REGULATION OF NEWLY EVOLVED ENZYMES. III EVOLUTION OF THE *ebg* REPRESSOR DURING SELECTION FOR ENHANCED LACTASE ACTIVITY

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ABSTRACT

The evolution of lactose utilization by *lacZ* deletion strains of *E. coli* occurs via mutations in the *ebg* genes. We show that one kind of mutation in the regulatory gene *ebgR* results in a repressor which retains the ability to repress synthesis of *ebg* enzymes, but which permits 4.5-fold more *ebg* enzyme synthesis during lactose induction than does the wild-type repressor. A comparison between the growth rate of various *ebg*+ strains on lactose and the amount of *ebg* enzyme synthesized by these strains shows that the rate of enzyme synthesis permitted by the wild-type repressor is insufficient for growth on lactose as a sole carbon source by a cell with the most active *ebg* lactase yet isolated. We conclude, therefore, that the evolution of lactose utilization requires both a structural and a regulatory mutation.

A number of investigators have described the selection of a new lactase enzyme in *lacZ* deletion strains of *E. coli* (WARREN 1972; CAMPBELL, LENGYEL and LANGRIDGE 1973; HALL and HARTL 1974). The new enzyme which permits *lacZ* deletion strains to utilize lactose as a sole carbon source was named *ebg*, for evolved β-galactosidase, and was shown to be unrelated to the classical *lacZ* β-galactosidase. *ebg* enzyme is the product of a gene, *ebgA*, located at 66 minutes on the new *E. coli* map (HALL and HARTL 1975; BACHMAN, LOW and TAYLOR 1976). The wild-type, or ancestral, allele of this gene is $ebgA^o$. It signifies ebg^o enzyme which is a β-galactosidase, in that it hydrolyzes the synthetic substrate ONPG (Orthonitrophenyl β-galactoside), but which is not an effective lactase *in vivo* (HARTL and HALL 1974). The $ebgA^o$ allele may be converted by a single step mutation to an $ebgA^+$ allele which specifiies an enzyme with sufficient lactase activity to permit lactose utilization (HALL, J. Bacteriol., in press). All such evolved enzymes are termed ebg^+ enzyme (CAMPBELL, LENGYEL and LANGRIDGE 1973; HALL and HARTL 1974, 1975). At least four forms of ebg^+ enzyme have been obtained. These forms are distinguishable from each other and from ebg^o enzyme on the basis of lactase activity and kinetic differences. ebg^o enzyme and three forms of ebg^+ enzyme have been described elsewhere (HALL, J. Mol. Biol., in press), and this communication describes the relevant properties of a fourth ebg^+ enzyme.

MATERIALS AND METHODS

Bacterial strains and culture conditions

All strains are *Escherichia coli* K12. Relevant genotypes of all strains are given in Table 1. Cells were grown in minimal salts medium (HALL and HARTL 1974) containing either 1.0 percent (w/v) sodium succinate, or 0.1 percent lactose and 2×10^{-4} M IPTG (isopropyl-β-D-thiogalactopyranoside) to induce synthesis of the lac permease. Amino acids, when required, were added to a concentration of 100 micrograms per ml. Cultures were maintained at 37° with constant vigorous aeration.

Enzyme activities, calculation of specific activities and specific synthesis

The preparation of cell extracts was described in HALL and HARTL (1974). Extracts contain only soluble protein; nucleic acids and nucleoproteins having been removed by streptomycin precipitation.

The standard assay for *ebg* enzyme activity measures the hydrolysis of ONPG and was described in HALL and HARTL (1975). The hydrolysis of lactose was assayed according to the method described by HALL (J. Mol. Biol., in press). In both cases, one unit = one nanomole of substrate hydrolyzed per minute at 37°.

Protein concentrations were determined from the A_{225} as described by HALL and HARTL (1975). Specific activity is units of activity per milligram of protein. Specific synthesis is the fraction of total protein in crude extracts which is *ebg* enzyme. It is calculated as the specific activity (ONPG) of a crude extract divided by the specific activity of the appropriate purified *ebg* enzyme. Specific activities of the various purified enzymes are given in HALL (J. Mol. Biol., in press).

Regulation of *ebg* enzyme synthesis is independent of the *ebgA* allele. Synthesis is subject

TABLE 1

Bacterial strains

Strain	Genotype
DS4680A	*ebgA*^{0}, *ebgR*+, *HfrC*, *spc*.
A2	*ebgA2*+, *ebgR2*, *HfrC*, *spc*.
A4	*ebgA4*+, *ebgR4*+*U*, *HfrC*, *spc*.
A42	*ebgA4*+, *ebgR42*, *HfrC*, *spc*.
1B1	*ebgA*^{0}, *ebgR1*, *HfrC*, *spc*.
5A1	*ebgA51*+, *ebgR*+, *HfrC*, *spc*.
PW16–8	*ebgA168*+, *ebgR26*+*U*, *HfrC*, *spc*. (Spon. double mutant of DS4680)
SJ-7	*ebgA*^{0}, *ebgR*+, *F*$^-$, *argG*, *metC*, *tolC*, *str*.
SJ-12	*ebgA52*+, *ebgR52*, *F*$^-$, *argG*, *metC*, *str*.
SJ-13	*ebgA51*+, *ebgR*+, *F*$^-$, *argG*, *metC*, *str*.
SJ-14	*ebgA4*+, *ebgR4*+*U*, *metC*, *argG*, *str F*$^-$.
SJ-14b	*ebgA4*+, *ebgR14*, *metC*, *argG*, *str F*$^-$. (*ebgR*$^-$ mutant of SJ-14)
SJ-17	*ebgA51*+, *ebgR1*, *F*$^-$, *str*. (Mating 1B1 × SJ-13)
SJ-18	*ebgA4*+, *ebgR*+, *F*$^-$, *str*, *argG*. Mating DS4680A × SJ-14b)
SJ-25	*ebgA168*+, *ebgR26*+*U*, *F*$^-$, *metC*, *str*. (Mating PW16–8 × SJ-7)

All strains are *Escherichia coli* K12 and carry *lacZ* deletion W4680. The derivations of most strains were given in HALL and HARTL (1975). Others are as shown.

All *ebgA* alleles including the designation "+" specify an enzyme that is effective in lactose hydrolysis. The alleles *ebgA2*+ and *ebgA4*+ specify indistinguishable enzymes (*ebg*+^{a} enzyme) based upon kinetic parameters on two substrates, range of substrate specificities, and thermal inactivation rates. All *ebgR* alleles designated "+" specify an active *ebg* repressor. The designation "U" is explained in the text. Other *ebgR* alleles specify an inactive repressor, and the strains are thus constitutive for *ebg* enzyme synthesis.

to control by a repressor which is the product of the nearby *ebgR* gene (HALL and HARTL 1975). In *ebgR*+ strains, synthesis is induced by lactose (or a lactose derivative), whereas *ebgR*− strains synthesize *ebg* enzyme constitutively (HALL and HARTL 1974, 1975). One of the more intriguing aspects of the *ebg* system is that synthesis of the ancestral (*i.e.*, wild-type) enzyme *ebg*^{0} is induced by lactose, although lactose is not a significant substrate of *ebg*^{0} enzyme (HALL, J. Mol. Biol., in press).

Growth rates

Cultures were grown overnight in minimal medium containing 2×10^{-4} M IPTG and sufficient succinate to limit cell density to 10^8 per ml. The culture was made 0.1% in lactose and shaken at 37°. Turbidity was monitored at 550 nm in a Spectronic 20 spectrophotometer. Growth rates are reported as α, the first order rate constant, in hr^{-1}.

RESULTS

Inducers of ebg^0 *enzyme synthesis*

When a *lacZ* deletion strain of *E. coli* K-12 is grown in glycerol or succinate medium containing both lactose and IPTG, synthesis of *ebg*^{0} enzyme is induced (HARTL and HALL 1974). The only role of IPTG in this induction process is to induce synthesis of the *lac* permease, and thereby to permit the cells to accumulate lactose (HALL and HARTL 1974).

We have screened analogs of lactose in order to determine whether they act as inducers of *ebgA*^{0}, Table 2. Three analogs are weak inducers, being only 5 to

TABLE 2

Induction of ebg^0 *enzyme in strain DS4680A* (ebgA^0 ebgR+)

Additions	Crude extract specific activity in units /mg ± 95% confidence interval
Non-inducers	
None	0.28 ± .058
IPTG, 2×10^{-4} M	0.30 ± .110
Melibiose*, 0.1%	0.20 ± .045
Galactose, 0.1%	0.20 ± .052
Sodium lactobionate*, 0.1%	0.20 ± .077
Glycerol-β-D-galactoside, 10^{-3} M	0.20 ± .059
Phenyl-β-D-galactoside*, 0.1%	0.13 ± .080
Methyl-β-D-thiogalactoside*, 10^{-3} M	0.34 ± .047
Galacturonic acid, 0.1%	0.22 ± .060
Weak inducers	
Methyl-β-galactoside, 0.1%	1.4 ± .279
Thiodigalactoside*, 10^{-3} M	1.3 ± .60
Lactulose*, 0.1%	3. ± 1.9
Strong inducers	
α-lactose*, 0.1%	29. ± 5.4
β-lactose*, 0.1%	33. ± 3.1
Strain 1B1 (*ebgA*^{0} *ebgR*−), constitutive	
None	535.8 ± 43.1

All cultures contained 1.0% (w/v) sodium succinate as a carbon source.
* Indicates that 2×10^{-4} M IPTG was added to the succinate minimal medium to induce the *lac* permease.

10% as effective as lactose. One of these, methyl-β-D-galactoside, is an inducer of the *lac* operon. The regulation of the *ebgA*^{0} gene is very different from that of the *lac* operon in that lactose analogs are not powerful inducers of the *ebgA*^{0} gene. Indeed, lactose induction of the *ebgA*^{0} gene results in only 5% of the maximal level of expression, as judged by comparison with an isogenic constitutive strain 1B1 (Table 2). This induction value may be compared with a value of 17% of maximal expression for the *lacZ* gene induced by lactose *in vivo* (JACOB and MONOD 1961).

An altered ebgR+ *allele*

HALL and HARTL (1974) pointed out that in thirty-one of thirty-four *ebg*+ isolates, synthesis of *ebg* enzyme was induced by lactose; the remainder were constitutive. The thirty-one inducible isolates had, therefore, apparently evolved solely via mutations in the *ebg* structural gene *ebgA*. At that time (HALL and HARTL 1975), there was no evidence that evolution had favored any changes in *ebg* regulatory functions other than occasional *ebgR*$^-$ mutants. Development of methods for purifying *ebg* enzyme (HALL, J. Mol. Biol., in press) has made it possible to examine this point more closely.

Because different *ebgA* alleles may specify enzymes with quite different specific activities, direct correlation of activity with the amount of *ebg* enzyme present is prohibited when dealing with more than one *ebgA* allele. The specific synthesis, as defined in MATERIALS AND METHODS, reflects only the *quantity* of enzyme; thus differences in specific synthesis are indicative of different regulatory states.

TABLE 3

Induction of ebg *enzyme in various strains*

Strain	Genotype	Induction state	Specific synthesis × 10^2 ± 95% confidence interval
DS4680A	*ebgA*^{0} *ebgR*+	Uninduced*	0.0022 ± .0003
DS4680A	*ebgA*^{0} *ebgR*+	Induced†	0.26 ± .05
A4	*ebgA4*+ *ebgR4*+*U*	Uninduced	0.0030 ± .0007
A4	*ebgA4*+ *ebgR4*+*U*	Induced	1.23 ± .25
5A1	*ebgA51*+ *ebgR*+	Induced	0.20 ± .038
SJ-18	*ebgA4*+ *ebgR*+	Uninduced	0.0027 ± .0005
SJ-18	*ebgA4*+ *ebgR*+	Induced	0.30 ± .07
SJ-26	*ebgA168*+ *ebgR26*+*U*	Induced	1.58 ± .16
		Constitutives	
A2	*ebgA2*+ *ebgR2*		4.61 ± 1.2
SJ-12	*ebgA52*+ *ebgR5*		5.1 ± 0.78
1B1	*ebg*^{0} *ebgR1*		4.84 ± 0.39
SJ-17	*ebgA51*+ *ebgR1*		4.6 ± 1.04
A42	*ebgA4*+ *ebgR42*		4.3 ± 0.82

* Uninduced: cultures grown in succinate minimal medium.
† Induced: cultures grown in succinate minimal medium containing 0.1% lactose and 2×10^{-4} M IPTG.

Table 3 shows the specific synthesis of *ebg* enzyme in a number of strains grown in the presence or absence of lactose. In the ancestral, or wild-type, strain DS4680A, lactose induction increased the specific synthesis 120-fold. Although the basal (uninduced) level of synthesis is the same in the *ebg*$^+$ strain A4, the specific synthesis during induction of strain A4 is 4.5-fold more than in strain DS4680A. The evolved strain A4 is clearly more inducible by lactose than the ancestral strain. This higher level of expression is not likely to be due to an up promoter mutation, since (a) the basal levels of DS4680A and A4 are virtually identical, and (b) *ebgR*$^-$ derivatives of the two strains (1B1 and A42, respectively) have very similar specific syntheses.

The altered level of expression of the *ebgA* gene of strain A4 could be either a property of the repressor protein (*ebgR* gene product), or a property of the *ebg* enzyme itself. If, for instance, *ebg*$^{+a}$ enzyme were more efficient at converting lactose to inducer (as *lacZ* β-galactosidase converts lactose to allolactose (JOBE and BOURGEOIS 1972), then the specific synthesis during lactose induction would be a property of the *ebgA*, not the *ebgR*, allele. In order to distinguish between these alternatives, we constructed strain SJ-18, which carries the *ebgA4*$^+$ allele present in strain A4 and the *ebgR*$^+$ allele present in strain DS4680A. Table 3 shows that the specific synthesis during lactose induction of strain SJ-18 is characteristic of strain DS4680A, demonstrating that the level of synthesis is determined by the *ebgR* allele present, *i.e.*, by the repressor. We have, therefore, designated the *ebgR* allele present in strain A4 as *ebgR4*$^+$*U*, "U" indicating an "up" repressor mutation.

Table 4 shows the response of the two repressors to the known inducers of *ebg* enzyme. There is little difference between the repressors' responses to weak inducers; however, evolution has favored a stronger response to lactose as an inducer during selection for utilization of lactose via the *ebg* enzyme.

The relationship between growth rate, ebg *lactase activity and specific synthesis*

Is the co-evolution of the repressor and the enzyme a chance event, or is co-evolution an obligatory feature of evolution of lactose utilization by *lacZ* deletion strains of *E. coli*? Table 5 shows the growth rates on lactose and estimated *in vivo* lactase activity for several *ebgA*$^+$ strains. *In vivo* lactase activities are estimated from (1) an estimated internal concentration of 53 mM lactose, (2) the calculated lactose activity of pure *ebg* enzymes at 53 mM lactose, and

TABLE 4

Inducers of strains DS4680A and A4

Inducer	Specific synthesis × 10^2 ± 95% confidence interval DS4680A (*ebgR*$^+$)	A4 (*ebgR4*$^+$*U*)
None	0.0022 ± .0003	0.0030 ± .0007
TDG	0.012 ± .0054	0.015 ± .0036
Methyl-β-galactoside	0.012 ± .0025	0.014 ± .0013
Lactulose	0.027 ± .017	0.056 ± .028
Lactose	0.26 ± .05	1.23 ± .25

TABLE 5

Estimated in vivo *lactase activities*

Strain	Enzyme	K_m* mM lactose	V_{max}* lactose units/mg	Velocity @ 53 mM lactose units/mg	Specific synthesis $\times 10^2$	Estimated V *in vivo* units/mg	Growth rate α hr^{-1}
A4	*ebg*+^{a}	62.5	4,180	1,920	1.22	23.5	0.10
SJ-12	*ebg*+^{b}	862.0	11,800	681	5.1	34.7	0.13
SJ-17	*ebg*+^{c}	244.0	5,130	916	4.85	42.1	0.15
A2	*ebg*+^{a}	62.5	4,180	1,920	4.61	84.4	0.37

V (*in vivo*) = Specific synthesis $\times$ V_{53}.

* Data from HALL, J. Mol. Biol., in press.

(3) the measured specific synthesis of *ebg* enzyme in each strain. The internal lactose concentration is calculated according to WINKLER and WILSON (1966) based upon 2.65 mM lactose in the growth medium. Lactose activities at 53 mM lactose are based upon the measured K_m (lactose) and V_{max} lactase activities of each enzyme (HALL, J. Mol. Biol., in press). Figure 1 shows a plot of growth rate (α) *vs.* estimated *in vivo* lactase activity. The regression line fits the equation

$$\alpha = (0.0046)\ (\text{Units/mg}) - 0.027 \qquad (1)$$

in which Units/mg is the estimated *in vivo* lactase activity, and α is the first order growth rate constant of a culture growing in a lactose minimal medium. The data in Table 5 fit equation (1) with a correlation coefficient $r = 0.99$. According to this equation, the minimal activity required for growth on lactose is 5.8 Units/mg; and a cell with 7.3 Units/mg lactase activity would have a doubling time of 100 hours ($\alpha = 0.0068\ hr^{-1}$).

Observations on several other strains tend to support this relationship. Strains DS4680A, 1B1 and 5A1 all fail to grow on lactose, and all synthesize less than the threshold level of 5.7 Units/mg lactase activity (see Table 6). Strain 5A1 is of particular interest because, although it was isolated in the same manner as other *ebg*$^+$ strains and it gives a positive indication of lactose fermentation on MacConkey-lactose indicator agar (Difco) containing 2×10^{-4} M IPTG, it nevertheless fails to utilize lactose as a carbon source. Table 6 shows that strain 5A1 has only 30% of the lactase activity required for lactose utilization. The equation also predicts that strain SJ-18, which carries the wild-type repressor gene but synthesizes *ebg*$^{+a}$ enzyme, will grow in lactose with a doubling time of 1900

TABLE 6

Predicted and observed growth rates in lactose minimal medium

Strain	Enzyme	Velocity @ 53 mM lactose units/mg	Specific synthesis $\times 10^2$	Estimated V *in vivo* units/mg	Predicted α hr^{-1}	Observed α hr^{-1}
DS4680A	*ebg*^{0}	82	0.271	0.22	0	0
1B1	*ebg*^{0}	82	4.600	3.85	0	0
5A1	*ebg*+^{c}	916	0.206	1.92	0	0
SJ-18	*ebg*+^{a}	1,920	0.308	5.91	0.00036	0

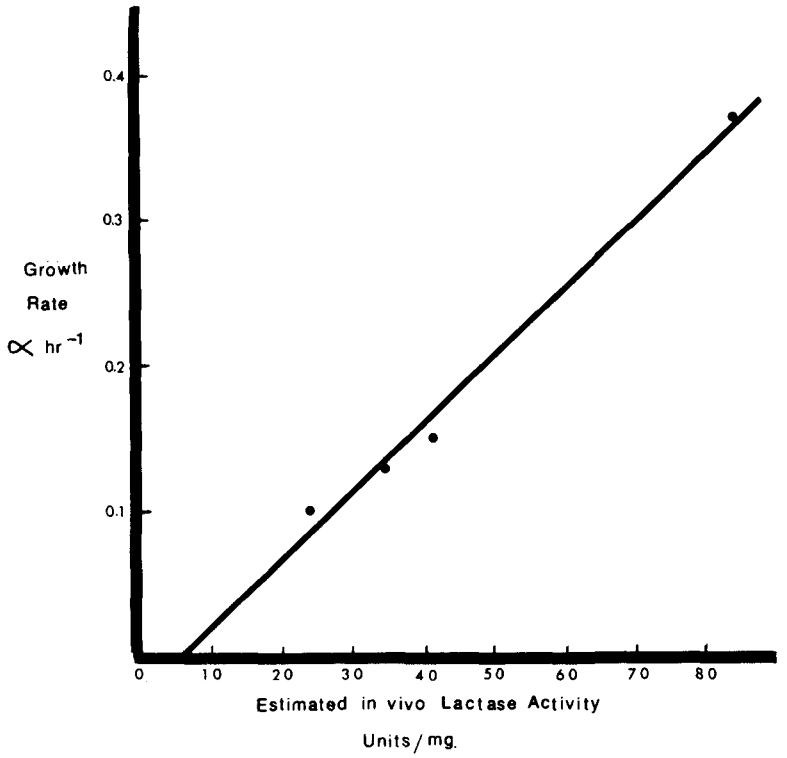

FIGURE 1.—Growth rate *vs. in vivo* lactase activity for four *ebgA*+ strains. Data are from the last two columns of Table 5.

hours, *i.e.*, no detectable growth under laboratory conditions. This prediction has been confirmed, in that strain SJ-18 fails to utilize lactose as a carbon source.

In the course of screening new *ebg*$^+$ isolates for unusual growth rates in lactose medium, we obtained strain SJ-26 which has a growth rate of $\alpha = 0.18$ hr^{-1}. Since strain SJ-26 was regulated, *i.e.*, *not* an *ebgR*$^-$ strain, it seemed likely that its *ebgA* allele specified a more active lactase than *ebg*$^{+a}$ enzyme. Equation (1) predicted that the crude extract lactase activity at 53 mM lactose would be 44.7 Units/mg.

The *ebg* enzyme was purified by previously described methods (HALL, J. Mol. Biol., in press), and the specific activity on ONPG and the K_m and V_{max} on lactose were determined (S.A. ONPG = 7150 Units/mg; K_m lactose = 49 mM; V_{max} lactose = 5400 Units/mg); the lactase activity at 53 mM lactose was calculated as 2807 Units/mg. Since these values are different from all previously examined *ebg* enzymes, the new enzyme is called *ebg*$^{+d}$. Measurements of crude extracts of strain SJ-26 showed that the specific synthesis $\times 10^2 = 1.58 \pm 0.165$. Thus, the estimated *in vivo* lactase activity is 0.0158 $\times$ 2807 Units/mg = 44.4 Units/mg, in close agreement with the predicted value of 44.7 Units/mg. The measured specific synthesis of 1.58% indicates that strain SJ-26, like strain A4, carries an

"up" repressor mutation and should, therefore, be designated *ebgA168*$^+$ *ebgR26*$^+$*U*.

From the equation above, a cell synthesizing the most active *ebg*$^+$ enzyme, *ebg*$^{+d}$, at the rate specified by the wild-type repressor (specific synthesis = 0.26%), would have a growth rate of $\alpha = 0.0069$ hr^{-1}, or a doubling time of 99 hours. Since even the most active *ebg* enzyme does not permit meaningful growth if regulated by a wild-type repressor, it is apparent that all *ebg*$^+$ strains must have, in addition to a mutation in the *ebgA* gene, a mutation in the *ebgR* gene as well. Co-evolution of the structural and regulatory *ebg* genes would thus appear to be a mandatory feature of the reacquisition of lactose utilization capability by *lacZ* deletion strains of *E. coli*.

DISCUSSION

The survey of various galactosides as inducers shows that there is virtually no overlap in inducer specificity between the *ebg* repressor and the *lac* repressor. Indeed, IPTG, TMG, melibiose and glycerol-galactoside, all of which are good inducers of the *lac* operon, are completely ineffective as inducers of *ebg* enzyme synthesis.

Since we do not yet know of other genes in the *ebg* operon and we can not directly assay *ebg* repressor-operator-ligand interactions, it can not be clearly demonstrated that lactose itself is the effective inducer. Because of the extremely low apparent affinity of lactose for the *ebg*^{0} enzyme (K_m = 1,150 mM, K_i = 1,780 mM as competitive inhibitor of ONPG hydrolysis), it seems unlikely that the actual inducer is a lactose derivative synthesized by *ebg*^{0} enzyme. It thus appears likely that lactose interacts directly with the *ebg*^{0} repressor. If lactose itself is the inducer, this emphasizes the dissimilarity between the *ebg* and *lac* repressors, since lactose is an anti-inducer of the *lac* operon (BARKLEY *et al.* 1975).

Evidence presented elsewhere has shown that a single mutation in the *ebgA* gene is sufficient to evolve lactase activity by the *ebgA* gene product (HALL, J. Bacteriol., in press). The evidence presented here suggests that although a single mutation is both necessary and sufficient to evolve lactase activity, it is not sufficient to evolve the capability of utilizing lactose as a carbon source. A mutant *ebg* repressor which permits an increased level of enzyme synthesis is required. The mutation may either be to *ebgR*$^-$, which permits constitutive synthesis at a rate 15-fold greater than that allowed by the wild-type repressor; or the mutation may be to *ebgR*$^+$*U* which maintains inducibility, but permits four- to six-fold more synthesis than the wild-type repressor. Among well over 100 independent *ebg*$^+$ isolates, 90% are regulated. All but one of these exhibit growth rates greater than $\alpha = 0.075$ hr^{-1}, and thus require a specific synthesis of at least 0.81%. Since this is threefold more synthesis than is permitted by the *ebgR*$^+$ allele in the ancestral strain, all these strains must have a mutant *ebgR* allele.

It would be expected that the most frequent *ebgR* mutation would be *ebgR*$^-$, on the grounds that relatively few mutations should increase the level of induction while maintaining repressor function, relative to the number of mutations

which would simply eliminate repressor function. The observation that 90% of the *ebg*$^+$ isolates are not *ebgR*$^-$ suggests that there is very powerful selection for the retention of regulation under our selective conditions.

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