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Fitness as a function of β-galactosidase activity in *Escherichia coli*

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Summary

Chemostat cultures in which the limiting nutrient was lactose have been used to study the relative growth rate of *Escherichia coli* in relation to the enzyme activity of β-galactosidase. A novel genetic procedure was employed in order to obtain amino acid substitutions within the *lacZ*-encoded β-galactosidase that result in differences in enzyme activity too small to be detected by ordinary mutant screens. The cryptic substitutions were obtained as spontaneous revertants of nonsense mutations within the *lacZ* gene, and the enzymes differing from wild type were identified by means of polyacrylamide gel electrophoresis or thermal denaturation studies. The relation between enzyme activity and growth rate of these and other mutants supports a model of intermediary metabolism in which the flux of substrate through a metabolic pathway is represented by a concave function of the activity of any enzyme in the pathway. The consequence is that small differences in enzyme activity from wild type result in even smaller changes in fitness.

1. Introduction

In the seminal paper, Kacser & Burns (1973) demonstrated that the net flux through a simple linear metabolic pathway should be a concave function of the activity of a single enzyme. Subsequent experimental and theoretical work by Kacser & Burns (1979, 1981) and others (Flint *et al.* 1981; Middleton & Kacser, 1983; Heinrich & Rapoport, 1983; Kacser & Beeby, 1984) has shown this result to be of wide application and profound importance. For example, the model has provided a compelling explanation of the recessive nature of inborn errors of metabolism at the molecular level (Kacser & Burns, 1981).

Within this general framework for considering and analysing complex metabolic systems comes hope for the elucidation of the relations between the kinetic behaviour of enzymes, the fluxes through the pathways, and the effects upon fitness. Already, some tentative inroads have been made. Kacser & Beeby (1984) have considered evolution in primitive cells, Heinrich & Rapoport (1983) have suggested an approach to understanding the design of metabolic systems, Middleton & Kacser (1983) have studied the relation between the activity of alcohol dehydrogenase and ethanol tolerance in *Drosophila*, and we have explored some of the implications of the theory in the context of the neutral theory of molecular evolution (Hartl, Dykhuizen & Dean, 1985).

Here we report on the relation between β-galactosidase activity and growth rate in *E. coli*, together with a novel genetic technique for obtaining amino acid substitutions that result in electrophoretic or thermolability differences from wild type.

2. Materials and Methods

Genetic manipulations. E. coli strain CSH10 (Miller, 1972) contains a nonsense mutation within the *lacZ* structural gene of β-galactosidase in the lactose operon that abolishes enzyme activity and growth on lactose. This strain was streaked out to yield single white colonies on MacConkey lactose indicator plates. After being left at 30 °C for one week, 21 red lactose-fermenting papillae were isolated from separate colonies. Using P1(*cml clr100*), eight of these putative mutants were transduced into strain DD320 (Dykhuizen & Davies, 1980), which carries a deletion covering the entire *lac* operon. This procedure ensures a uniform genetic background and screens out possible tRNA suppressors (no tRNA maps within two minutes of the *lac* operon).

Only a small amount of β-galactosidase activity is required for successful P1 transduction. A second β-galactosidase, designated evolved β-galactosidase and coded by the *ebgA* gene, has been successfully transduced despite having a β-galactosidase activity less than 0·5% of the fully induced wild-type enzyme (Hall, 1984). Therefore, a *lacZ* mutant resulting in an enzyme

Table 1. *Origin of β-galactosidase mutants*

Strain	Description	Source
Inducible *lac* operons		
TD1	Control	CSH64 (Miller, 1974)
TD10.1	Revertant	CSH10 (Miller, 1974)
TD10.2	Revertant	CSH10 (Miller, 1974)
TD10.3	Revertant	CSH10 (Miller, 1974)
TD10.4	Revertant	CSH10 (Miller, 1974)
Constitutive *lac* operons		
TD2	Control	JL3300 (Langridge, 1974)
TD3	Mutant	JL1190 (Langridge, 1974)
TD4	Mutant	JL1481 (Langridge, 1974)

with greater than 0·5% wild-type activity would also be expected to be transduced.

Two constitutive β-galactosidase mutations, JL1190 and JL1481 (Langridge, 1974), having substantially reduced enzyme activity, were transduced into the DD320 background to yield strains TD3 and TD4, respectively. As controls, the wild-type *lac* operon of CSH64 (Miller, 1974) and the constitutive isogenic operon of JL3300 were also transduced into DD320 to yield strains TD1 and TD2, respectively. Table 1 lists these and other strains used in this research.

Detection of substitutions. Amino acid substitutions differing from wild type were detected using vertical polyacrylamide gel electrophoresis and thermal inactivation studies. Overnight cultures grown in minimal Davis salts with 1% glucose (w/v) and 1×10^{-4} M isopropyl 1-thio-β-D-galactopyranoside (IPTG) were resuspended in the same volume of Z-buffer (Miller, 1974), sonicated and centrifuged to remove cellular debris. In order to detect small differences in electrophoretic mobility, extracts from putative variant strains and wild type (TD1) were loaded into alternate lanes on several gels. Only fresh extracts were used for all protocols. Gels of 7·5% polyacrylamide with 2·5% crosslinking were pre-run for 2 h at 15 V/cm at 12 °C in a continuous buffer of 0·1 M Tris-borate (pH 8.5). Samples were loaded in 40% sucrose, and electrophoresis was continued for 6 additional hours. The gels were then incubated at 37 °C for 1 h in phosphate–citrate buffer (pH 4.95) containing 0·2 g/l of 6-bromo-2-naphthyl-β-D-galactopyranoside, washed with distilled water, and stained with a 1 g/l solution of tetrazotized-*O*-dianisidine until dark blue bands appeared.

Heat inactivation studies were carried out at 58 °C. Samples were assayed as described by Miller (1974). About one-half of the total enzyme activity of the β-galactosidase of the wild-type strain (TD1) was lost in 1 h, whereas the heat-sensitive variants lost most of their enzyme activity after 45 min. This marked difference in thermolability was readily reproducible in unfractionated extracts. Net protein concentration had little effect on thermolability in these studies because β-galactosidase was stabilized by the presence of β-mercaptoethanol in Z-buffer (Miller, 1974).

Kinetics. The kinetic properties of the enzyme variants were studied by means of the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in Z-buffer, conducted at 37 °C with unfractionated extracts. The concentrations of cell protein in all extracts were estimated by the method of Bradford (1976).

The hydrolysis of lactose by the mutants was also studied, because ONPG is not the natural substrate of the enzyme. For these studies, unfractionated extracts were as described earlier but with 1% succinate (w/v) as a carbon source. These were carefully sonicated in Z-buffer, in both the presence and the absence of β-mercaptoethanol. No difference in activity per mg protein was detected (as judged by the hydrolysis of ONPG) and the experiments were pursued in its absence. The concentrations of lactose used were from 12 mM to 0·5 mM, except for TD4 for which the concentrations were twenty-fold higher.

The production of glucose at 37 °C was monitored by taking samples of a reaction mix (containing less than 1 μg/ml of cell protein) every 5 min, boiling them for 3 min to stop the reaction and estimating the concentration of glucose using the Glucose No. 15-UV diagnostic kit (Sigma Chemical Company). The kit uses glucose hexokinase in the presence of ATP to convert glucose to glucose-6-phosphate, which is then oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase in the presence of NADP. The reduction of NADP to NADPH is monitored at 340 nm. Every mole of NADP reduced is the equivalent of one mole of glucose produced. With extracts from the *lac* deletion strain DD320, no change in absorbance was detected. Although these studies demonstrated that TD3 and TD4 had less enzyme activity, those of TD10.3 and TD10.4 could not be distinguished from wild type (TD1).

The lactase activity of β-galactosidase was also investigated by continually monitoring the galactose produced by means of a coupled assay involving the production of NADH from the action of galactose dehydrogenase. The same lactose concentrations as mentioned earlier were used in the presence of 10 mM NAD and 10 μg/ml of galactose dehydrogenase. The production of NADH was measured at 340 nm. Estimates of V_{max} were made by assuming the equimolar production of galactose and NADH. The concentrations of cell protein in the reaction mixes were between 0·1 μg/ml and 0·03 μg/ml.

As the Lineweaver–Burke plot is known to be quite unreliable (Dowd & Riggs, 1964), the K_ms were estimated using the Eadie–Hofstee plot of velocity against ratio of velocity to substrate concentration. The negative value of the slope of this plot yields a less biased estimate of K_m, and because both axes contain a common variable (velocity), any error will enlarge the variance. Therefore, the tests of statistical significance are conservative. Estimates of V_{max} were obtained from the same data.

Chemostats. Relative growth rate, our operational measure of fitness, was investigated by means of competition between pairs of strains in chemostats in which the limiting nutrient was lactose, according to the procedure described by Dykhuizen & Hartl (Dykhuizen & Hartl, 1980, 1983; Hartl & Dykhuizen, 1981). From each revertant strain to be studied, a spontaneous mutation (*fhuA*) for resistance to bacteriophage T5 was selected in order to be used as a marker in the competition experiments. Chemostats were inoculated with equal proportions of the sensitive and resistant strains. Samples taken from the chemostat twice daily were serially diluted and plated onto LB agar in the presence or absence of the bacteriophage. The number of colonies formed in the absence of bacteriophage yields an estimate of the total number T of bacteria per sample, and the number of colonies formed in the presence of the bacteriophage yields an estimate of the number A of *fhuA* bacteria per sample. The number B of *fhuA*$^+$ cells was estimated by taking the difference, and the selection coefficient was estimated as the slope of the regression of $\ln(A/B)$ against time.

On limiting lactose, mutants constitutive for the synthesis of the *lac* operon proteins are rapidly selected for. IPTG was added to the chemostat media to a final concentration of 10^{-5} M, which averts this problem (Dykhuizen & Davies, 1980). Because an observation of selection in a chemostat might result from the variant β-galactosidase enzyme or from an unrecognized mutation in the genetic background, whenever selection was detected, the β-galactosidase genes in the competing strains were transduced again and the competition experiments carried out anew. Control experiments were also carried out in order to verify that the *fhuA* mutation itself does not affect growth rate.

3. Results

Mutant search. Three distinct amino acid substitutions resulting from spontaneous reversions of the same nonsense mutation were detected. Strains TD10.2 and TD10.3 had substitutions that result in enzymes with electophoretic mobilities that migrated fast and slow, respectively, relative to the wild-type enzyme of TD1. Strain TD10.4 had an enzyme that was thermally unstable at 58 °C. Another revertant, strain TD10.1, had an enzyme that was indistinguishable from wild type.

Competition studies. Revertant strains TD10.1 to TD10.4 were studied in competition against TD1, with lactose as the sole resource limiting growth rate. Within the resolution of the technique, the phenotypically wild-type revertant TD10.1 was selectively neutral. Therefore, any difference in fitness among the other revertant mutants must have been due to different amino acid substitutions in this nonsense codon. Of the remaining three revertants, the amino acid substitution in TD10.3 resulted in a decrease in fitness, that in TD10.2 was selectively neutral within the limits of resolution, and that in TD10.4 was favoured over wild type. Fig. 1 illustrates typical results obtained from competition between TD10.3 and TD1, and between TD10.4 and TD1. The selection coefficients were small but nevertheless within the limit of resolution of the technique, and in all cases they differed from zero at the 5% level. The mutations present in the constitutive Langridge strains, TD3 and TD4, were found to be selectively inferior to the control strain TD2. The failure to observe selection in direct competition between TD1 and TD2 demonstrated that constitutive synthesis of the *lac* operon proteins was equivalent to full induction by excess IPTG. Comparisons made between the absolute value of the slopes demonstrated that the *fhuA* mutations had no detectable effect on the fitnesses of the various β-galactosidase mutants, and therefore the pooled estimates of the selection coefficients are presented in Table 2.

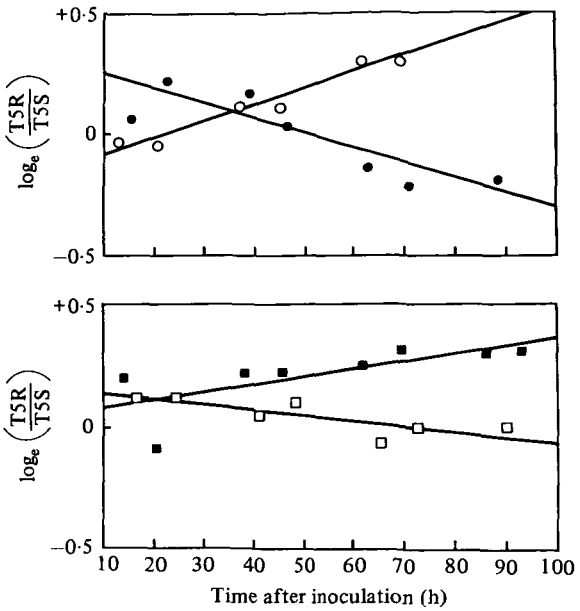

Fig. 1. Examples of weak selection under lactose limitation in chemostats with regression lines plotted. Open symbols are T5-sensitive variant strains against T5-resistant control strains; solid symbols are the reciprocal experiments. Circles, TD10.3 *vs* TD1. Squares, TD10.4 *vs* TD1.

Kinetic studies. The kinetic estimates for the hydrolysis of ONPG by the various β-galactosidase enzymes were based on six separate experiments. Significant heterogeneity of slopes was not found, and the pooled estimates are presented in Table 3. TD10.3, TD10.4, TD3 and TD4 had significantly different K_ms, suggesting that these mutants may have different activities on lactose.

The kinetic behaviour of the β-galactosidase of *K*12 is complicated by a number of side reactions, so it is necessary to investigate the kinetic mechanism proposed by Huber, Kurz & Wallenfells (1976). Their model, without certain trivial side reactions, is

Table 2. *Competitive abilities of β-galactosidase mutants*

Strain	s (h^{-1})	D (h^{-1})	Relative growth rate (1−s/D)
TD1	—	—	1
TD10.1	+0·0008	0·338	1·002
TD10.2	−0·0006	0·351	0·998
TD10.3	−0·0036*	0·326	0·989
TD10.4	+0·0021*	0·371	1·006
TD2	−0·0012	0·318	0·996
TD3	−0·017*	0·316	0·946
TD4	−0·066*	0·327	0·798

s, Selection coefficient; D, dilution rate of chemostat. *$P < 0·01$.

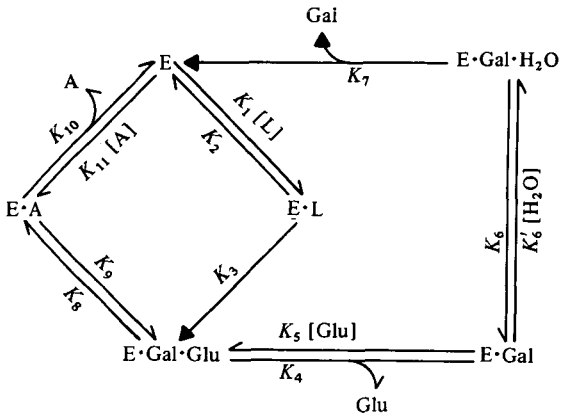

Fig. 2. The kinetic model of β-galactosidase. A, L, Glu and Gal are allolactose, lactose, glucose and galactose. E is the enzyme complex and K_i are rate constants. After Huber, Kurz & Wallenfells (1976).

presented in Fig. 2. Although allolactose is both a product and a substrate of β-galactosidase, these reactions do not provide an independent pathway for the hydrolysis of lactose because of the proposed common intermediate (E.Gal.Glu). Under physiological conditions the net rate of production of allolactose may be given as

$$d[A]/dt = K_{10}[EA] - K_{11}[E][A] \quad (1)$$

At steady state *in vivo* the production and consumption of allolactose will be approximately equal, so that equation 1 will be approximately zero. The differential equations describing the kinetic model can then be solved by the well-known method of assuming a quasi-steady state. Then, assuming that the concentration of lactose in the cell is very small, the expression for the activity of the enzyme *in vivo* is

$$\frac{V_{max}}{K_m} = \frac{K_1 K_3}{(K_2 + K_3)}[Et] \quad (2)$$

The result is not altogether surprising because at steady state the allolactose side reaction is at an equilibrium, so that any lactose converted to E.Gal.Glu must be hydrolysed to glucose and galactose. Therefore, the only rate constants of relevance are those involved with the binding and release of lactose and the irreversible conversion to the intermediate E.Gal.Glu.

However, under initial rate studies the allolactose side reaction is not at equilibrium. Under initial rate studies (with no glucose and no allolactose present), the K_m's for the production of glucose, galactose and allolactose are indistinguishable, but the V_{max} for the production of allolactose need not be equal to that for glucose or galactose. These experimental observations are accounted for by the model (Huber, Kurz & Wallenfells, 1976). The activity of the enzyme in terms of the production of allolactose and glucose or galactose is given by the following two equations,

$$\frac{V_{max}\,glu}{K_m} = \frac{V_{max}\,gal}{K_m} = \frac{K_1 K_3}{(K_2 + K_3)} \frac{K_4(K_9 + K_{10})}{(K_4 K_9 + K_4 K_{10} + K_8 K_{10})}[Et] \quad (3)$$

$$\frac{V_{max}\,allo}{K_m} = \frac{K_1 K_3}{(K_2 + K_3)} \frac{K_8 K_{10}}{(K_4 K_9 + K_4 K_{10} + K_8 K_{10})}[Et] \quad (4)$$

which, upon summing, yield

$$\frac{(V_{max}\,glu + V_{max}\,allo)}{K_m} = \frac{K_1 K_3}{(K_2 + K_3)}[Et] = \frac{V_{max}}{K_m} \quad (5)$$

Table 3. *Estimates of kinetic parameters of β-galactosidase mutants*

	ONPG		α-Lactose	
Strain	V_{max} (units)	K_m (mM)	V_{max} (units)	K_m (mM)
TD1	11·97±0·76	0·1933±0·0035	3·058±0·162	2·395±0·246
TD10.1	12·37±0·47	0·2037±0·0084	ND	ND
TD10.2	12·55±0·29	0·1941±0·0062	ND	ND
TD10.3	10·30±0·53	0·2254±0·0054	2·779±0·361	2·759±0·282
TD10.4	12·18±0·29	0·1697±0·0045	2·777±0·071	1·511±0·246
TD2	11·91±1·56	0·1904±0·0074	3·190±0·131	2·24±0·276
TD3	10·64±0·37	0·4554±0·0276	0·720±0·037	10·12±0·848
TD4	7·32±0·557	0·8096±0·0387	0·883±0·131	59·17±2·058

Units, Micromoles of product released min^{-1} mg^{-1} of unfractionated protein extract. ND, not determined.

Therefore, by studying the initial rates of production of allolactose, and glucose or galactose, an estimate of the *in vivo* activity of β-galactosidase can be found as the sum of the two maximum velocities divided by the Michaelis constant (see equations 3, 4 and 5).

In our kinetic studies of β-galactosidase, the production of glucose or of galactose but not of allolactose was estimated. Therefore, when comparing the activities of enzymes from the mutant strains we assume that the proportion of allolactose produced, $K_8 K_{10}/(K_4 K_9 + K_4 K_{10} + K_8 K_{10})$, is constant. If this proportion increases, the *in vivo* activity of the mutants relative to wild type will be underestimated; and, similarly, a decrease in this ratio would cause an overestimate. The kinetic estimates for the production of galactose from the hydrolysis of lactose are presented in Table 3. The estimates for the wild-type enzymes of TD1 and TD2 are similar to each other, and those from TD3 and TD4 are clearly significantly different from each other and wild type. These differences in activity appear to be far more pronounced on lactose than on ONPG, and whilst the data for TD10.3 and TD10.4 are not significantly different from the controls, they also deviate in the direction predicted from the competition experiments and the ONPG studies.

Table 4. *Estimates of kinetic parameters and growth rates of evolved β-galactosidase constitutive strains*

Class	V_{max} (units)	K_m (mM)	Growth rate (h^{-1})
K12[a]	32·6[b]	2·53	0·8
	28·7[c]	2·53	
ebg°	0·62	150	0·0
I	3·566 ± 0·375	22·0 ± 1·8	0·45
II	2·353 ± 0·298	59·0 ± 7·6	0·19
IV[d]	1·461 ± 0·101	0·824 ± 0·058	0·37
V	0·59	0·69	0·18

Units, Micromoles of product min^{-1} mg^{-1} of purified enzyme.
[a] Data obtained at 30 °C.
[b] V_{max} for glucose production.
[c] V_{max} for allolactose production.
[d] Hydrolysis activity only.

Data from studies on various alleles in the *ebg* gene for evolved β-galactosidase (Hall, 1984) are presented in Table 4, together with estimates for the *lacZ*-encoded β-galactosidase (Huber, Kurz & Wallenfells, 1976). These *ebg* classes represent mutants having enzymes of different kinetic properties. Only *ebg* class IV enzymes synthesize allolactose (Hall, 1982). This activity is about 10% of the hydrolytic activity of the same enzyme, and in view of the fact that allolactose activity accounts for such a small proportion of the total activity, the assumption of reversibility or irreversibility will have little effect. We shall assume that the model proposed for β-galactosidase is applicable to these class IV enzymes because the sequence of *ebg* structural gene has homology with that of *lacZ* (Stokes, Betts & Hall, 1985). The *lacZ* and *ebg* enzymes both account for about 5% of the total cellular protein when they are produced constitutively, so the ratio of *ebg* activity to that of *lacZ* should yield a consistent estimate of relative activity. Table 4 also presents the rates of growth of the various constitutive *ebg* strains and that of *E. coli* K12.

Flux theory. Table 5 presents the lactase activities and growth rates as percentages of wild type. The estimates from the mutant β-galactosidases are compared to the average of the controls TD1 and TD2, whilst those of the *ebg* mutants are compared to those of Huber, Kurz & Wallenfells (1976) – because the

Table 5. *Relative activities and relative growth rates of lactase mutants*

Strain	Lactase activity		Relative activity	Relative growth rate
	V_{max}/K_m	$V_{max}/(K_m+20)$		
TD1/TD2[a]	1·350	NA	100	100
TD10.3[a]	1·007	NA	74·6	98·9
TD10.4[a]	1·838	NA	136·1	100·6
TD3[a]	0·071	NA	5·3	94·6
TD4[a]	0·015	NA	1·1	79·8
K12[b,c]	24·229	NA	100	100
ebg°[b]	0·0004	NA	0·0017	0
I[b]	0·162	NA	0·669	56·3
II[b]	0·04	NA	0·1646	23·8
IV[b,c]	NA	0·077[d]	0·3185	46·3
V[b]	NA	0·029	0·1197	22·5

Data based on estimates presented in Tables 1, 2 and 3. NA = not applicable, as discussed in text.
[a] Units = 10^{-3} min^{-1} mg^{-1} of cell protein.
[b] Units = 10^{-3} min^{-1} mg^{-1} of purified enzyme.
[c] Activity calculated as the sum of allolactose and lactose activities.
[d] Activity based on a sum with transgalactosyl activity of 10% of the hydrolytic activity.

former are activities based on total cell protein, whereas the latter are based on purified extracts. The theory of Kacser & Burns (1973, 1979, 1984), which relates the flux to the activity of enzymes, assumes that all K_m's are larger than their respective substrate concentrations. However, the V_{max}'s of the *ebg* enzymes are so low that this approximation is probably invalid and the substrate concentrations probably exceed their K_m's. This will be especially true for *ebg* classes IV and V because their K_m's are rather low. The relation between the flux through a linear pathway as a function of the second enzyme, whose kinetics are saturable, may be derived as described in the appendix. With the assumption that the flux through the pathway is proportional to the rate of growth, μ, this approach yields the following equation,

$$\mu = \frac{\mu_{max} V_{max}/(K_m + L)}{Q + V_{max}/(K_m + L)}, \quad (6)$$

where μ_{max} is the maximum rate of growth, and $Q = V_{max}/(K_m + L)$ at $\mu_{max}/2$. These two terms, μ_{max} and Q, contain the concentrations of the first, second and final substrates of a linear pathway, along with the various thermodynamic and enzymic constants belonging to enzymes other than the one of interest (see the appendix for details). If the concentration of lactose, L, is less than the K_m then V_{max}/K_m should yield an accurate estimate of enzyme activity and μ_{max} and Q will be approximately constant. Based on the β-galactosidase data in Table 5, $\mu_{max} = 100{\cdot}6 \pm 0{\cdot}5$ and $Q = 0{\cdot}28 \pm 0{\cdot}01$.

Using the data of Winkler & Wilson (1966), Hall (1984) has estimated that the internal concentration of lactose in cells having only the lactase activity of *ebg* alleles is about 20 mM. This is of the order or less than the estimated K_m's of *ebg* classes I and II. The data from these two classes yield $\mu_{max} = 100{\cdot}53$ and $K_m = 0{\cdot}53$, and whilst no standard errors are reported because there are not enough degrees of freedom, note that the these estimates compare favourably with those from β-galactosidase data. Therefore, a low lactase activity, which is expected to increase the internal lactose concentration, does not appear to influence μ_{max} and Q to any great extent. In other words, μ_{max} and Q are approximately constant, even at rather high internal concentrations of lactose. The estimate of 20 mM for the internal concentration of lactose so greatly exceeds the K_m's of *ebg* classes IV and V that it has been incorporated into the estimated activities as prescribed in equation 6. Based on the combined *ebg* data the estimates of μ_{max} and Q are $100{\cdot}5 \pm 0{\cdot}1$ and $0{\cdot}42 \pm 0{\cdot}07$ respectively. These are not significantly different from those estimates made from the β-galactosidase data.

The relation between activity and growth rate is illustrated in Fig. 3. Although the curve was fitted by estimating μ_{max} and Q from an Eadie–Hofstee plot, the inset displays the more familiar double-reciprocal plot.

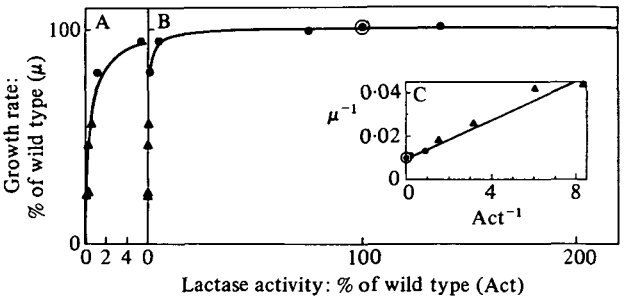

Fig. 3. Relative growth rate plotted as a function of relative lactase activity. ▲, *ebg* mutants; ●, β-galactosidase mutants; ⊙, wild type. (A) shows the fit of the theoretical curve in the 0–6% range: (B) shows the β-galactosidase mutants across the plateau: and the inset (C) displays the same data in the form of a double-reciprocal plot.

4. Discussion

Kacser & Beeby (1984) have postulated that growth rate is related to enzyme activity in a hyperbolic manner, because of the relation between enzyme activity and flux through the pathway. This hyperbolic relation appears to be a general property of many biochemical pathways (for examples, see Kacser & Burns, 1973, 1980; Flint *et al.* 1981). The data presented give further support for their hypothesis.

Using the combined set of *ebg* and *lacZ* data, one can calculate that a 1% increase in lactase activity would result in an increase in fitness of 0·000036, and a 10% increase in activity would yield a fitness advantage of 0·000326, relative to wild type. These calculations are based on the constitutive synthesis of the *lac* operon proteins. However, estimates of the effects of changes in the activity of β-glactosidase in inducible *lac* strains can be made by using the data for an inducible *lac* operon in competition for lactose with a constitutive strain (Dykhuizen & Davies, 1980). These calculations suggest that a 1% increase in the activity of β-galactosidase should yield a fitness advantage of 0·00015, and a 10% increase should yield an advantage of 0·00139, relative to the inducible wild type competing for lactose in chemostats.

However, under natural circumstances selection must be far less intense than that which occurs for limiting amounts of lactose in a chemostat, perhaps by a factor of 10 or even 100, if for no other reason that that *E. coli* inhabits an environment in which many alternative sources of carbon and energy are available. Such a view is supported by the observation that, in chemostats, an inducible strain grows at about half the rate of the constitutive strain in competition for lactose; and yet natural isolates of *E. coli* are rarely constitutive.

A similar curve presented earlier by Hartl, Dykhuizen & Dean (1985) was based on the ONPG data presented in Table 3. The curve presented in the present paper strengthens the argument that small differences in enzyme activity have negligible effects on

fitness, because Q and μ_{max} based on the natural substrate are so much smaller than found with ONPG.

A concave fitness function of the type illustrated in Fig. 3 suggests a rather startling principle. As selection acts to increase the activity of an inefficient enzyme, so a kind of law of diminishing metabolic returns sets in, until small variations in enzyme activity come to have negligible effects on fitness. Thus, selective neutrality might be considered as an inevitable consequence of the long-continued action of natural selection, a notion that is explored by Hartl, Dykhuizen & Dean (1985).

However, if Fig. 3 implies that mutations which have small effects on enzyme activity are selectively neutral or near neutral, the question as to the proportion of amino acid substitutions in this category remains open. Certainly, most nonsense and frameshift mutations abolish enzyme activity, and these are selectively deleterious. The situation with missense mutations is not so obvious. One of the three amino acid substitutions obtained by the reversion of CSH10 was selectively neutral in the competition experiments. The other two caused rather small selection coefficients. These may be neutral, or perhaps nearly so, under natural conditions. In any event, a sample based on the results of one codon is not sufficient to permit any generalisation. However, an extensive array of nonsense codons distributed throughout the *lacZ* gene is available, and will be used to answer this question.

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5. Appendix

Equation 6 was derived by considering a linear metabolic pathway of n reversible steps as given below,

$$S_0 \underset{E_0}{\rightleftharpoons} S_1 \underset{E_1}{\rightleftharpoons} S_2 \underset{E_2}{\rightleftharpoons} S_3 \underset{E_3}{\rightleftharpoons} \cdots \underset{E_{m-2}}{\rightleftharpoons} S_{n-1} \underset{E_{m-1}}{\rightleftharpoons} S_n \underset{E_n}{\rightleftharpoons}.$$

The flux across each of these steps is assumed to be of the following form,

$$F = \frac{\left[\frac{V_i}{K_i} S_i - \frac{V_i'}{K_i'} S_{i+1}\right]}{T_i}, \tag{7}$$

where F is the flux; S_i is the substrate concentration, S_{i+1} is the product concentration; V_i and K_i are the maximum velocity and Michaelis constant of the forward reaction; V_i' and K_i' are the maximum velocity and Michaelis constant of the reverse reaction; and where T_i is defined as

$$T_i = \left[1 + \frac{S_i}{K_i} + \frac{S_{i+1}}{K_i'}\right]. \tag{8}$$

Rewriting equation 7 and multiplying both sides by

$$\prod_{j=0}^{i-1} \left[\frac{V_j' K_j}{K_j' V_j}\right] \tag{9}$$

gives

$$F T_i \frac{K_i}{V_i} \prod_{j=0}^{i-1} \left[\frac{V_j' K_j}{K_j' V_j}\right] = S_i \prod_{j=0}^{i-1} \left[\frac{V_j' K_j}{K_j' V_j}\right] - S_{i+1} \prod_{j=0}^{i} \left[\frac{V_j' K_j}{K_j' V_j}\right]. \tag{10}$$

Summing over all steps yields

$$F\left(T_0 \frac{K_0}{V_0} + T_1 \frac{K_1}{V_1}\left[\frac{V_0' K_0}{K_0' V_0}\right] + \sum_{i=2}^{n-1} T_i \frac{K_i}{V_i} \prod_{j=0}^{i-1} \left[\frac{V_j' K_j}{K_j' V_j}\right]\right) = S_0 - S_n \prod_{j=0}^{n-1} \left[\frac{V_j' K_j}{K_j' V_j}\right]. \tag{11}$$

Rearranging to solve for F gives

$$F = \frac{S_0 - S_n \prod_{j=0}^{n-1} \left[\frac{V_j' K_j}{K_j' V_j}\right]}{\left(T_0 \frac{K_0}{V_0} + T_1 \frac{K_1}{V_1}\left[\frac{V_0' K_0}{K_0' V_0}\right] + \sum_{i=2}^{n-1} T_i \frac{K_i}{V_i} \prod_{j=0}^{i-1} \left[\frac{V_j' K_j}{K_j' V_j}\right]\right)}. \tag{12}$$

Note that when all steps are considered to be unsaturated, this equation reduces to that of Kacser & Burns (1973).

Enzymes downstream from step E_1 (β-galactosidase) are assumed to be unsaturated ($T_i = 1$ for $i > 1$). Thus everything under the summation sign is constant, as is the numerator. Expanding T_0 and T_1, and noting that S_2/K_1' must be very small, gives

$$F = \frac{C_1}{\left[1 + \frac{S_0}{K_0} + \frac{S_1}{K_0'}\right]\frac{K_0}{V_0} + \left[\frac{K_1 + S_1}{V_1}\right]\frac{V_0' K_0}{K_0' V_0} + C_2}. \tag{13}$$

Upon rearrangement this yields,

$$F = \frac{F_{max} \frac{V_1}{K_1 + S_1}}{Q + \frac{V_1}{K_1 + S_1}}, \tag{14}$$

where

$$F_{max} = \frac{C_1}{\left[C_2 + \left(1 + \frac{S_0}{K_0} + \frac{S_1}{K_0'}\right)\frac{K_0}{V_0}\right]}. \tag{15}$$

and

$$Q = \left[\frac{V_0 K_0'}{K_0 V_0'}\left[C_2 + \left(1 + \frac{S_0}{K_0} + \frac{S_1}{K_0'}\right)\frac{K_0}{V_0}\right]\right]^{-1}. \tag{16}$$

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