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Selective Disadvantage of Non-Functional Protein Synthesis in *Escherichia coli*

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Summary. Comparison of growth rates of isogenic strains that synthesize varying levels of β-galactosidase during continuous culture on non-inducing medium indicates that synthesis of low levels of non-functional protein has a small but possibly significant effect upon growth rate.

Key words: Selection/Continuous Culture/Non-Functional Protein Synthesis/*Escherichia coli*/Metabolic Evolution

INTRODUCTION

In 1964, Horowitz unified and extended Lewis' (1951) theory of the evolution of metabolic pathways. Lewis had suggested that new gene function could arise by a process involving the duplication of genetic material creating redundant genes that could then undergo mutation and selection to new function. Horowitz combined his earlier theory (1945) concerning the retrograde evolution of biosynthetic pathways with the gene duplication ideas of Lewis, when he imagined that as an essential nutrient in the medium was exhausted, a variant arises in the population which possesses an enzyme that could create the nutrient from a pre-existing immediate chemical precursor in the medium. Pathways arise in a retrograde manner as additional nutrients become exhausted and variants, able to synthesize the nutrient from precursors in the medium, are selected.

Catabolic pathways could arise by a similar process of gene

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duplication and mutation to new function thus allowing cells to catabolize immediate precursors of utilizable compounds.

The duplication and subsequent modification of genetic material, often entailing the expression of the duplicate gene(s) prior to their full assumption of a new role, is also a feature of virtually all models for the evolution of new protein function or specialization (e.g., Clarke, 1974; Rigby et al., 1974; Waley, 1969; Yčas, 1974; and Zuckerkandl, 1975).

It can be argued that these theories about the evolution of enzyme systems are not particularly attractive because the variants, with their wasteful excess synthesis of the duplicated gene products, would be culled from the population by selection. Establishment of such variants would depend either upon periodic selection and/or immediate isolation of the variant population. Some circumstantial evidence (Horiuchi et al., 1962) suggests that indeed high levels of excess protein synthesis (25% of the total cellular protein being non-functional) are detrimental to a cell. However, recent reviews of the evolution of enzyme systems in bacteria (Hegeman & Rosenberg, 1971; Clarke, 1974) suggest excess synthesis of proteins to play a major role in allowing cells to gain new metabolic capabilities. Additionally, comparisons of growth rates of cells in batch culture (A.G. Marr, personal communication) have suggested that cells performing wasteful synthesis at lower levels (~5% total cellular protein being non-functional) may not be stringently selected. Thus allowing such variants to successfully compete within the population.

We used the *lac* operon in *Escherichia coli* as a model to test the validity of the objection raised to the Horowitz-Lewis theory of pathway evolution and more recent models that entail synthesis of non-functional protein. We also hoped to gather information concerning growth rate regulation in bacterial cells (Maaløe, 1969) growing with long generation times.

MATERIALS AND METHODS

Strain Construction and Resultant Genotype. Dr. Jack Sadler kindly furnished us with a series of *lac* constitutive strains that were isogenic in all known respects, except the *lac* operator region, to W 42, a *lac* O^+ strain. The O^c mutations in D 531, D 533, D 534, and D 536 were produced spontaneously in the operator region of a *lac* operon on an F' *lac* episome. The O^c mutation in D 532 was produced by 2-amino purine mutagenesis. In order to minimize any additional genetic differences among the strains, this "D" series was constructed by the transfer into the F^- female of the original F' lac^+ episome. Recipient isogenicity for all of the crosses was insured by using samples, for the individual episome transfer experiments, of a culture of recipient cells cloned from a single colony. The

Table 1

Strain	Genotype
D 531	F' *lac-pro* B^+ i^+ O^c_{3-4} z^+ y^+ / Δ *lac-pro* B *rec* A^- str^s, spcR
D 532	" O^c_{116} "
D 533	" O^c_{103} "
D 534	" O^c_{10} "
D 536	" O^c_{12} "
W 42	" O^+ "

Table 2
Generation time and Z/B min at 20°C and 37°C for all strains on R-glycerol medium

	Z/B min[a]		Generation time (h)	
	20°C	37°C	20°C (±0.12)	37°C (±0.1)
531	0.420	0.415	6.7	2.0
532	0.099	0.101	6.6	2.0
533	0.092	0.099	6.6	2.1
534	0.120	0.123	6.7	2.1
536	0.038	0.040	6.6	2.1
W 42	0.001	0.001	6.6	2.0

[a] Z/B min is a measure of the constitutive rate of expression of β-galactosidase in a culture. It is the ratio of absorbance at 420 nm/ml of culture (*o*-nitrophenol released in the standard β-galactosidase assay) to the turbidity of the culture measured at 350 nm. The Z/B min has dimensions of specific activity (enzyme per mg cell material), and is a measure of cellular enzyme content as well as the rate of the lac gene products synthesis under steady-state conditions of growth in non-inducing medium.

donor sex factor was the same in each case (Table 1). The center column of Table 1 lists the genotype of the strains used in the study; those genetic symbols left of the slash represent the genotype of the F' *lac* O^c, while those to the right of the slash represent the chromosome. The recipient strain was *rec*A^- to prevent recombination. The *pro*B^+ marker on the episome insured the continual presence of the episome in cells grown in minimal media. Each of the mutants had a characteristic level of uninduced operon expression. The cells were grown in minimal glycerol medium and assayed for β-galactosidase. The activity of β-galactosidase in the culture per milliliter divided by the turbidity of the culture ($A_{350\ nm}$) gave the Z/B minimal values listed in Table 2.

Strain Storage and Maintenance. Stock suspensions as inocula for experiments were prepared by freezing 0.5 ml samples of high density R-glycerol cultures of the strains in vials containing 3 to 5 drops of dimethyl sulfoxide (DMSO). Such (5 to 6) small screw capped vials of each strain were prepared and stored at -70°C. Individual 5-bromo-4-chloro-3-indolyl-β-D-galactoside (BCIG) indicator medium cultures of each strain were maintained at 20°C for day to day use.

Media and Conditions of Cultivation. The minimal media utilized in this work were made from the mineral base (R-buffer) of Smith & Sadler (1971). In the turbidostat (Nephelostat) experiments described below and for cells growing under non-inducing conditions, 0.2% (v/v) filter-sterilized glycerol was added as sole carbon and energy source.

All liquid cultures were shaken at 150 cycles per minute in 125 ml flasks fitted with a 15 mm × 125 mm test tube side arm, on a rotary shaker or a reciprocating water bath shaker. Approximately 10% of the volume of the flask contained medium. Turbidity was used as a measure of growth. Periodically during an experiment the culture fluid was tipped into the side arm and its turbidity was measured in a Klett-Summerson colorimeter fitted with a number 66 filter.

Solid minimal media were prepared by adding 1.0% (w/v) Ionagar (Wilson Diagnostic Inc.) to the liquid media. The agar and mineral solutions were each prepared at double strength, autoclaved separately, and mixed just prior to dispensing.

BCIG indicator medium was used to differentiate colonies of constitutive mutants from those of W 42. This medium is based on R-buffer and has the additional ingredients per liter: 25 mg BCIG, 2.0 g disodium succinate, 2.5 g casamino acids and 10 g Ionagar. Plates were incubated at 37°C and examined after 24 h. W 42 produced a white colony on this medium, whereas the *lac* O^c mutants produced blue colonies.

Assay Conditions for β-Galactosidase. The assay procedure of Smith & Sadler (1971) was used, except that cells (0.5 to 1.0 ml of an exponentially growing culture) were lysed with 20 µl of a mixture: 1.0 ml toluene, 1.0 ml 10% (w/v) sodium lauryl sulfate (SLS), 1.0 ml 0.02 M $MnSO_4 \cdot H_2O$ and 5.0 ml β-mercaptoethanol (Putnam & Koch, 1975). Enzyme units per ml of assay culture (Z) were computed as net absorbance at 420 nm/(incubation time) (ml culture assayed), while Z/B is simply Z/absorbance at 350 nm per ml of the culture assayed.

The specific activity of pure β-galactosidase was determined utilizing these assay conditions and the value obtained agreed with previously published values (Craven et al., 1965).

Protein Determinations. The method of Lowry et al. (1951) was used to determine protein concentrations with bovine serum albumin as a standard. The method of Hu et al. (1959) was used to de-

termine the concentration of β-galactosidase when the specific activity of the pure enzyme was measured. Disc-gel electrophoresis (Ornstein, 1964) was used to ascertain the purity of the β-galactosidase.

Chemicals. All inorganic chemicals were of highest purity commercially available. From the Fisher Scientific Company, Fairlawn, New Jersey were obtained: dimethyl sulfoxide, disodium succinate, glycerol, SLS, TRIS, and toluene. Sigma Chemical Company, St. Louis, Missouri was the source of BCIG and β-mercaptoethanol; from Difco Laboratories, Detroit, Michigan, yeast extract, casamino acids (vitamin free) and agar. The Ionagar No. 2S came from Wilson Diagnostic Inc., Glenwood, Illinois; the bovine serum albumin from Pentex, Inc., Kankakee, Illinois and chloramphenicol was supplied by Calbiochem, San Diego, California.

The Nephelostat and Modifications. We felt there were several advantages to comparing the growth rates of strains in continuous culture apparatus with its constantly controlled conditions of medium composition, cell density and oxygen level.

The Lab-Line Mark I Nephelostat was used for all continuous culture experiments. Preliminary experiments resulted in several modifications of the basic apparatus: (1) the replacement of the 30 ml overflow collection tube with a 500 ml round bottomed flask, (2) the addition, to compensate for fluctuations in both the quality and quantity of air bubbled through the culture vessel, of an activated carbon filter and a flow controller, respectively, (3) the taping of a black cloth over the overflow assembly to prevent light in the room from interfering with the turbidity monitoring system inside the growth cabinet, (4) the addition of an auxillary recorder to monitor the culture density and the rate of addition of medium to the growth vessel, and finally (5) the growth vessel walls were treated with trimethyl silyl chloride to retard the growth of bacteria on the vessel wall. This treatment delayed wall growth, but did not prevent it. All experimental runs eventually were terminated by vessel wall growth.

Mixed Culture Growth Experiments and Data Treatment. Samples of both a minimal glycerol culture of a mutant and a minimal glycerol culture of W 42 were used to inoculate the growth vessel for each mixed culture experiment. During an experimental run, samples were periodically taken from the culture and plated on BCIG indicator medium. When growth occurred, the ratio of mutant to W 42 was determined. The flow rate of the system was also monitored and the cell generation time was calculated using the following assumptions and equations:

(a) in turbidostats the rate of which the culture vessel volume is replaced by new medium at constant flow rate (ω) is equal to the specific growth rate (α). $\omega = \alpha$.

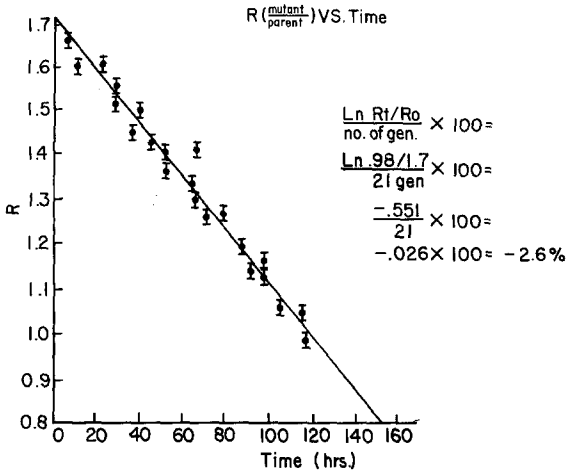

Fig.1
Ratio (R) of inducible parent to constitutive mutant 531 cells in the effluent of the Nephelostat as a function of time during growth on non-inducing glycerol minimal medium during a typical experiment. The calculation of the percent difference in growth rate is shown

(b) $\omega = W/V_o$, where W = flow rate (ml/min), and V_o is the culture volume.

(c) T = cell generation time (min) = $\ln 2/\alpha$

$$\frac{0.693}{\omega} = T$$

The percent difference in generation time between the mutant and W 42 was calculated using the equation of Baich & Johnson (1969):

$$\frac{\ln R_t/R_o}{\text{\# of generations } (R_o \rightarrow R_t)} \times 100 = \text{\% difference in generation times}$$

R_t = ratio mutant/W 42 at some final time

R_o = ratio mutant/W 42 at some initial time.

To accurately determine R_o and R_t for an experiment, a graph of R with respect to time was made, and a line representing the best fit through the data points was drawn and R_o and R_t were determined from that line (Figure 1). A typical experiment required 192 h. R (± 3%) was determined about 30 times. The cell generation time (± 10%) was calculated approximately 10 times. A linear regression analysis of the data indicated the line shown in Figure 1 had a Kendall correlation coefficient of +0.98 (Noether, 1971). Similar accuracy was obtained for all experiments.

RESULTS

Preliminary Experiments to Establish the Conditions of Growth in the Nephelostat. Preliminary experiments with the Nephelostat led to

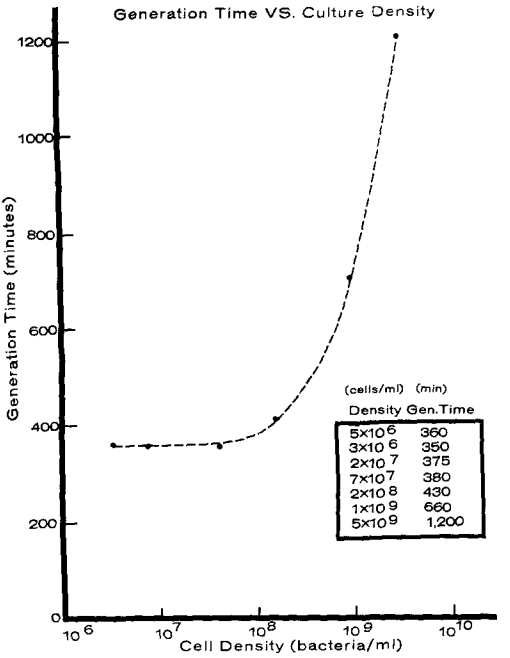

Fig.2
The effect of culture density upon growth rate of the parental strain in the continuous culture apparatus

three important observations concerning aspects of the basic methodology adopted. The first concerned additional effort to retard culture vessel wall growth, the second, the cell density used for all experiments, and the third, the need for preconditioning the strains to growth in the Nephelostat.

Since slower growing cultures develop wall growth more slowly (Kubitschek, 1970), and since it was important to prevent vessel wall growth in order to extend the sample gathering time, we attempted to grow the cells in the Nephelostat at 37°C, 35°C, 30°C, and 20°C. At all but the lowest temperature, wall growth occurred within 10 generations. The 20°C runs always lasted for at least 20 generations. Since Marr et al. (1964) had shown previously that *E.coli* K-12 strains grown at 20°C had the same regulatory pattern for the *lac* operon as 37°C grown cells, the 20°C temperature was adopted for all experiments.

It was shown that cultures of density greater than 5×10^9 cells per ml had greatly decreased growth rates in the Nephelostat (Figure 2), presumably because aeration became limiting, therefore a density of $2\text{-}3\times10^7$ cells per ml was used.

We noticed for cells not previously grown in the Nephelostat, that for 8±0.5 generations in the first two-membered mixed culture experiments, the relative growth rates of the two members fluctuated by as much as 15% from the post-eight generations rate. The final relative growth rate varied by < 5% for the rest of that, and of all other experiments. We interpreted this "adaptation" to involve the selection of "fitter" mutants (Atwood et al., 1951) of the strains that were able to grow faster in the Nephelostat. All strains were therefore condit-

Table 3
Percent total protein synthesized as products in the expression of the *lac* operon

Strain	β-galactosidase	M-protein[a]	thiogalactoside transacetylase[a]	Total
531	2.4	0.60	0.60	3.6
532	0.60	0.15	0.15	0.90
533	0.61	0.15	0.15	0.91
534	1.09	0.27	0.27	1.63
536	0.40	0.10	0.10	0.60
W 42	0.01	0.002	0.002	0.014

[a] It was assumed that equimolar amounts of M-protein and thiogalactoside transacetylase (TA) are produced during normal *lac* operon translation and that the ratio of β-galactosidase to M-protein and TA is 4:1:1 in K-12 strains of *E.coli* (Brown et al., 1967; Berg & Zabin, 1964).

ioned by growing them individually for 12 generations in the Nephelostat. Samples were frozen in DMSO and stored; only cells so treated were used as inocula in further experiments.

Measurement of Generation Times and ß-Galactosidase Levels in all of the Strains Following Growth at 20°C and 37°C in Minimal Glycerol. All strains were grown in minimal glycerol medium at 20°C and 37°C and both their minimum specific rate of synthesis (Z/B min, see footnote to Table 2) and their generation times were measured. Table 2 summarizes the results.

The Z/B minimal values indicate that experiments done at 20°C will yield cells with levels of *lac* operon expression very similar to the levels seen at 37°C grown cells. We were unable, as the results show, to detect any significant difference in growth rates between batch cultures of the strains, confirming earlier unpublished observations of A.G. Marr.

All strains were grown in R-glycerol at 20°C and measurements of the total cellular protein per ml of culture and the Z/B minimal values were made. Knowing the specific activity of pure β-galactosidase in the assay used allowed us then to calculate the amount of β-galactosidase per ml for all the strains grown in minimal glycerol. We then calculated the % total cellular protein wastefully synthesized as β-galactosidase in all of the strains. Table 3 summarizes the amount of non-functional protein in these six strains as the sum of all three *lac* gene products [β-galactosidase, M protein, and thiogalactoside transacetylase (TA)].

Mixed Culture Growth Experiments. Strains 531, 532, 533, 534, and 536 were grown individually with W 42 in mixed culture exper-

Table 4
Average % difference in generation time between the mutant strains and W 42 in R-glycerol at 20°C

	% Difference in generation time[a]	% Total cellular protein wasted
531 (ave. 3 expts)	-2.65±0.23	3.6 ±0.28
532 (ave. 3 expts)	-2.17±0.24	0.90±0.08
533 (ave. 6 expts)	-2.20±0.20	0.91±0.07
534 (ave. 3 expts)	-2.60±0.24	1.63±0.13
536 (ave. 8 expts)	-2.13±0.19	0.60±0.04

[a]The method of calculating this quantity is shown in Figure 1; the uncertainty associated with each value is the standard deviation.

Table 5
% Difference in generation time between 531, 533, and 536 compared to W 42 in R-glycerol at 20°C with and without chloramphenicol (1 µg/ml) (reduces growth rate at 20°C in R-glycerol for all strains by 15%)

Growth conditions	Strain		
	531	533	536
with chloramphenicol	-2.50	-2.30	-2.13
without chloramphenicol	-2.46	-2.17	-2.20

iments lasting at least 20 generations (8 days). Table 4 summarizes the results of these growth rate comparisons. There is no simple direct relationship between the level of *lac* operon expression in a mutant and its growth rate relative to W 42, the *lac* O^+ strain. W 42 grew ~2.3% faster than every mutant tested. Further experiments were altered by addition, midway in the experiment, of medium containing slightly inhibitory amounts (1 µg/ml) of chloramphenicol. This concentration of drug decreased the growth rate 15%. Since at low concentrations chloramphenicol specifically affects general protein synthesis in *E.coli* (Wisseman et al., 1954), it was assumed a change, after the addition of the chloramphenicol to the medium, in the relative growth rates of a mutant and W 42 to a rate reflecting the levels of *lac* operon expression in the two strains, would indicate that general protein synthesis had just become limiting and was not the normal intracellular growth rate limiting process. If no change occurred, then it can be concluded that the rate of general protein synthesis is not, and cannot be made to be, the growth rate limiting process. Table 5 summarizes these experiments. The general protein synthesis rate is clearly not the growth rate limiting process in these slowly growing cells

as there was essentially no change in the relative growth rate of these strains upon addition of medium containing chloramphenicol.

DISCUSSION

The interpretation of the mixed culture growth rate comparisons depends heavily on the fact that test cultures were isogenic with the parental strain in all respects except the particular *lac* O^C mutation. The only experimental step that might result in unforseen genetic changes is the use of an adaptation period of growth in the Nephelostat for each strain prior to mixing. However, "adaptation" took place in all cases in a virtually identical fashion with respect to time and growth rates, so that we assume that the same change(s) occurred in each strain. If W 42 is not isogenic in all respects with the test strains, this might account for the approximately 2% difference in generation time between all strains and the parent. We deem most significant the trend of growth rates among strains as a function of increasing wastefully synthesized cellular protein (Figure 3). Although a slight trend toward slower growth rates can be seen as wasteful protein synthesis increases, over the range examined the change is very small, and clearly not proportional to the extent of wasteful synthesis. It may be, however, that over many generations a small amount of wasteful synthesis is not selectively neutral.

The results of the mixed culture growth rate comparisons are consistent with an extension of Maaløe's (1969) constant efficiency hypothesis of growth rate regulation to cells growing with 7.0 h generation times. Both Maaløe and Nierlich (1974) have suggested the rate of synthesis of functional ribosomes to be the growth rate limiting process, although there also exists evidence contrary to this view (Koch, 1970). The 2.3-1.6% average difference in generation times between strain W 42 and strains 531, 532, 533, 534, and 536 may therefore reflect a faster ribosome synthesis rate in W 42. In failing to alter the relative growth rates upon the addition of chloramphenicol, additional support was obtained for the idea that the growth rate determining process involves the synthesis of some specific intracellular component (e.g. ribosomes), even for slowly growing cells.

Despite the fact that there is a measurable reduction in growth rate among strains with high levels of non-functional protein synthesis (Horiuchi et al.) there are several examples of levels of non-functional protein synthesis that do not measurably affect the growth rates of the strains involved. Table 6 lists examples of growth rate comparisons in populations of *E.coli*, one component of which is wastefully synthesizing *lac* operon proteins.

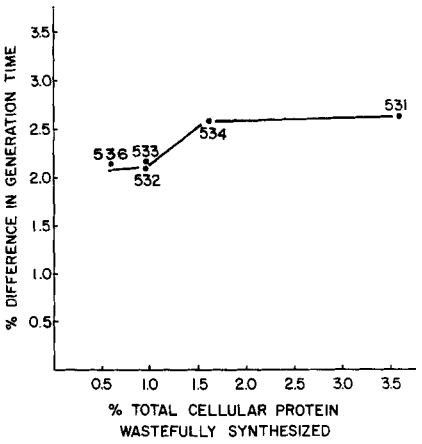

Fig.3
Relationship of relative growth rate to the level of *lac* operon expression in 5 strains examined

Table 6
Non-functional *lac* operon protein synthesis and its effects upon growth rate of *Escherichia coli*

Report	Method of inducing wasted synthesis	Level of *lac* operon expression (% total cellular protein)	Reduction in growth rate selection
Horiuchi et al., 1962	Genetic-multiple copies of the operon	25%	yes
Langridge, 1969	Genetic-multiple copies of the operon	≈5%	no
Marr, pers. comm.	Genetic-constitutive mutant	5%	no
Novick & Weiner, 1957	Gratuitous induction with thiomethyl-β-D-galactoside (TMG)	0.2-5%	no
Andrews & Hegeman	Genetic-O^c mutants	0.6-3.6%	no

A recent survey of the evolution of pathways (primarily catabolic pathways) in bacteria (Hegeman & Rosenberg, 1971) revealed that in a majority of the cases (59%) where it has been studied, the initial mutational step in gaining new catabolic potential (i.e., growth on a previously non-metabolizable carbon and energy source) involved the excess synthesis of an enzyme with poor but finite catalytic activity with the novel compound. The results outlined in Table 6 suggest that cells (variants) in a population would not necessarily be subject to stringent selection on the basis of a *low* level of excess protein synthesis, and thus objections to the Horowitz-Lewis theory of retrograde pathway evolution based on stringent variant counter-selection are probably not valid. Significantly, the survey of the evolution of new catabolic abilities in bac-

teria suggests, as did Horowitz and Lewis, that variants with constitutive low levels of catabolic enzyme synthesis may persist in a population of bacteria and provide a mechanism for that population to extend its metabolic capabilities through the selection of such variants.

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