-fold more effective as an inducer of *amiE* amidase. LEBLANC and MORTLOCK (1971) employed nitrosoguanidine as a mutagen, making it difficult to estimate the number of mutations that occurred. BRAMMAR, CLARKE and SKINNER (1967) employed both nitrosoguanidine and UV as mutagens, but they stated that formamide utilization arose spontaneously as well. Unfortunately, they did not indicate the relative frequency of constitutive and formamide-inducible mutants, either among the spontaneous or the mutagenized formamide utilizers.

The present study is, to the best of my knowledge, the first deliberate attempt to direct the evolution of a repressor to respond to a specific new inducer. The results presented show that the directed evolution of a specific repressor for a specific new function is possible. The frequency of these mutations is very low, on the order of $10^{-10}$. This mutation frequency is comparable to the rate of mutations to streptomycin resistance (HALL 1977). This is of some interest, because the rate of point mutations within the nearby *ebgA* gene was shown to be about 20-fold higher than this (HALL 1977). The simplest interpretation of the apparent disparity in mutations rates is that there are 20 or 30 sites within the $ebgA^o$ gene that can mutate to allow lactose hydrolysis; whereas, there are only one or two sites within the $ebgR^+$ gene that can mutate to allow the repressor to respond to lactulose as an inducer.

The $ebgR^{+L}$ mutation is pleiotropic, in that the evolved repressor exhibits an increased sensitivity to several natural $\beta$-galactosides. The changes in sensitivity to these inducers are not small. Methyl-galactoside becomes 10 to 40 times more powerful an inducer and galactose arabinoside, 30 to 90 times more powerful. Lactulose itself becomes 20 to 40 times more powerful. The increase in the effectiveness of lactose as an inducer is not as dramatic, because lactose was already an effective inducer of the wild-type repressor. Nevertheless, in strains 5A103 and 5A108 lactose induces up to 40% of the maximal rate of ebg enzyme synthesis as defined by the $ebgR^-$ allele. This makes the $ebgR^{+L}$ allele present in these strains the most sensitive to lactose yet discovered. The mutant allele $ebgR^{+U}$ (HALL and CLARKE 1977) is only two-thirds as sensitive to lactose as these $ebgR^{+L}$ alleles. It was previously pointed out (HALL and CLARKE 1977) that lactose was the only powerful inducer of ebg enzyme synthesis found in a survey of 12 natural and synthetic galactosides. Even the $ebgR^{+U}$ allele showed an increased response only to lactose. With the selection of $ebgR^{+L}$ alleles, lactose ceases to be the sole powerful inducer of ebg enzymes synthesis. We can now envision a clear series of mutations that could lead to the ability of *lacZ* deletion strains to utilize several $\beta$-galactosides as carbon sources, without sacrifice of cellular regulation. A mutation to an allele such as $ebgA51^+$ followed (or pre-

ceded) by a mutation to an $ebgR^{+L}$ allele results in a strain that is both lactose and lactulose positive. Subsequent mutations in *ebgA* that allow ebg enzyme to effectively hydrolyze methyl-galactoside or galactose-arabinoside would be immediately advantageous because the regulatory response to these sugars is already present. Thus, the $ebgR^{+L}$ mutations confer the potential for utilizing a wide variety of β-galactosides, without paying the high energetic cost of synthesizing an enzyme when it is not needed.

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